

Makoto Tominaga
Masahiro Takagi *Editors*

Thermal Biology

Temperature Sensing and
Temperature-Responding Systems

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Makoto Tominaga
Division of Cell Signaling
Okazaki Institute for Integrative
Bioscience (National Institute
for Physiological Sciences)
Okazaki, Aichi, Japan

Masahiro Takagi
School of Materials Science
Japan Advanced Institute of Science
and Technology
Nomi, Ishikawa, Japan

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Foreword

All biochemical and physiological processes exhibit temperature dependence, dictating how we and other organisms assess, respond, and adapt to our thermal environments. Indeed, it is fascinating to consider how different life forms have evolved to best thrive in their preferred temperature habitat, be that in a scalding thermal vent or a frigid snowcapped peak. At no time have such questions been more relevant than today, when we are experiencing rapid changes to our thermal world through climate change.

Like other sensory modalities, thermosensation begins with the process of stimulus detection, followed by transmission of this information along neural circuits to drive reflexive and adaptive physiological responses and behaviors. Given the ubiquitous nature of our thermal environment (be it hot or cold, we are never in a “no temperature” state), we sometimes take this critical and remarkable modality of thermosensation for granted. Thus, it is wonderful to see an excellent book devoted to this topic, one that covers recent progress in understanding the molecules and mechanisms that contribute to each of the essential steps of temperature sensation, ranging from thermosensitive ion channels involved in temperature detection to genetic and evolutionary pathways underlying thermal adaptation.

I would like to thank my old friend and colleague, Prof. Makoto Tominaga, for inviting me to introduce this book, which brings back many wonderful memories of our time together at UCSF in the early days of thermo-TRP channel discovery and characterization. The field has come a long way since then, but there are still many fundamental questions yet to be addressed. I hope that this book will inspire a new generation of sensory biologists, biophysicists, geneticists, and physiologists to further explore our thermal world and how we live in it.

San Francisco, CA, USA

David Julius

Preface

We are very pleased to now publish this Springer book entitled *Thermal Biology*.

Sensory function is fundamental for organisms to perceive external environmental stimuli, enabling them to respond to environment changes and maintain homeostasis. Temperature is one of the crucial environmental factors because it affects enzyme activities and biochemical reactions that regulate various physiological processes. Temperature also functions as a signaling factor in development and reproduction across various organisms. For animals, a key response to environmental temperature changes is thermoregulation, which is categorized into physiological and behavioral thermoregulations. Physiological thermoregulation is observed mainly in homeotherms, including shivering thermogenesis in skeletal muscles, non-shivering thermogenesis in brown adipose tissue, and vasodilation/vasoconstriction. In contrast, behavioral thermoregulation is a crucial physiological function conserved in a wide range of organisms including both homeotherms and ectotherms. Behavioral thermoregulation includes seeking comfort temperature zones, avoidance from extreme temperatures, and posture changes. Furthermore, temperature sensation is even more important in plants since they have to somehow adapt to the ambient temperature changes and cannot move for behavioral thermoregulation.

The progress in thermal biology research has been slow, partly because no temperature receptor proteins have been reported in mammals. As the readers know well, the Nobel Assembly at Karolinska Institute has decided to award the 2021 Nobel Prize in Physiology or Medicine jointly to David Julius and Ardem Patapoutian for their discoveries of receptors for temperature and touch. A gene for capsaicin receptor TRPV1 was isolated by the research group of David Julius in 1997, and it was clarified that TRPV1 is activated by noxious heat stimulus with a temperature over 43 °C, which received a lot of attention as the first temperature sensor. Other temperature-sensing proteins have also been isolated in the last couple of decades. With these advances in the thermal biology research, there was a large research project (Grant-in-Aid for Scientific Research on Innovative Areas) titled “Thermal Biology” (2015–2020) supported by the Japan Society for the Promotion of Science, in which both of us were involved.

This book provides a lot of recent research results from molecules to whole body and from plants, bacteria to humans by the researchers in the field of thermal biology in Japan, many of whom worked together in the

major research project, “Thermal Biology” described above. We believe that the readers would be the witnesses of this remarkable progress in the research field of thermal biology. Finally, we thank Springer and its staff for their assistance and encouragement.

Ishikawa, Japan
Nagoya, Japan

Masahiro Takagi
Makoto Tominaga

Contents

Part I Temperature-Sensing Mechanisms

1 Thermosensation and TRP Channels	3
Makoto Tominaga and Makiko Kashio	
2 Biomimetic Lipid Raft: Domain Stability and Interaction with Physiologically Active Molecules	15
Naofumi Shimokawa and Masahiro Takagi	
3 Cold Tolerance in the Nematode <i>Caenorhabditis elegans</i>	33
Atsushi Kuhara, Natsune Takagaki, Misaki Okahata, and Akane Ohta	
4 Temperature-Dependent Activation of Thermosensitive Transient Receptor Potential Channels	47
Kunitoshi Uchida	
5 Thermal Stress and Nuclear Transport	61
Shingo Kose, Yutaka Ogawa, and Naoko Imamoto	
6 Lipid-Mediated Mechanisms of Thermal Adaptation and Thermoregulatory Behavior in Animals	79
Kohjiro Nagao, Takuto Suito, Akira Murakami, and Masato Umeda	
7 Cellular Thermal Biology Using Fluorescent Nanothermometers	97
Kohki Okabe, Shingo Sotoma, and Yoshie Harada	
8 Temperature Dependent Activity of the Voltage-Gated Proton Channel	109
Yuichiro Fujiwara	
9 Sensory Processing of Cutaneous Temperature in the Peripheral and Central Nervous System	127
Kei Eto, Dennis Lawrence Cheung, and Junichi Nabekura	

Part II Temperature-Dependent Physiological Responses

- 10 Central Mechanisms of Thermoregulation and Fever in Mammals 141**
 Kazuhiro Nakamura
- 11 Inter-Organ Communication Involved in Brown Adipose Tissue Thermogenesis 161**
 Kei Takahashi, Tetsuya Yamada, and Hideki Katagiri
- 12 Circadian Clock and Body Temperature 177**
 Takahito Miyake, Yuichi Inoue, Yota Maekawa, and Masao Doi
- 13 Experimental Approaches Used to Investigate Voluntary Behavioral Thermoregulatory Responses 189**
 Airi Ido, Minami Kanai, Natsuko Hitora-Imamura, Hiroshi Nomura, and Masabumi Minami
- 14 Regulation of Neural Functions by Brain Temperature and Thermo-TRP Channels 199**
 Koji Shibasaki
- 15 β -Adrenergic Signal and Epigenomic Regulatory Process for Adaptive Thermogenesis 213**
 Yoshihiro Matsumura, Timothy F. Osborne, Ryo Ito, Hiroki Takahashi, and Juro Sakai
- 16 Mitophagy Responds to the Environmental Temperature and Regulates Mitochondrial Mass in Adipose Tissues 229**
 Shun-ichi Yamashita and Tomotake Kanki
- 17 Role of TRPA1 in Painful Cold Hypersensitivity 245**
 Takayuki Nakagawa and Shuji Kaneko
- 18 Thermal Adaptations in Animals: Genes, Development, and Evolution 253**
 Ako Agata and Tadashi Nomura

Part I

Temperature-Sensing Mechanisms

Thermosensation and TRP Channels

1

Makoto Tominaga and Makiko Kashio

Abstract

Somatosensory neurons can sense external temperature by converting sensation of temperature information to neural activity via afferent input to the central nervous system. Various populations of somatosensory neurons have specialized gene expression, including expression of thermosensitive transient receptor potential (TRP) ion channels. Thermosensitive TRP channels are responsible for thermal transduction at the peripheral ends of somatosensory neurons and can sense a wide range of temperatures. Here we focus on several thermosensitive TRP channels including TRPV1, TRPV4, TRPM2, TRPM3, TRPM8, TRPC5, and TRPA1 in sensory neurons. TRPV3, TRPV4, and TRPC5 are also involved in thermosensation in nonneuronal cells and tissues. In particular, we discuss

whether skin senses ambient temperatures through TRPV3 and TRPV4 activation in skin keratinocytes and the involvement of TRPM2 expressed by hypothalamic neurons in thermosensation in the brain.

Keywords

Thermosensitive TRP channel · TRPV1 · TRPV3 · TRPV4 · TRPM2 · TRPM3 · TRPM8 · TRPC5 · TRPA1 · TMEM79 · ANO1 · Dorsal root ganglion · Trigeminal ganglion

M. Tominaga (✉) · M. Kashio
Division of Cell Signaling, National Institute for
Physiological Sciences, National Institutes of Natural
Sciences, Okazaki, Japan

Thermal Biology Group, Exploratory Research
Center on Life and Living Systems (ExCELLS),
National Institutes of Natural Sciences,
Okazaki, Japan

Department of Physiological Sciences, The Graduate
University for Advanced Studies (SOKENDAI),
Okazaki, Japan
e-mail: tominaga@nips.ac.jp

1.1 Thermosensation

Changes in environmental temperatures affect various biological processes. As such, animals have evolved sophisticated physiological systems for sensing ambient temperature. Somatosensory neurons are generally assumed to have the ability to sense external temperature by converting temperature information into neural activity via afferent input to the central nervous system (CNS). Various populations of somatosensory neurons have specialized gene expression including expression of thermosensitive transient receptor potential (TRP) ion channels (Usoskin et al. 2015). Thermosensitive TRP channels are responsible for thermal transduction at the peripheral ends of somatosensory neurons and

can sense a wide range of temperatures. Activation of thermosensitive TRP channels promotes cation influx, leading to membrane depolarization that activates voltage-gated Na⁺ channels and in turn generates an action potential. This pathway is very simple, but represents a highly effective way to convert temperature stimulus into electro signals.

1.2 TRP Channels

A prototypical member of the TRP channel family was identified in the eye of *Drosophila* mutants that have *transient*, but not continuous, changes in *receptor potentials* in response to light stimulus (Montell and Rubin 1989). Accordingly, TRP channel research is most progressed in *Drosophila*. However, in mammals TRP channel family members and their functions differ substantially from those of *Drosophila*. There are 6 TRP subfamilies in mammals: TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPML (mucolipin), TRPP (polycystin), and TRPA (ankyrin).

1.3 Cloning of Capsaicin Receptor TRPV1

Research to clarify the mechanisms for detection of physical stimuli (thermal and mechanical stimuli) did not progress well until the late 1990s because the sensors involved were not yet known. In 1996 Oh et al. showed that capsaicin, a main pungent ingredient in “hot chili” peppers, activates nonselective cation channels in rat dorsal root ganglion (DRG) neurons both at a single-channel and whole-cell level (Oh et al. 1996), suggesting that the capsaicin receptor is an ion channel protein expressed in the plasma membrane of sensory neurons. The pungent sensations from foods containing agents like capsaicin are in fact pain rather than taste.

Meanwhile, the Julius group at University of California at San Francisco was working on nociceptive pathways and had identified for the first time several proteins, including ion channels, involved in nociception (Caterina et al. 1997). Given that the capsaicin receptor was known to have high Ca²⁺ permeability, the Julius group applied an expression cloning method with Ca²⁺-imaging using a fluorescence dye fura-2 to identify a protein activated by capsaicin that they termed vanilloid receptor subtype 1 (VR1) because the capsaicin structure includes a vanillyl moiety (Caterina et al. 1997). The work was featured in the October 1997 issue of *Nature* as the cover article (Fig. 1.1).

The name VR1 was updated to TRPV1, which is the member of the TRPV subfamily. In 1997, the ability of temperature stimulus to open ion channels had not yet been described. However, our experience with pungent and hot oral sensations evoked from foods containing capsicums suggested that heat stimulus might activate TRPV1. Our electrophysiological experiments revealed heat-evoked activation of TRPV1 did in fact occur with an apparent temperature threshold of around 43 °C, a temperature that is known to cause pain in humans and monkeys. Subsequently, painful acid stimuli were also shown to activate TRPV1 and a synergism among the effective stimuli of TRPV1 was demonstrated (Tominaga et al. 1998). For example, temperature thresholds for heat-evoked activation of TRPV1 could be lowered by mild acidic conditions. Temperature thresholds were also later reported to be decreased by the posttranslational modifications such as phosphorylation (Tominaga et al. 2001), which can explain why warm water, which does not normally cause painful sensation, can be a painful stimulus under inflammatory conditions. Furthermore, these stimuli (capsaicin, heat, and acid) activated TRPV1 in excised patch membranes, suggesting that they likely activate TRPV1 directly without need for any cytosolic components.

Fig. 1.1 The study describing the identification of the capsaicin receptor channel (to be renamed TRPV1) was featured as the cover article in the October 1997 issue of *Nature*

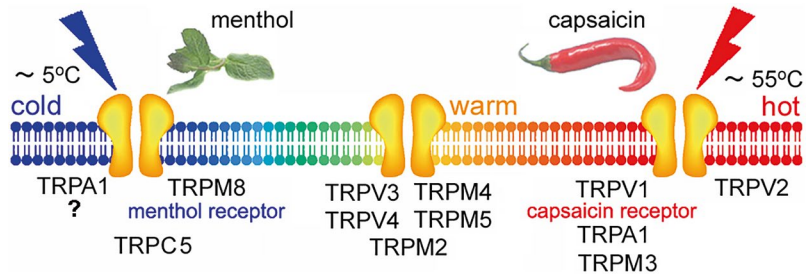


1.4 Thermosensitive TRP Channels

In mammals, the TRP ion channel superfamily can be divided into 7 families based on sequence similarity. Humans have 27 TRP ion channels. Since the initial cloning of TRPV1 in 1997, there are now 11 thermosensitive TRP channels (TRPV1, TRPV2, TRPV3, TRPV4, TRPM2,

TRPM3, TRPM4, TRPM5, TRPM, TRPC5, TRPA1) many of which belong to the TRPM and TRPV subfamilies (Fig. 1.2). Despite the large number of thermosensitive TRP channels, few have been demonstrated to be activated directly by heat. Moreover, even though high-resolution cryo-EM structures have been solved for all 11 thermosensitive TRP channels, the mechanism by which temperature opens these channels remains unclear.

Fig. 1.2 Eleven thermosensitive TRP channels in mammals



1.5 Noxious Heat Sensing by Thermo-sensitive TRP Channels

TRPV1 is expressed in unmyelinated non-peptidergic and peptidergic nociceptive neurons (Usoskin et al. 2015) and activated by various nociceptive stimuli including noxious high temperatures ($>43^{\circ}\text{C}$), capsaicin, and acidic pH (Caterina et al. 1997; Tominaga et al. 1998). TRPV1 is synergistically activated by these effective stimuli, and, accordingly, is known as a protein that explains a polymodal nociceptor. Moreover, the temperature sensitivity of TRPV1 is elevated and the temperature threshold for heat-evoked activation is decreased under inflammatory conditions, such that TRPV1 is activated at body temperature, which can contribute to inflammatory pain without external stimuli (Moriyama et al. 2005; Tominaga and Caterina 2004).

Heat-evoked TRPV1 activation in nociceptive neurons elicits defensive behaviors in mice to avoid tissue injury. In one study, DRG neurons from TRPV1 knockout (TRPV1KO) mice were not responsive to capsaicin, and had decreased response to acid (Caterina et al. 2000). Furthermore, the TRPV1KO mice had less pronounced pain-related behaviors after a capsaicin injection, although mechanosensitivity was not affected. DRG neurons from TRPV1KO mice had attenuated sensitivity to heat ($\sim 43^{\circ}\text{C}$), but retained sensitivity to higher temperatures ($\sim 55^{\circ}\text{C}$). Consistent with this result, TRPV1KO mice showed decreased defensive behaviors to noxious heat yet had responses to noxious thermal stimuli that were similar to wild-type mice,

suggesting the presence of redundant transduction processes for noxious heat detection. Another thermosensitive TRP channel, TRP subfamily M member 3 (TRPM3), was reported to be involved in noxious heat sensing. TRPM3 is strongly co-expressed with TRPV1 in small diameter sensory neurons (C fibers), and its activity is enhanced with increased temperature (Vriens et al. 2011). Like TRPV1KO mice, TRPM3KO mice have impaired heat sensitivity, but retain behavioral responses to noxious heat stimuli. Along with TRPV1 and TRPM3, TRP subfamily A member 1 (TRPA1) is now known to be involved in noxious heat sensing after having initially been reported to be involved in noxious cold sensing (Tominaga and Caterina 2004). There has been a long-standing debate regarding the temperature sensitivity of mammalian TRPA1 (Chen et al. 2013). When classified by chemical sensitivity, the functional expression of TRPV1, TRPM3, and TRPA1 in trigeminal ganglion (TG) neurons is highly overlapping (Vandewauw et al. 2018). The heat sensitivity in sensory neurons (45°C) and the behavioral responses to acute noxious heat were almost completely abolished in TRPV1/TRPM3/TRPA1 triple KO (TKO) mice, but not in double KO mice of any combination. Temperature preference from 5°C to 50°C and results from a two-temperature choice test ($30/45^{\circ}\text{C}$) were comparable between wild-type (WT) and TKO mice, suggesting an adaptive heat-sensing mechanism that occurs independently of TRPV1/TRPM3/TRPA1 activation. With acute noxious heat, the sensory nerve endings appear to recruit redundant thermosensitive TRP channels, including TRPV1, TRPM3, and TRPA1, to ensure the detection of noxious heat that has the potential to cause burn injuries. As

stated above, an earlier study reported that TRPA1 was cold-sensitive and not heat-sensitive (Story et al. 2003). Meanwhile, another study using TRPA1 incorporated into planar lipid bilayers observed a U-shaped activation curve in response to both cold and heat stimuli, and this signal was further enhanced by H₂O₂ application (Moparthi et al. 2016). In an analysis of TRPV1/TRPM3/TRPA1 TKO mice, heat-evoked TRPA1 activation was verified in TRPA1-transfected TKO sensory neurons (Vandewauw et al. 2018). Moreover, the heat-sensitivity of TRPA1 was indicated with heterologous expression of TRPA1 in CHO cells, in which the heat-evoked response was replicated in the presence of H₂O₂ and reduced by a TRPA1 inhibitor, suggesting that the cellular environment affects TRPA1 heat sensitivity. Furthermore, the heat-sensing ability of TRPA1 is well conserved in the evolutionary process from insects to birds (Saito and Tominaga 2015).

1.6 Innocuous Warmth Sensing by Thermosensitive TRP Channels

TRPM2 is a thermosensitive TRP channel that has sensitivity to innocuous warm temperatures (Kashio 2021). This temperature sensitivity is regulated by metabolic factors, including intracellular Ca²⁺, adenosine diphosphate ribose, and redox signals in the body temperature range (Kashio et al. 2012). Moreover, TRPM2 is expressed in many tissues including sensory neurons, and works as an ambient warm temperature sensor, independently of the noxious heat sensors TRPV1 and TRPM3 (Tan and McNaughton 2016). A very small population of sensory neurons having sensitivity to warm temperatures and that is not activated by agonists of thermosensitive TRPV1 and TRPM3 channels does exist. TRPM2KO mice could not sense temperature differences between 33 °C and 38 °C, whereas WT mice showed a preference for 33 °C. Thus, warmth sensing mediated by TRPM2 exists in a complicated cellular environment. This is evidenced by research indicating that TRPM2 is

activated by very high temperatures (>45 °C) in naïve conditions, and that channel activity is regulated by methionine oxidation (Kashio et al. 2012). Recently, another thermosensitive TRP channel, TRPM8, was reported to be involved in sensation of innocuous warm temperatures (Paricio-Montesinos et al. 2020). TRPM8 is also activated by cool temperatures (<27 °C) and menthol (McKemy et al. 2002; Peier et al. 2002a), which both cause cool sensations in humans. Temperature stimulation to the forepaw in an ex vivo skin-nerve preparation from mice revealed that C-fibers innervating the skin comprise “warmth-excited” and “warmth-inhibited” neural populations. TRPM8KO mice lacked the warmth-inhibited C-fibers. Moreover, in an in vivo study, WT mice trained to report changes in palm temperature acutely detected slight (>1 °C) temperature elevations from 22 °C or 33 °C, while TRPM8KO mice failed to respond to the temperature elevation even after training. The attenuated detection of warm temperatures observed in TRPM8KO mice was also recapitulated by acute TRPM8 inhibition with an antagonist, suggesting that TRPM8 inhibition by warm temperatures in warmth-inhibited neurons has an indispensable role in warm temperature detection. Warm temperature sensation that is dependent on TRPM2 and TRPM8 was also involved in a temperature-dependent masking behavior, a direct behavioral response to environmental changes (Ota et al. 2019). Another thermosensitive TRP channel, TRPV4, is involved in perception of innocuous warmth. TRPV4 is sensitive to warm temperatures (>27 °C) and is functionally expressed in DRG neurons, although higher expression levels were reported in skin keratinocytes (Guler et al. 2002; Liedtke et al. 2000). TRPV4KO mice selected warmer temperatures than did WT mice in a temperature preference test (Lee et al. 2005), whereas another study reported that TRPV4KO mice preferentially stayed in zones 29.0 °C to 31.9 °C, which was lower than that for WT mice (Ujisawa et al. 2022). In contrast, the abovementioned heat sensors TRPV1, TRPM3, and TRPA1 likely do not engage in innocuous warmth detection since TRPV1/TRPM3/TRPA1 TKO mice exhibited no

change in temperature preferences within the range of innocuous ambient temperatures (Vandewauw et al. 2018).

1.7 Cool Temperature Detection by TRPM8

Innocuous cool sensation is mediated by activation of TRPM8 (McKemy et al. 2002; Peier et al. 2002a), a nonselective cation channel sensitive to cool temperatures (<27 °C) and menthol. As with TRPV1, these stimuli synergistically activate TRPM8 and can modify temperature sensation (i.e., we experience a cooler sensation when cold and mint are combined). In the sensory system, TRPM8 is expressed in both unmyelinated, small diameter DRG neurons as well as myelinated, moderate diameter DRG neurons, which differs from the expression pattern for TRPV1 (Peier et al. 2002a). TG neurons prepared from TRPM8KO mice showed diminished activation in response to menthol and innocuous cool temperature (22 °C), but the response to a TRPA1/TRPV1 activator or noxious heat (45 °C) was not altered (Bautista et al. 2007). TRPM8KO mice showed attenuated cool-sensing behaviors in an acetone-evaporating test, and altered temperature preferences over a range of innocuous cool temperatures (Bautista et al. 2007; Dhaka et al. 2007). Importantly, the temperature preference and defensive responses to noxious cold <15 °C were conserved in TRPM8KO mice, suggesting that transducers of cool and noxious cold temperatures differ. TRPM8 activation by cool temperatures caused an analgesic effect in formalin-induced acute pain and nerve injury-induced chronic neuropathic pain (Dhaka et al. 2007; Knowlton et al. 2013).

1.8 Noxious Cold Sensing by Potential Thermosensitive TRP Channels

TRPA1 is activated by colder temperatures compared to temperature thresholds for TRPM8 activation (Story et al. 2003). However, as mentioned

above, whether TRPA1 is, in fact, involved in cold sensing remains unclear. Most (98%) TRPA1-positive TG neurons showed no cold (5 °C)-induced responses (Jordt et al. 2004). TG neurons with cold sensitivity can be segregated to two populations, menthol-sensitive with a moderate threshold (22 °C) and menthol-insensitive with a colder threshold (12 °C) (Bautista et al. 2007). The latter population of cold-activated neurons was reported to lack reactivity to TRPA1 agonists. Moreover, the ratio of these two populations was not affected by TRPA1 deficiency (Bautista et al. 2006). Behavioral studies comparing WT and TRPA1KO mice showed no difference in cold (<10 °C)-sensing behaviors. In addition, TRPA1/TRPM8 double KO mice showed no significant change in cold temperature preference and avoidance behaviors compared to TRPM8KO mice (Knowlton et al. 2010), making a contribution by TRPA1 to cold detection unlikely. Activation of TRPM8 by noxious cold was also reported for TRPM8KO mice, which have decreased behavioral responses to cold stimuli (Knowlton et al. 2013) and cold hypersensitivity induced by inflammation and nerve injury (Knowlton et al. 2011). Nonetheless, whether TRPM8 plays a role in pain or pain relief is poorly understood. Interestingly, the temperature preference in response to noxious cold (<15 °C) was greatly altered in mice having ablation of TRPM8-expressing neurons compared to TRPM8KO mice (Knowlton et al. 2013). In a two-temperature choice assay, TRPM8KO mice tended to avoid noxious cold temperatures (<15 °C) whereas mice with ablation of TRPM8-expressing neurons showed almost no avoidance of cold temperatures (10 ~ 15 °C). These data strongly suggest that additional cold transducing molecules that detect noxious cold temperatures are present in TRPM8-expressing sensory neurons. The TRPM8-expressing population of sensory neurons likely express other candidate cold sensors, including the sodium ion channel, Nav1.8, and the cyclic nucleotide-gated (CNG) cation channel alpha-3 (Usoskin et al. 2015; Luiz et al. 2019). In addition, TRP subfamily C member 5 (TRPC5) heterologously expressed in HEK293 cells had increased activity as tempera-

tures decreased from 40 °C to 14 °C (Zimmermann et al. 2011). Although TRPC5 was reported to be expressed in sensory neurons, the involvement of neural TRPC5 in cool/cold temperature detection is unclear. TRPC5KO mice showed no obvious changes in temperature preference from 5 °C to 30 °C, or cold (0 °C)-induced avoidance behaviors. A recent report indicated that TRPC5 in sensory neurons is involved in pathological pain induced by lysophosphatidylcholine production (Sadler et al. 2021). Further studies may clarify neural TRPC5 function in physiological/pathological thermosensation. In addition, a recent report indicated that cold-evoked activation of TRPC5 in nonneuronal odontoblasts is involved in cold-induced tooth pain (Bernal et al. 2021).

1.9 Temperature Sensation in the Brain

Although temperature stimuli are converted to electrical signals in the periphery and then transmitted to the CNS, the brain is known to sense temperatures as well. Some neurons in the preoptic area (POA) exhibit dramatic changes in firing rate upon a 1–3 °C change in local temperature (Boulant 1986). The molecular details of this warmth and cold sensitivity are not well understood. TRPM2 expressed in POA neurons was reported to play a key role in thermoregulation, particularly in response to fever (Song et al. 2016). Many mouse POA neurons express TRPM2 that is both thermosensitive and redox-sensitive. TRPM2 in warmth-sensitive POA neurons is activated above 38 °C within the high-fever temperature range. Selective activation of TRPM2-expressing neurons with a DREADD system in the POA caused hypothermia, presenting a useful paradigm for development of selective TRPM2 agonists for use as hypothermic agents. Further investigation is needed to better characterize the molecules involved in cold sensing by POA neurons.

The movement of mouse brain microglia was recently reported to be temperature-dependent and TRPV4 expressed in the microglia was shown to sense brain temperature (Nishimoto

et al. 2021). This result raises the novel possibility that many tissues or cells in humans could have thermogenic ability and in mice local temperature is detected by local cells such as microglia in the brain. Further studies are needed to explore these possibilities.

1.10 Thermosensitive TRP Channels of Nonneuronal Cells in Somatic Thermosensation

Mechanosensitive Piezo2 channels are functionally expressed in nonneuronal Merkel cells of ectoderm-origin (Maksimovic et al. 2014). Piezo2 activation by mechanical stimuli causes serotonin release from Merkel cells to sensory neurons during slow-adapting touch sensation (Chang et al. 2016). Somatic thermosensation appears to involve a similar system, in that non-neuronal cells are combined with sensory neurons. Keratinocytes are nonneuronal cells having an ectoderm origin that are reported to contribute to somatic thermosensation. The skin is a large organ, and represents the boundary between an organism and its environment, and consists almost entirely of keratinocytes, which respond to temperature stimulation via intracellular Ca^{2+} elevation. TRPV3 and TRPV4 are nonselective cation channels activated by warm temperatures, and are highly expressed in skin keratinocytes (Guler et al. 2002; Peier et al. 2002b). TRPV3 activation by warm temperatures stimulates keratinocytes to release ATP that subsequently activates ionotropic ATP receptors in peripheral nerves (Mandadi et al. 2009). Even though early studies reported that TRPV3 and TRPV4 are involved in thermosensation of innocuous warm stimuli (Guler et al. 2002; Moqrich et al. 2005), TRPV3/TRPV4 double KO mice showed normal behavioral responses to warm temperatures (Huang et al. 2011). Therefore, the functions of TRPV3 and TRPV4 in somatic thermosensation remain unclear. The temperature sensitivity of keratinocytes was also confirmed for cold temperatures (Sadler et al. 2020) and may be mediated by

Ca²⁺-release from intracellular stores, but not by cold-sensitive activation of TRPA1, TRPM8, or TRPC5. Interestingly, optogenetic inhibition of keratinocytes impaired defensive responses to noxious cold and heat. Moreover, nocifensive behaviors to noxious cold and heat were also blunted by intraplantar injection of apyrase, an enzyme that catalyzes ATP hydrolysis, and sensory neuron-specific knockout of the P2X4 receptor. These findings support a role for keratinocytes in somatic thermosensation mediated by purinergic signaling, even though the involvement of thermosensitive TRP channels is still unclear. In this regard, evidence for involvement of TRPV3 expressed in skin in temperature detection has been presented (Lei et al. 2023). Indeed, skin keratinocytes from TMEM79-deficient mice, which have high levels of TRPV3 protein, showed more TRPV3-mediated current responses, whereas TMEM79-deficient mice preferred a higher temperature than did wild-type mice in a Thermal Gradient Ring test (Ujisawa et al. 2022). Expression of the above-mentioned cold sensor, TRPC5, in odontoblasts was recently reported to be involved in cold-induced tooth pain, together with TRPA1 (Bernal et al. 2021). Teeth are composed of an outer enamel, dentin, and a pulp chamber. Odontoblasts are buried in the pulp-dentin boundary, and extend spindles toward the dentin-enamel boundary to secrete specific ions and proteins to maintain dentin structure. Odontoblasts are also considered to be involved in sensory function together with sensory neurons that innervate the odontoblasts. Despite a rigid structure, teeth can detect small temperature changes. In inflamed teeth in particular, cold temperature causes sharp, intense pain. To assess cold-induced, sensory nerve activity, an ex vivo jaw-nerve preparation was used for extracellular recording (Bernal et al. 2021). Cold-induced nerve excitability was higher in nociceptive neurons innervating the tooth compared to those in the skin. Cold-stimulation (10 °C) to a jaw-nerve preparation increased neural activity, and this response was suppressed by TRPA1 and/or TRPC5 inhibitors. In contrast, the response was not affected by TRPM8 inhibitors and activators, which

likely excludes a contribution from TRPM8. Consistently, the cold response was largely diminished in tooth nociceptors of TRPA1/TRPC5 double KO mice. In isolated sensory neurons innervating the tooth pulp region, cold-induced responses in neurons were mainly mediated by TRPM8 activity, but not by TRPA1 or TRPC5. In addition, TRPC5 expression was observed in the odontoblast layer rather than in sensory neurons. Taken together, these results indicate that the contribution of TRPC5 to the cold response could be due to its functional expression in odontoblasts rather than in sensory neurons. Interestingly, TRPC5 expression levels in human teeth are increased during pulpitis compared to those of healthy teeth. TRPC5 is also reported to be involved in inflammatory pain (Sadler et al. 2021), and thus may be an attractive therapeutic target for pathological tooth pain.

1.11 Interaction Between TRP Channels and Anoctamin1 to Regulate Temperature Sensation

TRP channels have high Ca²⁺ permeability (Gees et al. 2010), and Ca²⁺ entering cells through some thermosensitive TRP channels is reported to activate the Ca²⁺-activated chloride channel anoctamin1 (ANO1), also known as TMEM16A (Takayama et al. 2014; Derouiche et al. 2018; Yamanoi et al. 2023). TRPV1 and TRPA1, which both have high Ca²⁺ permeability, were also reported to form a functional complex with ANO1 (Takayama PNAS). In peripheral sensory neurons, opening of chloride-permeable channels causes chloride efflux due to high intracellular chloride concentrations, which is in contrast to CNS neurons that have very low amounts of intracellular chloride. Accordingly, interaction of TRPV1/TRPA1 with ANO1 causes further depolarization (Takayama et al. 2015) and in turn an increase in nociceptive signals. Cold sensation should be enhanced if TRPM8 functionally interacts with ANO1, although this possibility requires additional exploration.

1.12 Conclusions

Temperature represents environmental sensory inputs that can be detected by a range of organisms. Extreme low and high temperatures that can cause injury must be detected by the sensory system to trigger avoidance via defensive behaviors. Even in mild cold to warm temperatures, organisms must select optimal temperatures to maintain systemic metabolism for survival. Considering this continuous feature of temperature, it is important to answer questions about the mechanisms by which sensory systems classify different temperatures, including noxious cold, innocuous cool/warm, and noxious heat. Recent studies are progressively elucidating the mechanisms of thermo-TRPs, which together engage in somatic thermosensation, and are expressed not only in sensory neurons, but also in nonneuronal cells. Future studies are expected to answer these questions, particularly the molecular identity of noxious cold sensors, and to discover therapeutic strategies to control pathological conditions that involve somatic thermosensation.

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Biomimetic Lipid Raft: Domain Stability and Interaction with Physiologically Active Molecules

Naofumi Shimokawa and Masahiro Takagi

Abstract

The cell membrane, also called the plasma membrane, is the membrane on the cytoplasmic surface that separates the extracellular from the intracellular. It is thin, about 10 nm thick when viewed with an electron microscope, and is composed of two monolayers of phospholipid membranes (lipid bilayers) containing many types of proteins. It is now known that this cell membrane not only separates the extracellular from the intracellular, but is also involved in sensory stimuli such as pain, itching, sedation, and excitement. Since the “Fluid mosaic model” was proposed for cell membranes, molecules have been thought to be homogeneously distributed on the membrane surface. Later, at the end of the twentieth century, the existence of “Phase-separated microdomain structures” consisting of ordered phases rich in saturated lipids and cholesterol was suggested, and these were termed “Lipid rafts.” A model in which lipid rafts regulate cell signaling has been proposed and is the subject of active research.

This chapter first outlines the physicochemical properties and thermodynamic mod-

els of membrane phase separation (lipid rafts), which play an important role in cell signaling. Next, how physiologically active molecules such as local anesthetics, cooling agents (menthol), and warming agents (capsaicin) interact with artificial cell membranes will be presented.

It is undeniable that the plasma membrane contains many channels and receptors that are involved in the propagation of sensory stimuli. At the same time, however, it is important to understand that the membrane exerts a significant influence on the intensity and propagation of these stimuli.

Keywords

Signal transduction · Plasma membrane · Lipid raft · Phase separation · Membrane dynamics · Line tension · Physiological response

2.1 Introduction

2.1.1 Basic Structure of Plasma Membranes and Biomembranes

One of the main components in plasma membranes and biomembranes is a phospholipid. Phospholipid is a typical amphiphilic molecule,

N. Shimokawa · M. Takagi (✉)
School of Materials Science, Japan Advanced
Institute of Science and Technology,
Nomi, Ishikawa, Japan
e-mail: takagi@jaist.ac.jp

which consists of hydrophilic headgroup and hydrophobic chains. As headgroup structures, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), and many other structures are known, especially PC is the most abundant in plasma membranes. Hydrophobic group is composed of two hydrocarbon chains, such as myristoyl group, palmitoyl group, stearoyl group, and oleyl group.

Phospholipids self-assemble into various structures in water not to expose the hydrophobic groups to water, in other words, by hydrophobic interactions. Since the cross-sectional areas of headgroup and hydrophobic group in a phospholipid are roughly comparable, the molecular structure of phospholipid is cylinder (Israelachvili 2010), and such molecules tend to form bilayer structure. The bilayer has a structure in which two monolayers are bonded with hydrophobic groups facing each other, and the membrane thickness is about 5 - 10 nm corresponding to two phospholipid molecules. The plasma membrane has a bilayer structure, in which proteins are embedded, sugar chains are bound to the membrane surface, and the cytoskeleton is lined on the cytoplasmic side.

Plasma membrane is located on the outermost of animal cells and separates the inside and outside of the cells. This makes it possible to set different environments, such as ionic strengths, pH, and solute concentrations, inside and outside the cell. Furthermore, it is necessary to pass through the plasma membrane for the uptake/discharge of

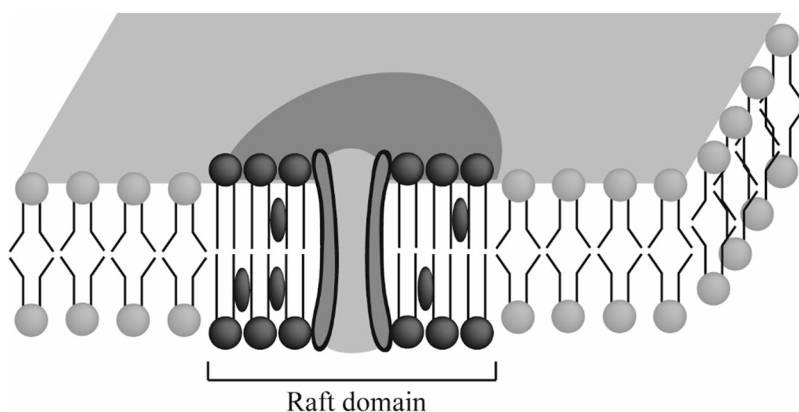
substances into/from the cell and the exchange of information. Therefore, plasma membranes play an important role in membrane trafficking and signal transduction (Alberts et al. 1989; Lehninger et al. 1993).

2.2 Lipid Rafts in Plasma Membranes

Although some models for describing the nature of plasma membrane have been proposed, we introduce two important models here. In 1972, Singer and Nicolson proposed the fluid mosaic model (Singer and Nicolson 1972). Plasma membranes are two-dimensional fluid membranes where the lipids and proteins diffuse. Proteins are embedded in the membranes and the membranes look like a mosaic. Therefore, the fluid mosaic model is a very epoch-making model that well incorporates the concept of fluidity in plasma membranes.

Simons and Ikonen proposed that the membrane components do not mix uniformly in plasma membranes, and sphingolipids and cholesterol-rich region called raft is formed (Fig. 2.1) (Simons and Ikonen 1997; Simons and Sampaio 2011). Functional membrane proteins are accumulated in the raft region, and various biocellular functions such as signal transduction and membrane trafficking occur as a consequence. At Keystone Symposium on Lipid Rafts in 2006, lipid raft is defined as “Membrane rafts are small (10–200 nm), heterogeneous, highly

Fig. 2.1 Schematic illustration of raft domain. Raft domain is composed of sphingolipids (dark headgroup and straight tails) and cholesterol (elliptical shape) and transmembrane protein is included



dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions.” (Pike 2006).

Here, we briefly introduce the history of raft concept (see the detailed history in a review (Lingwood and Simons 2010)). Yeagle found that cholesterol alters the membrane fluidity (Yeagle 1985). Based on this result, Ipsen et al. defined the intermediate fluidity formed by sphingolipids and cholesterol as liquid-ordered phase (Ipsen et al. 1987). Therefore, a liquid-ordered phase, which is a thermodynamical stable phase, was found in the 1980s. We explain about the liquid-ordered phase later. In the 1990s, the direct observation of the phase-separated structures in the lipid membranes began to take place with the development of optical microscopes. Keller’s group performed some pioneering studies on the phase separation based on physics (Veatch and Keller 2003, 2005a, b; Veatch et al. 2006). In this way, the studies about the phase separation in the model artificial cell membranes have been conducted. At the same time, the idea of detergent-resistant membrane (DRM) has been focused in vivo (Brown and London 1997; Schroeder et al. 1994; Field et al. 1995). The sphingolipid-rich membranes resist the solubilization of the nonionic detergent Triton X-100 at 4 °C, and such an extracted fraction is called DRM. The DRM includes sphingolipids, cholesterol, and some membrane proteins, and is recognized as raft region. In the 2000s, nano-scaled lipid domains on living cells were revealed with the further development of microscopes (Sengupta et al. 2011; Owen et al. 2012). Not only fluorescence resonance energy transfer (FRET), fluorescence correlation microscopy (FCS), and single-particle tracking (SPT) but also stimulated emission depletion (STED) and fluorescence photoactivation localization microscopy (FPALM), by Betzig et al. who won the Nobel Prize in Chemistry in 2014 (Betzig et al. 2006), were used to reveal the dynamic properties of nanoscopic domains of sphingolipids and cholesterol.

2.3 Thermodynamically Stable Membrane Phases and Phase Separation

As we have already mentioned above, phospholipids spontaneously form bilayer structures in aqueous phase. A closed bilayer structure is known as liposome or vesicle, in particular, micrometer-scaled liposome is called cell-sized liposome, which is used as a simple model for a cell and a carrier for water-soluble molecules.

As the temperature is changed, lipid membranes undergo a phase transition. At lower temperature, lipids are in the solid phase (gel phase). The hydrocarbon chains of the lipids are oriented, the lipids are tightly packed, and the membrane fluidity becomes low. On the other hand, at higher temperature, the lipids become a liquid phase (liquid crystalline phase). The orientation of the hydrocarbon chains is disturbed, the lipid packing becomes loose, and the membrane fluidity increases. This solid-liquid phase transition or order-disorder phase transition is called the main transition. Saturated lipids have straight tail structures because there are no double bonds in the hydrocarbon tails. Therefore, the attractions between hydrocarbon tails, such as hydrophobic interaction and van der Waals attraction, become stronger and the main transition temperature is high, for example, 24 °C for dimyristoylphosphatidylcholine (DMPC), 41 °C for dipalmitoylphosphatidylcholine (DPPC), and 55 °C distearoylphosphatidylcholine (DSPC). On the other hand, unsaturated lipids bend at the position of double bonds, and the steric repulsion between lipids is large. Therefore, it is difficult to pack densely. The main transition temperature is very low, and it is −17 °C for a typical unsaturated lipid, dioleoyl phosphatidylcholine (DOPC).

When mixed lipid membranes consisting of several lipids with different physical properties, such as saturated lipids and unsaturated lipids, are prepared, the compositional heterogeneous structures like raft domains can emerge spontaneously. This physicochemical phenomenon is interpreted as phase separation in the same way that water and oil do not mix together. First, we consider a

simple binary lipid system composed of saturated lipids and unsaturated lipids. At higher temperatures, these two components mix together. On the other hand, phase separation between saturated lipid-rich phase and unsaturated lipid-rich phase is found at lower temperature. The saturated lipid-rich and unsaturated lipid-rich phases are solid-ordered (S_o) phase and liquid-disordered (L_d) phase, respectively (Beakes et al. 2005; Gordon et al. 2006). Usually, the S_o phase forms domains and the S_o domains are surrounded by a continuous L_d phase. The shape of S_o domain is anisotropic and S_o domains diffuse in a continuous L_d phase by Brownian motion. If two S_o domains collide with each other, they will adhere but merge into one large domain.

Next, we consider a ternary lipid mixture consisting of saturated lipids, unsaturated lipids, and cholesterol (Chol). Chol transforms S_o phase into liquid-ordered (L_o) phase (Marsh 2010). Since raft domain includes saturated lipid and cholesterol, this L_o phase is regarded as a model for raft domain. When the total area of L_o phase is smaller than that of L_d phase in a liposome, L_o phase forms some domains surrounded by L_d phase in general. In the opposite case, L_d phase forms domains. The shape of L_o and L_d domains is circular by the interfacial tension (line tension) at the L_o/L_d phase boundary. When two such domains collide through Brownian motion, they will merge into one large domain.

2.4 Studies About Phase Separation in Lipid Membranes

The stability of phase-separated structures has been discussed under various conditions. Especially, such studies have been active in association with the development of direct observation techniques by fluorescent microscopy since 1990s. As pioneering studies, Keller's group reported the L_o phase formation and its stability based on physical approaches (Veatch and Keller 2003, 2005a, b; Veatch et al. 2006).

Based on these studies, the studies of the phase separation in lipid membranes have been

developed. The research about the critical phenomena in artificial lipid membranes and plasma membrane vesicles was performed from a physical viewpoint (Veatch et al. 2007, 2008). Since the correlation length of the compositional fluctuation near the critical point is short and the lifetime of domain is short, the behavior is similar to that of raft domain. Therefore, the relationship between the compositional fluctuation and raft domain is discussed.

A phenomenon in which the internal and external degrees of freedom of lipid membranes are coupled, such as the coupling between the membrane deformation and phase separation, is also interesting from physics. The budding of phase-separated domains is interpreted by the competition between the curvature energy and the interfacial energy at the domain boundary (Lipowsky 1993; Jülicher and Lipowsky 1993). Moreover, the deformation of phase-separated liposomes induced by the osmotic pressure or the addition of surfactant molecules and the pore formation induced by the phase separation in negatively charged lipid membranes are also explained by physical approaches (Yanagisawa et al. 2008; Hamada et al. 2007; Himeno et al. 2015).

Since some negatively charged phospholipids, such as phosphatidylserine, phosphatidylglycerol, and phosphatidylinositol, are present in plasma membranes to adsorb proteins on the membrane surface and control the channel activity (Alberts et al. 1989; Lehninger et al. 1993; Cabanos et al. 2017), the roles of the electrostatic interactions in negatively charged lipid membranes are investigated. The phase separation is suppressed in the lipid membranes containing negatively unsaturated lipids, and the addition of salt to the membranes progresses the phase separation (Shimokawa et al. 2010; Himeno et al. 2014). On the other hand, the phase separation progresses in the lipid membranes containing negatively saturated lipids. The phase behavior can be explained by some theoretical models based on the Poisson-Boltzmann theory (May et al. 2002; Shimokawa et al. 2011, 2016).

The domain pattern formation is one of the important topics from physics. The modulated phases (e.g., stripe and hexagonal phases) with a

characteristic length, which is determined by the competition between the short-range attraction and the long-range repulsion, are formed spontaneously. Some studies reported the formation of the modulated phases in the lipid membranes adsorbed on a solid substrate, in the lipid membranes containing hybrid lipids with one saturated hydrocarbon tail and one unsaturated hydrocarbon tail, and in the unsaturated fatty acids containing lipid membranes (Rozovsky et al. 2005; Konyakhina et al. 2011; Goh et al. 2013; Konyakhina et al. 2013; Shimokawa et al. 2017).

Since the composition and structure of the artificial lipid membranes are much simpler than those of plasma membranes, there is a large gap between these model systems and plasma membranes. Therefore, some approaches are taken in which the artificial lipid membranes are gradually complicated in order to imitate the plasma membranes. One of the important complexities in plasma membranes is asymmetric nature. Bilayer membrane consists of two monolayers and these monolayers have different lipid compositions in plasma membrane by flippase, phospholipid transporter (Alberts et al. 1989; Lehninger et al. 1993). Some studies prepared the liposomes with asymmetric lipid compositions and reveal the correlation of phase separation between two leaflets (Collins and Keller 2008; Cheng et al. 2009; Heberle et al. 2016; Chiantia et al. 2011; Hamada et al. 2008). Moreover, solute concentrations and ionic strengths inside and outside the cells are different. Such differences induce the osmotic pressure and the difference in the electrostatic interactions (Kubisch et al. 2016; Hamada et al. 2011; Wongsirajkul et al. 2020).

2.5 Physicochemical Mechanism of Phase Separation

The stability of phase separation can be interpreted by the competition between the interaction between lipids and mixing entropy. Here, we consider the Helmholtz free energy $F = U - TS$, where F is the free energy, U is the internal energy, T is the temperature, and S is the entropy. Since the entropic term becomes predominant at

higher temperature, lipids mix with each other, and the homogeneous phase is stabilized by increasing the mixing entropy. On the other hand, the attraction between same species becomes predominant at lower temperature, and phase separation emerges to reduce the internal energy.

There are several theoretical models to explain the phase separation behavior. Here, we introduce a simple macro-phase separation model. We consider a binary lipid system consisting of A-lipid and B-lipid, and the undeformable membrane lying in the xy plane. The mole fraction of A-lipid is ϕ ($0 < \phi < 1$), and that of B-lipid is $1 - \phi$. Since we assume that two types of lipids have same cross-sectional area, the mole fraction is the same as the areal fraction. The free energy per lipid molecule is given by

$$f(\phi) = \phi \ln \phi + (1 - \phi) \ln(1 - \phi) + \chi \phi(1 - \phi),$$

where χ is the interaction parameter and is generally proportional to the inverse of temperature. f and χ have the unit of energy scaled by $k_B T$ (k_B is the Boltzmann constant and T is the temperature). This model is known as Flory-Huggins (FH) theory, which was proposed to describe the phase separation in polymer solution (Flory 1942; Huggins 1942). The phase diagram in the (ϕ, χ) -plane can be obtained by minimizing the free energy with respect to ϕ and the result is shown in Fig. 2.2. The boundary between the phase-separated state and homogeneous phase in the phase diagram is called the binodal line, and it is expressed by

$$\chi_{\text{bi}} = \frac{1}{2\phi - 1} \ln \frac{\phi}{1 - \phi}$$

for FH theory. The phase-separated region can be divided into two regions, unstable region and metastable region, by the spinodal line expressed as

$$\chi_{\text{sp}} = \frac{1}{2\phi(1 - \phi)}.$$

The binodal line and the spinodal line are in contact with each other at one point, which is the critical point $(\phi_c, \chi_c) = (0.5, 2)$. The phase transi-

tion through the critical point is the second-order phase transition, otherwise it is the first-order phase transition with discontinuity. When the phase transition from the homogeneous phase to the unstable region in the phase-separated state by decreasing the temperature, the spinodal decomposition is observed as phase separation dynamics. On the other hand, the nucleation and growth occur when the phase transition to the metastable region. The sequential microscopic images of these phase separation dynamics are shown in Fig. 2.3 (Veatch and Keller 2003).

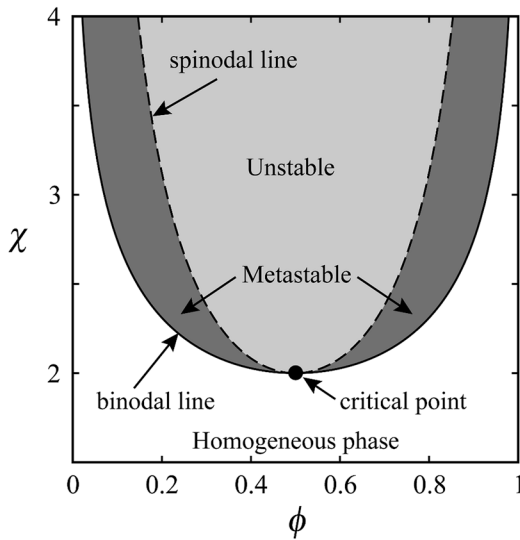


Fig. 2.2 Phase diagram obtained from FH theory. White, dark gray, and light gray regions are homogeneous phase, metastable region, and unstable region, respectively. Solid and dashed lines are binodal and spinodal lines, respectively. Filled circle is critical point

2.6 Line Tension: Interfacial Tension at Phase-Separated Domain Boundary

When the phase separation takes place, the phase-separated two phases contact each other at the domain boundary. The interfacial tension at this boundary is called line tension. The line tension becomes an important physical quantity to discuss the stability of domains. The interfacial energy (line energy) can be obtained by integrating the line tension along the domain boundary. If the line tension is constant along the domain boundary, the line energy is simply obtained by the product of the line tension and domain boundary length. In order to reduce the line energy, the domain boundary length should be shortened. Therefore, the L_o and L_d domains become circular shaped. Moreover, two domains merge into one domain by the collision to reduce the total domain boundary length. On the other hand, the shape of S_o domain is not circle due to the anisotropy of line tension (Gordon et al. 2006; Pimpinelli and Vallain 1998). In addition, S_o domains do not merge due to very slow lipid diffusion.

The height mismatch caused by the membrane thickness difference between the domain and non-domain regions is considered as the origin of line tension (García-Sáez et al. 2007). In an ordered phase, the hydrocarbon tails of lipids are ordered, and the membrane thickness is thick. However, the membrane thickness in a disordered phase is thin. Therefore, the membrane

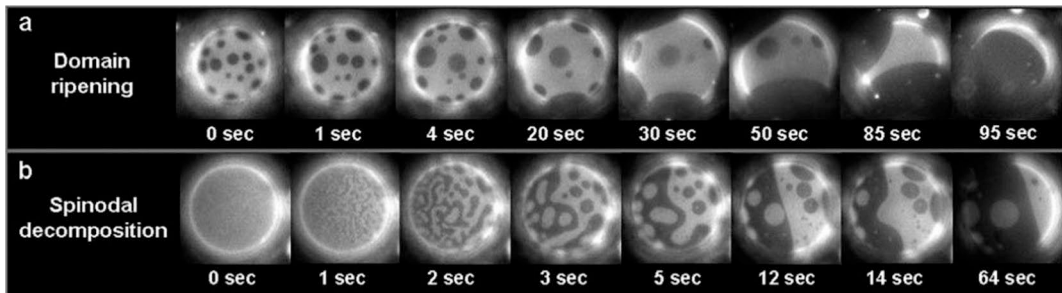


Fig. 2.3 Fluorescent microscopic images of phase separation dynamics, domain ripening (nucleation and growth), and spinodal decomposition. (Reproduced in part from the study by Veatch and Keller (2003) with permission)

height mismatch is present at the domain boundary. A part of the hydrophobic region is exposed to water due to this height mismatch, and the energy penalty by the hydrophobic interaction becomes an origin of line tension. On the other hand, even if there is no height mismatch at the domain boundary, it was reported that the line tension does not vanish (Georgieva et al. 2015). Although the height mismatch is important on the line tension, the interactions between lipids also play a crucial role in the line tension.

The line tension can be measured by micropipette aspiration and thermal fluctuation of domain shape, and a typical value is roughly 3 pN (Tian et al. 2007; Stottrup et al. 2007; Esposito et al. 2007). Honerkamp-Smith et al. measured the line tension from the phase-separated domain fluctuation in the vicinity of the critical point (Honerkamp-Smith et al. 2008). As the temperature increases and approaches the critical temperature T_c , the compositional difference between the coexisting two phases becomes smaller. This behavior also can be seen in FH theory explained above. The order parameter m indicating the compositional difference between two phases is expressed as $m \simeq (T_c - T)^\beta$. The line tension γ is roughly determined by the compositional difference, that is $\gamma \simeq (T_c - T)^\nu$. The line tension decreases as the temperature approaches the critical temperature and eventually vanishes. Since the line tension is smaller near the critical point, the shape of liquid domain always fluctuates due to thermal noise. Honerkamp-Smith et al. measured these critical exponents and indicated that the critical behavior in lipid membranes is in the universality class of the two-dimensional Ising model. Within 2D Ising model, ν and β are known to be 1 and 1/8, respectively.

2.7 Interactions Between Physiologically Active Molecules and Receptors

As one of the regulatory mechanisms of physiological functions in the living body, there is an intracellular signal transduction by a receptor present in the plasma membrane. Receptors spe-

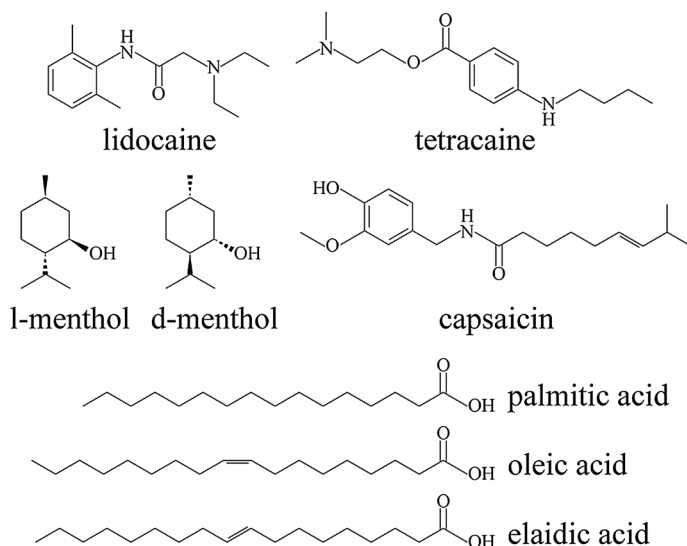
cifically recognize the stimuli by physiologically active molecules and bind to them. As a result, the information by the physiologically active molecules is transmitted intracellularly. In this way, the relationship between physiologically active molecules and receptors has been supported as a key–keyhole relationship.

Since many physiologically active molecules are hydrophobic, they can be incorporated into the hydrophobic regions of the plasma membranes. Consequently, the physical properties of the membranes, such as fluidity, rigidity, curvature, and thermal stability, are altered. In addition, some receptors are known to be present in the raft domains (Martens et al. 2004). The formation of phase-separated raft domains is directly linked to the membrane physical properties. Therefore, the following mechanism is considered; (i) physiologically active molecules are incorporated into the hydrophobic region of the membranes, (ii) the membrane physical properties are changed, (iii) the stability of raft domains is also changed, and (iv) the functions of the receptors in the raft domains are affected. Such an indirect interaction between the physiologically active molecules and receptors via the lipid membranes is different from the picture of a one-to-one correspondence such as a key–keyhole relationship.

Studies in the lipid membranes containing some physiologically active molecules have been performed. For example, Muddana et al. performed the observation of the phase separation in lipid membranes with vitamin E (VE), surfactant molecule Triton-X 100 (TX), and benzyl alcohol (BA) (Muddana et al. 2012). VE suppresses the phase separation, while TX and BA progress it. Especially, VE reduces the line tension at phase-separated domain boundary and it was reported that VE may suppress the virus infection (Yang et al. 2015, 2016).

From the next section, we discuss the effects of some physiologically active molecules on the phase separation of the lipid membranes, such as local anesthetics (lidocaine and tetracaine), 1/-d-menthol, capsaicin, and fatty acids (palmitic acid, oleic acid, and elaidic acid) as shown in Fig. 2.4.

Fig. 2.4 Chemical structures of physiologically active molecules



2.8 Physiologically Active Molecules: Local Anesthetics

Local anesthetic (LA), which is one of the important drugs in modern surgical medicine, is used to suppress pain. The mechanism for pain suppression by LAs is the deactivation of sodium ion channel and the inhibition of action potentials of neural cell membranes (Becker and Reed 2012; Ragsdale et al. 1996; Scholz 2002). In addition, LAs alter the physical properties of lipid membranes (Paiva et al. 2012; Bandejas et al. 2013; Serro et al. 2014). Therefore, there may be an indirect interaction between LAs and the channel proteins through the change of the membrane physical properties (Tsuchiya and Mizogami 2013; Komai and McDowell 2005).

The phase separation in lipid membranes containing LAs, such as lidocaine and tetracaine, has been performed (Sugahara et al. 2015, 2017). Dioleoyl phosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), and cholesterol (Chol) were used as lipids, and, at room temperature, the S_o phase that is rich in DPPC and L_d phase that is rich in DOPC coexist in DOPC/DPPC/Chol = 50:50:0 as shown in Fig. 2.5a, and the L_o phase that is rich in DPPC and Chol and L_d phase that is rich in DOPC coexist in DOPC/DPPC/Chol = 40:40:20 as shown in Fig. 2.5b. Although the phase separation in DOPC/DPPC/

Chol = 50:50:0 with LAs was observed, LAs seemed to give little effect on the formation of phase separation. On the other hand, as LAs concentrations increase, the phase separation between the L_o and L_d phases was suppressed for DOPC/DPPC/Chol = 40:40:20 with LAs. Especially, this tendency was more clearly seen in tetracaine than lidocaine. To reveal the suppression of phase separation by LAs, a visualization experiment of membrane fluidity was performed using a fluorescent probe, Laurdan. The membrane fluidity difference between the coexisting two phases became smaller as LAs concentrations increase. Therefore, the physical properties of these two phases became closer and the homogeneous phase could appear.

Further, the temperature dependence in the phase separation formation was clarified (Sugahara et al. 2017). As we have already mentioned above, the phase separation disappears at higher temperature. The miscibility temperature, which is defined as the temperature at which the fraction of phase-separated liposome reached 50%, was measured by a fluorescent microscopy. The miscibility temperature for DOPC/DPPC/Chol = 50:50:0 without any LAs was 33.1 °C (Fig. 2.5b and c). However, the temperature was not dramatically changed by the addition of any LAs; 32.9 °C (lidocaine 10%), 32.1 °C (lidocaine 20%), 32.7 °C (tetracaine 10%), and 31.8 °C

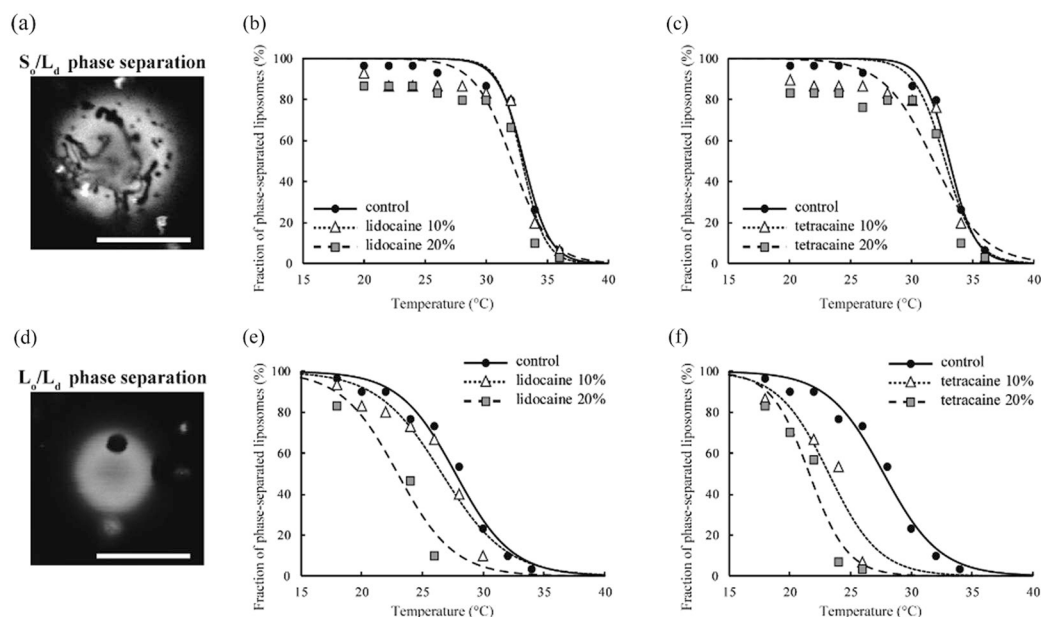


Fig. 2.5 (a) Representative fluorescent microscopic images of the S_0/L_d phase separation. Scale bars: 10 μm . (b, c) Fractions of phase-separated liposomes composed of DOPC/DPPC/lidocaine and DOPC/DPPC/tetracaine, respectively, as a function of temperature. (d) Representative fluorescent

microscopic images of the L_0/L_d phase separation. Scale bar 10 μm . (e, f) Fractions of phase-separated liposomes composed of DOPC/DPPC/Chol/lidocaine and DOPC/DPPC/Chol/tetracaine, respectively, as a function of temperature. (Reproduced from the study by Sugahara et al. 2017)

(tetracaine 20%) as shown in Fig. 2.5b and c. On contrast, the miscibility temperature for DOPC/DPPC/Chol = 40:40:20 was significantly changed by LAs; 27.7 °C (control without any LAs), 26.6 °C (lidocaine 10%), 23 °C (lidocaine 20%), 23.2 °C (tetracaine 10%), and 21.6 °C (tetracaine 20%) as shown in Fig. 2.5e and f. Therefore, the thermostability of phase-separated structures was decreased by the presence of LAs. Moreover, tetracaine strongly decreased the thermostability of the L_0/L_d phase separation than lidocaine. In addition, from the domain boundary fluctuation, the line tension values for LAs-containing phase-separated lipid membranes were measured with changing the temperature. LAs decreased the line tension at the boundary between the L_0 phase and L_d phase. As the temperature increased, the line tension tended to be lower slightly.

The main transition temperature is also influenced by the addition of LAs and can be measured by a differential scanning calorimetry (DSC). It was presumed that the asymmetric

peaks could be described as a linear combination of two independent transitions. The peak temperature shifts obtained from the peak deconvolution are summarized in Fig. 2.6. Since the S_0 phase and LA-rich phase were formed in binary mixtures composed of DPPC and LAs, it was found that LAs cannot be partitioned into the DPPC-rich S_0 phase easily. In ternary mixtures composed of DPPC, Chol, and LAs, the peak temperature for the L_0 phase was decreased as the LA's concentrations increased. On the other hand, the peak temperature for the S_0 phase was decreased slightly. Moreover, these effects were more pronounced for tetracaine than for lidocaine. Therefore, the LAs were partitioned into the L_0 phase rather than the S_0 phase. Thus, most of the LAs were partitioned into the L_d phase for the S_0/L_d phase separation and some amount of LAs were included in the L_0 phase for L_0/L_d phase separation. Therefore, LAs did not give crucial effects on the S_0/L_d phase separation and

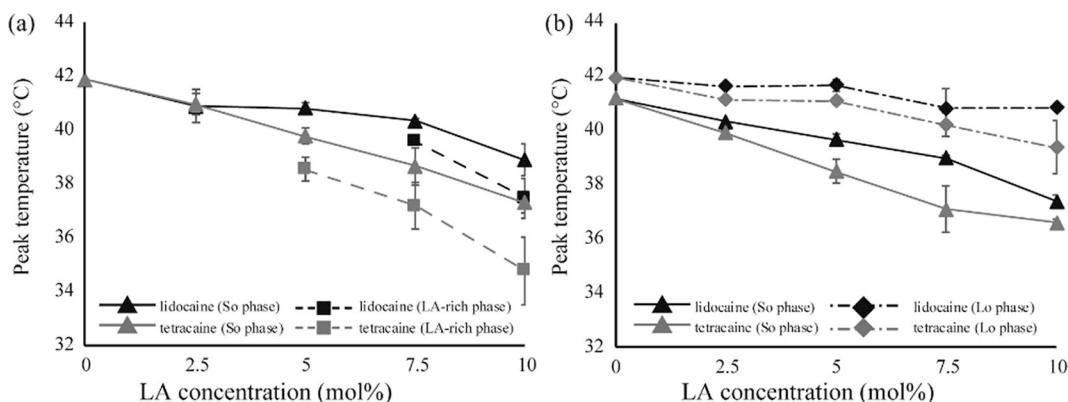


Fig. 2.6 (a, b) Shifts in peak temperature obtained from DSC measurements in DPPC/LA and DPPC/Chol/LA mixtures, respectively. (Reproduced from the study by Sugahara et al. 2017)

decreased the miscibility temperature and the line tension in the L_o/L_d phase separation.

Generally, tetracaine is known as a powerful and toxic LA than lidocaine (Onizuka et al. 2011). Based on the experimental results, tetracaine strongly suppressed the phase separation and decreased the main transition temperature than lidocaine. The correlation between pharmacological strength and membrane phase behavior was reported for *n*-alcohol, one of a general anesthetic, and the longer and more hydrophobic *n*-alcohol had stronger anesthesia (Gray et al. 2013). From an LA oil/water partitioning experiment, the anesthesia and the hydrophobicity of LA molecule were correlated (Strichartz et al. 1990). The strength of anesthesia should be discussed based on not only the hydrophobicity of LA molecules, but also the interaction between the L_o phase and LA molecules.

LAs reduced the stability of the L_o phase, which mimics a raft region. If sodium ion channels are present in the raft, the LAs may destroy the raft and inhibit the channel function. It is predicted that this process hinders signal transduction. A recent study of *Drosophila* has found that general anesthetics destroy the nanodomains of ganglioside, GM1 (Pavel et al. 2020). It has been reported that domain disruption releases the enzyme phospholipase D2 (PLD2) and inhibits neuronal firing. This is one mechanism to explain the function of LAs and needs further verification in the future.

2.9 Physiological Active Molecules: Menthol and Capsaicin

Menthol was used widely in a variety of industrial applications, such as toothpaste, chewing gum, and cigarettes (Lee and Glantz 2011; Klausner 2011; Salgado and Glantz 2011; Anderson 2011). The most characteristic property of menthol is its cooling sensation. Transient receptor potential (TRP) channels are involved in temperature sensing. Menthol is one of the channel active molecules and plays an important role in temperature sensing. The protein transient receptor potential cation channel subfamily M member 8 (TRPM8) senses temperature changes and provides a cooling sensation with menthol. Therefore, TRPM8 is known as the cold and menthol receptor 1 (CMR1). Menthol exhibits a dual behavior depending on the temperature, and enhances the cold sensation at room temperature, while it enhances the warm sensation above 37 °C (Macpherson et al. 2006; Cliff and Green 1994; Green 1985).

Since menthol has asymmetric carbons, there are some optical isomers. (1*R*, 2*S*, 5*R*)-2-Isopropyl-5-methylcyclohexanol (l-menthol) can be found in nature, causes a cooling sensation (Liedtke and Heller 2007; Chuang et al. 2004), and is used industrially as described above. On the other hand, (1*S*, 2*R*, 5*S*)-2-isopropyl-5-methylcyclohexanol (d-menthol) rarely exists in

nature and does not cause a cooling sensation. Hall et al. reported l-menthol showed more potent positive modulation of GABA_A currents than d-menthol, indicating the stereoselectivity of l-menthol on GABA_A (Hall et al. 2004). On the other hand, a stereoselectivity of menthol could not be detected on the $\alpha 7$ -nACh receptor (Abrar et al. 2013). Therefore, chirality of the molecules becomes an important factor for the functioning of the molecules in the biological processes. Although the importance of chirality in proteins have been discussed, the interactions of optical isomers in lipid membranes have not been fully understood.

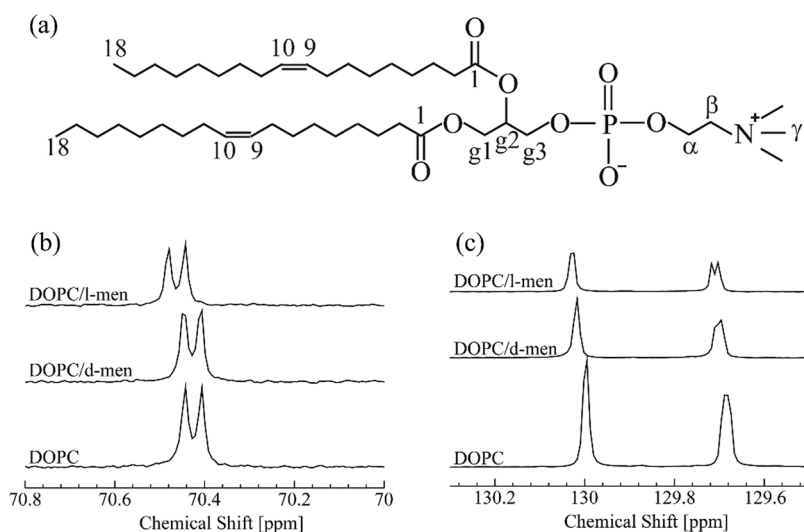
The NMR spectroscopy was performed to make clear the difference in the interactions in lipid membranes between l-menthol and d-menthol (Gusain et al. 2017). The measurement condition was DOPC/l- or d-menthol = (10:1) in CDCl₃ at 15 °C. ¹³C-NMR spectra of DOPC titrated with l- or d-menthol were displayed in Fig. 2.7. The peak around 70.4 ppm was assigned to the g2 position of DOPC as shown in Fig. 2.7a. This peak was not changed by the addition of d-menthol. However, the significant chemical shift change could be seen for l-menthol (Fig. 2.7b). Since the g2 position of DOPC was in hydrophilic part, l-menthol interacted with the headgroup of DOPC. On the other hand, the peaks at 129.7 ppm and 130 ppm

corresponding to C10 and C9 positions of DOPC, respectively, were affected by both l- and d-menthol (Fig. 2.7c). This result indicated that l- and d-menthol interacted with the hydrophobic group of DOPC. Therefore, l-menthol showed the hydrophilic and hydrophobic interactions with DOPC, while d-menthol only showed the hydrophobic interaction with DOPC (Fig. 2.8).

The phase behavior of DOPC/DPPC/Chol membranes with menthol was observed by fluorescent microscopy (Gusain et al. 2017). l- and d-Menthol did not give any crucial effects on the S₀/L_d phase separation in DOPC/DPPC/Chol = 50:50:0 and the L_o/L_d phase separation in DOPC/DPPC/Chol = 40:40:20. In addition, the miscibility temperatures for these two systems also did not change by both l- and d-menthol. For the L_o/L_d phase separation in DOPC/DPPC/Chol = 35:35:30, d-menthol progressed the phase separation and increased the miscibility temperature, while l-menthol suppressed the phase separation and decreased the miscibility temperature.

At DOPC/DPPC/Chol = 50:50:0 and 40:40:20, d-menthol could not be partitioned into the hydrophobic regions of the closely-packed S₀ and L_o phases and was included in the loosely packed L_d phase. Therefore, d-menthol did not affect the formation of S₀ and L_o phases in these lipid compositions. On the other hand, l-menthol could interact with lipids through both hydrophilic and

Fig. 2.7 (a) Systematic numbering of the chemical structure of DOPC. (b) Peaks correspond to g2 in DOPC obtained from ¹³C NMR spectra of DOPC at 15 °C. (c) Peaks around 129.7 ppm and 130 ppm correspond to C10 and C9 in DOPC, respectively, obtained from ¹³C NMR spectra of DOPC at 15 °C. (Reproduced in part from the study by (Gusain et al. 2017))



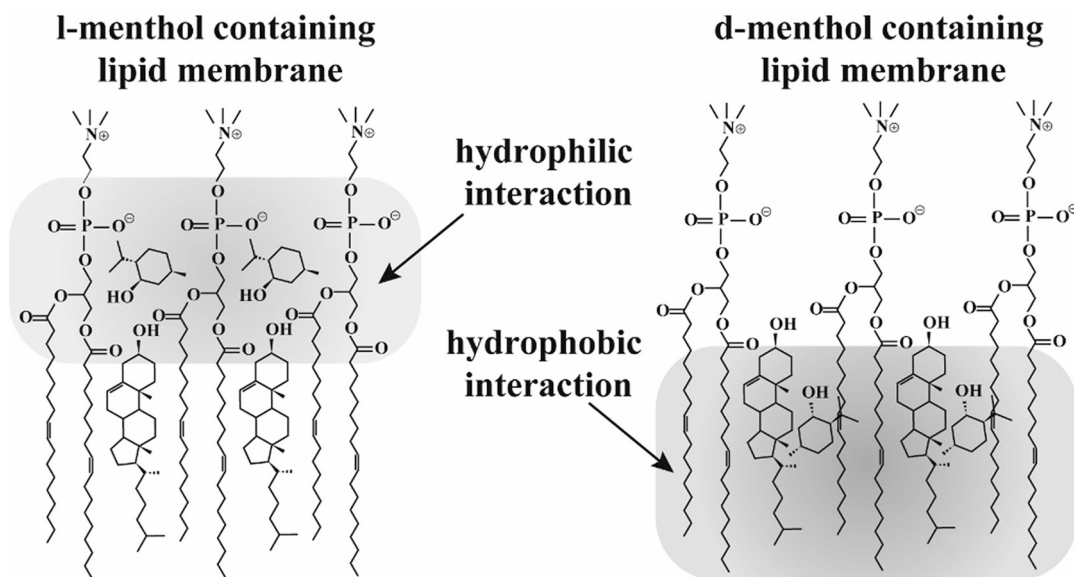


Fig. 2.8 Schematic illustration of interaction between lipid membranes and l- and d-menthol

hydrophobic interactions. Therefore, some amount of l-menthol located in hydrophilic part of lipid membranes. Since the hydrophilic head-group structures of DOPC and DPPC were identical (phosphatidylcholine, PC), l-menthol mainly interacted with DPPC-rich phase where the lipid density was high. The hydrophilic part of such a high-density lipid phase was crowded by the entry of l-menthol and this crowding caused the steric repulsion. However, the attraction between DPPC molecules was so strong that the steric repulsion did not inhibit the domain formation. As a result, the significant difference of phase behavior by the addition of l-menthol could not be found.

On the other hand, there was a large difference between l- and d-menthol in DOPC/DPPC/Chol = 35:35:30. The phase behavior by d-menthol could be explained by assuming the attraction between d-menthol and Chol. Generally, the L_o/L_d phase separation became unstable as the Chol concentration increases. The miscibility temperature decreased by the increase of Chol (Veatch and Keller 2003, 2005a, b; Veatch et al. 2006; Wongsirajkul et al. 2020). Therefore, the L_o phase for DOPC/DPPC/Chol = 35:35:30 was packed looser than that for DOPC/DPPC/Chol = 40:40:20. As already men-

tioned above, d-menthol was mainly partitioned into the L_d phase. By the attraction between d-menthol and Chol, Chol in the L_o phase migrated into the L_d phase. This Chol depletion from the L_o phase stabilized the L_o phase. The attraction between d-menthol and Chol should be investigated by some additional experiments in the future. l-Menthol interacted with the hydrophilic group of the L_o phase. The hydrophilic group was very crowded by the DPPC headgroup, the hydroxy group of Chol, and l-menthol, resulting in the strong steric repulsion. Since the L_o phase in DOPC/DPPC/Chol = 35:35:30 was loosely packed, l-menthol disrupted the L_o domain.

The temperature and the presence of Chol have some impacts on the lipid packing. At lower temperature or lower Chol concentration, the lipid membrane structure was stabilized corresponding to the cold sensitization. Under these conditions, l-menthol stabilized the lipid membrane structure through the hydrophilic interactions. On the other hand, the domain structure was disrupted at higher temperature or higher Chol concentration. These results were expected to correspond to the dual behavior in which l-menthol showed a cooling sensation at low concentrations and a warming sensation at high

concentrations. It was reported that some TRP channels are sensitive to the Chol concentration (Lui et al. 2006). Therefore, the hydrophilic interaction between lipids and l-menthol depending on Chol concentration might play an important role in the activation of TRPM8.

Capsaicin (8-methyl-*N*-vanillyl-6--nonenamide), which can be obtained from chili peppers, is a compound inducing warming sensation. It was known that capsaicin selectively binds to transient receptor vanilloid 1 (TRPV1), which is a heat-activated calcium ion channel opening between 37 °C and 45 °C. TRPV1 can open below 37 °C by the capsaicin binding and it causes the warming sensation (Caterina et al. 1991). The phase separation in capsaicin-containing lipid membranes was also examined (Sharma et al. 2019). The phase separation was suppressed in DOPC/DPPC/Chol = 40:40:20 as the capsaicin concentration increases. Since the phase separation disappeared at higher temperature, it was considered that capsaicin made the lipid membranes similar to the state where the temperature is high.

2.10 Physiologically Active Molecules: Fatty Acids

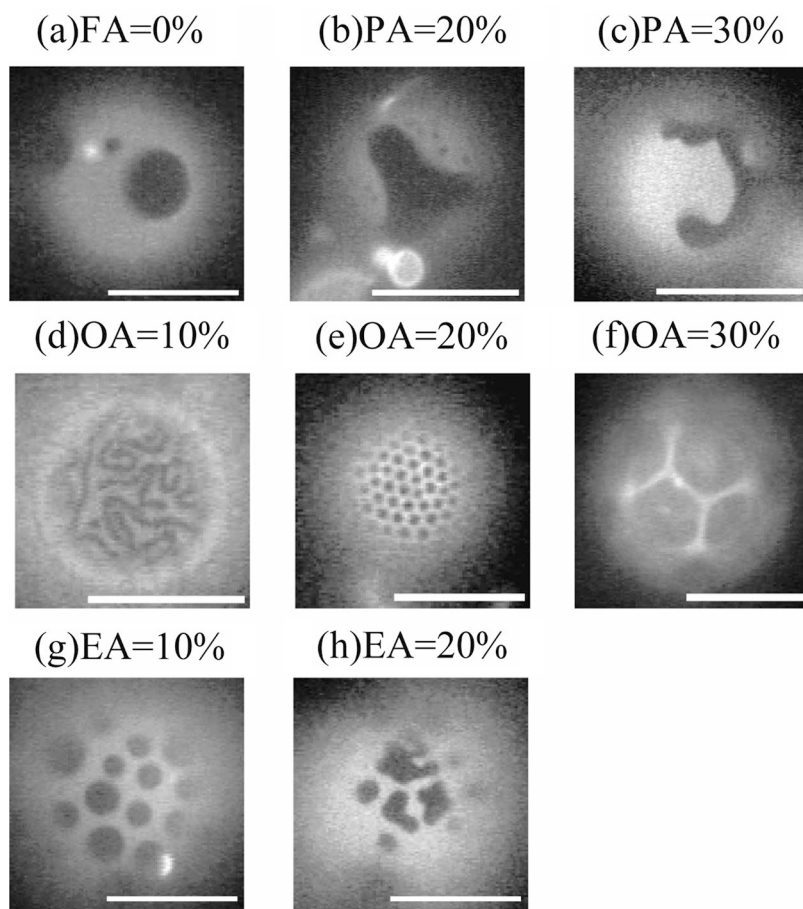
As discussed so far, additive molecules incorporated into the lipid membranes, such as physiologically active molecules, strongly affected the phase separation. The lipid compositions in cell membranes are modulated by our daily diet. The health effects by fatty acids (FAs) are often discussed. An increase in saturated FA in the diet leads to an increase in low-density lipoprotein (LDL) cholesterol (Mensink et al. 2003), and this may become a risk factor for arteriosclerosis and coronary stenosis (Turpeinen et al. 1979; Blankenhorn et al. 1990; Watts et al. 1994). In contrast, an increase in unsaturated FA (*cis*-isomer) causes an increase in high-density lipoprotein (HDL) cholesterol, leading to decreased risk of cardiovascular disease (Mensink et al. 2003). A *trans*-FA increases LDL cholesterol and decreases HDL cholesterol and may cause cardiovascular disease, brain infarction, and cancer

(Mensink et al. 1990; Ascherio et al. 1999; Baylin et al. 2003). Therefore, these fatty acids may also have some important impacts on the phase separation.

The phase separation in DOPC/DPPC/Chol = 40:40:20 with a saturated FA, palmitic acid (PA), was observed by fluorescent microscopy (Shimokawa et al. 2017). Since the tail structures of DPPC and PA were identical, DPPC molecules were gradually replaced with PA molecules. Although the L_o/L_d phase separation could be observed until DOPC/DPPC/Chol/PA = 40:30:20:10, the S_o/L_d phase separation became predominant at DOPC/DPPC/Chol/PA = 40:20:20:20 and 40:10:20:30. This result indicated that PA transferred the L_o phase to the S_o phase. Next, the effect of an unsaturated fatty acid, oleic acid (OA), was examined. Because the tail structure of OA was the same as that of DOPC, DOPC was replaced with OA. From DOPC/DPPC/Chol/OA = 30:40:20:10 to 10:40:20:30, some specific domain patterns (modulated phases), such as stripe, hexagonal, and polygonal domains were found as shown in Fig. 2.9. Although some domains had anisotropic shapes, they were considered to be in the L_o phase because the domain boundary fluctuated. From the domain boundary fluctuation, the line tension values were measured quantitatively and were reduced from 3.2 pN to 1.3 pN by the addition of OA. The line tension reduction was attributed in part to the formation of the modulated phases, which have long domain boundary length. In the following, DPPC was replaced with a *trans*-FA, elaidic acid (EA) in the same manner. At DOPC/DPPC/Chol/EA = 40:20:20:20, the fractions of the L_o/L_d and S_o/L_d phase separation were almost identical; the S_o phase formation was promoted as in PA. In order to reveal the effects of FAs on the L_o phase, the main transition temperatures of DPPC/Chol/FAs were measured by DSC. Consequently, PA and EA, which promoted the domain rigidification, increased the transition temperature. In contrast, OA, which reduced the line tension at the L_o/L_d domain boundary, decreased it.

Since PA and EA had straight molecular structures, they could be partitioned into the closely

Fig. 2.9 Representative fluorescent microscopic images. (a) DOPC/DPPC/Chol = 40:40:20 without any FAs. (b) DOPC/DPPC/Chol/PA = 40:20:20:20. (c) DOPC/DPPC/Chol/PA = 40:10:20:30. (d) DOPC/DPPC/Chol/OA = 30:40:20:10. (e) DOPC/DPPC/Chol/OA = 20:40:20:20. (f) DOPC/DPPC/Chol/OA = 10:40:20:30. (g) DOPC/DPPC/Chol/EA = 40:30:20:10. (h) DOPC/DPPC/Chol/EA = 40:20:20:20. Scale bars: 10 μm . (Reproduced in part from the study by Shimokawa et al. 2017)



packed L_o phase and interacted with DPPC strongly. Due to the inclusion of these FAs in the L_o phase, Chol was excluded from the L_o phase to L_d phase. Therefore, the Chol concentration in the L_o phase decreased, and the phase transformed into the S_o phase. Some OA molecules were also partitioned into the L_o phase. However, the chain ordering in the L_o phase was disturbed by the presence of OA with the kink structure in its molecular structure. The difference in the physical properties between the L_o and L_d phases (dipole density, curvature, membrane thickness, and chain ordering) became smaller and the line tension was reduced.

FAs strongly affected the Chol localization. In other words, FAs contributed the formation and stability of raft domains. Cytoplasmic protein is palmitoylated by palmitoyltransferase; thus the protein is conjugated with PA. The hydrophobic-

ity of the protein is increased, and the protein is localized in the plasma membranes. It was reported that this protein migrates into the raft region (Levental et al. 2010). In addition, this palmitoylation can be mimicked using artificial lipidated proteins in liposomes and living cells (Uchida et al. 2022). Not only do FAs modulate the membrane ordering, but also FA-conjugated proteins are involved in raft domains.

2.11 Future Perspective

As explained above, liposomes are extremely useful as cell membrane models. Liposomes, which are artificial membranes, can change their composition, allowing them to mimic various conditions of cell membranes, such as stressful environments and aging. Furthermore, liposomes

can also mimic the organelle membranes that exist within cells, and have a very wide range of applications. For example, there is a report of mimicking the endoplasmic reticulum membrane, which has very low cholesterol content, and studying the interaction between amyloid β , the cause of Alzheimer's disease, and the model endoplasmic reticulum membrane under stress conditions (Nakatani et al. 2022). Thus, cell-sized liposomes are a useful tool for visualizing the properties of various bioactive substances and the stresses that cause diseases.

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Cold Tolerance in the Nematode *Caenorhabditis elegans*

3

Atsushi Kuhara, Natsune Takagaki,
Misaki Okahata, and Akane Ohta

Abstract

Organisms receive environmental information and respond accordingly in order to survive and proliferate. Temperature is the environmental factor of most immediate importance, as exceeding its life-supporting range renders essential biochemical reactions impossible. In this chapter, we introduce the mechanisms underlying cold tolerance and temperature acclimation in a model organism—the nematode *Caenorhabditis elegans*, at molecular and physiological levels. Recent investigations utilizing molecular genetics and neural calcium imaging have unveiled a novel perspective on cold tolerance within the nematode worm. Notably, the ASJ neuron, previously known to possess photosensitive properties, has been found to sense temperature and regulate the sperm and gut cell-mediated pathway underlying cold tolerance. We will also explore *C. elegans*' cold tolerance and cold acclimation at the molecular and tissue levels.

A. Kuhara (✉) · N. Takagaki · M. Okahata · A. Ohta
Faculty of Science and Engineering, Graduate School
of Natural Science, Institute for Integrative
Neurobiology, Konan University,
Okamoto, Higashinada-ku, Kobe, Japan

AMED-PRIME, Japan Agency for Medical Research
and Development, Tokyo, Japan

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pathway · Cold stimulus · Temperature
sensing · Potassium channel · Trimeric
G-protein · Endoribonuclease · Sperm

3.1 *Caenorhabditis elegans* As a Model Organism for Temperature Response

While a recent study predicts that global warming will elevate the average worldwide temperature by 1.8–4 °C within the next century (Solomon et al. 2007), localized cooling occurrences, such as the freezing of Serbia's Danube River in 2017, are not unprecedented. Even as far back as the seventeenth century, there are records of brief glacial periods. An illustrative case is the freezing of London's River Thames from 1645 to 1715, which was likely triggered by the Maunder Minimum sunspot activity (Eddy 1976). Clearly, mechanisms related to cold and heat tolerance and their acclimation are imperative for the survival of all creatures. One extensively studied mechanism involves the modification in composition of fatty acid in membranes of the cell. An augmentation on the proportion in unsaturated fatty acids aids in sustaining flexibility of membrane in the cell at lower temperature, which is a

critical factor in both plant and animal cold tolerance. To prevent the cytosol from freezing, numerous cells accumulate sugars like trehalose (Goto et al. 1993; Sakurai 2012). While researchers have extensively examined these adaptive “outputs,” the molecular systems responsible for processing temperature “input” are only just starting to garner academic attention.

Caenorhabditis elegans is one of the powerful model animals in the analysis of neurology, system biology, and developmental biology (Brenner 1974; Riddle et al. 1997; Wood and The Community of *C. elegans* Researchers 1988). *C. elegans* hermaphrodites possess only 959 cells, which include 302 neurons. All cell lineages are completely determined (Sulston et al. 1983). Most *C. elegans* worms are hermaphroditic and possess a life cycle of about 3.5 days at 20 °C. A single hermaphroditic individual can lay around 300 progenies over its lifetime, and spontaneous X chromosome nondisjunction may result in male progeny useful in hermaphrodite strain crossing. In 1998, ahead of other multicellular organisms, genomic DNA sequencing had already been published in the genome database for *C. elegans* (*C. elegans* Sequencing Consortium 1998). Owing to this, fundamental analytical tools such as genome database and gene structure prediction programs were developed for later use in creating the human genome database.

Utilizing well-established techniques in molecular biology and genetics, *C. elegans* has been employed as a model organism to investigate numerous biological processes (Barr 2003). To illustrate, processes like RNA interference, apoptosis and microRNA were initially uncovered by researchers working with *C. elegans*. Furthermore, these findings extend to analogous processes in more complex life forms, given that numerous fundamental molecular and physiological mechanisms remain conserved from *C. elegans* to humans evolutionarily. For instance, within the *C. elegans* nervous system, electron microscopy has unveiled the existence of approximately 600 axonal gap junctions and 5000 chemical synapses (White et al. 1986). *C. elegans* capitalizes on this comparatively simple nervous system to react to a multitude of environmental

cues such as taste, smell, touch, and temperature (Bargmann et al. 1993; Bargmann and Horvitz 1991; Chalfie et al. 1985; Dusenbery et al. 1975; Hedgecock and Russell 1975; Mori and Ohshima 1995). Moreover, by utilizing laser ablation to selectively eliminate individual neurons, researchers have been able to pinpoint specific cells necessary for sensation (Bargmann et al. 1993; Bargmann and Horvitz 1991; Chalfie et al. 1985; Mori and Ohshima 1995). It is due to these rationales that *C. elegans* is also adopted as a model organism in the exploration in temperature response.

3.2 Cold Tolerance and Temperature Acclimation of Nematode *C. elegans*

Thermal responses have been analyzed by using tolerance and behavior phenotypes in *C. elegans* (Ohta et al. 2023; Okahata et al. 2022; Ohta and Kuhara 2013; Ohnishi et al. 2019). One of the most studied behaviors in temperature responses is thermotaxis—a temperature-seeking behavior. Worms move to previous grown temperature and move isothermally on a thermal gradient ranging from about 15–25 °C upon being raised at a certain temperature with food. Conversely, *C. elegans* avoids the grown temperature if conditioned without food. The neural circuitry and associated molecular mechanisms underlying thermotaxis have been studied in depth (Aoki and Mori 2015; Mori et al. 2007; Ohta and Kuhara 2013). *C. elegans* can grow within the range of 15–25 °C, and under cultivation above 28 °C, these worms produce dauer larva that can tolerate high temperature. However, worms do not transform dauer larva in cold conditions, and cold tolerance has remained largely unexplored.

C. elegans has the ability of cold tolerance depending on the various grown temperatures (Fig. 3.1) (Murray et al. 2007). For instance, wild-type worms cultivated at 15 °C can survive well after being transferred to 2 °C for 48–96 h, while 20/25 °C-grown worms cannot (Ohta et al.

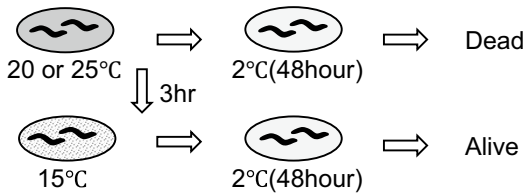


Fig. 3.1 Cold Tolerance and Temperature Acclimation of *Caenorhabditis elegans*. After being grown at 25 °C, wild-type N2 *C. elegans* animals are unable to survive at 2 °C for 48 h. However, worms cultivated at 15 °C can endure the same cold stimulus. Worms grown at 25 °C can survive at 2 °C after being grown at 15 °C for 3 h

2014, 2023; Sonoda et al. 2016; Takeishi et al. 2020; Okahata et al. 2022; Ohnishi et al. 2019). This resistance, cold tolerance, changes within only 2–3 h of exposure of a thermal stimulus. For example, 20–25 °C-grown animals normally do not survive at 2 °C, while 20–25 °C-grown animals can survive at 2 °C after exposure to 15 °C for 3 h (Fig. 3.1) (Ohta et al. 2014; Ujisawa et al. 2014a).

3.3 Light and Chemical Sensory Neurons Perceive Temperature Changes Underlying Cold Tolerance and Acclimation

In order to ascertain the tissues contributing to cold tolerance, various tissue-specific mutants were tested, and a mutant abnormal in neurons showed heightened cold tolerance following cultivation at 20 °C. Some mutants with impaired myosin in body muscle or a collagen in the cuticle did not exhibit this abnormality, and mutants for a cGMP-gated channel (CNGC) encoded by *tax-4* gene, expressed in ten pairs of sensory neurons, displayed an atypical increment of cold resistance. A homeodomain transcription factor encoded by *pros-1* gene, homologous to mammalian Prox1 and *Drosophila* prospero, was involved in the development of glial cells enveloping sensory neurons located at the head. Knocking down the *pros-1* led to anomalous sensory neuron morphology as well as cold tolerance abnormality (Kage-Nakadai et al. 2016).

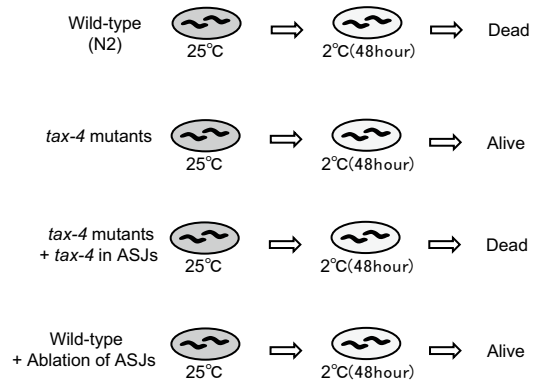


Fig. 3.2 ASJ sensory neuron's role in cold tolerance. The abnormal cold tolerance seen in *tax-4* mutants was restored by expressing its wild-type gene specifically in the ASJ thermosensory neuron. Laser ablation of the ASJ sensory neuron of wild-type led to an unusual enhancement of cold tolerance

The supranormal cold tolerance in CNGC mutant *tax-4* was restored by the expression of the wild-type CNGC exclusively in a single pair of the ASJ sensory neurons (Fig. 3.2). Moreover, the use of laser ablation on the ASJ neurons induced an increment of cold tolerance, meaning the negative regulatory role of ASJ sensory neurons in cold tolerance (Fig. 3.2) (Ohta et al. 2014). The ASJ sensory neurons were previously reported as light and pheromone-sensing neurons (Liu et al. 2010), and to explore whether ASJ also detects temperature, calcium imaging employing a gene encodable indicator of calcium named “cameleon” was utilized to monitor neural activity. These calcium imaging experiments unveiled the responsiveness of ASJ to temperature stimulation, and the thermal response of ASJ was decreased in the mutant lacking a CNGC, TAX-4. This abnormality was restored by expressing the wild-type CNGC TAX-4, in ASJ neurons, indicating that CNGC TAX-4 is required for transduction of thermal signaling in ASJ. ASJ temperature response was determined to be unaffected in the mutant impairing synaptobrevin essential for synaptic transmission, meaning that a neurochemical input from other neurons is not essential for the temperature response of ASJ. Collectively, these experiments posit that ASJ sensory neurons assume a pivotal role in

detecting environmental temperature (Ohta et al. 2014; Ujisawa et al. 2014b).

3.4 G-Protein Signaling Essential for Photo-Sensation in the ASJ Plays an Indispensable Role in Cold Tolerance

The process of phototransduction in the ASJ sensory neuron has been previously elucidated (Fig. 3.3) (Liu et al. 2010). During this sequence, multiple G-protein alpha subunits ($G\alpha$), guanylyl cyclase (GC), and phosphodiesterase (PDE) collaborate to receive light through the photoreceptor LITE-1. Receiving photo-stimuli by LITE-1 leads to the activation of $G\alpha$ encoded by *goa-1* and *gpa-3* genes, GC encoded by *daf-11* and *odr-1* genes, and the cGMP-gated channel encoded by *tax-2* and *tax-4* genes, sequentially. PDE encoded by *pde-1*, *pde-2*, and *pde-5* genes serves as a negative regulator of photo-sensation by hydrolyzing cGMP (Liu et al. 2010) (Fig. 3.3). Mutations in these $G\alpha$, GC, and PDE cause enhanced cold tolerance that is resemble phenotype to that of the CNGC mutant (Ohta et al. 2014). However, the mutants lacking the pho-

toceptor protein encoded by *lite-1* gene show normal phenotype in cold tolerance (Ohta et al. 2014), meaning that while photo-sensory-signaling proteins are essential for cold tolerance, photoreceptors are not.

At the sensory terminus in the dendrite of ASJ sensory neuron, three types of $G\alpha$ proteins encoded by *goa-1*, *gpa-1*, and *gpa-3* genes are localized (Ujisawa et al. 2016). Single mutants for *goa-1* and *gpa-1* display abnormally heightened cold tolerance. Moreover, their $G\alpha$ double mutant animals exhibit even greater cold tolerance than either single mutant, implying additive actions of these $G\alpha$ genes. Mutations in *gpa-3* do not show significant cold tolerance abnormalities. However, the *gpa-3* mutation enhances the abnormal *goa-1* mutant phenotype, and triple mutants, *goa-1*; *gpa-3*; *gpa-1*, demonstrate more pronounced cold tolerance abnormalities than either their double mutant strains. It is evident that these three $G\alpha$ proteins cooperate in performing of cold tolerance (Ohta et al. 2014; Ujisawa et al. 2016).

Although PDE functions as a negative regulator of ASJ light signaling, various PDE mutations reduce ASJ activity during temperature response (Fig. 3.4) (Ujisawa et al. 2016). These findings are similar to the phenomena of vertebrate photoreceptors where defective PDE led to a decrease

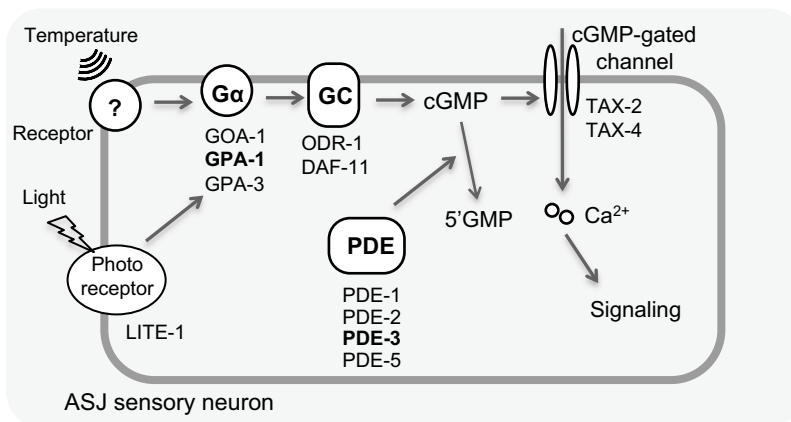


Fig. 3.3 Model of light and temperature signaling during cold tolerance regulated by the ASJ sensory neuron. The signaling pathways for temperature and light are believed to share common and function-specific molecules. The names of molecules described in bold indicate those spe-

cific to temperature signaling. $G\alpha$, GC, and PDE, among other molecular components, may also play central roles in temperature signaling, as temperature response was not completely eliminated in mutant models. The identity of the temperature receptor remains unknown

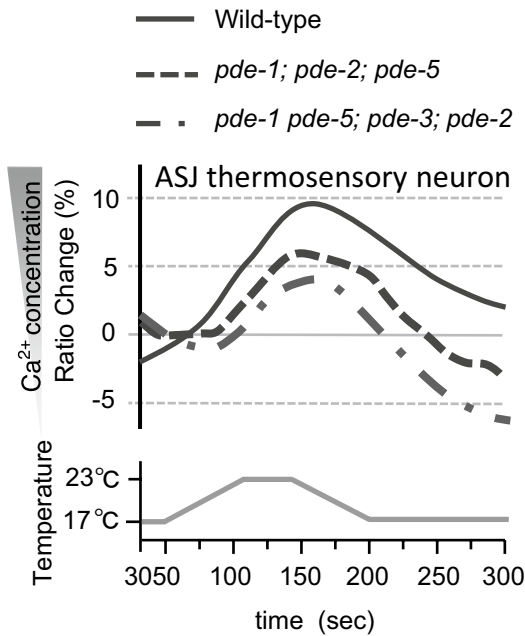


Fig. 3.4 Measurement of ASJ temperature responsiveness in PDE mutants. Changes in intracellular Ca^{2+} were measured using the yellow/cyan fluorescence ratio obtained from the yellow chameleon (YC3.60) indicator

in GC activity and halted signaling due to excessive calcium influx into sensory neurons (Koch and Stryer 1988; Nakatani and Yau 1988). These and other investigations propose that temperature and light sensation in *C. elegans* might share common molecular mechanisms (Ujisawa et al. 2016).

Although temperature receptor molecules of ASJ thermosensory neuron have not yet been found, new temperature receptor acting in ASG thermosensory neurons involved in cold tolerance was recently identified. DEG-1 encoding a degenerin/epithelial sodium channel, known as a mechanosensory molecule in *C. elegans*, functions as a thermoreceptor in a pair of head sensory neuron, ASG (Takagaki et al. 2020). This neuron, in turn, regulates downstream AVH and AIN interneurons required for cold tolerance. The human homologue of DEG-1, MDEG1, also acts as a temperature receptor (Takagaki et al. 2020). This means that the analysis of *C. elegans*' cold tolerance has facilitated the identification of human thermoreceptor.

3.5 The Function of the Calcium-Dependent Endoribonuclease ENDU-2 in Cell Autonomous and Nonautonomous Roles Within Cold Tolerance

In the cold resistance, a pair of ASJ sensory neurons in the head detects changes in temperature (Ohta et al. 2014). Subsequently, insulin released from synaptic site of these ASJ neurons binds to the insulin receptor encoded by *daf-2* gene in the gut and neurons (Ohta et al. 2014). This insulin-signaling pathway governs downstream gene expression through the FOXO-type transcription factor encoded by *daf-16* gene. Furthermore, the genes that modulate cold tolerance were screened and identified by DNA microarray analysis and observation of phenotype of many mutants (Ohta et al. 2014). The expression levels of the *endu-2* gene increases following a temperature change, and *endu-2* mutants not only exhibit abnormal cold tolerance but also a shorter lifespan and reduced brood size (Ujisawa et al. 2018; Ohta et al. 2014). ENDU-2 is homologous to the human calcium-dependent endoribonuclease EndoU, which has been studied at the cellular level but still has an unclear systemic role. Biochemical analysis has demonstrated that ENDU-2 exhibits binding and ribonuclease (RNase) activity similar to human EndoU (Ujisawa et al. 2018).

To identify the responsible tissue(s) for *endu-2*-dependent temperature resistance, studies were performed to explore the cold tolerance of *endu-2* mutants with specific tissue abnormalities (Ujisawa et al. 2018). ENDU-2 was found to be expressed in the gut, muscle cells, and neurons. Abnormally heightened cold resistance of *endu-2* mutants is restored by introducing wild-type *endu-2* gene in muscle cells and the ADL pair of head sensory neurons known for their role in chemo-sensation. The ADL sensory neurons are recognized for detecting aversive chemicals, and calcium imaging experiments were performed to elucidate whether the ADL neurons can also detect thermal changes. These experiments revealed that ADL sensory neurons indeed

respond to environmental temperature, and this responsiveness is impaired in the *endu-2* loss-of-function animal (Fig. 3.5). Furthermore, the abnormal thermal response in *endu-2* animal is restored by the expression of wild-type *endu-2* gene specifically in either the ADL neurons or muscle cells (Fig. 3.5). The significance of these findings lies in the revelation that ADL thermal response in wild-type is controlled by cell autonomous function of ENDU-2 within the ADL and cell nonautonomous function of ENDU-2 in muscle cell.

Transcriptome analysis, comparing RNA profiles between wild-type and *endu-2* animals, indicates that ENDU-2 alters the expression levels of apoptotic genes like the caspase gene, *ced-3* (Ujisawa et al. 2018). The *ced-3* animals display an abnormal decrement of cold tolerance due to

defective ENDU-2 function in ADL neurons and muscle cells, whereas the heightened cold tolerance of *endu-2* animals is suppressed by the mutation in *ced-3* gene. These investigations provide evidence for ENDU-2 function mediated by ADL neurons and muscles through CED-3, caspase (Ujisawa et al. 2018).

In *C. elegans*, apoptotic signaling and the CED-3, caspase, regulate remodeling of synapses in neural cells (Ujisawa et al. 2018). The mutation of *ced-3* reduces the number of synapses in the axon at the dorsal nerve (Meng et al. 2015). Conversely, *endu-2* mutation elevates this number, while *ced-3* mutation decreases it. These findings collectively suggest that calcium-dependent endoribonuclease ENDU-2 operates a remodeling of synapses via caspase CED-3 (Fig. 3.6), and that the ENDU-2 plays a key role in the expression of caspase to promote remodeling of synapses and enhance cold tolerance (Fig. 3.7) (Ujisawa et al. 2018).

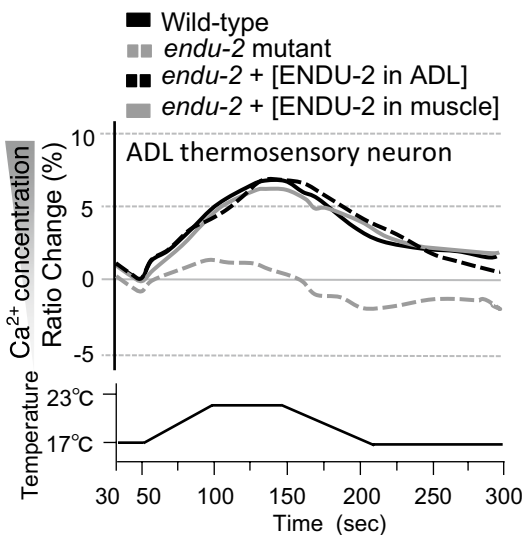


Fig. 3.5 ENDU-2 Expression in sensory neurons and muscle cells rescue ADL neuron's response to thermal stimulus in *endu-2* mutants. Calcium concentration in wild-type ADL neurons varied according to temperature. In contrast, the response significantly decreased in *endu-2* mutants. The abnormal phenotype of *endu-2* mutants was restored by expressing wild-type *endu-2* in the ADL neurons, as shown in *endu-2* + [ENDU-2 in ADL]. Additionally, specific expression of *endu-2* in muscle cells rescued the abnormal phenotype of *endu-2* mutants, as shown in *endu-2* + [ENDU-2 in muscle]. Changes in intracellular Ca^{2+} were measured using the yellow/cyan fluorescence ratio of the yellow chameleon (YC3.60) indicator

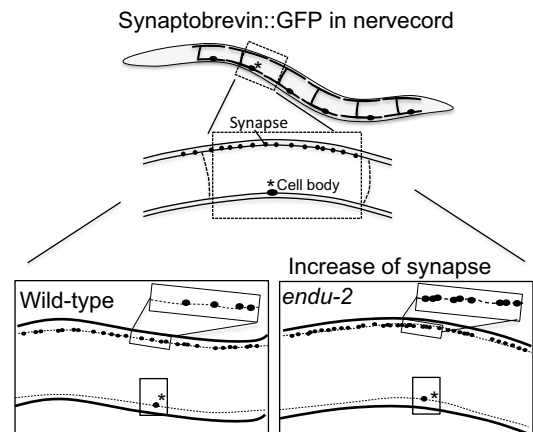


Fig. 3.6 ENDU-2 on synaptic remodeling. The upper diagram illustrates synapses in the dorsal and ventral nerve cords, with asterisks (*) indicating DD2 motor neuron cell bodies. The number and density of synapses in the dorsal nerve cord were examined using the presynaptic marker synaptobrevin SNB-1::GFP. The *endu-2* mutation led to an increase in the number and density of synapses in the dorsal nerve cord, as shown in the right panel. Synapses of DD neurons in the ventral nerve cord are eliminated by apoptotic signaling molecules during development from the larval stage to young adulthood. This process involves the remodeling of new synapses in the dorsal cord during adulthood, during which ENDU-2 negatively regulates synaptic remodeling

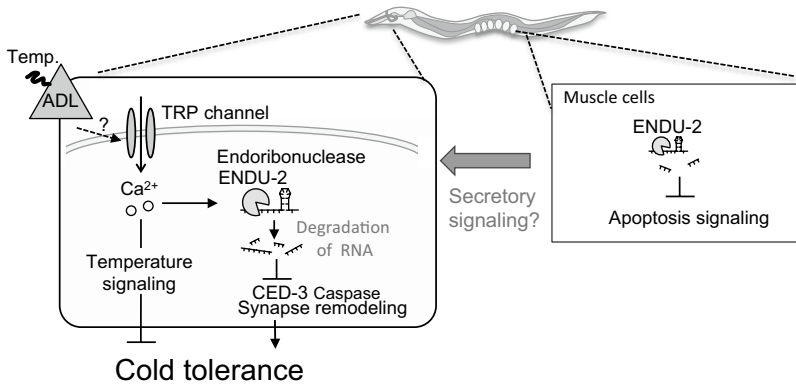


Fig. 3.7 ENDU-2-linked cold resistance model. The collaboration of ENDU-2 and thermo-TRP channels yields an influence on the neural dynamics within the ADL sensory neuron. Notably, ENDU-2 acts as a negative regulator to the CED-3-related synaptic remodeling taking place in the dorsal nerve cord. Apoptotic signaling occurring in

muscle cells and ADL neurons might decrease a neural function of ADL thermosensory neurons. The intricate signaling network directed by ENDU-2 orchestrates pivotal procedures for cold endurance, at both cell-autonomous and cell-nonautonomous manner

A recent report described that RNase ENDU-2 exhibits characteristics of a secreted protein (Qi et al. 2021). While the precise mechanisms governing the secretion of ENDU-2 remain to be fully elucidated, it has been demonstrated that ENDU-2 can be transported to different tissues beyond its originating cells (Qi et al. 2021). According the expression of *endu-2* is observed in gut cells, the extracellular release of RNase ENDU-2 from gut cells could potentially exert nonautonomous influence on ADL thermosensitivity.

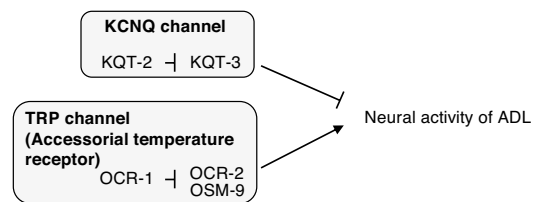


Fig. 3.8 Model of molecular mechanism regulating ADL neuron activity. The TRP channel subunit OCR-1 negatively regulates OCR-2 and OSM-9, while the KCNQ-type potassium channel subunit KQT-2 negatively regulates KQT-3

3.6 Cold Acclimation and ADL Thermo-Responsivity Are Modulated by Environmental Oxygen Concentration via KCNQ Potassium Channel, KQT-2

The expression of *kqt-2* gene, which encodes a mammalian KCNQ-type potassium channel, is upregulated upon temperature decrease (Sugi et al. 2011). The *kqt-2* mutant exhibits an abnormal increase in cold acclimation upon temperature decrease (Okahata et al. 2019). The *kqt-2* gene is expressed in the two pair of head sensory neurons, ADL and ASK, and in the intestine, and

its transcript functions to positively regulate ADL activity (Okahata et al. 2019). In general, KCNQ-type potassium channels are known to function to decrease neural activity, whereas KQT-2 paradoxically increases ADL activity under temperature change. Structurally, KCNQ2 and KCNQ3 form KCNQ homo-channels or hetero-channels that regulate neuronal excitability by generating M currents. Similar to mammalian KCNQ channels, *C. elegans* KQT-2 and KQT-3 possibly form a KQT-2/KQT-3 heteromeric channel that inactivates neural activity in ADL sensory neuron. Formation of KQT-2/KQT-3 heteromeric channels could reduce functional potassium channel activity in ADL sensory neurons (Fig. 3.8) (Okahata et al. 2019). TRP channels OSM-9, OCR-2, and OCR-1 regulate cold acclimation and

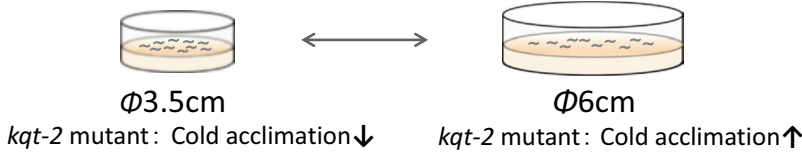


Fig. 3.9 Cold acclimation of animals cultivated on different sizes of culture plates. Abnormal cold acclimation of *kqt-2* mutant animals depends on the size of the cultur-

ing plate, whether $\phi 3.5$ cm or $\phi 6.0$ cm. The *kqt-2* mutant animals exhibit enhanced cold acclimation after cultivation on a $\phi 6.0$ cm agar medium plate

modulate the ADL thermo-responsivity (Ujisawa et al. 2018), in which OSM-9 and OCR-2 act as accessory thermo-receptors (Ohnishi et al. 2020). *ocr-1* single mutants display abnormal ADL excitation (Okahata et al. 2019), while ADL activity is decreased in *ocr-1*; *osm-9*; *ocr-2* triple mutants (Ujisawa et al. 2018). Conversely, *osm-9*; *ocr-1* and *ocr-2*; *ocr-1* double mutants display normal ADL excitability, suggesting that defective *osm-9* and *ocr-2* somehow block the deleterious nature of mutant *ocr-1*. Thus, the OCR-1 TRP channel subunit regulates temperature responsiveness of ADL by acting as a negative regulator of OSM-9 and OCR-2 (Fig. 3.8) (Okahata et al. 2019). The calcium TRP channels and the KQT potassium channels appear to modulate neuronal activity by forming subunits in ADL neurons, respectively. Recent report proposes a dual thermosensing machinery through a G protein-coupled receptor (GPCR) encoded by *srh-40* gene and TRP channels OSM-9 and OCR-2 in the ADL neurons (Ohnishi et al., 2024).

The *kqt-2* mutant exhibited stronger cold acclimation abnormality when animals were cultivated on medium-sized agar plate than on smaller plates (Fig. 3.9) (Okahata et al. 2019). The ADL sensory neurons not only responded to temperature, but also to the nematode pheromone, ascaroside. The responsiveness of the ADL to ascaroside pheromone is indirectly affected by oxygen information from upstream URX neurons known as oxygen-sensing neuron via RMG interneurons. (Fenk and de Bono 2017).

The *gcy-35* mutant defective in the oxygen receptor of URX neuron exhibits the decrement of cold acclimation. Furthermore, although *kqt-2* mutants show abnormal cold acclimation and decreased ADL thermal responsiveness, the *gcy-35* mutation suppresses both abnormal cold acclimation and decrement of ADL thermal

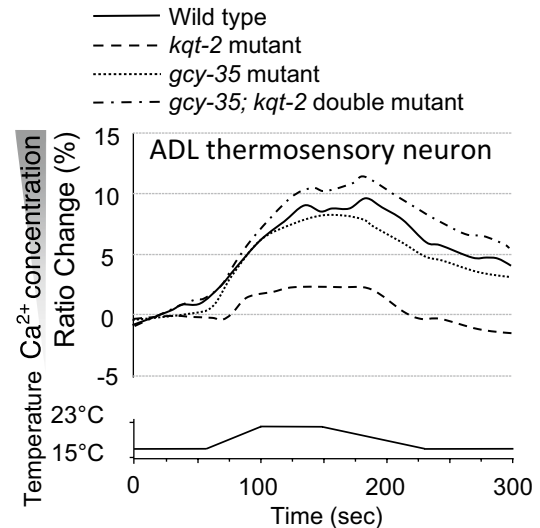


Fig. 3.10 Suppression of abnormal Ca^{2+} thermal response in *kqt-2* mutant ADL neurons by oxygen receptor mutation. Ca^{2+} imaging showed that Ca^{2+} thermal response in *kqt-2* mutant ADL neurons becomes significantly lower, as depicted in “*kqt-2* mutant.” This abnormally low thermosensitive Ca^{2+} response was restored to wild-type levels by introducing the GCY-35 oxygen receptor mutation in URX sensory neurons, as shown in “*gcy-35*; *kqt-2* double mutant.” Changes in intracellular Ca^{2+} concentration were measured using the yellow/cyan fluorescence ratio obtained from the YC3.60 indicator

responsivity of *kqt-2* mutant (Fig. 3.10) (Okahata et al. 2019). Thus, it is possible that ambient oxygen signaling from URX neuron and temperature signaling in ADL are integrated into the ADL sensory neuron to modulate cold acclimation via a simple neural circuit (Fig. 3.11).

C. elegans must detect, process, and respond to information about its environment using a limited number of neurons. Here, we have proposed a simple neural circuit that makes use of oxygen information detected by the URX in combination with downstream temperature information detected by the ADL to regulate cold acclima-

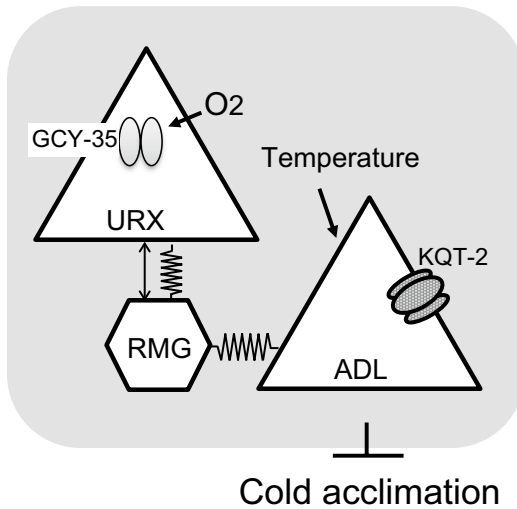


Fig. 3.11 Neural circuit model integrating temperature sensation and oxygen signaling. URX sensory neurons are believed to indirectly regulate temperature signaling during cold acclimation. The sensation of high O_2 levels by URX neurons through the oxygen receptor GCY-35 generates signals that travel via RMG and ADL neurons to facilitate cold acclimation. This is likely achieved by inhibiting ADL excitability. When KQT-2 function is lost in the ADL, neuronal activity in response to temperature changes is diminished, enhancing the ability to acclimate to cold. If GCY-35 function is compromised, signaling from URX neurons mimicking low O_2 conditions occurs. Consequently, this boosts ADL excitability sufficiently to counteract the inhibitory effect of nonfunctional KQT-2. As a result, the hyper-normal cold acclimation seen in *kqt-2* mutants is suppressed either by a *gcy-35* mutation or low O_2 levels

tion. It is likely that higher organism also employs mechanisms in which a single neuron regulates multiple types of sensory information.

3.7 Tissue Networks That Regulate Cold Tolerance

Downstream of ASJ thermosensory neurons, pivotal in the pathway regulating cold tolerance, lies a network of tissues (Fig. 3.12) (Ohta et al. 2014). This pathway intersects with aging-related mechanisms, as evidenced by shared molecular components like FOXO-type transcription factors and AGE-1/PI3 kinase (Savory et al. 2011). Upon temperature stimulus, ASJ sensory neurons release insulin-like molecules that are received by the insulin receptor DAF-2 in

gut cells and neurons. In the gut, the insulin receptor controls downstream PI3 kinase and AKT kinase, impeding translocation of FOXO-type transcription factor encoded by *daf-16* gene to the nucleus. Consequently, FOXO-dependent gene expression is suppressed, which enhances cold tolerance. Variation in *C. elegans* cold tolerance links to changes in phospholipid saturation, suggesting DAF-16's probable target is a desaturase gene (Murray et al. 2007; Savory et al. 2011).

Cold tolerance also involves glycerol accumulation in the gut (Liu et al. 2017). Cold stimulus activates Gs type trimeric G protein α subunit and protein kinase A, which boost a hormone-sensitive lipase encoded by *hosl-1* gene. This lipase governs glycerol accumulation, a by-product of lipid hydrolysis that is crucial for cold resistance (Liu et al. 2017). In mice, long-chain acyl-CoA dehydrogenase (VLCAD) is vital for cold stress survival; VLCAD deficiency reduces mouse cold resistance. VLCAD heightens TMEM135, a novel mouse protein. In *C. elegans*, TMEM135 overexpression enhances cold stress survival (Exil et al. 2010), while its deletion reduces the storage of fat, longevity, and activity of mitochondria (Exil et al. 2010).

The insulin receptor signaling influences stress tolerance responses, including cold (Gal et al. 2004). However, mutations affecting stress responsive gene products like MAP kinases encoded by *jnk-1*, *kgh-1*, and *kgh-2* genes, the heat shock factor encoded by *hsf-1* gene, and the heat-shock proteins encoded by *hsp-16.2*, *hsp-16.41*, and *hsp-16.48* genes do not alter cold tolerance (Ohta et al. 2014). Metabolism links cold resistance and stress, as cold-adaptive responses coincide with dietary restriction; choline supplementation counters such responses (Klapper et al. 2016).

Cold shock reduces pigmentation, gut and gonad sizes, and vulvar morphology, with subsequent recovery (Robinson and Powell 2016). The *fshr-1* mutant, harboring a faulty G-protein coupled receptor, displays strong cold-shock resistance and abnormal pigmentation (Robinson and Powell 2016). Intestine sensitivity to cold influences aging and stress responses. A cold receptor

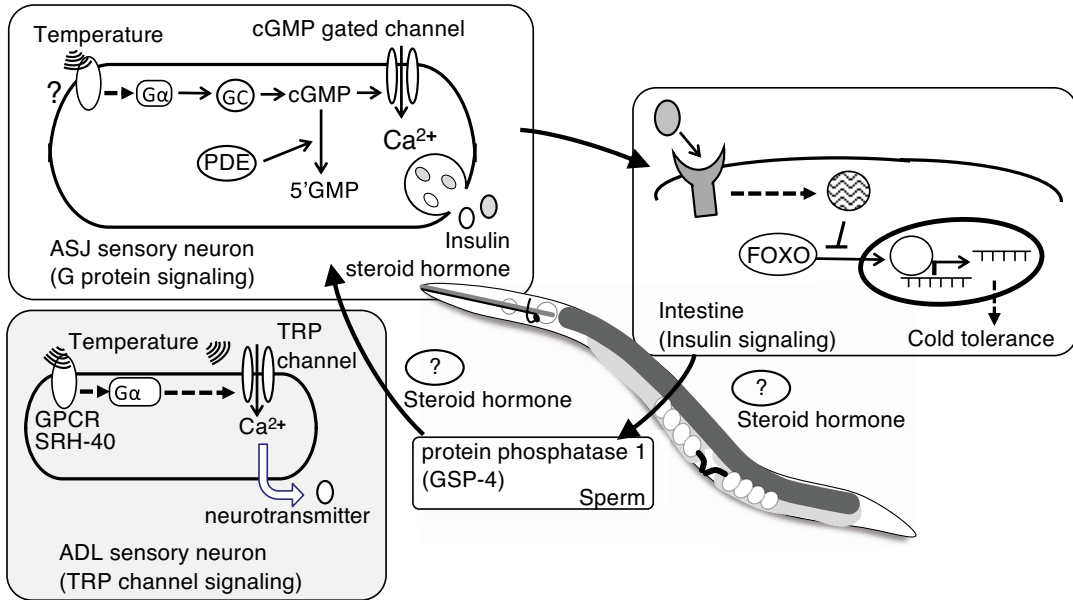


Fig. 3.12 Tissue networks and cold tolerance. The regulation of cold acclimation and tolerance involves a tissue network comprising nerve cells, gut cells, and sperm. The detection of temperature by sensory neurons, ASJ and ADL, leads to release the insulin and steroid hormones

from ASJ neurons. These hormones are received by the gut, which in turn impacts sperm via nuclear hormone receptors. These receptors indirectly affect temperature signaling in ASJ neurons through an unidentified feedback system

TRP channel encoded by *trpa-1* and downstream protein kinase C encoded by *pkc-2* are vital in these processes (Xiao et al. 2013). Yet, these TRP channel and PKC mutants do not exhibit defective cold tolerance (Ohta et al. 2014), indicating TRPA-1's role in intestine signaling is separate from cold resistance (Xiao et al. 2013).

DNA microarray analysis with insulin receptor *daf-2* mutants under temperature changes has helped to isolate downstream genes involved in insulin signaling for intestine (Sonoda et al. 2016). Expression of several sperm-related genes is altered in insulin receptor mutant, and mutation in a sperm-specific protein phosphatase PP1 encoded by *gsp-4* gene in particular displays abnormal cold resistance (Sonoda et al. 2016). Calcium imaging studies have revealed that *gsp-4* mutants have the decreased ability to respond to thermal stimuli of ASJ thermosensory neuron (Sonoda et al. 2016). Sperm, therefore, negatively regulates ASJ temperature sensation. Quantitative PCR and epistasis analyses further suggest that the gut can influence sperm gene expression, which in turn can influence neuronal

activity of ASJ thermosensory neuron (Sonoda et al. 2016).

Consider this proposed cold tolerance model based on our current understanding of the tissue networks and feedback systems involved (Fig. 3.12) (Sonoda et al. 2016). Under this model, cold tolerance begins when a thermal stimulus activates thermosensory neurons, ASJ and ADL, at the head. The ASJ communicates with the gut via insulin and steroid hormones, gut induces changes in sperm gene expression, and the resulting alteration in sperm activity either directly or indirectly influences neuronal activity of the ASJ thermosensory neurons. Moreover, this model poses a hypothesis that cold tolerance could be a heritable phenotype given the involvement of germ cells. As such, multigenerational studies investigating the epigenetic consequences of cold tolerance are of current interest.

A recent study has revealed a sequence of interactions between neural circuit and gut function in cold tolerance (Motomura et al. 2022): ASJ thermosensory neurons activate PVQ interneurons located at the tail, which, in turn, stimu-

late RMG interneurons through glutamate-dependent neurotransmitter signaling. RMG interneurons subsequently facilitate the secretion of a neuropeptide encoded by *flp-7* from a neuron. FLR-7 is then received by a neuropeptide receptor encoded by *npr-22* in the gut, and NPR-22 activates the triglyceride lipase encoded by *atgl-1*, resulting in the decrement of gut fat. This reduction in gut fat leads to decreased cold tolerance and cold acclimation (Motomura et al. 2022).

3.8 Diversity of Nematode Cold Tolerance and Acclimation: Natural Variation and Insights

C. elegans wild-type strains exhibit diverse cold tolerance and acclimation traits across the globe (Okahata et al. 2016). For instance, the AB1 wild-type strain originally isolated in Australia demonstrates rapid acclimation to cooler temperatures, in contrast to the slower acclimation of the CB4856 strain from Hawaii. The genomic sequencing of *C. elegans* has facilitated the identification of DNA polymorphisms responsible for this variability. Employing next-generation DNA sequencing and SNPs mapping, Okahata et al. have successfully mapped the specific chromosomal region (chromosome I) where the pertinent polymorphism is located (Okahata et al. 2016).

Nematodes, which are among the most abundant creatures on earth, display a wide range of distribution patterns. Certain species of nematodes, such as *Meloidogyne hapla*, known as the northern root-knot nematode, show survival abilities across temperatures ranging between -15 and 27 °C within a year. Remarkably, freezing *M. hapla* during the J2 developmental stage significantly enhances its cold tolerance, similar to the cold acclimation observed in *C. elegans* (Wu et al. 2018). Notably, the DAW1, *Panagrolaimus* sp., known as an Antarctic nematode survives routine intracellular freezing (Wharton and Brown 1991; Wharton and Ferns 1995). Cold-acclimated DAW1 individuals exhibit substantially higher trehalose levels compared to their

non-acclimated counterparts. Furthermore, the cold exposure prompts strong upregulation of genes required for trehalose synthesis (Seybold et al. 2017).

Parasitic nematodes have evolved by altering combinations of genetic polymorphisms to adapt to the body temperature of the host organism and to allow them to survive outside the body. Illustrating extreme adaptation, the *Marshallagia marshalli*, a parasitic nematode, thrives even in freezing temperatures below -30 °C (Carlsson et al. 2013). Moreover, *Steinernema feltiae* and *Heterorhabditis bacteriophora*, entomopathogenic nematodes, exhibit survival at -13 °C through cryoprotective dehydration (Ali and Wharton 2013). Variations in proliferation rates and cold tolerance have been observed between parasitic and nonparasitic nematodes (Anderson and Coleman 1982). However, the molecular and biochemical mechanisms underlying these distinctions remain predominantly unexplored. The prospect of identifying genes responsible for the inherent variability in cold tolerance and acclimation among different strains of *C. elegans* offers the potential to uncover novel insights into the molecular and physiological aspects of adaptability to cold acclimation, as well as the evolutionary development of temperature adaptation.

3.9 Conclusion

Cold tolerance and temperature acclimation are crucial mechanisms for the survival and proliferation of organisms in the face of constantly changing environmental conditions. Recent molecular and genetic analyses have established that the cold tolerance and temperature acclimation of *C. elegans* is a valuable model for investigating the integration of neural function, stress-responsive genes, and networks of various tissues. The feedback system between sperm and neurons serves as an illustrative example of the new insight. The advancements in bioimaging and optogenetics have also greatly contributed to elucidation of neuronal functions underlying the cold tolerance at the physiological level. Although much remains to be uncovered, future research

endeavors are bound to unveil the underlying fundamental molecular mechanisms of temperature sensation in relation to tolerance and acclimation.

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Temperature-Dependent Activation of Thermosensitive Transient Receptor Potential Channels

Kunitoshi Uchida

Abstract

Temperature detection is essential for the survival and perpetuation of any species. Thermoreceptors in the skin sense the body temperature and also the temperatures of the ambient air and the objects. In 1997, Dr. David Julius and his colleagues found that a receptor expressed in small-diameter primary sensory neurons was activated by capsaicin (the pungent chemical in hot pepper). This receptor was also activated by temperature above 42 °C. That was the first time that a thermal receptor in primary sensory neurons has been identified. This receptor is named transient receptor potential vanilloid 1 (TRPV1). Now, 11 thermosensitive TRP channels are known. In this chapter, we summarize the reports and analyze thermosensitive TRP channels in a variety of ways to clarify the activation mechanisms by which temperature changes are sensed.

Keywords

TRP channel · Thermosensitivity · Cation channel · Patch-clamp recording · 10-degree

temperature coefficient · Lipid bilayer membrane

4.1 Introduction

Temperature detection is essential for the survival and perpetuation of all species. In particular, temperature sensation is essential for maintaining body temperature and the avoidance of injury. Thermoreceptors in the skin sense the temperature of the ambient air and the objects that we touch. Thermal nociception is excited by extremes of temperature as well as by strong mechanical stimuli. One group of thermal nociceptors is excited by noxious heat (temperature above 45 °C). A second group responds to noxious cold (that which cools the skin below 5 °C). Innocuous temperature changes can be detected in skin and/or primary sensory neurons. Primary sensory neurons detect temperature information and transmit it to the brain. One such innervates the skin or viscera and the other projects to the dorsal horn of the spinal cord. Somatosensory temperature information is transmitted to the thalamus via anterolateral system. Axons that cross the midline in the medulla to form the medial lemniscus are termed second-order neurons. As these axons ascend through the brainstem, they shift laterally, joining fibers of the spinothalamic tract in the midbrain. Then, the third-order neurons innervated to the primary

K. Uchida (✉)
Division of Environmental and Life Sciences, School
of Food and Nutritional Sciences, University of
Shizuoka, Shizuoka, Japan
e-mail: kuchida@u-shizuoka-ken.ac.jp

somatosensory area of the cerebral cortex. Temperature information is also transmitted to the preoptic area (POA) of the hypothalamus, which is a center for unconscious autonomic control of body temperature through the lateral parabrachial nucleus (LPB). Temperature information inputs from dorsal horn neurons cause direct activation of POA-projecting LPB neurons (Nakamura 2011). In the brain, it is thought that changes in internal temperature are detected by specialized thermoreceptors located throughout the body core, including the viscera, brain, and spinal cord and the most sensitive site is the POA. Localized heating or cooling could induce feedback responses to maintain the narrow range of the desired body temperature.

Primary sensory neurons are divided into three types based on the diameter of the fiber and cell body, and the conduction velocity of action potential. Specifically, (1) A β , 6–12 μm of fiber diameter and 36–72 m/s conduction velocity; (2) A δ , 1–6 μm of fiber diameter and 4–36 m/s of conduction velocity; (3) C, 0.2–1.5 μm of fiber diameter and 0.4–2 m/s of conduction velocity. Thermosensation is thought to be involved in the A δ and C fibers supporting pain and itch sensations. It is likely that Ruffini corpuscles also act as thermoreceptors that respond for a prolonged period; so in the case of deep burns, pain will continue, as these receptors will not be burned off (Kenshalo and Bergen 1975). The end-bulbs of Krause are also thought to be thermoreceptors sensitive to cold and activated by temperatures less than 20 °C (Kent 1998). The findings of the

expression and functions of thermosensor in A δ and C fibers can conclude that free nerve ending of A δ and C fiber are major contributor for temperature sensing.

In 1997, Dr. David Julius and colleagues found that a receptor expressed in small-diameter primary sensory neurons was activated by capsaicin (the source of pungent flavor in hot peppers), and they named this receptor the vanilloid receptor 1 (VR-1) (Caterina et al. 1997). They also found that VR-1 was also activated by temperatures above 42 °C, the first finding of a thermal receptor in primary sensory neurons. Given that VR-1 belongs to the transient receptor potential superfamily, it was called TRP vanilloid 1 (TRPV1). Since that initial discovery, many researchers have found the ion channels have thermosensitivity. Many of them belong to the TRP channel family (Table 4.1). Other channels such as TMEM16 (anoctamine, a Ca²⁺-activated Cl⁻ channel), TREK-1 (a two-pore domain K⁺ channel), and DEGyENaC (degenerin/epithelial Na⁺ channel) have since been revealed. Evidence that ion channels act in temperature sensation is supported by their high 10 °C temperature coefficient (Q_{10}). This parameter describes the rate of change of a chemical or biological reaction when temperature is increased by 10 °C. The rate of any reaction is affected by temperature and usually results in a doubling of the reaction rate. When this happens, the Q_{10} value for the reaction is 2. On the other hand, thermosensitive ion channels exhibited much higher Q_{10} values than 10.

Table 4.1 Properties of thermosensitive TRP channels

	Temperature threshold	Tissue distribution	Other stimuli
TRPV1	>42 °C	Sensory neuron, brain, skin	Capsaicin, proton, sanshool, allacin, camphor, resiniferatoxin, vanillotoxin, 2-APB, propofol, anandamide, arachidonic acid metabolic products (by lipoxygenases), NO, extracellular cation
TRPV2	>52 °C	Sensory neuron, brain, spinal cord, lung, liver, spleen, colon, heart, immunocyte	Probenecid, 2-APB, cannabidiol, mechanical stimulation
TRPV3	>32 °C	Skin, sensory neuron, brain, spinal cord, stomach, colon	Camphor, carvacrol, menthol, eugenol, thymol, 2-APB
TRPV4	>34 °C	Skin, sensory neuron, brain, kidney, lung, inner ear, bladder	4 α -PDD, bisandrographolide, citric acid, arachidonic acid metabolic products (by epoxigenases), anandamide, hypoxosmolality, mechanical stimulation (cyclic) /ADP ribose, hydrogen peroxide, intracellular Ca ²⁺
TRPM2	>36 °C	Brain, immunocyte, pancreas, etc.	Ca ²⁺ store depletion, pregnenolone sulfate, nifedipine, clotrimazole
TRPM3	Warm—heat	Brain, sensory neuron, pancreas, eye	Intracellular Ca ²⁺
TRPM4	Warm	Heart, liver, immunocyte, etc.	Intracellular Ca ²⁺
TRPM5	Warm	Taste cell	Intracellular Ca ²⁺ , steviol glycosides
TRPM8	<26 °C	Sensory neuron	Menthol, icilin, eucalyptol
TRPC5	Cold	Brain, sensory neuron, liver, heart, kidney	G _{q/11} -coupled receptors, diacylglycerol, Gd ³⁺
TRPA1	<17 °C	Sensory neuron, inner cell	Allyl isothiocyanate, carvacrol, cinnamaldehyde, allacin, acrolein, icilin, tetrahydrocannabinol, menthol (10–100 μ M), formalin, hydrogen peroxide, alkalization, intracellular Ca ²⁺ , NSAIDs, anesthesia, octanol/ hexanol, etc.

4.2 TRP Channels

Most transient receptor potential (TRP) channels are nonselective cation channels. In mammals, TRP channels are divided into six subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin), and TRPA (ankyrin). TRP channels are expressed in many tissues and have a wide variety of physiological functions, including detection of various physical and chemical stimuli in vision, taste, olfaction, hearing, touch, and thermosensation (Venkatachalam and Montell 2007). Functions of TRP channels include the following: (1) sensors for external and internal environments, (2) signal transduction as a signal integrator, and (3) cation transport. The gene encoding the capsaicin receptor as a noxious heat sensor, which is now called TRPV1, was isolated from a rodent sensory neuron cDNA library in 1997 and was considered to be a breakthrough for research concerning temperature sensing (Caterina et al. 1997). To date, 11 of the TRP channels have been reported to have thermosensitivity, and hence are called thermosensitive TRP channels (Table 4.1). These channels belong to the TRPV, TRPM, TRPA, and TRPC subfamilies, and their temperature thresholds for activation are in the range of physiological temperatures. TRPV1, TRPV2, and TRPM3 are activated by elevated temperatures, whereas TRPM8, TRPA, 1 and TRPC5 are activated by cool and cold temperatures. TRPV3, TRPV4, TRPM2, TRPM4, and TRPM5 are activated by warm temperatures. Thermosensitive TRP channels usually function as “multimodal receptors” that respond to various chemical and physical stimuli. Activation of thermosensitive TRP channels by temperature changes and ligands cause membrane depolarization and increases in intracellular Ca^{2+} concentrations in many cell types. Thermosensitive TRP channels expressed in sensory neurons and skin can act as ambient temperature sensors. In addition, thermosensitive TRP channels are also expressed in tissues that are not exposed to dynamic temperature changes, suggesting that these channels can also sense internal temperatures or have physiological roles not related to

temperature detection. Recently, activation mechanisms of thermosensitive TRP channels by ligands have been clarified, and many experimental approaches have been applied to clarify the mechanisms by which TRP channels are activated by temperature changes.

4.2.1 TRPV1

As mentioned above, TRPV1 was the first channel that was discovered to act as a thermosensor in mammals. TRPV1 is expressed in small and middle diameter ($A\delta$ and C) primary sensory neurons and also in the brain (Caterina et al. 1997; Toth et al. 2005). To characterize the temperature-dependent current activation of ion channels, it is common to use an Arrhenius plot in which log values of current are plotted against reciprocal values of absolute temperature. The plot provides the precise temperature threshold at the flex point of the curve. With TRPV1, the threshold of heat-evoked activation is 42 °C and the Q_{10} value of TRPV1 is more than 25 in heterologous expression systems, i.e., $Q_{10} = 26$ (Vyklicky et al. 2003) and $Q_{10} = 37$ (Susankova et al. 2007). The temperature threshold for TRPV1 is thought to be a fixed value, but it can be shifted by other stimuli and modulators. Protein kinase C, a signal kinase downstream from Gq-coupled receptors, phosphorylates Ser residues at 502 and 800 of TRPV1 resulting in a shift of temperature threshold from 42 to 30–35 °C (Numazaki et al. 2002; Mandadi et al. 2004). Mg^{2+} substantially lowers the activation threshold temperature by interaction with the extracellular side of TRPV1 (Cao et al. 2014; Yang et al. 2014).

To understand the activating mechanisms of the TRPV1 channel, many mutational analyses have been conducted as well as analyses of chimeras formed by swapping domains between TRP channels. There are a few reports in which TRPV1 mutants have been impaired only in temperature-dependent activation. Replacement of the negative amino acid residue at E600 to a neutral or positive amino acid (E600Q and E600K) abolishes the activating current by protons and potentiates heat-induced activation of

TRPV1 (Jordt et al. 2000). A less severe perturbation to the turret region of the TRPV1 channel by deletion of its first ten amino acids (Δ G603-S612) substantially and specifically affects heat activation and not capsaicin activation of the channel (Cui et al. 2012). In addition, within the S6 inner helix domain of TRPV1 (Y666-G683), the mutations L669A, Y671A, and M677A elicit large effects on heat-dependent activation but only modest effects on capsaicin- and voltage-dependent activation. The heat response of S512Y mutant (abolished but the effect of capsaicin) remained unaffected (Sutton et al. 2005). Thus, it is possible that different mechanisms might underlie between capsaicin-induced and heat-induced activation of TRPV1.

It is possible that some of the residues differentially affect allosteric coupling between the activation site(s) (capsaicin-binding site, temperature or voltage sensor) and the movement of the gate (Susankova et al. 2007). The analysis of data from a TRPV1 chimera could support this concept. Exchanging the intracellular C-termini between TRPV1 and TRPM8 was found to switch their sensitivity to heat or cold, and Q727 and W752 in the C-terminus of TRPV1 is the minimum portion that is able to turn TRPM8 into a heat receptor (Brauchi et al. 2007). Based on the results from systematic chimeric analysis between TRPV1 and TRPV2, the intracellular segment between the ankyrin repeat domain and transmembrane domain 1 was proposed to serve as the thermal sensor for TRPV1 (Yao et al. 2011). Since the voltage-gated K^+ channel has no temperature-induced activating ability, Zhang et al. analyzed a chimera in which the pore region of a voltage-gated K^+ channel was replaced by the pore region of TRPV1. Moving the TRPV1 pore into the voltage-gated K^+ channel retained exquisite sensitivity to noxious heat (Zhang et al. 2018). On the other hand, some reports demonstrated that the TRPV1 S1-S4 membrane domain, which is involved in chemical activation, participates in the temperature-dependent activation of TRPV1 based upon NMR analysis. This domain undergoes a non-denaturing temperature-dependent transition with a high thermosensitivity (Sosa-Pagan et al. 2017; Kim et al. 2020).

There are alternative mechanisms of heat-evoked activation of TRPV1. For example, other molecules may indirectly activate TRPV1. Patwardhan et al. revealed that oxidized linoleic acid metabolites 9- and 13-hydroxyoctadecadienoic acid (9- and 13-HODE) are formed in mouse and rat skin biopsies by exposure to noxious heat. Both 9- and 13-HODE and their metabolites, 9- and 13-oxoODE, activated TRPV1 and therefore constitute a family of endogenous TRPV1 agonists (Patwardhan et al. 2010). In contrast, based on the results from single channel analysis, intracellular molecules are not necessary to activate TRPV1 by heat. For example, heat-induced TRPV1 channel opening was observed in excised patch membrane in an inside-out configuration (Tominaga et al. 1998). In addition, heat-induced activating currents were also observed in artificial lipid bilayer membranes with purified TRPV1 protein (Cao et al. 2013). The Q_{10} value of activating current was 20 in a lipid bilayer membrane with purified TRPV1 protein. In addition, the activating temperature threshold of TRPV1 in an artificial lipid bilayer was 43 °C (Cao et al. 2013), and that was similar to that measured with patch-clamp recording with heterologous expression of TRPV1 to mammalian cells. Sun et al. also showed that TRPV1 protein exhibited heat-evoked channel opening in a planar lipid bilayer. Moreover, the Q_{10} value of open probability was 18 and the Q_{10} value of the conductance of TRPV1 was about 2 (Sun and Zakharian 2015). Temperature-induced currents of TRPV1 are caused by channel opening, but not by conductance. Cholesterol depletion by methyl- β -cyclodextrin caused pore dilation of the TRPV1 channel, but did not affect heat-induced activation of TRPV1 (Jansson et al. 2013). Since they used lipid membranes consisting of POPE, POPC, and/or POPG, and cholesterol was not contained in those artificial bilayer lipid membrane, cholesterol might not be essential for heat-induced activation of TRPV1.

From the structural analysis of TRPV1 by Cryo-EM, a proposed mechanism for vanilloid agonist action has been developed (Gao et al.

2016). The cofactor phosphatidylinositol binds to the vanilloid pocket to stabilize the channel in its closed state. A vanilloid agonist can displace phosphatidylinositol to facilitate formation of a salt bridge between R557 and E570, consequently pulling the S4–S5 linker away from the channel's central axis to open the lower gate. Heat may open the channel through a similar mechanism involving thermal displacement of resident phosphatidylinositides (Gao et al. 2016). The report that phosphatidylinositol negatively regulates TRPV1 activation can support this concept (Cao et al. 2013). On the other hand, phosphatidylinositides are essential for TRPV1 activation by heat as well as capsaicin (Lukacs et al. 2013; Sun and Zakharian 2015).

Like capsaicin, heat-induced desensitization also occurs in TRPV1. However, its mechanism is different from capsaicin-induced desensitization. Ca^{2+} -dependent processes are involved in capsaicin-induced desensitization of TRPV1, but heat-induced desensitization is observed even in the absence of extracellular Ca^{2+} (Joseph et al. 2013). From structural comparisons of TRPV1 and TRPV3, it was found that the C-terminal domain of TRPV1 contains two distinct segments that differentially regulate capsaicin- and heat-induced activation and desensitization (Joseph et al. 2013). In addition, temperature-sensitive interaction between the N- and C-terminal domains of mouse TRPV1 drives a conformational rearrangement in the pore leading to heat-induced desensitization (Luo et al. 2019). Mg^{2+} and Ba^{2+} cause a Ca^{2+} -independent desensitization that specifically prevents heat-induced channel activation, while capsaicin-induced desensitization of TRPV1 is Ca^{2+} -dependent (Cao et al. 2014).

4.2.2 TRPV2

TRPV2 is activated by higher temperatures with a threshold of 52 °C. It is expressed in myelinated A β - and A δ -fibers in dorsal root ganglia (Caterina et al. 1999). Whereas TRPV2 is thought to be involved in the detection of a much higher range of noxious heat, TRPV2KO mice did not show

phenotypes related to heat sensation (Park et al. 2011). TRPV2 is also expressed in the brain, spinal cord, colon, heart, immunocytes, and adipose tissue. Heat activation of the channel exhibits strong use-dependence. That is, prior heat activation can profoundly alter its subsequent temperature responsiveness, causing decreases in both temperature activation threshold and amplitude of temperature-dependence activation (Liu and Qin 2016).

While rodent orthologs of TRPV2 are heat sensitive, human TRPV2 is heat-insensitive (Neeper et al. 2007). Oxidation of methionine residues activates the high-threshold heat-sensitive ion channel TRPV2. The threshold for activation dropped from 50 to 37 °C after methionine oxidation (Fricke et al. 2019). Furthermore, methionine oxidation causes heat (~50 °C)-induced activation in heterologous expression system of hTRPV2.

4.2.3 TRPV3

TRPV3 is activated by warm temperatures. The activating temperature threshold of TRPV3 is 31 °C, which is close to the threshold for warm-sensitive neurons. The Q_{10} of the current induced by elevating temperature is in the range 17–22 (Xu et al. 2002). TRPV3 is expressed in skin, brain, spinal cord, stomach, and colon. Warm temperatures may be detected by TRPV3 expressed in skin keratinocytes. It transmits signals to primary sensory neurons by releasing ATP (Mandadi et al. 2009). Another candidate for transmission is NO; nitrite and TRPV3 are required for normal innocuous temperature behaviors (Miyamoto et al. 2011). TRPV3 knockout mice showed strong deficits in responses to innocuous from room temperature to 35 °C and noxious heat (Moqrich et al. 2005). In contrast, a later study showed that the effect of TRPV3 knockout on thermosensing was dependent on the background strain used and neither TRPV3 nor TRPV4 appeared to be involved in noxious heat sensation (Huang et al. 2011).

Temperature-induced TRPV3 opening can be observed in an outside-out configuration (Xu et al. 2002). Moreover, temperature-dependent

opening was also observed at 32 °C in lipid bilayer membranes of DPPC with purified TRPV3 protein (Kol et al. 2013), indicating that intracellular molecules are not necessary to activate TRPV3 by heat. Analysis of TRPV3 mutants demonstrated the involvement of the N-terminus with the sixth transmembrane domain in temperature-dependent activation of TRPV3. Five single-point mutations located in the putative sixth transmembrane domain of TRPV3 exhibited specific abolishment of heat-activation without effects on chemically induced activation or voltage-dependency (Grandl et al. 2008). TRPV3 temperature sensitivity can be highly dynamic and use-dependent. Upon initial activation, the channel exhibits a high-temperature threshold in a noxious temperature range above 50 °C. Activation by warm temperature was observed in response to second activation. The membrane-proximal domain on the N terminus, located between the ankyrin repeats and the first transmembrane segment, was involved in this use-dependency (Liu and Qin 2017).

4.2.4 TRPV4

TRPV4 is activated by warm temperatures. The activating temperature threshold of TRPV4 was 34 °C, and the Q_{10} value of temperature-dependent current was >10 (Guler et al. 2002; Watanabe et al. 2002). TRPV4 is predominantly expressed in the epithelial cells and also in peripheral neurons and the brain (Alessandri-Haber et al. 2003; Shibasaki et al. 2007). Some data from TRPV4 knockout mice indicate that TRPV4 can contribute to the detection of warm temperatures in vivo (Todaka et al. 2004; Lee et al. 2005). In contrast, a study showed that neither TRPV3 nor TRPV4 appeared to be involved in noxious heat sensation (Huang et al. 2011).

Temperature-evoked channel opening was observed in cell-attached recording but not in excised inside-out recording (Watanabe et al. 2002). Cell swelling activates TRPV4 by means of the PLA2-dependent formation of arachidonic acids, and its subsequent conversion to

5',6'-epoxyeicosatrienoic acid by means of a cytochrome P450 epoxygenase-dependent pathway. Heat-induced activation of TRPV4 is achieved through PLA2- and a cytochrome P450 epoxygenase-independent pathway (Vriens et al. 2004). Mutation of Y555 within transmembrane domain 3 impairs TRPV4 activation by heat (Vriens et al. 2004, 2007). The tyrosine residue within the N-terminus of TRPV4 has a crucial role in activation by heat, mechanical stress, and hypotonic cell swelling, but not by synthetic ligands (Wegierski et al. 2009). The reported lack of activation was fully recovered in the presence of PIP₂ (Garcia-Elias et al. 2013).

4.2.5 TRPM8

In 2002, Mckemy and colleagues characterized and cloned a menthol receptor from trigeminal sensory neurons. It was also activated by thermal stimuli in the cool to cold range (McKemy et al. 2002). Based on the analysis of TRPM8 knockout mice, TRPM8 is the principal detector of environmental cold in vivo (Bautista et al. 2007; Colburn et al. 2007).

The activating temperature threshold of TRPM8 is 26 °C. The Q_{10} value of current is 24, and the unitary conductance is 60 pS at 10 °C and 75 pS at 30 °C (Brauchi et al. 2004). Unlike other TRP channels, the activating temperature threshold of TRPM8 is relative. An increase in the exposed temperature from 30 to 40 °C increased the temperature thresholds for TRPM8 activation from 28 to 34 °C in heterologous expression systems of TRPM8. In addition, ambient temperature did not affect the temperature threshold of TRPM8 in the absence of PIP₂ (Fujita et al. 2013).

Like TRPV1, the gating mechanisms of some thermosensitive TRP channels could be explained using an allosteric mechanism for both voltage- and temperature-dependence (Brauchi et al. 2004). This concept has been thoroughly discussed in regard to TRPM8 (reviewed by Voets et al. (2007)). The C-terminus of TRPV1 confers heat-dependent regulation on the TRPM8 pore module, whereas the TRPM8 cytosolic module confers cold-depen-

dent regulation on the TRPV1 pore module. TRPM8 has a modular C-terminal domain that is able to determine channel phenotype regarding temperature sensitivity (Brauchi et al. 2006; Diaz-Franulic et al. 2020). Another possible candidate for the cold-sensitive domain is the pore loop domain of TRPM8 (Pertusa et al. 2018).

Cold-induced currents are also observed in lipid bilayer membranes with purified TRPM8 protein (Zakharian et al. 2010). The Q_{10} value of open probability is 40, and the Q_{10} value of conductance is 3. The temperature activating threshold of purified TRPM8 protein is between 18 and 21 °C, which is lower than that in a heterologous expression system. Phosphatidylinositides are necessary for activation of TRPM8 by not only cooling but also menthol (Zakharian et al. 2010). Removing cholesterol by treatment with M- β CD increases the temperature threshold without changes in the Q_{10} value of current (Morenilla-Palao et al. 2009).

4.2.6 TRPA1

Another cold sensor was found in 2003. TRPA1 is expressed in primary sensory neurons and is activated by cold temperatures. The activation temperature threshold of TRPA1 is 17 °C, which is lower than that of TRPM8 (Story et al. 2003). TRPA1 is also activated by mustard oil (Bandell et al. 2004).

Although many reports have demonstrated that TRPA1 is involved in cold-induced nociception in vivo (Kwan et al. 2006; Karashima et al. 2009), whether TRPA1 itself is a cold sensor or not is still mystery. Mouse TRPA1 was not activated by cold temperatures (Nagata et al. 2005). One possible mechanism is that TRPA1 activation occurs via intracellular Ca^{2+} . Application of Ca^{2+} to the intracellular side of the membrane patch directly activated single-channel or macroscopic TRPA1 currents, and intracellular Ca^{2+} increases during cooling in heterologous systems (Doerner et al. 2007; Zurborg et al. 2007). On the other hand, Sawada et al. demonstrated that single-channel opening of mouse TRPA1 by cold was observed in inside-out con-

figuration and the activation temperature threshold was 17 °C (Sawada et al. 2007). Karashima et al. also demonstrated that cold-activated TRPA1 currents were readily detected using whole cell, cell-attached, and excised membrane patch clamp methods under complete Ca^{2+} -depleted conditions (Karashima et al. 2009). Temperature-dependency is thought to be different between species. Rodent TRPA1 was activated by cold, but human TRPA1 was not (Chen et al. 2013). The current–voltage relationships for rat and human TRPA1 were superimposable at either 24 or 8 °C, and unitary conductance at 8 °C was reduced for both channels. That is, for rat TRPA1, the unitary conductance was 93 pS at 24 °C and 35 pS at 8 °C. For human TRPA1, the unitary conductance was 87 pS at 24 °C and 30 pS at 8 °C. Cold-induced channel opening was also observed in lipid bilayer membranes with purified human TRPA1, indicating that TRPA1 is intrinsically cold-sensitive (Moparthy et al. 2014). The Q_{10} value of open probability is 40 and conductance is 50 pS at 10 °C and 46 pS at 20 °C.

The transmembrane domain 5 segment and the linker between transmembrane domain 5 and 6 determine cold sensitivity (Chen et al. 2013). The directionality of temperature activation in the mouse TRPA1 ion channel is inverted by single-point mutations in ankyrin repeat domain 6. Mutant channels have high temperature sensitivity of voltage-dependent activation, specifically of channel opening, which is reminiscent of other heat-activated TRP channels. All mutations are located in ankyrin repeat six, which identifies this domain as a sensitive modulator of thermal activation (Jabba et al. 2014). In contrast, human TRPA1 without its N-terminal ARD ($\Delta 1$ –688 hTRPA1) is intrinsically cold-sensitive, and cold-sensing properties of human TRPA1 reside outside the N-terminal ARD (Moparthy et al. 2014).

Moparthy et al. also demonstrate that TRPA1 also act as heat sensor (Moparthy et al. 2016). They showed that redox modification and ligands affect human TRPA1 cold and heat sensing properties in lipid bilayer and whole-cell patch-clamp recordings. The Q_{10} value of open probability by heat was 6 and the conductance did not depend on the temperature; the values were 35 pS at

25 °C and 26 pS at 40 °C. Heat-induced activation of mouse TRPA1 was also observed in heterologous expression system (Sinica et al. 2019).

Prolyl hydroxylase causes sensitization of TRPA1 to reactive oxygen species (ROS). Prolyl hydroxylase inhibition provides cold-insensitive human TRPA1 with ROS-dependent cold sensitivity (Miyake et al. 2016). Another possibility is that TRPA1 is modulated by the local lipid environment (Startek and Talavera 2020). After cooling to 12 °C, *Escherichia coli* LPS and allyl isothiocyanate induced robust Ca^{2+} responses in a heterologous expression system of mTRPA1. The amplitudes of the responses to these stimuli were significantly lower in cells treated with the cholesterol scavenger methyl β -cyclodextrin or with the sphingolipids hydrolyzer sphingomyelinase.

4.2.7 TRPM2

In 2006, TRPM2 was shown to be activated by warm-temperature sensor in the presence of cyclic adenosine 5'-diphosphate ribose (cADPR). The activation temperature threshold of TRPM2 with cADPR was 35 °C, and the Q_{10} value of TRPM2 current with ADPR, cADPR, and β -nicotinamide-adenine dinucleotide was 33–45 (Togashi et al. 2006). TRPM2 is expressed in immune cells and the pancreas. Recently, it was reported that TRPM2 was expressed in primary sensory neurons that detected ambient warm temperature (Vilar et al. 2020). Moreover, TRPM2 was expressed in a subpopulation of hypothalamic neurons in the preoptic area that is a center for sensing and regulation of body temperature (Song et al. 2016).

Reactive oxygen species have the ability to activate TRPM2 synergistically. For example, hydrogen peroxide-induced activation of TRPM2 was enhanced by temperature elevation (Kashio et al. 2012). The novel calmodulin-binding motif in its NudT9H domain contributes to the temperature sensitivity of TRPM2. Temperature-dependent activity with ADPR was abolished in the W1355A/I1368A mutant in which anchor residues involved in calmodulin binding were replaced by alanine (Gatkowski et al. 2019).

4.2.8 TRPM3

TRPM3 is expressed in primary sensory neurons and detects noxious heat. In heterologous expression system, the Q_{10} value of heat-evoked current of TRPM3 α 2 was 7.2 and there was no activation threshold from 25 to 40 °C (Vriens et al. 2011). In *Xenopus* oocyte recording, the Q_{10} of current for TRPM3 α 2 was 55 and the activation temperature threshold was 41 °C. Other variants TRPM3 γ 2 and γ 3 did not respond to heat (Uchida et al. 2019).

The weak temperature-dependent activation of TRPM3 α 2 was observed in artificial lipid bilayer membranes, and the Q_{10} value of open probability was 5 (Uchida et al. 2016). The Q_{10} value of conductance with a ligand was less than 2. Whereas PIP_2 was necessary for activation of TRPM3 α 2 by pregnenolone sulfate or by heat, PIP_2 was not necessary for activation by nifedipine (Uchida et al. 2016).

4.2.9 Other TRP Channels

TRPM4 and TRPM5 are Ca^{2+} -activated monovalent-cation permeable channels. In the presence of intracellular Ca^{2+} , activities of TRPM4 and TRPM5 are dramatically enhanced by temperature elevation from 15 to 35 °C. The Q_{10} value of TRPM5 current with intracellular Ca^{2+} was 10 and that of TRPM4 was 8 (Talavera et al. 2005).

TRPC5-positive neurons range in size from small- to medium- as well as large-diameter neurons. The activation temperature threshold of TRPC5 was 25 °C, and the Q_{10} value of activating current was 10. Unlike TRPM8, which inactivates over time at a constant temperature, TRPC5 did not inactivate significantly in persistent colder temperatures (Zimmermann et al. 2011).

4.3 Remarkable

To understand the activation mechanisms of thermosensitive TRP channels in response to temperature changes, electrophysiological studies have included the analysis of mutants and chimeric

channels, whole-cell and single-channel recordings, and lipid bilayer membranes. Moreover, analysis of the structure of TRP channels at near-atomic resolution by Cryo-EM has yielded new insights into gating mechanisms of TRP channels induced by chemicals. However, understanding the gating mechanisms of thermosensitive TRP channels is insufficient. Lipid bilayer membrane methods mimic natural cell membranes with minimal components, and they can illuminate the functions of TRP channel protein with greater precision because temperature affects all biomolecules. Molecular dynamics can also enhance our understanding of its mechanisms. Some thermosensitive TRP channels could be intrinsically temperature-gated ion channels, whereas others could be indirectly modulated by molecules that have temperature dependency. Not only TRP channels, but also other ion channels have been reported to have thermosensitivity. For example, anoctamin 1 and 2, Ca^{2+} -activated Cl^- channels exhibit heat-induced activation (Cho et al. 2012). The activation temperature threshold of anoctamin 1 is 44 °C and the Q_{10} value of the activating current is 19 without intracellular Ca^{2+} (Cho and Oh 2013). Leak K^+ channels have thermosensitivity and an increase in temperature from 22 to 42 °C stimulates the current amplitude of TREK-1 (Maingret et al. 2000), and TRAAK and TREK-1 control both cold and heat pain perception in mice (Noel et al. 2009). Cold temperature markedly increased the constitutive activities of DEG/ENaC (Askwith et al. 2001). One of the voltage-gated sodium channels is cold resistant, and Nav1.8 in nociceptors is critical for the perception of cold pain and pain in the cold (Zimmermann et al. 2007). Thus, many types of ion channels with low homology have thermosensitivity. Although we cannot deny the possible existence of shared thermosensitive modules in such channels, temperature-dependent gating mechanisms could diverge.

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Thermal Stress and Nuclear Transport

5

Shingo Kose, Yutaka Ogawa, and Naoko Imamoto

Abstract

Nuclear transport is the basis for the biological reaction of eukaryotic cells, as it is essential to coordinate nuclear and cytoplasmic events separated by nuclear envelope. Although we currently understand the basic molecular mechanisms of nuclear transport in detail, many unexplored areas remain. For example, it is believed that the regulations and biological functions of the nuclear transport receptors (NTRs) highlights the significance of the transport pathways in physiological contexts. However, physiological significance of multiple parallel transport pathways consisting of more than 20 NTRs is still poorly understood, because our knowledge of each pathway, regarding their substrate information or how they are differently regulated, is still limited. In this report, we describe studies showing how nuclear transport systems in general are affected by temperature rises, namely, thermal stress or heat stress. We will then focus on Importin α family members and unique transport factor Hikesi, because these two NTRs are affected in heat stress. Our present review will provide an additional view to

point out the importance of diversity of the nuclear transport pathways in eukaryotic cells.

Keywords

Nuclear transport · Importin · Importin α · Hikesi · HSP (Heat shock protein) · HSF1 · Heat stress · Stress granule

5.1 I. Introduction

The segregation of the nucleus and cytoplasm is a key feature of eukaryotic cells that enables the regulation of the complexity of cellular systems that evolved after karyogenesis (Dickmanns et al. 2015; Lusk and King 2017; Nguyen et al. 2019). The nuclear and cytoplasmic contents of each cell are precisely configured; however, they dynamically change upon extracellular stimulation, environmental change, or cellular state change. A number of studies have shown that the deregulation of nucleocytoplasmic partitioning of molecules affects cellular activities that are linked to numerous human diseases (Hung and Link 2011; Mor et al. 2014). For example, tumor suppressors (p53, p73, and FOXO1), antiapoptotic proteins (SIRT1, NPM, and AP1) and growth regulators (p21, p27, Rb, BRCA1, I κ B, and APC), which are localized in the nucleus in normal cells, are mislocalized to the cytoplasm in many cancer cells (Kau et al. 2004; Turner et al.

S. Kose (✉) · Y. Ogawa (✉) · N. Imamoto (✉)
Cellular Dynamics Laboratory, RIKEN Cluster for
Pioneering Research, Wako, Saitama, Japan
e-mail: skose@riken.jp; yogawa@riken.jp;
nimamoto@riken.jp

2012; Hill et al. 2014; Taylor et al. 2019). This translocation ablates their nuclear functions, allowing uncontrolled cell division. The localization defects of these molecules in cancer cells are often caused by upregulation of nuclear export machinery. Thus, the nuclear export mediators of these molecules are now recognized as potential therapeutic targets (Sun et al. 2013; Gravina et al. 2014; Senapedis et al. 2014; Fung and Chook 2014; Das et al. 2015; Matsuura 2016; Delman et al. 2019).

Another example is the mislocalization of nuclear RNA-binding proteins, such as TAR DNA-binding protein 43 (TDP-43), fused in sarcoma (FUS), which plays an important role in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) pathology, a devastating adult-onset neurodegenerative disorder (Boeynaems et al. 2016; Kim et al. 2017). These proteins are mislocalized from their normal nuclear compartment to the cytoplasm and undergo liquid-liquid phase separation (LLPS), forming TDP-43 liquid droplets or stress granules. As a consequence, the nuclear function of these RNA-binding proteins declines, and their cytoplasmic function is increased, which leads to cell toxicity that promotes ALS and FTLD pathology. The mislocalization of proteins is considered as a result of nucleocytoplasmic transport defects, which are the common features of ALS and FTLD caused by Cu/Zn superoxide dismutase (SOD1) mutation and C9orf72 hexanucleotide repeat expansion (HRE), which affect nuclear transport machinery functions (Zhang et al. 2015; Jovičić et al. 2015; Freibaum et al. 2015, for review see Guo et al. 2019; Hutten and Dormann 2020; Fallini et al. 2020). Furthermore, recent evidence shows a direct link between the formation of LLPS-formed cytoplasmic droplets and nucleocytoplasmic transport defects (Mikhaleva and Lemke 2018; Zhang et al. 2018; Gasset-Rosa et al. 2019; Aizawa et al. 2019): Nucleocytoplasmic transport defects not only cause the cytoplasmic accumulation of RNA-binding proteins that facilitate the formation of TDP-43 liquid droplets and stress granules, but also cause critical nucleocytoplasmic factors to

be incorporated into the LLPS, forming cytoplasmic droplets, which in turn facilitate the nuclear transport decline.

In this chapter, after providing an overview of nucleocytoplasmic transport systems, we will describe a series of studies on nuclear transport systems that are affected by thermal stress. The study embraces our work and, because of its uniqueness, we hope to provide new perspective, not only for use in the field of nucleocytoplasmic transport, but also for use in diverse fields of biological science.

5.2 II. Overview of Nucleocytoplasmic Transport

Nuclear-cytoplasmic compartmentalization is established by the double lipid bilayer of the nuclear envelope (NE) and selective permeability of nuclear pore complexes (NPCs) embedded in the NE (Cautain et al. 2015). All molecules must traverse NPCs to enter and exit the nucleus. NPCs possess a sieve-like barrier with a Stokes radius of ~2.6 nm that is mainly composed of phenylalanine-glycine (FG)-rich repeats and allow passive diffusion of proteins smaller than ~40 kDa (Mohr et al. 2009). The majority of larger proteins traverse the NPC barrier in an active and facilitated manner with the help of nuclear transport receptors (NTRs). All known NTRs show hydrophobic properties, which are important for interaction with the FG domain that reside in the central channel of NPCs (Cook et al. 2007; Schmidt and Görlich 2016; Christie et al. 2016). NTRs are continuously shuttled between the nucleus and cytoplasm by traversing NPCs in a facilitated manner.

The basic concept of the nuclear transport mechanism was established based on an understanding of how cargos accumulate into one compartment after moving from another compartment against a concentration gradient (Görlich and Kutay 1999; Weis 2003). An NTR binds cargos in one compartment and releases them in different compartments. In nuclear import, an NTR binds

cargo in the cytoplasm and releases it in the nucleus, while in nuclear export, NTR binds cargo in the nucleus and releases it in the cytoplasm. This process is coupled to the input of metabolic energy typically achieved by the small GTPase Ran system. RCC1, the chromatin-bound Ran guanine-nucleotide exchange factor (RanGEF), generates RanGTP in the nucleus, whereas cytoplasmic Ran GTPase-activating protein (RanGAP) generates RanGDP in the cytoplasm, creating a steep RanGTP gradient across the NE (Izaurre et al. 1997; Kaláb et al. 2006). RanGTP directly binds to NTRs and switches their affinity with cargos in a manner opposite of Importin and Exportin system.

Importins bind their cargo at low RanGTP levels in the cytoplasm, whereas Exportins recruit their cargo at high RanGTP levels in the nucleus. Importin cargo complexes traverse NPCs and then disassemble in the nucleus with increased RanGTP levels, whereas RanGTP-Exportin cargo complexes traverse NPCs and then disassemble in the cytoplasm where RanGTP is converted to RanGDP. The traversing of import and export complexes across the NPC is facilitated, while Importins and Exportins use metabolic energy supplied by the Ran GTPase system for directional transport of their cargos against the respective concentration gradients.

5.2.1 Nuclear Transport Pathways

For nuclear import and export, macromolecules generally require specific transport signals, namely, a nuclear localization signal (NLS) or a nuclear export signal (NES) that binds to appropriate NTRs to traverse NPCs. In human cells, it has been estimated that approximately 35% of proteins expressed in cells enter the nucleus at some point during the cell cycle (Kumar et al. 2002), and the majority of these proteins are considered to be carried by 20 members of Importin β family NTRs, which include Importins and Exportins. The most well-studied NLS is the classical basic NLS (cNLS: Dingwall and Laskey 1991; Lange et al. 2007), which binds Importin α

(Adam and Gerace 1991; Görlich et al. 1994; Imamoto et al. 1995b; Weis et al. 1995), which is an adaptor protein that binds exclusively to Importin β (Adam and Adam 1994; Imamoto et al. 1995a; Görlich et al. 1995; Radu et al. 1995) through its Importin β -binding (IBB) domain (Cingolani et al. 1999). There are seven Importin α proteins in human cells that show overlapping but distinct functions (Goldfarb et al. 2004; Miyamoto et al. 2016). The ternary complex of cNLS bound to the Importin α/β heterodimer traverses NPCs from the cytoplasm to the nucleus. In the nucleus, the cNLS cargo is released from the complex when RanGTP binds to Importin β , and Importin α binds to its export factor CAS (Exportin 2) (Kutay et al. 1997) complexed with RanGTP. Importin β and Importin α recycle back from the cytoplasm. The most well-studied NES is a leucine-rich NES (Fischer et al. 1995; Wen et al. 1995) that binds to CRM1 (Exportin 1) complexed with Ran GTP (Stade et al. 1997; Fornerod et al. 1997; Fukuda et al. 1997), and this ternary complex traverses the NPC from the nucleus to the cytoplasm. Other identified NLSs are PY-NLS with a C-terminal proline-tyrosine dipeptide for Transportin 1 and Transportin 2 (Lee et al. 2006), the SR domain for Transportin SR (Kataoka et al. 1999), the IK-NLS for yeast Kap121p (Kobayashi and Matsuura 2013), and the β -like Importin-binding (BIB) domain that binds to Importin 5, Importin 7, and Importin β (Jakel and Görlich 1998). Only a limited number of cargos have been identified for many other Importin family NTRs. On the other hand, certain NTRs do not belong to the Importin β family, such as NTF2 (Paschal and Gerace 1995) and Hkeshi (Kose et al. 2012), which are dedicated to carrying specific cargo, i.e., RanGTP and HSP70, respectively.

It is unclear why cells need to have such a large number of NTRs. The *S. cerevisiae* genome encodes 14 Importin β families, and the human genome encodes 20 Importin β families (Chook and Suel 2011; Kimura and Imamoto 2014). A higher degree of similarity is observed between family member orthologues in different species than between paralogs in the same species, sug-

gesting that each member of the Importin β family shows critical cell functions conserved among species (Quan et al. 2008). Although there is low sequence identity among Importin β family members (20% ~ 30% sequence similarity), they all show structural similarity called HEAT repeats. Studies focused on individual cargo types indicate that each Importin β family member recognizes a unique set of proteins or RNAs but with some redundancy. Family members show spatiotemporally different expression patterns during development, although the physiological significance of these patterns is not clear. Recent attempts to understand the cargo spectrum of Importin β family NTRs using proteomic approaches combined with different experimental setups (in vitro nuclear transport assay, selective inhibitors, proximity labeling, or affinity purification) have provided further information on the functions of NTRs based on their cargo information. For example, the cargo spectrum of CRM1 was explored in-depth, and the analysis revealed that the majority of CRM1 cargos show strong bias toward cytoplasmic localization, suggesting that CRM1 is essential for maintaining compartment identity through their active nuclear export and by back sorting a variety of proteins to the cytoplasm from the nucleus (Kirli et al. 2015). Comprehensive cargo analysis of 12 Importins (Kimura et al. 2017) and 16 NTRs (three Importin α family members, five Importins, three Exportins, and NTF2, NXT1, and NXT2; Mackmull et al. 2017) led to the identification of a significant number of new cargo candidates and showed that cargos of the same NTR are functionally related to one another and that the predominant protein groups in a cargo cohort differ among the NTRs, but there is also redundancy. Cargo candidates of Importin 13, which is known to mediate both nuclear import and export, were identified and validated intensively. Among the 255 cargo candidates identified, 117 proteins were suggested as potential export cargos, and 94 proteins among these potential export cargos were also exported by CRM1, suggesting that there is significant redundancy between these two NTRs (Baade et al. 2018; Baade and Kehlenbach 2019).

5.3 III. Ran-Dependent Transport System Under Heat Stress Conditions

The nuclear transport system is affected by various stressors. In many cases, it involves localization changes of Ran and Importin α 1 (KPNA2). Under oxidative and UV stress conditions, nuclear Ran leaks into the cytoplasm, and after removal of the stressors, the Ran gradient recovers (Czubryt et al. 2000; Miyamoto et al. 2004; Yasuda et al. 2006; Datta et al. 2014; Chatterjee and Paschal 2015). On the other hand, under hyperosmotic stress conditions, the Ran distribution is transiently disrupted, however, it recovers after 0.5–1 h period (Kelley and Paschal 2007). On the other hand, Importin α 1 (KPNA2) translocates into the nucleus under the oxidative and UV stress condition, whereas its localization is not affected under the hyperosmotic stress. Similar to these stressors, heat stress is known to cause the nuclear translocation of Importin α 1 (KPNA2) (Miyamoto et al. 2004; Furuta et al. 2004; Kodiha et al. 2008). In addition, Importin α 1 (KPNA2), Importin α 4 (KPNA3), and Importin α 5 (KPNA1) are partially incorporated in stress granules (Fujimura et al. 2010; Mahboubi et al. 2013). These localization changes of Ran and Importin α under exposure to stressors seem to cause the downregulation of Importin α / β -dependent import efficiencies.

5.3.1 Temperature Dependencies of the Nuclear Transport System

In the case of the heat stress response, however, the situation is somewhat different. Whereas studies of the heat stress response have been mainly conducted by comparing physiological conditions of 37 °C and severe stress conditions of 42 °C or higher temperature, which often induce cell cycle arrest or apoptosis, cellular responses under mild heat stress conditions in the fever range are different (Park et al. 2005). In particular, little is known about how the nuclear transport system is regulated at each temperature.

To answer this question, we recently reported precise threshold temperatures of the inhibition of the main Ran-dependent transport pathways, i.e., Importin α/β and Transportin 1-dependent import and CRM1 (Exportin 1)-dependent export (Ogawa and Imamoto 2018). First, we developed a new temperature shift assay method for the incubation of suspension cultured cells at various temperatures (37 ~ 45 °C) with high accuracy and reproductivity using a PCR thermal cycler. Then, we developed stable cell lines expressing a reporter gene for each nuclear transport pathway, i.e., GFP-fused Importin α/β or Transportin 1-dependent NLS (SV NLS or M9 NLS) or CRM1-dependent NES (Rev NES). Under physiological conditions, these NLS and NES reporters are localized in the nucleus and the cytoplasm, respectively. Importantly, as these reporters are small enough to pass through NPCs freely (Paine et al. 1975), when their Ran dependent active transport pathways are completely inhibited, they are localized equally throughout the cytoplasm and the nucleus. We applied these cell lines to the temperature shift assay and determined the threshold temperatures of transport inhibition. The temperature at which the ratio changed by 10% was defined as the threshold temperature. Interestingly, the threshold temperature for the inhibition of GFP-SV NLS was 41.5 °C whereas that of GFP-M9 NLS and GFP-Rev NES was 42.6 and 43.8 °C, respectively. Additionally, Hsc70 and Hsp70, members of the 70-kDa heat shock protein family, which are representatives

of nucleus-translocating proteins in response to temperature rise (Welch and Feramisco 1984), start their nuclear translocations at 39.9 °C. These results indicate that whole nuclear transport system responses to temperature rises with a step-wise mechanism (Fig. 5.1).

5.3.2 Temperature Sensitivity of Importin $\alpha 1$

Because even for the transport pathways under the control of the Ran gradient, the threshold temperatures are different, additional other control mechanisms are assumed to potentially exist. In fact, whereas some studies have reported the partial leakage of Ran from the nucleus under high temperatures (Furuta et al. 2004; Yasuda et al. 2006), our quantification analysis showed that this leakage is limited (Ogawa and Imamoto 2018). Similarly, the localization of Importin β was not affected. As the threshold temperature of GFP-SV NLS, which is transported by Importin α/β , is lower than those of other transport pathways, we focused on the nuclear translocation of Importin $\alpha 1$ according to temperature rise. Indeed, the threshold temperature of the nuclear translocation of endogenous Importin $\alpha 1$ was 41.5 °C and matched that of the inhibition of the Importin α/β -dependent nuclear import. Therefore, Importin $\alpha 1$ is assumed to contribute to inhibition at lower temperature than other Ran-dependent transport pathways. Additionally,

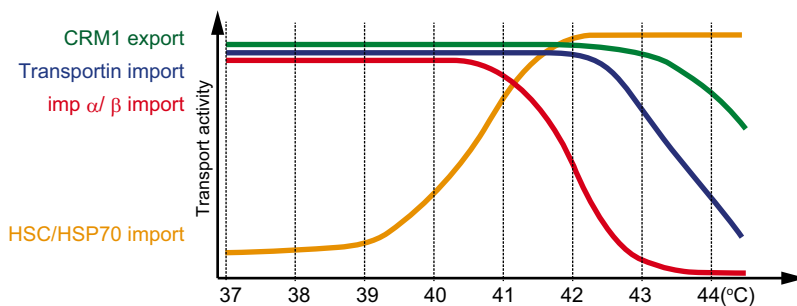


Fig. 5.1 Model for temperature-dependent transport modulations. As the environmental temperature rises, nuclear translocations of HSP70 occur at 39.9 °C followed by the inhibition of Importin α/β -dependent import

at 41.5 °C. Transportin-dependent import and CRM1-dependent export are maintained through higher temperatures (42.6 °C and 43.8 °C, respectively)

nuclear accumulated Importin α 1 is less mobile, and a large part of it is sequestered by binding to the intranuclear structures (Furuta et al. 2004; Yasuda et al. 2012; Ogawa and Imamoto 2018). Therefore, we hypothesized that Importin α 1 is more sensitive to temperature rises and, as a result, starts to be denatured at lower temperature than other transport factors. In fact, Importin α 1 preincubated at high temperatures showed inhibited *in vitro* binding with NLS substrates and reduced import efficiency.

In summary, the hypersensitivity to temperatures of Importin α 1 partially contributes to the regulation of the nuclear transport system in a stepwise response to temperature rises. This outcome implies that cells respond differently to various levels of stress. For instance, nucleocytoplasmic shuttling proteins bearing both Importin α 1/ β -dependent NLSs and CRM1-dependent NESs may change their localizations according to temperature rises (Fig. 5.2). These proteins may translocate into the cytoplasm at temperature ranging from 42 °C to 43 °C because Importin α 1/ β -dependent import is inhibited at this temperature, whereas CRM1-dependent export remains active. On the other hand, Transportin 1-dependent import is active up to 43 °C. Thus, the types of localization signals might specify the localization changes of proteins according to the extent of the heat stress.

Whereas little is known about the physiological roles of the multistep inhibition of Ran-dependent transport upon temperature rises, one possibility is that it is involved into the DNA damage response. Reportedly, Mre11, Rad50, and Nbs1, which are involved in double-strand break (DSB) repair, translocate from the nucleus to the cytoplasm via CRM1-dependent export pathway at 42–45 °C (Seno and Dynlacht 2004), whereas these proteins remain associated with the site of double-strand breaks in the nucleus at 41 °C (Krawczyk et al. 2011; Velichko et al. 2013). Interestingly, the nuclear localization and proper formation of the nuclear foci of Nbs1 were dependent on Importin α 1 (Tseng et al. 2005; Teng et al. 2006). Therefore, this localization control may be due to the dysfunction of Importin α 1 at temperatures higher than 42 °C and the thermostability of CRM1-dependent export.

5.3.3 Potential Transport Pathways Responding to Temperature Rises

As Importin β family consists of up to approximately 20 members depending on the species (Kimura and Imamoto 2014), the question remains: how do other transport factors respond to temperature rises? A Cellular thermal shift

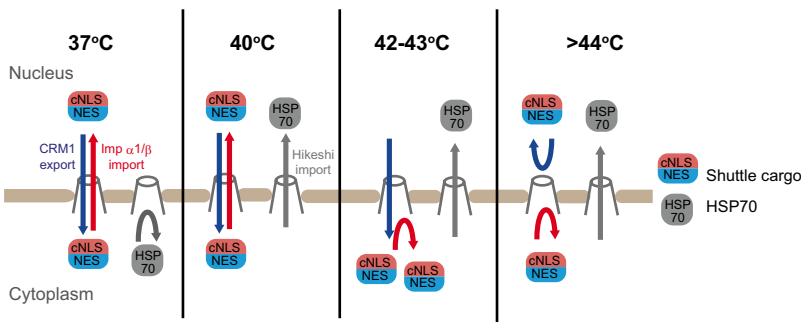
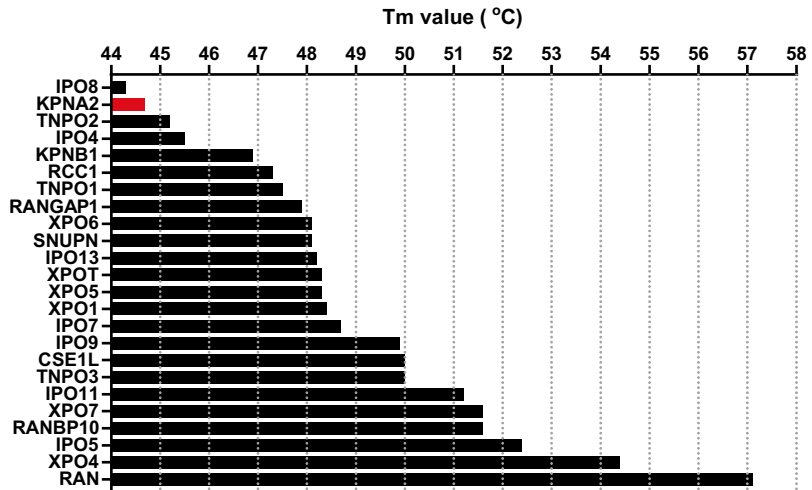


Fig. 5.2 Model for temperature-dependent localization change. The localizations of shuttle proteins bearing both classical basic NLS (cNLS) and leucine-rich NES are affected by the temperature sensitivities of both Importin α / β and CRM1-dependent transport pathways. At 37 °C, such shuttle proteins localize in both the cytoplasm and

the nucleus. In the range from approximately 42 °C to 43 °C, only Importin α / β -dependent pathway is inhibited, resulting in these proteins likely being excluded from the nucleus. At temperatures higher than 44 °C, both import and export pathways are inhibited. In contrast, the import of HSP70 is activated above 40 °C

Fig. 5.3 T_m values of nuclear transport factors. The T_m values of nuclear transport factors in intact cells were obtained from the data of Table_S2_GO_analysis (Savitski et al. 2014). The red bar shows the T_m value of Importin $\alpha 1$ (KPNA2)



assay (CETSA), which was recently developed (Martinez Molina et al. 2013), provides valuable clues to understanding the overall thermal stability of the whole nuclear transport system. CETSA is a method to quantify the amount of proteins in the soluble fraction of cell lysates heated to various temperatures using Western blotting or mass spectrometry. The denaturation temperature (T_m) of the target protein can be determined from changes in its amount in the soluble fraction according to temperature rises. Savitski et al. (2014) demonstrated comprehensive T_m values for 5299 proteins from the human K562 chronic myeloid leukemia cell line in combination with neutron-encoded isobaric mass tagging reagents (TMT) (Werner et al. 2014). Figure 5.3 shows the T_m values of Importin β family and Ran-related proteins, as measured in their experiments. These results imply that, similar to Importin α/β -dependent import, transport pathways depending on IPO8 (Importin 8), TNPO2 (Transportin 2), and IPO4 (Importin 4) are sensitive to temperature rises. A comprehensive understanding of the temperature-dependent nuclear transport system requires further investigations of, in particular, nuclear proteome at each temperature. However, it is still technically challenging because denatured proteins at each temperature should be taken into account.

5.4 IV. Heat Stress Response

Diverse cellular stresses, including the thermal stress, induce structural instability of folded proteins, leading to protein dysfunction and protein aggregation. To maintain proteins in a functional state, molecular chaperones need to survey and correct the structure of unfolded proteins. In general, molecular chaperones are upregulated by stress-inducible responses (reviewed in Lindquist 1986), and then those stress responses increase cellular protein-folding capacity, which adjusts protein homeostasis (proteostasis) and supports the recovery of cellular functions.

5.4.1 Activation of HSF1

Heat shock factor 1 (HSF1) is known to be the master transcription factor of the heat stress response in eukaryotes. Activation of HSF1 is regulated by multistep processes (reviewed in Anckar and Sistonen 2011). In response to elevated temperature, HSF1 recognizes a consensus heat shock element (HSE) DNA-binding site, consisting of extended sequence repeats of nGAAn (Kroeger and Morimoto 1994; Trinklein et al. 2004), and then activates the mRNA expression of many genes encoding molecular chaper-

ones. Since mammalian HSF1 possesses a potent bipartite NLS, HSF1 exists predominantly in the nucleus under normal conditions, presumably as an inactive monomer with low affinity for DNA (Mercier et al. 1999; Vujanac et al. 2005). In response to a variety of cellular stresses, such as heat shock, HSF1 is converted into a transcriptionally active homotrimer that can bind the promoter regions of heat shock genes (Sarge et al. 1993; Xiao et al. 1991).

Although detailed mechanisms for switching between the active and inactive forms of HSF1 remain obscure, evidence has shown that the HSF1 molecule itself senses elevated temperature. Increased temperature induces the unfolding of human HSF1 in the regulatory region, causing HSF1 trimerization and DNA-binding activity (Hentze et al. 2016). However, human HSF1 activation in different organisms occurs at different temperatures (Baler et al. 1993; Clos et al. 1993). Furthermore, the threshold temperature for HSF1 activation is different between organs in the same individual (Sarge et al. 1995; Gothard et al. 2003). Therefore, oligomerization and activation of HSF1 is likely to be regulated by multistep factors, such as several posttranslational modifications, including phosphorylation and protein interactors.

5.4.2 Nuclear Stress Granules

In response to heat stress, HSF1 rapidly forms granules in the nucleus, called nuclear stress granules (nSGs), nuclear stress bodies, or HSF1 foci (Sarge et al. 1993; Cotto et al. 1997; reviewed in Biamonti and Vourc'h 2010). These nSGs disappear gradually during the attenuation of the stress response. Interestingly, nSGs do not form at promoter regions of heat shock proteins; however, human HSF1 binds to noncoding satellite III repeat sequences at pericentric q11-q12 regions of human chromosome 9 (Jolly et al. 2004). Since the kinetics of the appearance of nSGs in heat-stressed cells are closely correlated with the transcriptional induction of heat shock genes, the formation of nSGs has been considered a marker of the heat shock response and HSF1 activation. However, single-cell-based analyses recently showed that

HSF1 foci dissolution rather than formation coincides with the transcriptional activation of HSF1 during the heat stress response and cell survival (Gaglia et al. 2020). In response to stress, nascent HSF1 foci are formed with dynamic properties via LLPS. However, the biochemical characterization of these HSF1 foci gradually changed during prolonged stress, and eventually, HSF1 is incorporated into indissoluble gel-like condensates. In these cells, activation of HSF1 is suppressed, leading to cellular apoptosis induced by the maladaptation to stress.

5.4.3 Stress Granules and Phase Separation During the Thermal Stress

Despite many studies on the heat stress response, how cells sense environmental temperature at the molecular level remains an important unsolved issue. However, recent studies on phase separation have suggested the possibility of a novel mechanism for sensing intracellular temperature changes (Wallace et al. 2015; Riback et al. 2017). Heat stress causes not only protein unfolding and aggregation but also translation inhibition of newly synthesized proteins and triggers the formation of cytoplasmic SG-containing proteins and mRNAs (reviewed in Wlozin and Ivanov 2019). A highly conserved poly(A)-binding protein (Pab1 in yeast; PABPC1 in humans) is a common component of SGs. Riback et al. revealed that yeast Pab1 undergoes phase separation in response to physiological changes in both temperature and pH to forms hydrogels and SGs (Riback et al. 2017). Notably, Pab1 mutations that interfere with phase separation inhibit cell fitness during prolonged stress, indicating that the process of Pab1 phase separation is adapted to cellular thermal stress.

5.4.4 Nuclear Import of HSP70 and Its Cochaperones Under Heat Stress Conditions

Cytosolic 70-kDa constitutive heat shock cognate protein and heat shock protein (Hsc70/

Hsp70: HSP70) belong to a major family of molecular chaperones for maintaining cellular proteostasis (reviewed in Kim et al. 2013; Rosenzweig et al. 2019). HSP70 interacts with unfolded or misfolded proteins, prevents their aggregation, and promotes refolding and solubilization of denatured proteins under both pathophysiological and proteotoxic stress conditions. The chaperone cycle of HSP70 is coupled with the ATPase cycle of HSP70, which is regulated by two cochaperones, J domain proteins (JDPs) (the HSP40 family), and nucleotide exchange factors (NEFs) (Fig. 5.4). The ATP-form of HSP70 has weak affinity for its substrates. JDPs facilitate the hydrolysis of ATP to ADP during HSP70 activation and thereby trigger the synergistic tight binding of HSP70 to its substrates. In turn, NEFs accelerate the release of ADP from HSP70 and then rebind ATP, leading to release and subsequent refolding of the substrate.

5.4.5 The Hikeshi-Mediated Nuclear Import of HSP70

HSP70 is predominantly localized in the cytoplasm. However, in response to thermal stress, HSP70 rapidly migrates from the cytoplasm into the nucleus and nucleolus under heat stress conditions (Pelham 1984; Velazquez and Lindquist 1984; Welch and Feramisco 1984), although Importin β -mediated nucleocytoplasmic transport is suppressed in response to heat stress (Furuta et al. 2004; Miyamoto et al. 2004; Kodiha et al. 2008, also see above). Previously, we identified an alternative import pathway of HSP70 under heat stress conditions via the nuclear import carrier Hikeshi (Kose et al. 2012). Hikeshi efficiently mediates the nuclear import of HSP70 under stress conditions. As far as we know, HSP70 (Hsc70/Hsp70) is a sole transport cargo of Hikeshi. Hikeshi does not belong to the

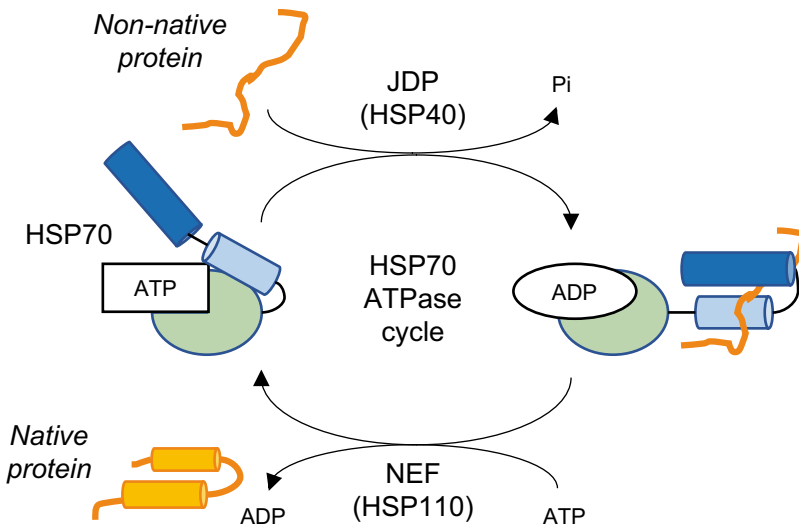


Fig. 5.4 Chaperone cycle of HSP70. The ATPase cycle of HSP70 is regulated by J-domain proteins (JDPs) (HSP40) and nucleotide exchange factors (NEFs) (HSP110/BAG). ATP-bound HSP70 is open for binding and refolding substrate proteins. JDPs stimulate the ATPase activity of HSP70 and facilitate the tight interactions between substrates and ADP-bound HSP70. NEFs induce the nucleotide exchange of HSP70, and then,

ADP-HSP70 is converted to ATP-HSP70 with low affinity for the substrate, resulting in substrate release and refolding. Interestingly, the interaction between Hikeshi and HSP70 also depends on the nucleotide state of HSP70. Hikeshi binds to ATP-HSP70 with high affinity and carries ATP-HSP70 into the nucleus. Hikeshi interacts with ADP-HSP70 with low affinity, possibly dissociating from HSP70

Importin β family. It is an evolutionarily conserved protein that was acquired in eukaryotic cell genomes (Kose et al. 2022).

All Importin β family members have a flexible structure containing HEAT repeats derived from huntingtin, elongation factor 3, the A subunit of protein phosphatase 2A and TOR1 (Andrade and Bork 1995, reviewed in Yoshimura and Hirano 2016) (Fig. 5.5). A single HEAT motif (approximately 40 amino acids in length) contains two antiparallel α -helices linked by a loop. The structural flexibility of the HEAT repeats plays a crucial role in molecular interactions during nucleocytoplasmic transport processes, including the recognition and release of transport cargos, FG-nucleoporins (FG-Nups), and RanGTP (reviewed in Christie et al. 2016).

Hikeshi does not contain HEAT repeats. The crystal structure of human Hikeshi suggests that Hikeshi forms an asymmetric homodimer induced by a linker region and has an intrinsically flexible C-terminal domain (CTD) (Song et al. 2015) (Fig. 5.6). Since the instability of Hikeshi dimer formation leads to disruption of the formation of a stable Hikeshi/HSP70 complex, the asymmetric dimer formation of Hikeshi

is thought to be important for the recognition of the ATP form of HSP70. The N-terminal domain (NTD) of Hikeshi contains hydrophobic pockets. Hydrophobic pocket mutations significantly decreased the NPC passage activity of Hikeshi, therefore these hydrophobic pockets in the NTD are likely to involve FG-Nup recognition. Interestingly, the extended loop (E-loop), specifically the Phe97 residue, in the NTD overlies the hydrophobic pocket, therefore intramolecular movement of the E-loop may play an important role in the regulatory recognition of FG-Nups and migration through the NPC.

Importin β family members interact with the Ran GTPase to regulate substrate recognition. In contrast to Importin β family molecules, Hikeshi does not interact with Ran (Kose et al. 2012) and utilizes another regulatory mechanism; Hikeshi-mediated transport is coupled to the ATPase cycle of HSP70. Since Hikeshi binds efficiently to the ATP form of HSP70, not to its ADP form, Hikeshi mediates the nuclear import of the ATP form of HSP70 more efficiently than ADP form of HSP70. Actually, cochaperone J domain protein HSP40 (DNAJB1, DNAJA1), which facilitates the ATP hydrolysis of HSP70, inhibits the binding of

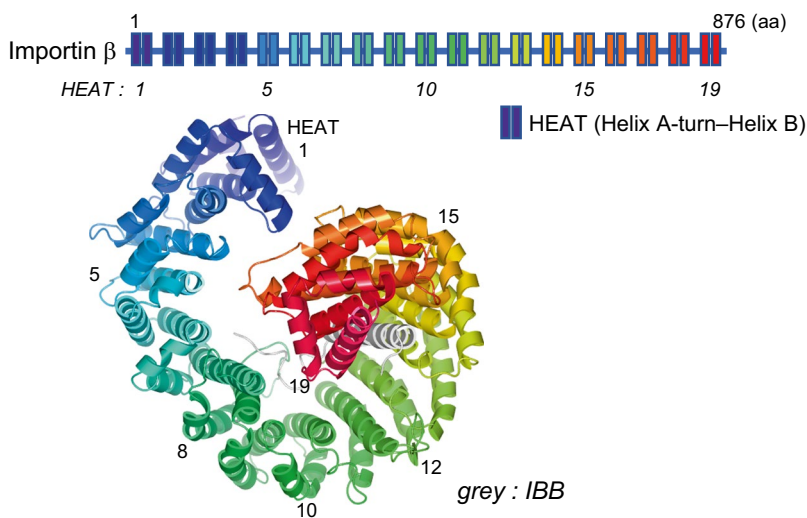


Fig. 5.5 Structure of human Importin β (PDB: 1QGK). All Importin β family members are composed of structures containing HEAT repeats of two antiparallel α -helices linked by a loop. The structural flexibility of HEAT repeats plays a crucial role in molecular interactions

during nucleocytoplasmic transport processes. The ribbon represents a human Importin β containing 19 HEAT repeats, colored from blue to red. The Importin β -binding (IBB) domain of Importin α is shown in grey

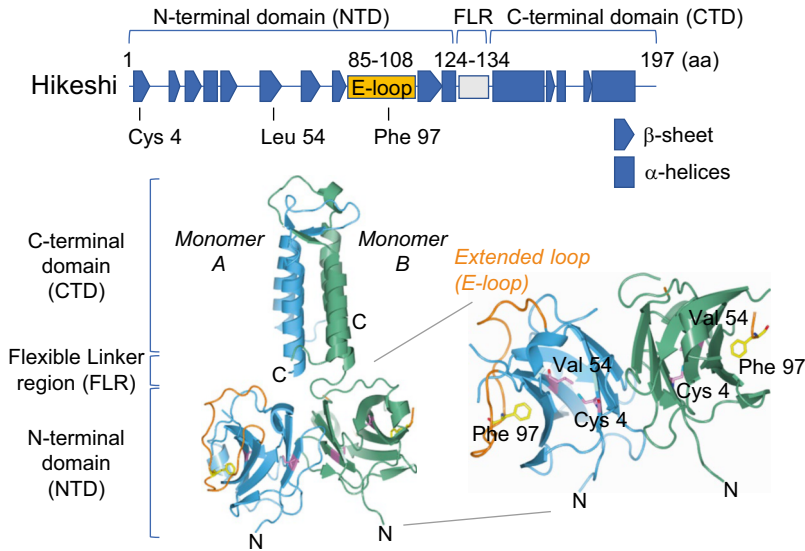


Fig. 5.6 An asymmetric dimer structure of human Hikeshi (PDB: 3WVZ). The Hikeshi monomer is composed of the N-terminal domain (NTD) with an extended loop (E-loop) and the C-terminal domain (CTD) linked by a flexible linker region (FLR). The ribbon represents an asymmetric dimer structure of human Hikeshi. Hikeshi forms an asymmetric homodimer via the FLR and an intrinsically flexible CTD and recognizes the ATP form of HSP70. The NTD contains hydrophobic pockets, which

presumably function in FG-Nup recognition. The Phe97 residue in the E-loop is fully buried in the hydrophobic pocket. Therefore, intramolecular movement of the E-loop may play an important role in the regulatory recognition of FG-Nups required for NPC passage. The homozygosity for a single missense mutation, p. Cys4Ser or p. Val54Leu, in the human Hikeshi/C11orf73 gene, causes leukodystrophies (Edvardson et al. 2016; Vasilescu et al. 2017)

Hikeshi to HSP70. On the basis of these observations, Hikeshi is thought to sense the whole allosteric conformational change of HSP70 and specifically recognize the ATP form of HSP70, which is assumed to be free of substrate.

5.4.6 The Physiological Significance of the Hikeshi-Mediated Nuclear Import Pathway

Depletion of Hikeshi inhibits the nuclear import of HSP70 under stress conditions and, as a result, it severely impairs recovery from cellular stress damage (Kose et al. 2012; Yanoma et al. 2017). Our previous results showed that exogenously expressed classical basic NLS-tagged HSP70 in Hikeshi-depleted cells significantly suppressed nuclear damage caused by thermal stress. Therefore, the nuclear functions of HSP70 systems are likely to be important for cell homeostasis and viability.

As mentioned above, Hikeshi plays an important role in counteracting proteotoxic stress. However, Hikeshi may function in various cellular processes under physiological conditions. Indeed, Hikeshi dysfunction in development causes lethal defects. Hikeshi-knockout mice are lethal just after birth (Fernández-Valdivia et al. 2006, our unpublished results). Homozygosity for a single missense mutation in the human Hikeshi (C11orf73) gene causes leukodystrophies, which are genetic white matter disorders affecting the formation or maintenance of myelin (Edvardson et al. 2016; Vasilescu et al. 2017). The fibroblasts of two patients harboring p. Cys4Ser or p. Val54Leu amino acid substitution expressed undetectable levels of the Hikeshi mutant protein, suggesting that these mutations induce an unstable Hikeshi protein. The nuclear accumulation of HSP70 in the fibroblasts of these patients in response to heat shock stress was inefficient.

Although dysfunctional Hikeshi leads to serious effects at both the cellular and organismal lev-

els, our knowledge of Hikeshi function is very limited. We have recently reported that the effects of Hikeshi depletion on cell viability after proteotoxic stress are different in two human cultured cell lines, HeLa (derived from human cervical carcinoma) and hTERT-RPE1 (human telomerase reverse transcriptase-immortalized retinal pigment epithelial 1) cell lines (Rahman et al. 2017). Hikeshi depletion in HeLa cells causes a decrease in resistance against proteotoxic stress, leading to apoptosis during recovery from stress. However, Hikeshi depletion increased the viability of hTERT-RPE1 cells after exposure to proteotoxic stress. Surprisingly, we found that, in response to stress, p21 activation following p53 activation is upregulated to a greater extent in Hikeshi-knockout hTERT-RPE1 cells than in wild-type hTERT-RPE1 cells, probably causing a positive antiapoptotic effect. Similar to its effect in hTERT-RPE1 cells, Hikeshi knockout also increased the survival rate of mouse embryonic fibroblasts (MEFs) after thermal stress was induced (our unpublished results). Furthermore, we recently reported that depletion of Hikeshi induces a reduction in nuclear HSP70 and the activation of HSF1 under nonstressed conditions and disturbs the heat shock response and nuclear proteostasis (Kose et al. 2022). These evidences clearly show the importance of nuclear function of HSP70.

Hikeshi, as well as stress-inducible HSP70, is not an essential gene in the cell. However, Hikeshi may affect diverse cellular and developmental functions through its interaction with the molecular chaperone HSP70 and thus contribute to proteostasis and tissue homeostasis. Further studies are needed to understand the details of the physiological functions and molecular mechanisms of Hikeshi.

5.4.7 Subcellular Distribution of Cochaperones of HSP70 Under the Heat Stress Conditions

HSP70 presumably functions in nuclear protein quality control (PQC) systems under heat stress conditions. In addition to HSP70, some of the

diverse members HSP70 cochaperones are known to be enriched in the nucleus upon exposure to proteotoxic stress (reviewed in Shibata and Morimoto 2014). DNAJA1, a major human J domain protein, is localized in the cytoplasm under both normal and heat stress conditions, whereas DNAJB1 accumulates to high levels in the nucleus and nucleolus under heat stress conditions (Hattori et al. 1993; Terada and Mori 2000). Although NEFs (HSPH1, HSPH2) are localized to both the nucleus and cytoplasm (Hageman et al. 2011), Hsp110 β , which is a splicing variant of HSPH1, accumulates in the nucleus under mild heat shock conditions (Saito et al. 2007).

The HSP70/HSP90 organizing protein HOP (or murine stress-inducible protein 1/mSTI1) is known to act as a scaffold protein to form the HSP70/HOP/HSP90 complex. Hop is predominantly localized in the cytoplasm. However, by treatment with leptomycin-B, which is an inhibitor of the nuclear export receptor CRM1, HOP accumulates in the nucleus, suggesting that HOP shuttles between the cytoplasm and nucleus (Longshaw et al. 2004). Furthermore, in response to heat stress, Hop accumulates in the nucleus (Daniel et al. 2008).

The cytosolic E3 ligase C-terminus of the HSP70-interacting protein CHIP mediates the ubiquitin-dependent degradation of substrates bound to HSP70 or HSP90 (Connell et al. 2001). CHIP also accumulates in the nucleus in response to heat stress and confers resistance against cellular stress by promoting HSF1 activity (Dai et al. 2003). Furthermore, CHIP negatively regulates the basal levels of HSP70 via the ubiquitylation of HSP70 during recovery to stress. Therefore, CHIP provides the mechanism for fine-tuning the protein levels of HSP70 to reflect the status of cellular proteostasis (Qian et al. 2006).

5.5 V. Conclusions and Perspectives

The molecular mechanism of nuclear transport has been described in great detail, and now we understand the structures of transport machinery

(NTR and NPC) at atomic or near-atomic resolution. Despite these advances, our knowledge of the significance of the diversity of transport pathways in eukaryotic cells remains very limited. Although attempts to understand the cargo spectrum are ongoing, contradictions have emerged; for example, the overlap of identified cargos between different studies is rather limited, probably due to diverse experimental setups, which indicates the complexity of the studies needed to process large fractions of the cellular proteome. There is increasing evidence showing that each NTR recognizes specific cargo groups, but there is significant redundancy for some NTRs. In addition, many analyses have indicated that every NTR can potentially recognize from 100 to 400 different cargos without consensus signals or structures. The determination of NTR cargos specificity is among the fundamental open issues that needs to be addressed.

Studies of the temperature sensitivities of transport systems have revealed the physiological significance of the diversity of transport pathways from different aspects when considering the complexity of cargo. Although one might think that the determined temperature thresholds that activate or inhibit transport activity among each transport pathway are subtle (i.e., the threshold for Importin α is 41.1°C, while the threshold for CRM1 is 43.8 °C), we recognize that these threshold temperatures are accurate (the error is within 0.1 °C) and highly reproducible. This stability suggests that “temperature” may become an important element for regulating the activity of independent transport pathways. Among the transport pathways examined, Importin- α - and Hikeshi-mediated transport pathways, whose activity is altered under “fever-ranged” mild heat stress conditions, are particularly interesting when considering the physiological significance of their temperature sensitivity. In addition, it is important to understand the molecular mechanisms of temperature sensitivities; what makes Importin α more temperature sensitive than other HEAT repeat structure proteins, and what is the temperature sensor for Hikeshi transport?

The discovery of Hikeshi has raised new problems. Although the mechanisms of proteostasis

in the cytosol and endoplasmic reticulum have been considerably elucidated, those in the nucleus remain relatively unclear (reviewed in Shibata and Morimoto 2014). Many molecular chaperones are located in or are relocated to the nucleus in response to various proteotoxic stresses, including heat stress. However, few nucleocytoplasmic transport receptors of these molecules have been identified. Further understanding of the regulated nucleocytoplasmic transport systems will increase our knowledge of nuclear protein quality control (PQC) systems, especially in higher eukaryotic organisms.

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Lipid-Mediated Mechanisms of Thermal Adaptation and Thermoregulatory Behavior in Animals

Kohjiro Nagao, Takuto Suito, Akira Murakami, and Masato Umeda

Abstract

Temperature affects a variety of cellular processes because the molecular motion of cellular constituents and the rate of biochemical reactions are sensitive to temperature changes. Thus, the adaptation to temperature is necessary to maintain cellular functions during temperature fluctuation, particularly in poikilothermic organisms. For a wide range of organisms, cellular lipid molecules play a pivotal role during thermal adaptation. Temperature changes affect the physicochemical properties of lipid molecules, resulting in the alteration of cell membrane-related functions and energy metabolism. Since the chemical structures of lipid molecules determine

their physicochemical properties and cellular functions, cellular lipids, particularly fatty acid-containing lipid molecules, are remodeled as a thermal adaptation response to compensate for the effects of temperature change. In this chapter, we first introduce the structure and biosynthetic pathway of fatty acid-containing lipid molecules, such as phospholipid and triacylglycerol, followed by a description of the cellular lipid-mediated mechanisms of thermal adaptation and thermoregulatory behavior in animals.

Keywords

Phospholipid · Triacylglycerol · Fatty acid desaturase · Polyunsaturated fatty acid

K. Nagao (✉)

Laboratory of Biophysical Chemistry, Kyoto Pharmaceutical University, Kyoto, Japan

Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto, Japan; e-mail: nagao-k@mb.kyoto-phu.ac.jp

T. Suito · A. Murakami

Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto, Japan

M. Umeda

Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto, Japan

HOLO BIO Co., Ltd., Kyoto, Japan

6.1 Structure and Biosynthesis of Fatty Acid-Containing Lipid Molecules

Temperature is one of the most pervasive environmental factors that affect the physiology, behavior, and evolution of organisms. When animals, plants, and other poikilothermic organisms are exposed to thermal changes, alterations in the chemical structures of cell membrane-forming lipids occur to maintain the cellular functions. Additionally, temperature changes also affect the metabolism of energy-storing lipids. In this sec-

tion, we describe the structure and biosynthetic pathway of fatty acid-containing lipid molecules, such as phospholipid and triacylglycerol, that play a pivotal role in the thermal adaptation of animals.

6.1.1 Physicochemical Properties of Cell Membrane

The cell membrane is a bilayer structure composed of membrane proteins and lipid molecules including phospholipids, glycolipids, and sterols. The chemical structures of lipids influence the physicochemical properties of cell membrane that affect the structure, activity, and localization of membrane proteins (van Meer et al. 2008). For example, the polar head groups and hydrophobic tails of phospholipids affect membrane fluidity or viscosity, which reflects the degree of alignment and mobility of the molecules comprising the lipid bilayer. The dynamics and packing of membrane lipids are also affected by temperature, thereby changes in ambient temperature strongly influence the membrane fluidity: the membrane fluidity increases when the environmental temperature is high and decreases when the temperature is low (Fig. 6.1a). Since the appropriate fluidity of cell membranes are required for a broad range of cellular functions, a variety of

remodeling events in structure as well as organization of membrane lipids occur as a thermal adaptation response.

Phospholipids are essential biological molecules that constitute the cell membranes. Phospholipids contain two fatty acids bound at the *sn*-1 and *sn*-2 positions of a glycerol backbone (Fig. 6.1b). These fatty acids vary in hydrocarbon chain length (ranging from 12 to 22) and the number and position of double bonds. The *sn*-3 position of the glycerol backbone of phospholipids contains a polar head group (e.g., phosphocholine, phosphoethanolamine, phosphoserine, phosphoinositol, or phosphoglycerol) attached by a phosphodiester linkage. Because the chemical structure of the phospholipid determines its physicochemical properties and cellular functions, cells can regulate diverse biological functions by modulating combinations of polar head group and fatty acyl chains in the phospholipid molecules (Harayama and Riezman 2018).

One of the most essential determinants for physicochemical properties of phospholipids is the number of double bonds in the fatty acyl chains (Stubbs and Smith 1984). In animals, most double bonds occur in a *cis* configuration that immobilizes the hydrocarbon chain and creates a bent structure. Fatty acids containing one double bond in hydrocarbon chain are known as mono-unsaturated fatty acids (MUFAs). One of the

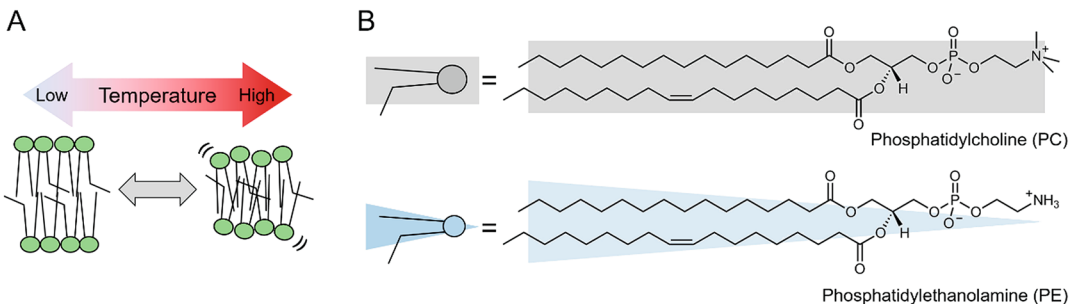


Fig. 6.1 Structure and physicochemical properties of phospholipids. (a). The effect of changes in temperature on the physicochemical properties of cell membranes. Membrane fluidity is increased when the environmental temperature is high because of the increased mobility of lipids and decreased when the environmental temperature is low. (b). Phosphatidylcholine (PC), the main phospholipid in eukaryotic cell membranes, is cylindrically shaped

and is preferred for the formation of bilayer membranes. By contrast, phosphatidylethanolamine (PE), which has a small polar head group, is cone-shaped and is unfavorable to form a stable bilayer structure. When the proportion of unsaturated fatty acids in the acyl chain of phospholipids increases, the structure of the lipid bilayer becomes disordered

most abundant MUFA in animal is oleic acid (C18:1 *n*-9) that contains one double bond at the $\Delta 9$ -position of the hydrocarbon chain. Fatty acids with two or more double bonds in hydrocarbon chain are known as polyunsaturated fatty acids (PUFAs). For example, linoleic acid (C18:2 *n*-6) has an 18-carbon length hydrocarbon chain with two double bonds at the $\Delta 9$ and $\Delta 12$ positions. The double bond content of the phospholipid acyl chains increases when organisms are exposed to cold temperatures. As described later, the control of membrane fluidity by changing the double bond content in the phospholipid acyl chains is critical to maintain cellular homeostasis and adapt to varying environmental temperatures (Los and Murata 2004).

6.1.2 Biosynthesis of MUFAs by $\Delta 9$ -Fatty Acid Desaturase

Extracellular fatty acids, such as those released from lipoprotein particles, can be incorporated into cells to control the fatty acid composition of membrane phospholipids (Spector 2001). Additionally, *de novo* lipid synthesis also has a considerable effect on the intracellular pool of fatty acids. Saturated fatty acids, such as palmitic acid (C16:0), can be synthesized from acetyl-CoA by reactions mediated by acetyl-CoA carboxylase and fatty acid synthase. Saturated fatty acids are converted to unsaturated fatty acids by introducing double bond(s). Fatty acid desaturases mediate the introduction of a double bond into the acyl chain of acyl-CoA and play a central role in the biosynthesis of unsaturated fatty acids (Los and Murata 1998). There are many fatty acid desaturases that vary in substrate specificity and preference for the site of double bond introduction. Among them, $\Delta 9$ -fatty acid desaturase ($\Delta 9$ -desaturase), which introduces a *cis* double bond at the $\Delta 9$ -position of acyl-CoA, is a rate-limiting enzyme in the biosynthesis of MUFAs, which are used as biosynthetic precursors of phospholipids, triacylglycerols, wax esters, cholesterol esters, and PUFAs (Ntambi and Miyazaki 2004). Because the content of unsaturated fatty acids in phospholipids affects membrane fluidity,

$\Delta 9$ -desaturase plays a critical role in the control of physicochemical properties of cell membrane. Additionally, since membrane fluidity is affected by environmental temperature, the expression of $\Delta 9$ -desaturase in a variety of organisms (e.g., bacteria, yeast, and fish) is induced at low temperatures as a thermal adaptation response (Aguilar et al. 1998; Nakagawa et al. 2002; Tiku et al. 1996).

6.1.3 Regulation of $\Delta 9$ -Desaturase Expression by Gene Transcription Control

In mammalian cells, the expression of $\Delta 9$ -desaturase stearoyl-CoA desaturase 1 (SCD1) is regulated at the transcriptional level and mediated by sterol regulatory element-binding protein, liver X receptor, and carbohydrate-responsive element-binding protein (ALJohani et al. 2017). Furthermore, the regulatory mechanism for the expression of Ole1, a $\Delta 9$ -desaturase in *Saccharomyces cerevisiae*, has been reported. The expression of Ole1 is regulated at the level of transcription and through mRNA stability (McDonough et al. 1992; Gonzalez and Martin 1996). The regulation of *Ole1* gene transcription is mediated via two *cis* elements in the *Ole1* promoter: the low-oxygen response element and the fatty acid-regulated sequence (Choi et al. 1996; Vasconcelles et al. 2001). The two endoplasmic reticulum (ER)-localized transcription factors, Mga2 and Spt23, positively regulate Ole1 expression, and the processing of Mga2 and Spt23 is controlled by the cellular unsaturated fatty acid content. Mga2 is necessary for the induction of Ole1 expression at low temperatures (Nakagawa et al. 2002), suggesting that the mechanism for sensing membrane fluidity is also involved in sensing the environmental temperature. In fact, in prokaryotic *Bacillus subtilis*, a model has been proposed in which the transmembrane protein, DesK, senses temperature changes through changes in membrane thickness and regulates the expression of $\Delta 9$ -desaturase Des (Cybulski et al. 2002). In the proposed model, the thickness of phospholipid bilayer

increases at low temperatures, which forces a cluster of polar amino acid residues of DesK into the hydrophobic environment of membrane. This conformational change in DesK activates its autokinase activity. Phosphorylated DesK then activates DesR, which is a transcriptional activator of Des.

6.1.4 Regulation of $\Delta 9$ -Desaturase Expression by Protein Degradation Control

Although the expression of $\Delta 9$ -desaturases is primarily regulated at the gene transcription level, these enzymes may also be regulated through protein degradation. The transmembrane helices, cytosolic loops, and C-terminal domains of $\Delta 9$ -desaturases are well conserved among eukaryotes including human, mouse, zebrafish, and yeast; however, the N-terminal domains of the $\Delta 9$ -desaturases exhibit low homology. Additionally, the N-terminal domain is not essential for the catalytic reaction of $\Delta 9$ -desaturases. Therefore, $\Delta 9$ -desaturases may have varying motifs for posttranslational control in the N-terminal domain.

SCD1 is a short-lived protein, and its half-life in the microsome fraction is just a few hours (Ozols 1997; Heinemann and Ozols 1998; Oshino and Sato 1972). Since SCD1 transcription is strictly controlled, its short life is advantageous for tuning SCD1 expression. Although full-length rat SCD1 protein has a half-life of a few hours, SCD1 mutants lacking the N-terminal domain (residues 1–26 or 1–44) are stable (Mziaut et al. 2000). Additionally, a fusion protein comprising the N-terminal 33-residue of SCD1 and green fluorescent protein has a short half-life (Mziaut et al. 2000), indicating that the N-terminal domain of SCD1 functions as a degradation signal. The N-terminal domain of SCD1 contains a PEST sequence, which is a tag for rapid protein degradation, which is enriched in proline, glutamic acid, serine, and threonine residues (Kato et al. 2006). Proteasome inhibitors suppress the degradation of SCD1 proteins (Kato et al. 2006). Furthermore, the polyubiquitination of SCD1

and the interaction between SCD1 and AAA-ATPase p97 are induced by proteasome inhibitor treatment (Kato et al. 2006). Thus, it is believed that the degradation of the SCD1 protein is mediated through the ER-associated degradation pathway.

The strict regulation of $\Delta 9$ -desaturase expression is important to maintain the appropriate physicochemical properties and functions of cell membranes. As described above, mechanisms for the regulation of the expression levels of $\Delta 9$ -desaturases have been elucidated. However, it remains unclear how the expression of $\Delta 9$ -desaturase is regulated in response to the changes in the unsaturation of membrane lipid acyl chains in animals. Although the degradation of SCD1 protein is not affected by the levels of unsaturated fatty acids (Kato et al. 2006), we found that the expression level of *Drosophila melanogaster* $\Delta 9$ -desaturase DESAT1 is post-translationally regulated in response to changes in fatty acid composition (Murakami et al. 2017). In *Drosophila* S2 cells, changes in DESAT1 activity directly affect the physicochemical properties of the cell membrane for the following reasons: (i) DESAT1 is the sole fatty acid desaturase in S2 cells (Murakami et al. 2017), (ii) *Drosophila* cannot de novo synthesize sterols from acetyl-CoA (Clark and Block 1959), (iii) the acyl chains of the phospholipid molecules in S2 cells contain only a trace amount of PUFAs (Matsuo et al. 2019). Therefore, S2 cells are useful for the analysis of the mechanisms that regulate the function and expression of $\Delta 9$ -desaturase. Similar to mammalian $\Delta 9$ -desaturases, DESAT1 protein is rapidly degraded in S2 cells and exhibits a protein half-life of 2 h (Murakami et al. 2017). Supplementing the culture medium with unsaturated fatty acids causes a rapid incorporation of added fatty acids into phospholipid molecules and enhances the degradation of DESAT1 protein. Moreover, $\Delta 9$ -desaturase inhibitors decrease the unsaturation of the acyl chain of the membrane phospholipids and suppress the degradation of DESAT1 protein. Thus, it is evident that changes in the composition of phos-

pholipid acyl chains are responsible for regulating DESAT1 expression.

Two sequential proline residues (Pro2 and Pro3) in the N-terminal sequence of DESAT1 protein were found to be responsible for the unsaturated fatty acid-dependent degradation of DESAT1 protein. Single alanine substitutions and a double alanine substitution in the second and third residues of DESAT1 protein abolished the responsiveness of DESAT1 protein degradation to the level of fatty acid unsaturation of phospholipids (Murakami et al. 2017). Thus, we have designated the N-terminal sequential two proline residues (Pro2 and Pro3) of DESAT1 protein as a di-proline motif, which is important for the control of DESAT1 protein degradation in response to changes in cellular unsaturated fatty acid levels. The two typical calpains of *Drosophila*, calpain A and calpain B, that contain Ca^{2+} - and lipid-binding motifs are involved in the di-proline motif-dependent degradation of DESAT1 protein. Comparing the sequences of $\Delta 9$ -desaturases among different species, most $\Delta 9$ -desaturases have a single proline residue at the second or third residue; however, DESAT1 contains the di-proline motif. *Drosophila* DESAT2, which differs from DESAT1 in physiological function and substrate preference, lacks the di-proline motif (Nagao et al. 2019). Introducing the Pro2-Pro3 sequence into the N-terminus of mouse SCD1 promotes the unsaturated fatty acid-dependent degradation of mouse SCD1 in S2 cells. This indicates that the di-proline motif also functions in the context of mammalian $\Delta 9$ -desaturases (Murakami et al. 2017).

6.1.5 Biosynthetic Pathway of PUFAs

Figure 6.2 shows the pathways for PUFA synthesis. Several fatty acid desaturases that introduce a double bond at $\Delta 5$, $\Delta 6$, $\Delta 9$, $\Delta 12$, and ω -3 positions of acyl chain are involved in PUFA synthesis. Although $\Delta 9$ -desaturases are present in most organisms (Tiku et al. 1996; Meesters et al. 1997; Stukey et al. 1989; Thiede et al. 1986),

fatty acid desaturases for PUFA synthesis are not present in all organisms. In plants and algae, MUFAs such as oleic acid can be further desaturated to form PUFAs including linoleic acid and α -linolenic acid (C18:3 *n*-3) (Harwood 1996). Additionally, some animals including *Caenorhabditis elegans* and the American cockroach are able to synthesize linoleic acid and α -linolenic acid de novo (Borgeson et al. 1990; Zhou et al. 2011).

PUFAs containing 20 or more carbons are known as long-chain PUFAs (LC-PUFAs). Particularly, arachidonic acid (C20:4 *n*-6; AA), eicosapentaenoic acid (C20:5 *n*-3; EPA), and docosahexaenoic acid (C22:6 *n*-3; DHA) play a pivotal role in a variety of physiological processes. Although linoleic acid and α -linolenic acid must be obtained from the diet or other environmental sources, mammals and many types of fish are able to synthesize *n*-6 LC-PUFAs (e.g., AA) and *n*-3 LC-PUFAs (e.g., EPA and DHA) from linoleic acid and α -linolenic acid, respectively. Linoleic acid and α -linolenic acid are converted by $\Delta 6$ -desaturase to γ -linolenic acid (C18:3 *n*-6) and stearidonic acid (C18:4 *n*-3), respectively. Next, C18:3 *n*-6 and C18:4 *n*-3 are converted by fatty acid elongase to dihomogamma-linolenic acid (C20:3 *n*-6) and eicosatetraenoic acid (C20:4 *n*-3), respectively. Finally, C20:3 *n*-6 and C20:4 *n*-3 are converted to AA and EPA by $\Delta 5$ -desaturase, respectively. In mammals, DHA is produced from EPA by four additional steps. EPA is elongated to docosapentaenoic acid (C22:5 *n*-3), which is then elongated to form tetracosapentaenoic acid (C24:5 *n*-3). Next, C24:5 *n*-3 is converted to tetracosahexaenoic acid (C24:6 *n*-3), and two carbons are removed by β -oxidation to form DHA. The same four steps are also used to synthesize C22:5 *n*-6 from AA. Moreover, $\Delta 4$ -desaturase, which directly converts C22:5 *n*-3 to DHA, has been identified in several plants and nematodes (Meesapyodsuk and Qiu 2012; Spychalla et al. 1997).

C. elegans represents a useful model for analyzing the function of PUFAs. Since *C. elegans* possesses $\Delta 5$, $\Delta 6$, $\Delta 9$, $\Delta 12$, and ω -3 desaturases, PUFAs can be synthesized de novo from saturated fatty acids. Mutant strains of fatty acid

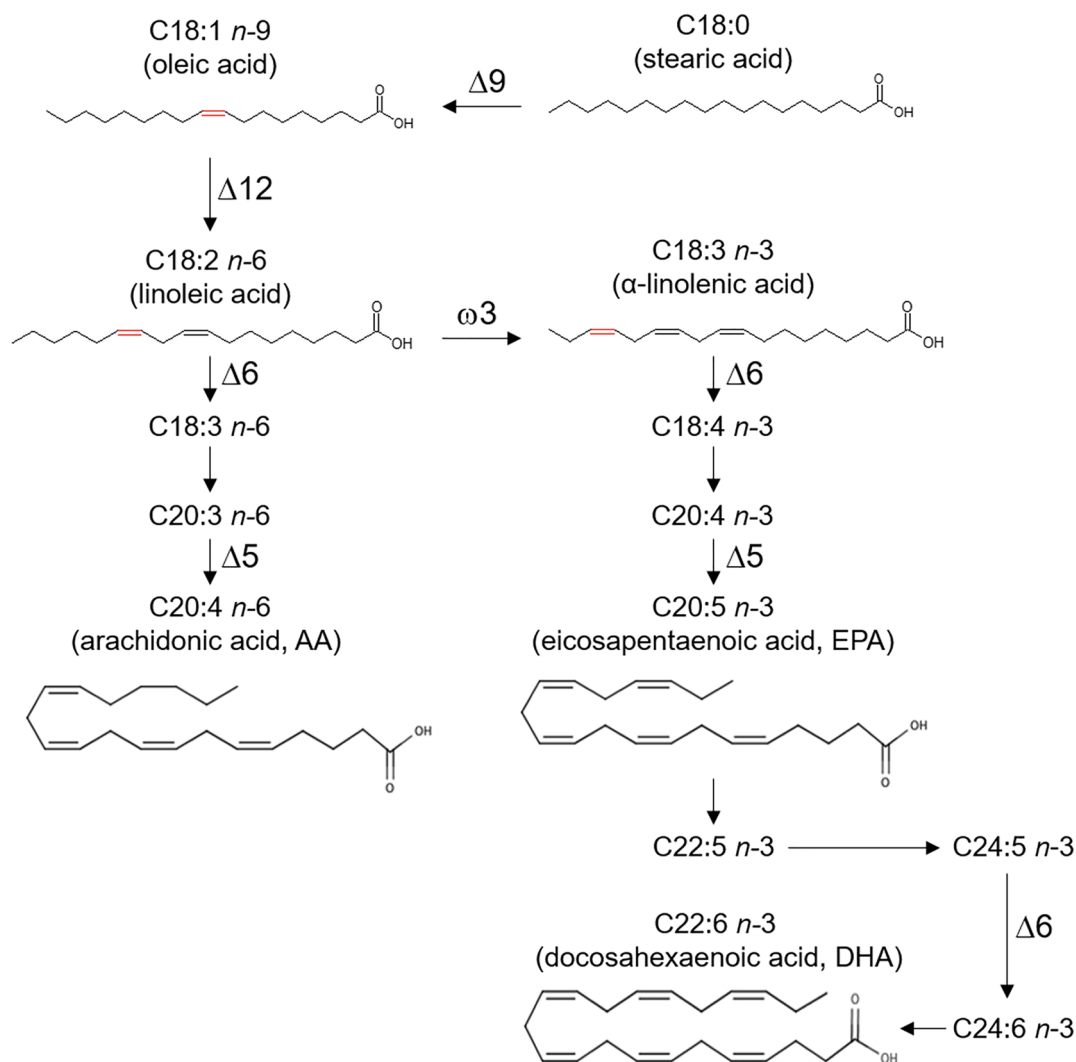


Fig. 6.2 Biosynthesis pathway for PUFA. The biosynthetic pathway of *n*-6 LC-PUFAs and *n*-3 LC-PUFAs is shown. *n*-6 LC-PUFAs, such as AA, can be synthesized

from linoleic acid. *n*-3 LC-PUFAs, such as EPA and DHA, can be synthesized from α -linolenic acid

desaturase have been used for functional studies of PUFAs (Vasquez et al. 2014). Furthermore, transgenic animals with *C. elegans* desaturase genes have been used to analyze the functions of PUFAs in vivo. For example, FAT-1 transgenic mice, which can introduce the double bond at the ω -3 position of *n*-6 PUFAs (Kang et al. 2004), have been used to study the physiological roles of *n*-3 PUFAs in the cardiovascular and neurological systems.

6.1.6 Function of PUFAs as Constituents of Cellular Lipid Molecules

Several lysophospholipid acyltransferases that are responsible for the synthesis of PUFA-containing phospholipids have been identified. Lysophosphatidic acid acyltransferase 3 (LPAAT3) is involved in the production of DHA-containing phospholipid molecules in mice. LPAAT3-deficient mice exhibit severe male infertility with abnormal sperm morphology and

disordered disk morphology in photoreceptor cells because of impaired membrane deformability (Shindou et al. 2017; Iizuka-Hishikawa et al. 2017). Lysophosphatidylcholine acyltransferase 3 (LPCAT3) incorporates AA into phospholipids. LPCAT3-deficient mice show extensive triacylglycerol accumulation and dysfunction in enterocytes resulting from the abnormal lipoprotein assembly in the liver and enterocytes (Hashidate-Yoshida et al. 2015).

The functions of membrane proteins can be controlled by PUFA-containing phospholipids. For example, the activity of rat TRPV4 is enhanced by *n*-3 PUFA-containing phospholipids through the modulation of cell membrane fluidity (Caires et al. 2017). The activity of Na⁺/K⁺ ATPase can also be controlled by PUFA-containing phospholipids (Cornelius et al. 2015; Bhatia et al. 2016). Furthermore, structural analyses revealed that membrane phospholipids specifically bind to the surface of membrane proteins (Gao et al. 2016). Thus, it is likely that acyl chains of PUFA-containing phospholipids are important for regulating the structure and function of membrane proteins through the direct binding and/or modulation of physicochemical properties of cell membrane. Additionally, PUFA-containing phospholipids are thought to function as modulators of membrane properties during temperature changes. Since temperature changes alter the physicochemical properties and functions of the cell membrane, the regulation of content of PUFA-containing phospholipids can contribute to the thermal adaptation response.

PUFAs also affect the physicochemical properties and metabolism of triacylglycerol molecules. An analysis using differential scanning calorimetry demonstrated that the melting point of PUFA-containing triacylglycerols is lower than that of MUFA-containing triacylglycerols. Moreover, PUFA-rich fish oils contain a liquid fraction even below −110 °C (Tolstorebrov et al. 2014).

6.1.7 Function of PUFAs as Signaling Molecules

PUFAs and their metabolites also function as signaling molecules. In mammals, PUFAs are uti-

lized as precursors for lipid mediators formed through the enzymatic oxidation mediated by cyclooxygenases (COXs), lipoxygenases (LOXs), or cytochrome P450 monooxygenases (CYPs). AA is a common precursor of many eicosanoids. When cells are stimulated, AA is released from membrane phospholipids by phospholipase A2 (Murakami et al. 2011) and metabolized by either COXs, LOXs, or CYPs. The COX pathway produces prostaglandins (PGs) and thromboxanes. The LOX pathway results in the production of leukotrienes, lipoxins, and hydroxyeicosatetraenoic acids (HETEs). HETEs and epoxyeicosatrienoic acids are produced by the CYP pathway. AA-derived eicosanoids are involved in a broad range of physiological processes including inflammation, cardiovascular homeostasis, and ischemia-reperfusion injury (Arita 2012; Stables and Gilroy 2011; Smyth et al. 2009).

Recently, *n*-3 LC-PUFAs, particularly EPA and DHA, have been shown to play beneficial roles in several diseases including arthritis, colitis, and metabolic syndrome. It is believed that *n*-3 LC-PUFAs have two main roles in the down-regulation of *n*-6 LC-PUFAs-induced responses. The first is to serve as an alternative substrate for COX or LOX, which results in the suppression of AA-derived PG (i.e., PGI₂ and PGE₂) production (Schmitz and Ecker 2008). The second is conversion to anti-inflammatory and protective mediators such as the resolvins (Serhan et al. 2004). These lipid mediators exert potent responses via several targets including G-protein-coupled receptors and transient receptor potential (TRP) channels (Krishnamoorthy et al. 2010; Ji et al. 2011).

6.2 Fatty Acid-Containing Lipids in the Thermal Adaptation of Animals

Because temperature affects the physicochemical properties of cell membrane, the remodeling of chemical structure of phospholipid acyl chains contributes to the maintenance of cell membrane fluidity in response to changes in temperature, known as homeoviscous adaptation. Additionally, the alteration of acyl chain composition of triac-

ylglycerol molecules during the fluctuation of temperature also has contributions to the thermal adaptation through the modulation of energy metabolism. Fatty acid desaturases that introduce a double bond into the fatty acid moiety of acyl-CoA play a crucial role in the thermal adaptation of animals by controlling the chemical structure of acyl chains in phospholipid and triacylglycerol molecules. The role of fatty acid desaturases in the thermal adaptation has been studied in various organisms including bacteria (*Escherichia coli* and *Bacillus subtilis*), fungi (*Saccharomyces cerevisiae*), higher plants (*Arabidopsis thaliana*), and protozoans (*Tetrahymena thermophila*). Additionally, the cellular lipid-mediated mechanisms of adaptation to changes in temperature have also been studied in fish and insects. In this section, we discuss the various roles of fatty acid-containing lipid molecules in the thermal adaptation of fish and insects by presenting our recent findings.

6.2.1 Lipid Molecules-Mediated Thermal Adaptation in Marine Fish

Although many fish species have adapted to relatively stable thermal habitats, the maintenance of physiological function is particularly acute for fish species that experience significant challenges to thermal homeostasis. Sellner and Hazel first demonstrated that cold acclimation increased the rate of unsaturated fatty acid synthesis in trout hepatocytes up to twice the rate of hepatocytes from warm-acclimated trout (Hazel and Sellner 1979). Hazel's group found that the induction of desaturase activity was not especially important in trout but was critical for cyprinid fish in which MUFAs play a more prominent role in cold acclimation. They further demonstrated that salmonids incorporate a greater proportion of LC-PUFAs into membrane phospholipids during cold acclimation.

The remodeling of phospholipid acyl chains occurs in various fish species during thermal acclimation, in which the levels of PUFAs, rather than MUFAs, are most commonly elevated.

Particularly, certain LC-PUFAs, such as EPA and DHA, were preferentially incorporated into both phospholipids and triacylglycerol in cold-acclimated teleost species. Most marine fish species acquire LC-PUFAs from the aquatic food web in which marine phytoplankton are the primary producer of LC-PUFAs. In aquatic food webs, *n*-3 LC-PUFAs produced by marine phytoplankton are rich in zooplankton and fish. This allows for physiological adaptation to vertical migration by maintaining the membrane order under low temperature and high-pressure conditions in the deep sea (Gladyshev et al. 2013; Pond et al. 2014).

6.2.2 Homeoviscous Adaptation in Freshwater Fish Cell Membranes

Freshwater phytoplankton contain high levels of linoleic acid and α -linolenic acid, in contrast to marine phytoplankton that produce high levels of EPA and DHA (Twining et al. 2016). It is thought that most freshwater and diadromous fish species synthesize LC-PUFAs from C18-PUFAs. This is in stark contrast to marine fish species that live in LC-PUFA-rich environments and lack evolutionary pressure to retain their ability to endogenously produce LC-PUFAs (Twining et al. 2016). Compared with wild marine fish, freshwater fish contain lower amounts of LC-PUFAs, such as EPA and DHA, and higher amounts of C18-fatty acids (Henderson and Tocher 1987). However, limited information on the detailed molecular species of freshwater fish lipids is available.

Lake Biwa (Tabata et al. 2016), an ancient lake in Japan, is home to more than 50 species of fish, but there has been no detailed lipid analysis of these fish species. *Gymnogobius isaza* is a freshwater goby endemic to Lake Biwa and inhabits the lake bottom (30–100 m depth; approximately 8 °C water temperature throughout the year) (Fig. 6.3a). *G. isaza* is adapted to inhabit pelagic habitats and exhibits a strong swimming ability and significant diel vertical migration from the bottom of the lake to near the

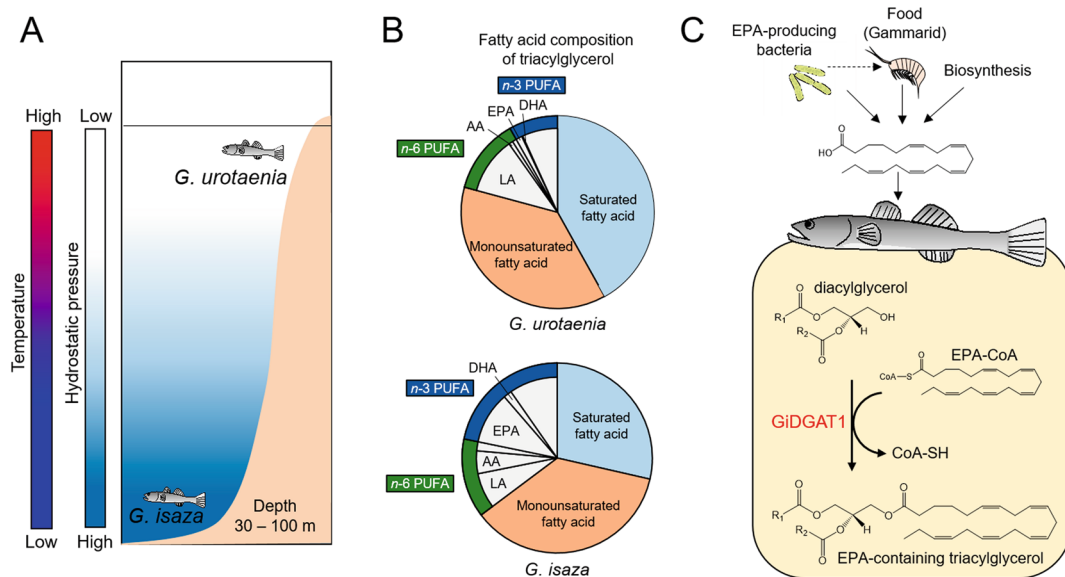


Fig. 6.3 Unique fatty acid composition of the triacylglycerol molecules in *G. isaza*. (a) *G. isaza* inhabits the lake bottom (30–100 m depth; approximately 8 °C water temperature throughout the year), whereas *G. urotaenia* lives on the shore of Lake Biwa. (b) Fatty acid composition of liver triacylglycerol molecules of *G. isaza* and *G. urotaenia*. (c) Putative biosynthetic pathway of EPA-containing triacylglycerol in *G. isaza* (Suito et al. 2018). LA, linoleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

surface in summer and autumn. Since the surface water temperature during the migration season (16 °C–23 °C) is higher than that of the lake bottom (8 °C), *G. isaza* is expected to experience not only hydrostatic pressure but also significant daily fluctuations in temperature.

We recently reported that LC-PUFAs constitute up to 50.6% and 46.0%, respectively, of the phospholipid acyl chains in the liver and muscle of *G. isaza* (Suito et al. 2018). Particularly, n-3 PUFAs comprise up to 41.2% and 32.7% of the total phospholipid fatty acids in the liver and muscle of *G. isaza*, respectively. The most unique feature of the phospholipid acyl chain of *G. isaza* is its significantly higher EPA content in both the liver (15.8%) and muscle (14.6%) compared with that reported in freshwater fish species. Ackman et al. reported the fatty acid composition of phospholipids in five major Indian carps that share a common food niche in the lentic community (Ackman et al. 2002). *Labeo rohita* muscle exhibited the highest n-3 PUFA content (28.2%) among these carps but had lower EPA content (3.7%) (Ackman et al. 2002). Lei et al. analyzed

the lipid composition of five wild freshwater fish from Poyang Lake, China (Lei et al. 2012). The EPA content in the phospholipid acyl chains in these wild freshwater fish accounted for 1.19–7.54% of the total phospholipid acyl chains (Lei et al. 2012). Kozlova et al. analyzed the fatty acid composition of the phospholipids of two pelagic cottoid fish caught at a depth of 150–350 m north of Lake Baikal (Kozlova and Khotimchenko 2000). The EPA content of the liver and muscle phospholipids in these two cottoid fish accounted for 2.6–11.4% of the total phospholipid acyl chains (Kozlova and Khotimchenko 2000). Thus, it is widely believed that DHA, not EPA, is the major LC-PUFA of phospholipids in most fish species including marine fish. Therefore, higher EPA content (14.6%) in total phospholipid acyl chains of *G. isaza* muscle compared with the DHA content (11.5%) is a unique characteristic of the fatty acid profile of *G. isaza* phospholipids (Suito et al. 2018).

In the synthesis pathway of EPA, C18:4 n-3 is produced by $\Delta 6$ -desaturation of α -linolenic acid

(Fig. 6.2). C18:4 *n*-3 is then elongated to C20:4 *n*-3, which is desaturated at the Δ 5-position to yield EPA. DHA synthesis from EPA requires two additional elongation steps, a second Δ 6-desaturation step, and a chain-shortening step. The main diet of *G. isaza*, a freshwater gammaride (Briones et al. 2012), contains large amounts of LC-PUFAs with scarce amount of the intermediates of the biosynthetic reaction, including C22:5 *n*-3. Because relatively high levels of the intermediates of the biosynthetic pathway of *n*-3 LC-PUFAs are detected in the phospholipid acyl chains of *G. isaza*, some *n*-3 LC-PUFAs found in the phospholipids of *G. isaza* are likely to be synthesized by the fish.

6.2.3 Triacylglycerol Molecules in Thermal Adaptation of Fish

Triacylglycerol molecules also play an important role in thermal adaptation. The triacylglycerol content of both the liver and muscle of *G. isaza* was significantly higher than that in *G. urotaenia*, a closely related goby species that lives on the shore of Lake Biwa (Fig. 6.3a) (Suito et al. 2018). Because triacylglycerol is an important source for energy metabolism, high triacylglycerol content may support the powerful swimming ability of *G. isaza* to predate pelagic zooplankton and benthic gammarid amphipods. A unique feature of *G. isaza* triacylglycerol is a very high content of *n*-3 PUFAs, which account for 22.0% and 27.0% of the total triacylglycerol acyl chains in the liver and muscle, respectively (Fig. 6.3b). The *n*-3 PUFA content in both the liver and muscle of *G. isaza* is higher than that of *G. urotaenia*. EPA is the most abundant PUFA in the acyl chain of triacylglycerol molecules in the liver (10.5%) and muscle (14.1%) of *G. isaza*. The reported values for EPA content in the triacylglycerol acyl chains range from 0.1% to 6.5% in the liver and muscle of two pelagic cottoid fishes in Lake Baikal (Kozlova and Khotimchenko 2000), from 0.4% to 4.9% in five fish species of Indian carp (Ackman et al. 2002), and from 0.94% to 3.26% in five fish species in Poyang Lake (Lei et al. 2012). Thus, the fatty acid profile of *G. isaza* tri-

acylglycerol molecules is unique compared with that reported in freshwater fish species. The triacylglycerol molecules in oxidative muscle of Antarctic fish are enriched in EPA (13.0%) and DHA (12.0%), which are effectively used as fuels to support aerobic energy metabolism (Crockett and Sidell 1993). The significantly higher content of *n*-3 PUFAs in *G. isaza* triacylglycerol molecules may reflect adaptation to the benthopelagic life in Lake Biwa.

In *G. isaza* triacylglycerol molecules, EPA is incorporated at either the *sn*-1 or *sn*-3 position. This is in sharp contrast to the observation that *n*-3 LC-PUFAs are more likely to be incorporated at the *sn*-2 position of the marine fish triacylglycerol molecules. Incorporation of fatty acid at the *sn*-3 position of diacylglycerol is mediated by acyl-CoA:diacylglycerol acyltransferase (DGAT). Most vertebrate genomes contain at least two *DGAT* genes, *DGAT1* and *DGAT2*, which have different structural and physiological functions (Liu et al. 2012). Mouse *DGAT1* is a membrane protein that resides in the ER and is ubiquitously expressed in various tissues including the small intestine, adipose tissue, and testis (Yen et al. 2008). *DGAT1* deletion in mice causes a reduction in adiposity and confers resistance to obesity (Smith et al. 2000). Mouse *DGAT2* is a membrane protein that is intracellularly localized to the ER and lipid droplet (Yen et al. 2008). Mouse *DGAT2* is expressed in most tissues, whereas the high-level expression of *DGAT2* is observed in the adipocytes and liver. Deficiency of *DGAT2* in mice causes a decrease in plasma triacylglycerol fatty acids and glucose, resulting in lethality shortly after birth. *DGAT1* and *DGAT2* play different roles in triacylglycerol metabolism as *DGAT1* failed to compensate for deficiencies in *DGAT2*-deficient mice. We have isolated the coding sequences for GiDGAT1 and GiDGAT2 from a liver cDNA library of *G. isaza* and expressed them in a *DGAT*-deficient yeast strain (Suito et al. 2018). GiDGAT1-expressing yeast produces triacylglycerol molecules with higher proportions of linoleic acid, α -linolenic acid, and EPA compared with GiDGAT2-expressing yeast. Particularly, the proportion of EPA in the acyl chain of the triacylglycerol mol-

ecules of GiDGAT1-expressing yeast is 2.4 times higher than that of the GiDGAT2-expressing yeast. This indicates that GiDGAT1 has a higher potential to incorporate EPA into triacylglycerol molecules compared with GiDGAT2. Marine fish oils, such as cod liver oil and tuna oil, have been reported to contain more DHA than EPA (Ackman 1967). Freshwater fish triacylglycerol molecules also exhibit similar fatty acid profiles (Ackman et al. 2002; Lei et al. 2012; Kozlova and Khotimchenko 2000). By contrast, the EPA content was 6.2 times higher than the DHA content in the triacylglycerol from the liver of *G. isaza* (Fig. 6.3b) (Suito et al. 2018). It is unlikely that low levels of DHA in triacylglycerol result from an inadequate supply of DHA because the proportion of DHA in the acyl chain of liver phospholipids is 17.9% in *G. isaza*. The incorporation of EPA into triacylglycerol molecules in GiDGAT1-expressing yeast was 2.6 times higher than that of DHA, but a comparable incorporation of EPA and DHA into triacylglycerol molecules was observed in GiDGAT2-expressing yeast. Although further in vivo evaluation is required, it is likely that GiDGAT1 is involved in the production of EPA-enriched triacylglycerol molecules in *G. isaza* (Fig. 6.3c) (Suito et al. 2018).

6.2.4 Homeoviscous Adaptation in *Drosophila* Cell Membranes

The fruit fly *D. melanogaster* represents a useful genetic model system in embryology, physiology, immunology, population genetics, social biology, and cell biology (Gonzalez 2013). Although studies using *Drosophila* have revealed many cellular systems and functions conserved among multicellular organisms, the cellular lipids of insects, including *Drosophila*, have unique characteristics compared with those of other eukaryotes. For example, the most abundant phospholipid in *Drosophila* is phosphatidylethanolamine (PE) (Guan et al. 2013), while the most abundant phospholipid in mammals is phosphatidylcholine (PC) (Fig. 6.1b). PE, a class of phospholipid enriched in *Drosophila*, is a cone-shaped

lipid that favors non-bilayer structures in the hexagonal II and cubic phases. Thus, PE-rich membranes tend to exhibit bending fluctuations and generate intrinsic negative curvature. Moreover, the local enrichment of PE plays an important role in various biological processes including cell division, membrane fusion, and viral replication. More than 50% of the fatty acids in *Drosophila* are less than 18 carbons in length. The contents of LC-PUFAs, such as AA, EPA, and DHA, are remarkably low in *Drosophila* (Shen et al. 2010).

Although LC-PUFA contents are low, *D. melanogaster* may be used as a model system for evaluating the contributions of C18-PUFAs, such as linoleic acid and α -linolenic acid, to cold adaptation. Adaptation to low temperatures increased the proportion of PUFA-containing phospholipids in *Drosophila* cell membranes, similar to that of other species. For example, a 1 day exposure at 18 °C (7 °C lower than normal rearing conditions) caused an increase in the proportion of PE and PC molecules with more than and equal to two double bonds in *Drosophila* larvae (Suito et al. 2020). The breeding of *Drosophila* at 12 °C altered dietary preference from yeast to PUFA-containing plant foods, thus affecting the composition of the membrane lipids (Brankatschk et al. 2018). Moreover, these changes in membrane lipid composition in cold-exposed flies altered membrane fluidity and improved their development and survival in a cold environment (Brankatschk et al. 2018).

6.2.5 Role of PUFAs in *Drosophila* Behavioral Thermoregulation

As feeding behavior is switched to regulate PUFA content in response to changes in temperature (Brankatschk et al. 2018), other temperature-related behaviors may be affected by PUFA content. Thermoregulation is achieved by temperature sensing followed by an effector response. An essential part of the effector reactions in *D. melanogaster* is behavioral thermoregulation, which is the movement of animals to environments with more comfortable temperatures. This occurs because the body temperature of flies is

strongly affected by environmental temperature. Previously, we reported that the dystroglycan mutant fly, *atsugari*, exhibited a cold preference phenotype with enhanced mitochondrial oxidative energy metabolism (Takeuchi et al. 2009). However, the lipid molecule-mediated mechanisms that influence behavioral thermoregulation are not fully understood. Additionally, although dietary PUFA supplementation has been reported to affect the *Drosophila* visual system and cold survival, the effects of changes in PUFA content on whole-body or a specific tissue on thermoregulatory behavior remain unclear.

For evaluation of the role of PUFAs that are increased in cold-exposed flies, we established a transgenic *Drosophila* strain of *C. elegans* $\Delta 12$ -desaturase FAT-2 (Suito et al. 2020). To express FAT-2 in a tissue-specific manner, we used the GAL4-upstream activating sequence (UAS) system in which the yeast transcription activator, GAL4, with a specific promoter and/or enhancer enables the transcription of the downstream sequence of UAS in a tissue/cell-specific manner. In our FAT-2 transgenic flies, the coding sequence of the *Fat-2* gene is aligned downstream of UAS. Ubiquitous expression of FAT-2 driven by the Tub-GAL4 driver caused an increase in the proportion of linoleic acid content of the total phospholipid acyl chains from 6.3% to 36.4%. An increase of the linoleic acid-containing phospholipid molecules in whole-body was also induced by tissue-specific expression of FAT-2 in fat body or gut. Thus, it is apparent that FAT-2 can be functionally expressed in these transgenic flies.

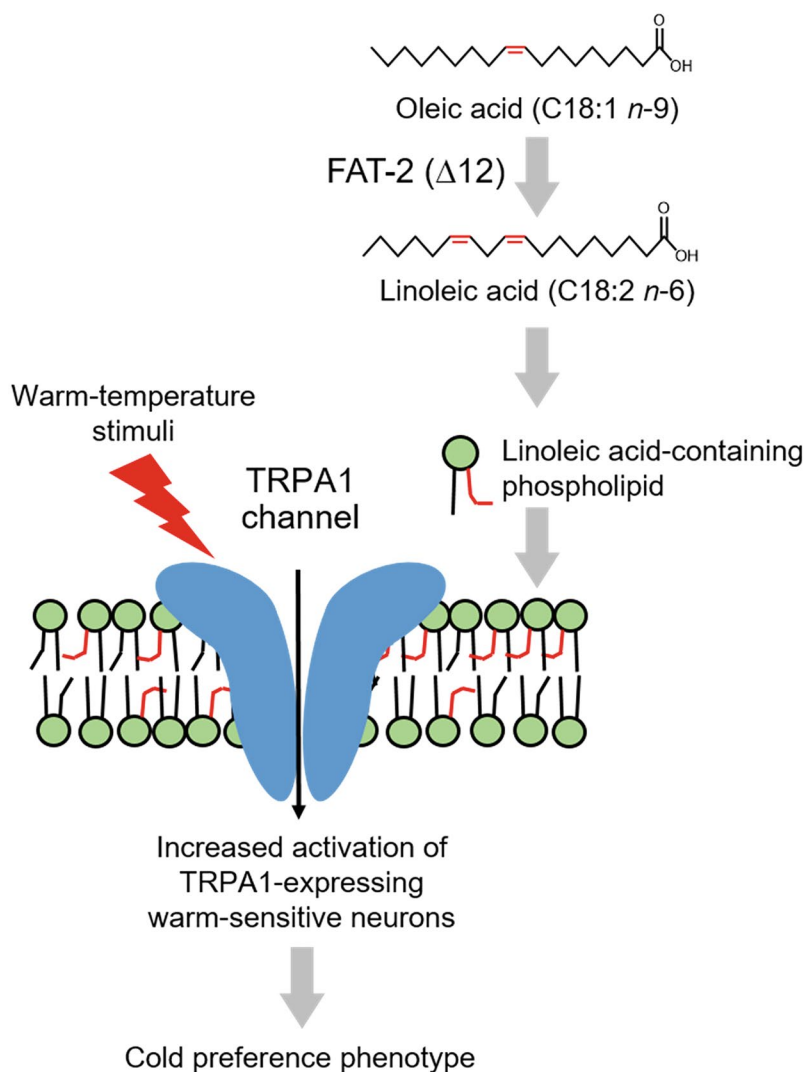
Tissue-specific expression of FAT-2 in the fat body or gut caused a decrease in the preferred temperature of larvae, indicating that an increase in the proportion of linoleic acid-containing phospholipids results in a cold preference phenotype in *Drosophila* larvae. The neuronal expression of FAT-2 also reduced the preferred temperature of the third instar larvae of *Drosophila* by 3.5 °C. A significant increase in the proportion of linoleic acid-containing phospholipids was observed in the central nervous system of FAT-2-expressing larvae, indicating that the composition of the neuronal lipid mole-

cules influences the thermoregulatory behavior of *Drosophila*.

In the *D. melanogaster* studies, a variety of thermosensitive receptors were identified by combining genetic approaches, electrophysiological techniques, and the analysis of temperature preferences. Members of the TRP channel family are the most widely studied thermosensitive receptors, which sense a variety of environmental cues including physical and chemical stimuli (Venkatachalam and Montell 2007). Among TRP channel members, the TRPA1 channel is well known for its ability to detect unfavorable temperatures and noxious chemicals (Saito and Tominaga 2017). The thermosensitivity of mammalian TRPA1 channels remains controversial. However, it is evident that the *Drosophila* TRPA1 channel is activated by warm temperatures (Viswanath et al. 2003). The *Drosophila* TRPA1 channel was identified as a thermosensitive receptor that regulates thermoregulatory behavior. Additionally, TRPA1 is expressed in anterior cell neurons of adult flies, and TRPA1-deficient flies exhibit reduced aversion to high temperatures (Hamada et al. 2008). The *Drosophila* TRPA1 channel is also important for the high temperature-induced rolling behavior and the determination of temperature preferences in larvae (Luo et al. 2017; Sokabe et al. 2016). Similar to the effect of neuronal expression of FAT-2, FAT-2 expression induced by the TRPA1-GAL4 driver also caused a significant decrease in the preferred temperature of the larvae (Suito et al. 2020). Additionally, the response of TRPA1-expressing neurons to the increase of temperature was potentiated by FAT-2 expression. Therefore, linoleic acid-containing membrane lipids affect the thermoregulatory behavior of *Drosophila* larvae by enhancing the activation of TRPA1-expressing neurons in response to temperature stimuli (Fig. 6.4) (Suito et al. 2020).

Motter et al. reported that rat TRPA1 expressed in HEK293T cells exhibited strong activation following treatment with AA, EPA, and DHA, but only weak activation with linoleic acid (Motter and Ahern 2012). They also reported that *Drosophila* TRPA1 expressed in HEK293T cells was not activated by DHA (Motter and Ahern

Fig. 6.4 The putative models for FAT-2-induced regulation of thermoregulatory behavior. Expression of FAT-2 in TRPA1-expressing neurons causes the biosynthesis of linoleic acid. Biosynthesized linoleic acid is incorporated into membrane-forming phospholipid molecules. The linoleic acid-containing phospholipid molecules enhance the warm temperature-induced activation of the TRPA1 channel. The enhanced activation of the TRPA1 channel results in the cold preference of larvae (Suito et al. 2020)



2012). The activation of TRPA1 by PUFAs was not mediated by any known ligand-binding domains (Motter and Ahern 2012). Our analysis demonstrated that non-esterified linoleic acid does not function as an activator of *Drosophila* TRPA1 (Suito et al. 2020). We also reported that the expression of FAT-2 in neurons did not affect response to the TRPA1 activator, allyl isothiocyanate (AITC). Furthermore, it was reported that the pore and N-terminal regions upstream of the ankyrin repeats of TRPA1 are required for thermal activation, which is distinct from the domain related to activation by AITC. Thus, it is likely that the increased activation of TRPA1-expressing

neurons in FAT-2-expressing flies is mediated by the regulation of temperature-sensing specific mechanism. Several TRP channels were shown to have the phosphatidylinositol-binding domain that is involved in the regulation of channel activity. Additionally, structural analysis revealed the presence of a phospholipid-binding motif in the TRPA1 channel (Paulsen et al. 2015; Suito et al. 2020). Therefore, it is likely that the function and activity of the TRPA1 channel are regulated by direct interaction with membrane phospholipid molecules. The activities of the TRP channels are also highly sensitive to changes in the physico-chemical properties of the lipid bilayer mem-

brane, such as membrane tension that could be controlled by membrane phospholipid composition (Caires et al. 2017). Therefore, we propose that PUFA-containing phospholipids in neural cell membrane modulate the thermosensitivity of the *Drosophila* TRPA1 channel through direct interaction with the channel and/or by modulating the physicochemical properties of the phospholipid membrane (Suito et al. 2020).

The cold preference of larvae is also induced by FAT-2 expression under control of GAL4-thermoreceptor constructs that are involved in cold sensation. These include TRP-GAL4, TRPL-GAL4, iav-GAL4, and R11F02-GAL4. It was reported that the activities of TRP channels can be modulated by PUFAs (Ciardo and Ferrer-Montiel 2017). For example, linoleic acid inhibits the response of mammalian TRPM8 to menthol and the response of mammalian TRPV1 to capsaicin (Parnas et al. 2009). This raises the possibility that cold sensation-associated thermoreceptors could be desensitized or inhibited by linoleic acid produced by FAT-2 in *Drosophila* larvae. These findings imply that PUFAs affect the various steps of thermoregulatory behavior for the comprehensive adaptation against temperature changes in *Drosophila*.

6.3 Perspective

As discussed above, the alteration of lipid composition and the role of lipid-metabolizing enzymes in the thermal adaptation process have been extensively studied in a variety of animals. However, it is unclear how cells and organisms sense the environmental temperature changes for lipid-mediated thermal adaptation. The proteins that detect the changes in temperature, such as thermosensitive channels (e.g., TRP channels), may be involved in temperature sensing for lipid-mediated thermal adaptation. Alternatively, the sensor proteins that detect changes in the physicochemical properties of cell membrane may also contribute to temperature sensing because temperature changes affect the fluidity, tension, and thickness of the phospholipid bilayer. Although most of the information related to the

mechanism of thermal adaptation have been established in genetic model organisms, unique adaptation and robust tolerance to temperature changes are also observed in non-model organisms. Technological advances such as next-generation DNA sequence technology and genome editing tools (e.g., CRISPR/Cas9 and TALEN) will facilitate the analysis and manipulation of the expression and function of specific genes in non-model organisms. These advances provide a unique opportunity to explore novel temperature sensors for lipid-mediated thermal adaptation even in non-model organisms. Future research using these technologies may provide insight into how cells and organisms respond to changes in environmental temperature through the remodeling of fatty acid-containing lipid molecules for the maintenance of biological processes.

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Cellular Thermal Biology Using Fluorescent Nanothermometers

7

Kohki Okabe, Shingo Sotoma, and Yoshie Harada

Abstract

It has been known that cells have mechanisms to sense and respond to environmental noxiousness and mild temperature changes, such as heat shock response and thermosensitive TRP channels. Meanwhile, new methods of measuring temperature at the cellular level has recently been developed using fluorescent nanothermometers. Among these thermometers, fluorescent polymeric thermometers and fluorescent nanodiamonds excel in the properties required for intracellular thermometry. By using these novel methods to measure the temperature of single cells in cultures and tissues,

it was revealed that spontaneous spatiotemporal temperature fluctuations occur within cells. Furthermore, the temperature fluctuations were related to organelles such as mitochondria and cellular and physiological functions, revealing a close relationship between intracellular temperature and cellular functions.

Keywords

Intracellular temperature · Cellular thermogenesis · Fluorescent polymeric thermometer · Fluorescent nanodiamond · Nitrogen-vacancy center

K. Okabe (✉)
Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan

JST, PRESTO, Saitama, Japan
e-mail: okabe@mol.f.u-tokyo.ac.jp

S. Sotoma (✉)
Faculty of Molecular Chemistry and Engineering, Kyoto Institute of Technology, Kyoto, Japan
e-mail: shsotoma@kit.ac.jp

Y. Harada
Institute for Protein Research, Osaka University, Osaka, Japan

Center for Quantum Information and Quantum Biology, Osaka, Japan

Premium Research Institute for Human Metaverse Medicine (WPI-PRIME), Osaka University, Osaka, Japan
e-mail: yharada@protein.osaka-u.ac.jp

7.1 Introduction to Cellular Thermometry

Adaptation to temperature changes in the environment is an essential function of life. The body temperature of endotherms is kept higher than that of the environment by spontaneous heat generation (Geneva et al. 2019; Cannon and Nedergaard 2004). It fluctuates greatly in association with various physiological activities, such as circadian rhythms and menstrual cycle (Refinetti and Menaker 1992; Lee 2016). Medical studies have shown an increase in heat production in cancerous tissue (Karnebogen et al. 1993; Monti et al. 1986), localized infections (Waterman et al. 1969), febrile seizures (Dube et al. 2007), and malignant hyperthermia (Rosenberg et al. 2015).

Cells possess various systems to detect environmental temperature changes. To date, temperature-related cell biology has primarily investigated responses to noxious changes in environmental temperature. First, heat shock responses serve as an adaptive system to temperature changes in external environments (Lindquist 1986; Velichko et al. 2013). Also, adaptive alterations in gene expression by heat shock proteins and translation through stress granule formation are the essence of this defense mechanism (Richter et al. 2010; Grousl et al. 2009). Second, several ion channels, including thermosensitive transient receptor potential (TRP) channels, are activated temperature-dependently (Montell and Caterina 2007; Voets et al. 2005). These functions propagate information regarding temperature change, either noxious or mild, in external environments or plasma membranes into the interior of cells as electrical signals. This is the mechanism through which humans can sense environmental temperature changes. Interestingly, a part of the TRP channels is expressed deep inside the brain, which is considered less vulnerable to external temperature change, suggesting that these channels can sense the changes in temperature inside brain tissue. These facts show that the temperature variation at the cellular level inside living bodies could contribute to physiological functions.

Despite the fundamental involvement of temperature in life systems, little is known about whether spontaneously produced heat in cells helps serve their base functions. Because temperature is a fundamental physical quantity that governs biochemical reactions, it is easily imagined that spontaneous or adaptive thermogenesis inside cells influences intracellular reactions. Notably, such spontaneous temperature variation-dependent cellular functions could be a new type of intracellular signaling, which was previously only understood through molecular interactions.

To answer this unexplored question, new tools to investigate intracellular thermal changes in live cells or animals have attracted considerable attention (Okabe et al. 2018; Zhou et al. 2020; Okabe et al. 2021). Fluorescence thermometry is a unique and powerful method to measure intracellular temperature variations. Recently, many

intracellular fluorescent thermometers have been developed, some of which can visualize subcellular temperature changes. In addition, various molecular species have developed fluorescent thermometers, each with a different temperature sensing principle. Among them, fluorescent polymeric thermometers (FPTs) and fluorescent nanodiamond (FND) excel in the properties required for intracellular temperature measurements, such as high sensitivity, visualization of temperature distribution, and noninterference with intracellular molecules; hence, they should contribute to thermal biology. This chapter describes these two representatives of fluorescent thermometers that have enabled intracellular thermometry, and how they have contributed to emerging biology.

7.2 Fluorescent Polymeric Thermometer for Intracellular Temperature Mapping

7.2.1 Fluorescent Polyacrylamides for Molecular Thermometers

Polyacrylamides, such as poly-*N*-Isopropylacrylamide (NIPAM) or poly-*N*-propyl-acrylamide (NNPAM), are classically well-known for their temperature-dependent structural changes (Pelton 2000), which is why they have opened the door to developing molecular thermometers. A unique characteristic of the temperature sensitivity of polyacrylamides is a reversible collapse transition related to the lower critical solution temperature around 33 °C, which enables a sharp response to a temperature change. Combining NIPAM and NNPAM with a water-sensitive fluorophore, such as benzofurazans (Uchiyama et al. 2017), Uchiyama et al. have developed highly sensitive FPT (Fig. 7.1). At low temperatures, a thermo-responsive polyacrylamide sequence in FPT assumes an extended structure with the hydration of amide linkages, where water-sensitive benzofurazan can be quenched by neighboring water molecules. At higher temperatures, which weaken hydration, the polyacrylamide sequence undergoes phase

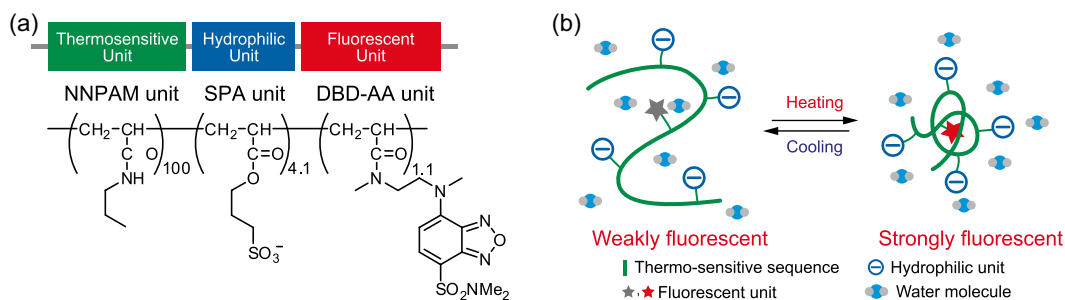


Fig. 7.1 Fluorescent polymeric thermometer, FPT. Chemical structures (a) and functional diagram (b). Reprinted with permission from Ref. (Okabe et al. 2012)

transition to shrink because of hydrophobic interactions among the NNPAM units, releasing water molecules and strong fluorescence from benzofuran. Furthermore, the modifications of fluorescent polyacrylamide, such as the brightness of fluorescence unit or the range of temperature response, can be adjusted by optimizing the fluorophore ratio or synthesizing a block copolymer containing more than one polymer having different temperature sensitivity, respectively, which improve their thermosensing properties. By adding a hydrophilic unit and optimizing the unit composition (i.e., thermosensitive, fluorescent, and hydrophilic units), we have established FPTs applicable to living cells (Fig. 7.1).

In 2009, the first demonstration of intracellular thermometry was performed using a fluorescent nanogel thermometer (FNT), a type of FPT, and an epifluorescence microscope (Gota et al. 2009). The gelation of fluorescent polyacrylamide minimized the interaction of the fluorescent unit with cellular components and maximized the structural response to temperature change. By measuring the total fluorescence intensity of the FNT microinjected into living single COS7 cells, we have tracked the average temperature inside single living cells. The calibration curve for the fluorescence response of FNT to a change in the medium temperature achieved a temperature resolution as low as 0.18 °C. Intracellular thermometry with FNT on apoptotic cells showed a significant intracellular temperature increase (1.8 °C for 2 h), which uncovered the existence of a temperature variation inside single living cells for the first time.

7.2.2 Temperature Mapping with FPT Inside Single Cells

In contrast to the excellent sensitivity in measuring the temperature change using FNT, the dot-like distribution of intracellular FNT hinders the measurement of temperature distribution in single cells. This challenge motivated us to develop a novel FPT with a lower molecular weight that evenly distributes inside cells. Furthermore, the fluorescence intensity of cells filled with FNT or FPT can be affected by variation in their local concentration, illuminating light intensity, and photobleaching. To overcome these limitations, we adopted the fluorescence lifetime of FPT as a temperature-dependent variable because it is independent of fluctuations under these experimental conditions. We then applied FPT to the time-correlated single-photon counting (TCSPC)-based fluorescence lifetime imaging microscopy (FLIM), which permits an accurate intracellular temperature mapping inside single living COS7 cells. Strikingly, this revealed an inhomogeneous temperature distribution, such as higher temperature in the nucleus, mitochondria (in part), and centrosome than the surrounding cytoplasm even in single steady-state cells (Fig. 7.2) (Okabe et al. 2012). Notably, the intracellular temperature gradient related to cellular events, including the cell cycle, attracted significant attention in biology. Moreover, these results strongly suggest the intrinsic relationship of the intracellular temperature with cell functions.

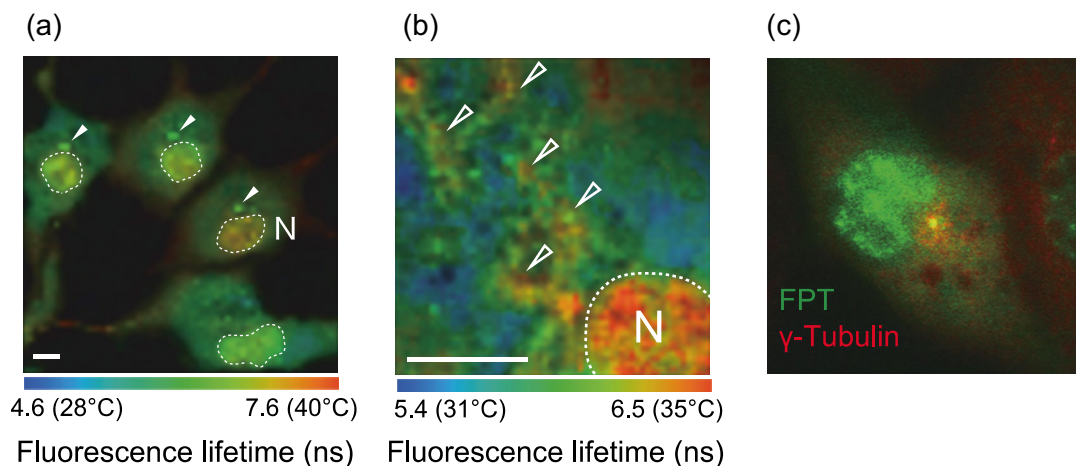


Fig. 7.2 Temperature mapping in living COS7 cells with FPT. **(a,b)** Confocal fluorescence lifetime image of FPT obtained with FLIM. The nucleus (dotted line with N in A) and local area near the mitochondria (outlined arrowhead in b) showed higher temperature than the surround-

ing cytoplasm. **(c)** Higher temperature near the centrosome (arrowhead). Merged epifluorescence images of FPT (green), the centrosome visualized with γ -tubulin antibody (red). Bars represent 10 μ m. Reproduced with permission from Ref. (Okabe et al. 2012)

7.2.3 Applications to Biology and Physiology

7.2.3.1 Brown Adipose Tissue (BAT) Cells

Mitochondria is a major thermogenic organelle in a cell. The proton gradient is maintained at the mitochondrial membranes, whose energy is used in ATP production; this energy can be converted into heat by uncoupling proton transport and ATP production (Enerback et al. 1997; Matthias et al. 2000). Chemical uncouplers, such as carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), are widely used as a model of proton gradient cancelation in various cell lines (Paulik et al. 1998; Terada 1981). For example, we have reported that FNT and FPT captured the temperature rise by around 1 °C in COS7 cells induced by FCCP (Fig. 7.3a) (Gota et al. 2009; Okabe et al. 2012). Interestingly, mitochondrial thermogenesis can be observed in the time course (i.e., the stimulated state) and spatial distribution (i.e., the steady state).

In brown adipocytes, uncoupling protein 1 (UCP1), which is selectively expressed in this

tissue, generates heat by canceling the proton gradient of the mitochondrial membrane. Stimulation of a β_3 -adrenoreceptor, such as nor-epinephrine, is a physiological stimulus for activating UCP1 in this thermogenic tissue (Cannon and Nedergaard 2004). The biological application of intracellular thermometry using FNT revealed the intracellular temperature elevation (~ 1.29 °C) in brown adipocytes upon β_3 -adrenoreceptor selective agonist treatment (Fig. 7.3b) (Hattori et al. 2016). However, the temperature increases upon this stimulation in BAT cells of an apoptosis signal-regulating kinase 1(ASK1) deficiency, which leads to impaired metabolic responses, including UCP1 expression in BAT and oxygen consumption of mice, was significantly restricted (~ 0.52 °C) (Fig. 7.3b). Furthermore, recently, an involvement of protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) in this UCP1-dependent thermogenesis in BAT was shown using this method (Kato et al. 2020). These results have proven the causal correlation between intracellular thermogenesis and metabolic, physiological responses of individuals.

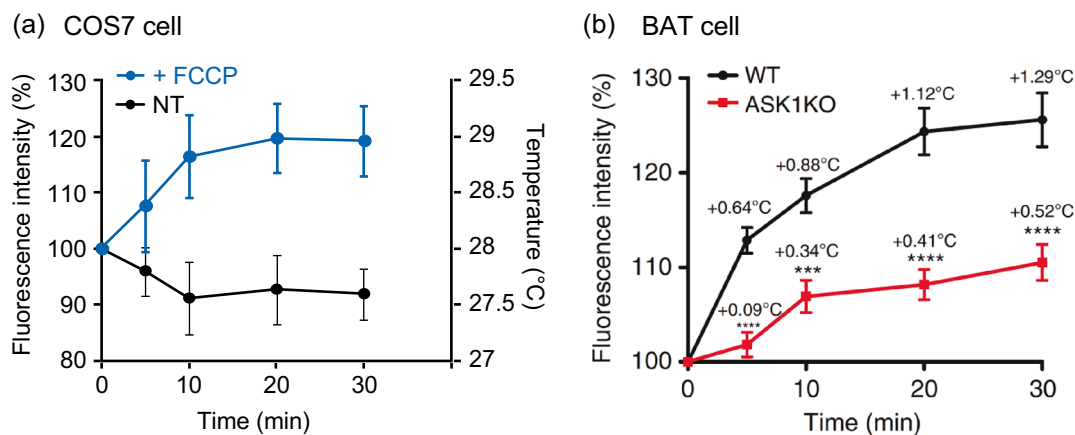


Fig. 7.3 Monitoring of temperature increase in mitochondria-stimulated cells with FNT. **(a)** The temperature increase in COS7 cells upon chemical uncoupler FCCP treatment. **(b)** Suppressed thermogenesis in apoptosis signal-regulating kinase 1 (ASK1) knockout mouse

brown adipocyte tissue (BAT) cells. CL316,243, a β_3 adrenoceptors-specific agonist, was used to induce metabolic response in BAT cells. Reproduced with permission from Ref. (Hattori et al. 2016)

7.2.3.2 Mouse Brain Slice (Hoshi et al. 2018)

We applied intracellular thermometry to neural function. Cerebral edema is a severe brain disease in which brain tissue rapidly expands due to ischemia, such as cerebral infarction. Although many mechanisms of cerebral edema development are unknown, we investigated the involvement of intracellular temperature fluctuations in edema because hypothermia is clinically applied. Also, the relation of the temperature-sensitive ion channel TRPV4 (White et al. 2016), which is strongly activated above 34 °C, is reported in this disease. To measure the temperature inside brain neurons, the cell membrane permeabilizing FPT (Hayashi et al. 2015) was introduced into mouse acute brain slices, and FLIM analyzed the fluorescence lifetime. As a result, FPT in the brain slices responded sensitively to temperature changes in the culture medium, indicating the feasibility of temperature imaging inside nerve cells. Next, the brain slices that were subjected to ischemic stimulation showed an increase in nerve cell temperature of approximately 2 °C compared

with the untreated state (Fig. 7.4). By imitating this, when a high-temperature stimulus was applied to the brain slice, an increase in size, a symptom of cerebral edema, was observed. This result suggested that the spontaneous temperature rise induced by ischemia in nerve cells should cause cerebral edema.

Furthermore, we assumed the neural activity as the cause of this fever and examined the possibility that glutamic acid is involved in the temperature rise due to ischemia. First, glutamate stimulation increased the intracellular temperature in the brain slice by approximately 2 °C. Additionally, treatment with a glutamate receptor inhibitor suppressed the increase in brain temperature due to ischemic treatment. These results revealed that neural activity-dependent intracellular thermogenesis is responsible for the temperature rise during ischemia. Furthermore, these results revealed the interesting property of thermal signaling that temperature changes associated with the spontaneous activity of cells induce severe diseases; also, temperature changes inside cells activate temperature-sensitive molecules in the cell membrane.

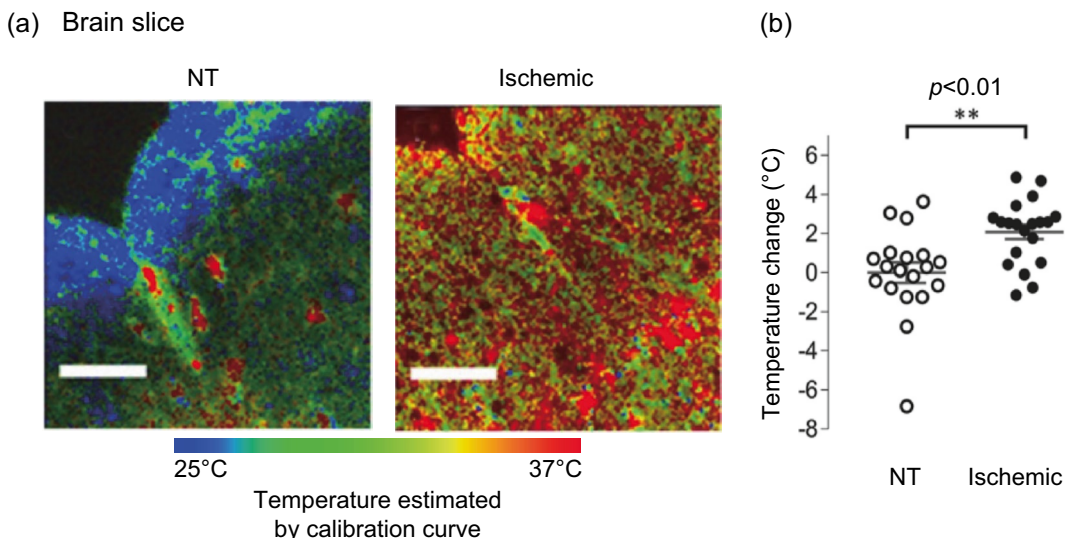


Fig. 7.4 Imaging of ischemia-provoked temperature increase in mouse neuronal slice with FPT. (a) Temperature mapping of mouse neuronal slices. Bars

represent 50 μm . (b) Temperature increase upon ischemic stimulation. Reproduced with permission from Ref. (Hoshi et al. 2018)

7.3 Nanodiamonds for Intracellular Temperature Sensing

7.3.1 Nitrogen-Vacancy Center

The nitrogen-vacancy center (NVC) is a crystal defect in a diamond composed of a nitrogen atom and an adjacent vacancy center (Fig. 7.5a) (Doherty et al. 2013; Jacques et al. 2009). NVCs have two stochastic charge states: neutral (NV^0 , electron spin: $S = 1/2$) and negatively charged (NV^- , $S = 1$) (Doherty et al. 2013), with the energy diagram of NV^- (Fig. 7.5b) (Jarmola et al. 2015). Both NVCs emit in the near-IR region when excited by green light (Fig. 7.5c); hence, nanodiamonds containing NVCs are often called fluorescent nanodiamonds (FNDs) (Gaebel et al. 2004; Yu et al. 2005). Since FND fluorescence shows neither photobleaching nor photoblinking, they are ideal for long-term and single-molecule imaging applications. Among these NVCs, special attention is paid to NV^- centers because of their unique magneto-optical properties (Fig. 7.5b) (Hsiao et al. 2016; Hui et al. 2017). The ground state of NV^- is a spin-triplet, and the spin sublevels, $m_s = 0$ and $m_s = \pm 1$, are split by

zero-field splitting D (ca. 2.87 GHz), with the spin sublevels $m_s = \pm 1$ degenerating in the absence of an external magnetic field. Microwave (MW) irradiation at this frequency induces electron spin magnetic resonance between $m_s = 0$ and ± 1 spin levels, resulting in a reduced fluorescence emission due to spin-dependent intersystem crossing. The optical readout of these spin quantum states is known as optically detected magnetic resonance (ODMR, Fig. 7.5d) (Doherty et al. 2013; Jelezko and Wrachtrup 2006; Schirhagl et al. 2014; Degen et al. 2017). ODMR can be used to measure temperature, which is the main topic of this review, and magnetic and electric field strength (Dube et al. 2007; Rosenberg et al. 2015; Lindquist 1986).

7.3.2 Temperature Measurements with FNDs

7.3.2.1 ODMR

In the ODMR spectrum (Fig. 7.5d), MW frequency-dependent changes in NV^- -fluorescence intensity provide valuable information, including sample temperature. By monitoring the temperature-dependent shift of

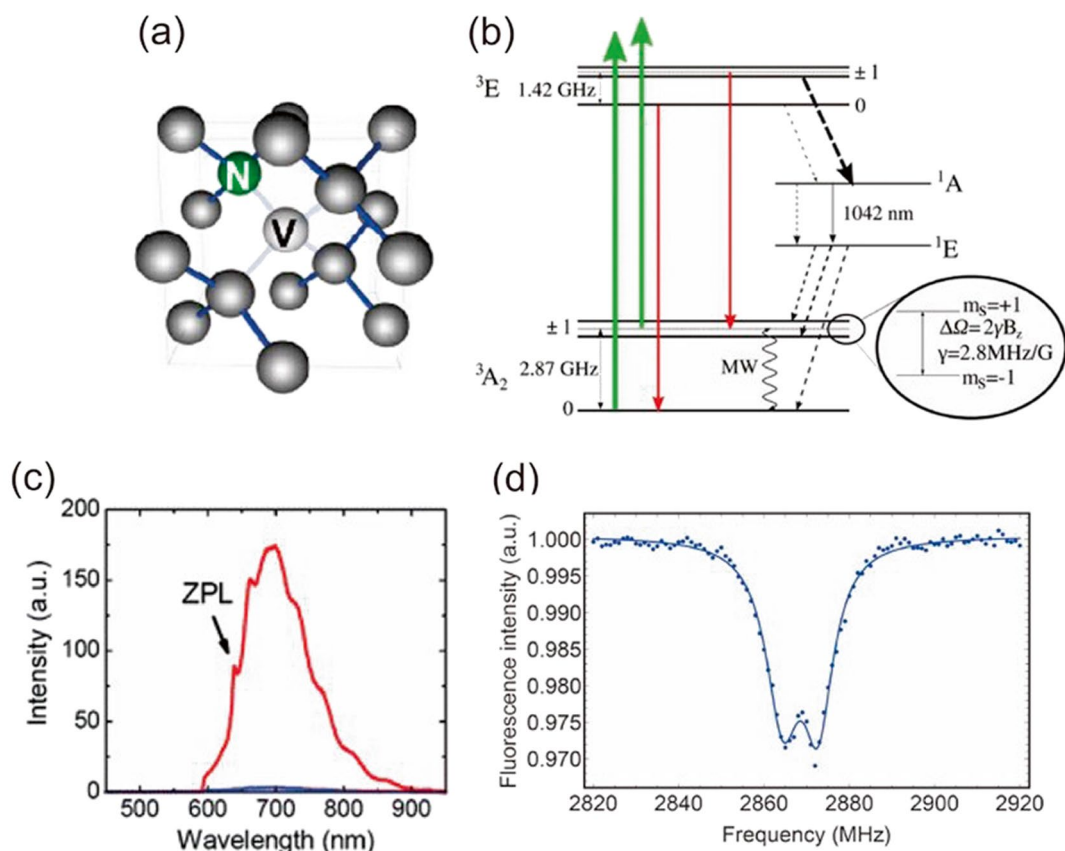
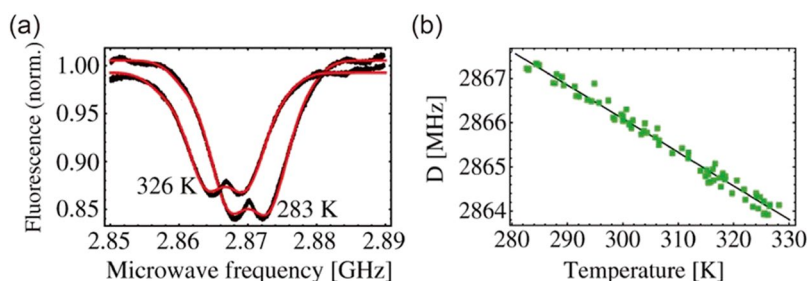


Fig. 7.5 (a) Structure of a NVC in diamond crystal. N: nitrogen atom, V: vacancy center. Modified with permission from Ref. (Jacques et al. 2009). (b) Energy level diagram of NV⁻. Modified with permission from Ref.

(Jarmola et al. 2015). (c) Fluorescence and (d) ODMR spectra of FNDs. ZPL: Zero-phonon line. Reproduced with permission from Ref. (Yu et al. 2005) and (Sekiguchi et al. 2018), respectively

Fig. 7.6 (a) Temperature-dependent shifts of an ODMR spectrum. (b) Frequency shifts of the ODMR peak center around 280–330 K. Modified with permission from Ref. (Acosta et al. 2010)



zero-field splitting D , measuring the temperature around FNDs is possible. Acosta et al. first demonstrated that NV⁻ can serve as a temperature sensor (Acosta et al. 2010). They measured the temperature-dependent shift in D from 280 to 330 K for an NV⁻ center ensemble from

0.01–15 ppm and reported a thermal shift of $\Delta D / \Delta T = -75 \text{ kHz/K}$ (Fig. 7.6).

For measurements of biologically relevant temperatures, it is problematic if biological factors beyond temperature influence the temperature sensing ability of FNDs. However, Sekiguchi

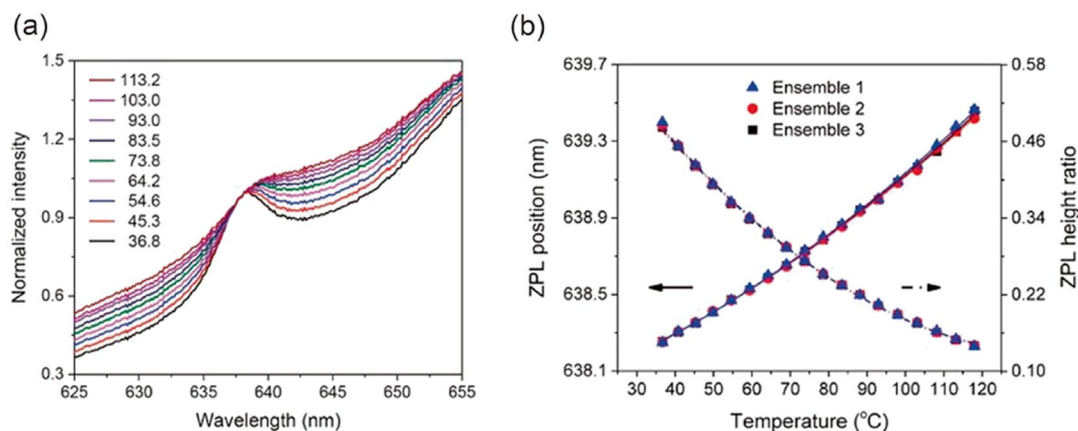


Fig. 7.7 (a) Comparisons of the fluorescence spectra in the ZPL region of NV⁻ centers in PHEMA-embedded FNDs at 36.8 °C–113.2 °C. The spectra were normalized to the intensity at 638.161 nm. (b) Changes in ZPL position and height ratios over 35 °C–120 °C for three repre-

sentative FND ensembles embedded in the PHEMA films. Curves represent the best fits of the experimental data using second-order polynomials. Modified with permission from Ref. (Hui et al. 2019)

et al. demonstrated that the thermosensing ability of FNDs is barely influenced by environmental factors, including pH, ion concentration, viscosity, molecular interactions, and organic solvent presence (Sekiguchi et al. 2018). Thus, FNDs serve as robust temperature sensors inside single cells.

7.3.2.2 All-Optical

The fluorescence spectra of FNDs are characterized by a zero-phonon line (ZPL) at 637 nm (Fig. 7.5c), whose frequency shifts can be used to measure temperature (Chen et al. 2011). It is also possible to monitor the change in ZPL height concerning its phonon sideband using a ratiometric technique developed by Plakhotnik et al. (Plakhotnik et al. 2015). Such all-optical methods are preferable for bioapplications because they do not require a metal wire for inducing MW frequency, which must be placed close to the FNDs for ODMR method. The temperature-dependent change in ZPL frequency is relatively small (0.015 nm/K⁻¹); however, the accuracy of temperature measurements can reach sub-kelvin levels due to the lack of FND fluorescence photobleaching. For example, Hui et al. reported that the temperature sensitivities using ZPL shifts and height changes of FNDs embedded in poly(2-hydroxyethyl methacrylate) (PHEMA) were 0.46–1.1 K Hz^{-1/2} and 0.15–0.62 K Hz^{-1/2}, respec-

tively, over the temperature range of 35–120 °C (Fig. 7.7) (Hui et al. 2019).

7.3.3 Recent Bioapplications

Precise measurements of local temperature in living systems were reported for the first time by Kucsko et al (Kucsko et al. 2013). To minimize measurement times, instead of measuring the whole ODMR spectrum, they monitored temperature changes at four points within the ODMR spectrum, which allowed for 4-s temperature measurements with a precision of 0.1 K in HeLa cells. Temperature sensing has also been performed for primary cortical neurons by Simpson et al. (Simpson et al. 2017) and adipose tissue-derived stem cells by Yukawa et al. (Yukawa et al. 2020). The latter study shows that intracellular temperature influences growth factor production and the degree of differentiation into adipocytes and osteocytes. Recently, Fujiwara et al. demonstrated temperature measurements in *C. elegans* (Fujiwara et al. 2020). Since FNDs move quickly inside *C. elegans*, Fujiwara et al. developed a fast particle-tracking algorithm. In addition, to exclude artifacts caused by fluorescence intensity fluctuations arising from the worm's body structure, they also established an error-correction filter. The resulting system allows for real-time

temperature measurements using mobile FNDs inside living adult *C. elegans* with a precision of around ± 0.22 °C. Then, this system was successfully employed to determine the internal temperatures of worms under pharmacological treatments using a mitochondrial uncoupler (i.e., FCCP) meant to induce non-shivering thermogenesis (Fig. 7.8).

Thanks to their physicochemical robustness, FNDs can be hybridized with various nanomaterials. Thus, FND-gold nanohybrids have immense potential as nanodevices for hyperlocalized hyperthermia and other nanothermometry applications. Tsai et al. employed an all-optical method, similar to that described above, to con-

duct thermometric measurements using FND/gold nanorod hybrids (FND-GNR) that acted as dual-function nanoheaters and thermometers to probe the local thermostability of subcellular components in live cells (Tsai et al. 2017). These FND-GNRs were used along with optical microscopy to visualize and characterize the thermally induced rupture of membrane-tunneling nanotubes formed between HEK293T cells upon membrane disruption and retraction. They found that the rupture temperatures arising due to local and global heating were 28 ± 2 °C and 38 ± 1 °C, respectively.

The concerns with using FND/GNR hybrids include that FND/GNR stoichiometry is chal-

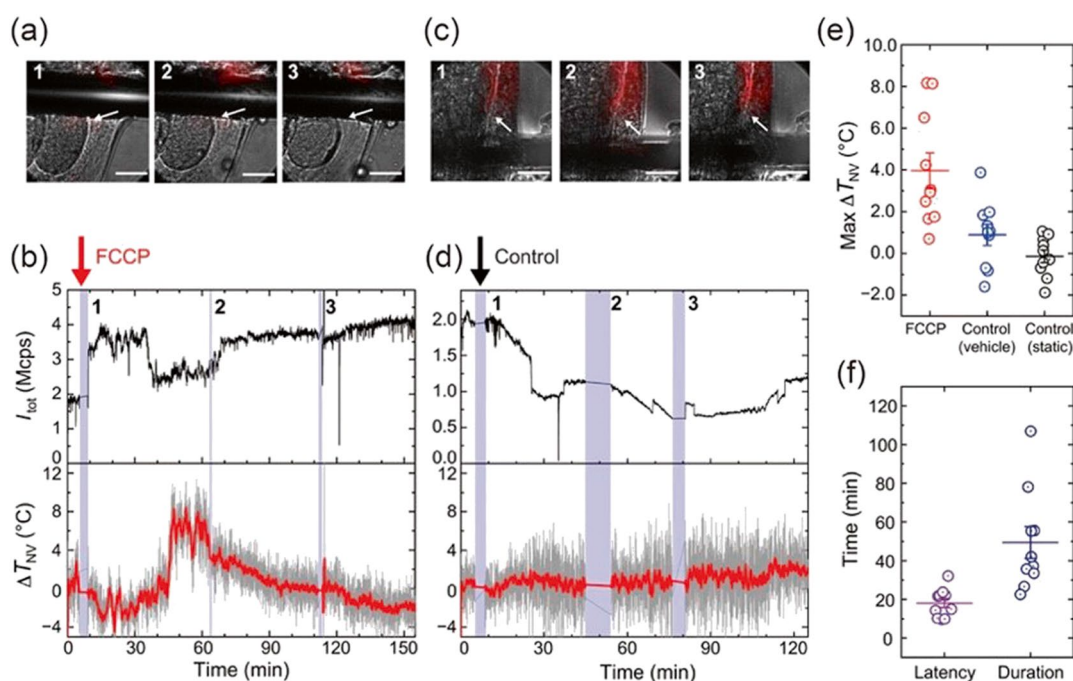


Fig. 7.8 (a and c) Merged photos of NDs during FCCP stimulation (60 μM) and vehicle control experiments. Red scale, red fluorescence; gray scale, bright field. The numbers in the upper left-hand corner of each image correspond to specific times at which each picture is captured during measurements shown in (b) and (d). Scale bars, 20 μm . (b and d) I_{tot} (top) and ΔT_{NV} (bottom) during FCCP stimulation and vehicle control experiments. The blue shaded regions represent periods when no temperature measurements were performed and in which the photographs in (a) and (c) were obtained. ΔT_{NV} was calculated using the equation $dD/dT = -65.4 \text{ kHz} \cdot ^\circ\text{C}^{-1}$ for both types of experiments. (e) Statistical plots of the maximum ΔT_{NV} for FCCP stimulation (red), vehicle control (blue),

and static control (black, no solution added). $n = 10$ for all data. Mean values $\pm$ SE were 4.0 °C $\pm$ 0.9 °C, 0.9 °C $\pm$ 0.5 °C, and -0.1 °C $\pm$ 0.3 °C for the FCCP, vehicle control, and static control experiments, respectively. All measurements were performed at a constant temperature of 23 °C with fluctuations of less than 0.25 °C. Probed NDs were located within 20 μm of the antenna, with a mean distance of 8.9 μm . (f) The latency and durations of responses to increased temperature responses of ΔT_{NV} for the FCCP stimulus, whose means $\pm$ SEs of 18.1 ± 2.3 and 49.4 ± 8.22 min, respectively. Reprinted with permission from Ref. (Fujiwara et al. 2020)

lenging to control; also, GNRs can be deformed by heating, making quantitative, reproducible experiments difficult. To overcome this, Sotoma et al. used polydopamine (PDA) as a heater and performed intracellular thermal conductivity measurements (Sotoma et al. 2020). PDA coating is a facile coating method inspired by adhesive protein composition in mussels and has strong photothermal properties. PDA-coated FNDs (FND-PDA) were fabricated to create a two-in-one nanoheater/thermometer (Fig. 7.9a, b) with high particle uniformity. Additionally, the PDA layer thickness, which is related to the heating efficiency, can be easily controlled by changing the reaction conditions. Once fabricated, the temperature increase in FND in FND-PDA caused by laser irradiation was examined, reflecting the thermal conductivity of the surrounding medium. Indeed, the temperature increase in air, water, and oil is markedly correlated with literature values of thermal conductivity (Fig. 7.9c). Next, the intracellular thermal conductivities of HeLa and MCF-7 cells were examined, with the mean conductivities of two separate cell lines being $0.11 \pm 0.04 \text{ W m}^{-1} \text{ K}^{-1}$ (95% confidence) with similar measurement accuracies, which were significantly smaller than that of water (Fig. 7.9c).

7.4 Future Perspective

The applications of these fluorescent thermometers in cells, tissues, and even in vivo are now developing a new field of thermal biology (Okabe et al. 2021). This emerging technique of intracellular thermometry has started uncovering the thermal properties of intracellular environments, temperature variations, and heat propagation in time and space. Considering the diverse results of intracellular temperature changes associated with cell organelles and physiological functions discovered so far, local intracellular temperature changes should be involved in cellular functions and physiological phenomena in addition to the well-known body temperature control by brown adipocyte thermogenesis. Furthermore, the existence of a mechanism in which spontaneous intracellular heat generation in brain neurons activates its temperature-sensitive molecule, TRPV4 (Hoshi et al. 2018), indicates that spontaneous heat production in cells may regulate the functions of other molecules. Further investigation into the sensing and use of intracellular heat in physiological phenomena will reveal the mechanism of thermal signaling (Okabe et al. 2021): the conformational changes in proteins or enhancement of enzymatic activities via local temperature change might influence signal

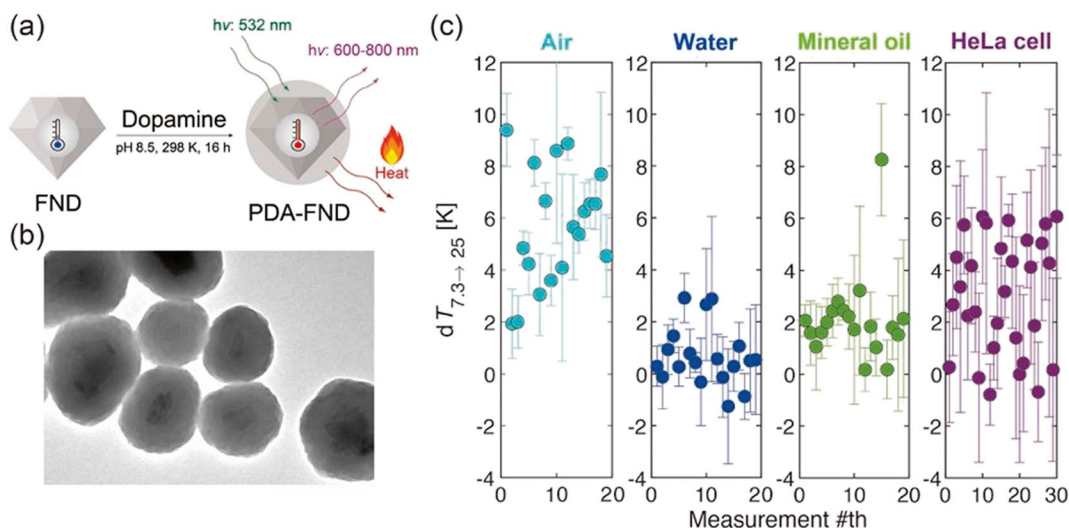


Fig. 7.9 (a) Schematic illustration of dual functional PDA-FNDs prepared from FNDs. PDA-FNDs function as a luminescent nanothermometer, while PDA releases heat in a light-dependent manner. (b) TEM images of PDA-FNDs. Images are $751 \text{ nm} \times 534 \text{ nm}$. (c) Plots and error

bars indicating $\Delta T \pm$ standard deviation for each particle in different environments measured when increasing laser power from 7.3 mW to 25 mW. Modified with permission from Ref. (Sotoma et al. 2020)

transduction (Chuma et al. 2024). This spontaneous temperature variation-assisted thermal signaling will be a new trend not limited to thermal biology, where the elucidation of the mechanisms of environmental adaptation was the main focus, but also in molecular biology, which has been discussed predominately in the chemical interaction between molecules. Furthermore, thermal signaling should facilitate unexplained physiological phenomena, such as thermoregulation, biorhythms, biometabolic regulation, cancer progression, and development (Chuma et al. 2024). Thus, fluorescent thermometry in single living cells will contribute to revolutionary understandings of the relationship between temperature and life soon.

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Temperature Dependent Activity of the Voltage-Gated Proton Channel

8

Yuichiro Fujiwara

Abstract

Voltage-gated proton channel (Hv) has activity of proton transport following electrochemical gradient of proton. Hv is expressed in neutrophils and macrophages of which functions are physiologically temperature-sensitive. Hv is also expressed in human sperm cells and regulates their locomotion. H⁺ transport through Hv is both regulated by membrane potential and pH difference across biological membrane. It is also reported that properties of Hv such as proton conductance and gating are highly temperature-dependent. Hv consists of the N-terminal cytoplasmic domain, the voltage sensor domain (VSD), and the C-terminal coiled-coil domain, and H⁺ permeates through VSD voltage-dependently. The functional unit of Hv is a dimer via the interaction between C-terminal coiled-coils assembly domain. We have reported that the coiled-coil domain of Hv has the nature of dissociation around our bodily temperature and mutational change of the coiled-coil affected temperature-sensitive gating, especially its temperature threshold. The temperature-sensitive gating is assessed from two separate points: tem-

perature threshold and temperature dependence. In this chapter, I describe physiological roles and molecular structure mechanisms of Hv by mainly focusing on thermosensitive properties.

Keywords

Voltage-gated proton channel · Structure–function relationship · Temperature-dependent gating · Temperature threshold · Phagosomal ion channel · Voltage sensor · pH sensor · Coiled-coil

8.1 Introduction

The proper balance of pH in the extracellular fluid is crucial for the normal physiology of the body, and cellular metabolism. Bodily pH homeostasis depends on pH of cellular microenvironment. pH is regulated by many species of membrane proteins including proton pumps, transporters, and channels. Voltage-gated proton channel (Hv) mediates robust activities of proton transport along electrochemical gradient of protons across biological membranes. The voltage-dependent H⁺ current was first “discovered” in snail neurons (Thomas and Meech 1982), and Hv currents were later described in mammalian blood cells (Decoursey 2003) and in human sperm cells (Lishko et al. 2010). Physiological

Y. Fujiwara (✉)

Molecular Physiology & Biophysics, Faculty of Medicine/Graduate School of Medicine, Kagawa University, Miki-cho, Kagawa, Japan
e-mail: fujiwara.yuichiro@kagawa-u.ac.jp

roles of Hv have been most intensively studied in mammalian phagocytes (Demaurex and Petheo 2005; Decoursey 2003). H^+ export through Hv is required for charge compensation due to the export of electrons at the production of superoxide anion by NADPH oxidase (Henderson et al. 1987; Decoursey 2003) (Fig. 8.1). Hv also plays an important role in species preservation functions, and alkalization of sperm cytoplasm via the H^+ export through Hv triggers the sperm locomotion (Lishko et al. 2010).

cDNA of Hv was first cloned from mouse and sea squirt by bioinformatics approach, and its structure consists only of the voltage sensor domain with similarity to that of voltage-gated ion channels (Ramsey et al. 2006; Sasaki et al. 2006). Hv has simple molecular architecture consisting of the N-terminal cytoplasmic domain, four transmembrane segments, and the cytoplasmic coiled-coil motif. It is one of the smallest cation channels so far identified from mammalian genomes (Koch et al. 2008). Voltage-gated H^+ channel 1 (Hv1) is a protein that in humans is

encoded by the HVCN1 gene. HVCN1-deficient mice exhibit significantly reduced production of reactive oxygen species in neutrophil (Okochi et al. 2009; Ramsey et al. 2006), proving that Hv1 plays an important role in phagocyte function (Okochi et al. 2016).

Hv is known to be activated by various factors such as membrane potential, the pH difference between intracellular and extracellular, unsaturated fatty acids, and the membrane stretch, suggesting multiple sensor and effector functions. One of the outstanding features of Hv is its high temperature sensitivity (DeCoursey and Cherny 1998). Q_{10} of activation gating of Hv is larger than in other voltage-gated ion channels (Decoursey 2003). The high Q_{10} property of Hv suggests its physiological function, and in fact, phagocytes that abundantly express Hv have several temperature-dependent functions in bacterial killing, release of bioactive substance, chemotaxis, apoptosis, and proliferation. Hv has also been shown to be present in cancer cells, sperm cells, and phytoplankton (Taylor et al. 2011). In this chapter, I focus on temperature-dependent mechanisms of Hv with relevance to its cellular physiology.

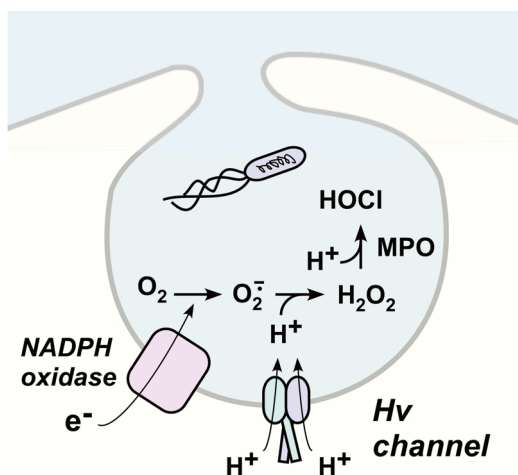
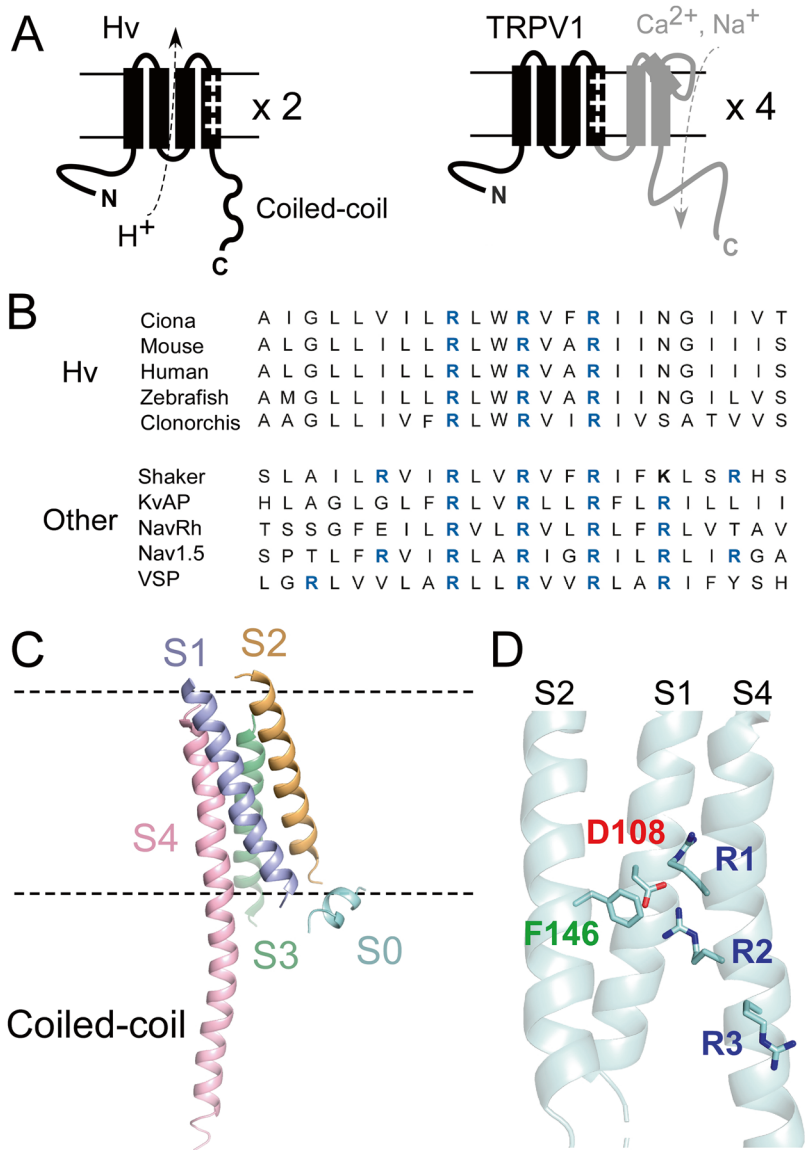


Fig. 8.1 Hv in phagosomes. Physiological coupling between Hv and NADPH oxidase in neutrophil membrane. NADPH oxidase transfers electrons across the cell membrane and phagosomal membranes and leaves protons in the cytoplasm. This leads to charge imbalance, thus making membrane depolarized and making cytoplasm acidic. Hv cancel this charge imbalance and maintain the cytoplasmic pH neutral. In phagosomes, proton is also required for making H_2O_2 from superoxide anions

8.2 Biophysical Properties of Hv

Hv consists of the voltage-sensor domain and exhibits voltage-dependent gating as in other voltage-gated ion channels (Fig. 8.2a, b). One unique feature is that its conductance–voltage relationship is shifted by change in either intracellular or extracellular pH. In acidic pH in intracellular side or in alkalic pH in extracellular side, Hv can be activated by physiological range of membrane potential. Thanks to the nature of pH-sensitivity of voltage-dependent gating, Hv is opened only when electrochemical gradient drives outward H^+ current. As observed in other ion channels, Hv is also activated by unsaturated fatty acid; arachidonic acid (DeCoursey and Cherny 1993; Kawanabe and Okamura 2016) and endocannabinoids (Lishko et al. 2010) greatly enhance the activity of the voltage-gated H^+ current. The activation of Hv is also facilitated by

Fig. 8.2 Structure of Hv. (a) Molecular designs of Hv and thermosensitive TRPV1 channel. Hv contains a cytoplasmic coiled-coil domain, which mediates dimerization. TRPV1 forms a tetramer and forms a pore in the center. (b) Multiple alignments of amino acid sequence in S4 from Hv channels and the voltage-gated channel superfamily. (c) Overall structure of the mouse chimeric Hv1 protein is shown as a monomer. (d) Spatial relationship between Asp108, Phe146 and three Arg in S4. The S3 helix has been omitted to allow core structure to be viewed



membrane stretch (Pathak et al. 2016). Relationships between these sensor functions and the membrane events in phagocytosis are expected to be elucidated.

An important feature of gating of Hv is its high sensitivity to heat. Temperature dependence of activation kinetics was described in bone marrow-derived mast cell (Kuno et al. 1997) and then intensively studied in alveolar type II cells of rat lung using whole cell patch clamp recording (DeCoursey and Cherny 1998). Notably, Q_{10} of activation kinetics of Hv is two to three times

larger than that in other voltage-gated ion channels. Q_{10} of activation kinetics recorded from human neutrophils is about 6–9, which is significantly larger than 3–5 in other voltage-gated ion channels (DeCoursey and Cherny 1998). Increasing temperature accelerates both the activation and deactivation kinetics, and the activation energy, E_a , is approximately 30–38 kcal/mol greater than for most other ion channels (DeCoursey and Cherny 1998). Q_{10} values were slightly different in other cell types that endogenously express Hv currents, such as human

neutrophil, THP-1 cell (human macrophage cell line), and BV-2 cell (murine microglia cell line) probably reflecting some variations of molecular nature of Hv among these cell types, possibly phosphorylation or stoichiometry. The temperature-sensitive activation of Hv is consistently observed in the heterologous expression system (Fujiwara et al. 2014; Fujiwara et al. 2012). This heat-sensitive gating property evokes the dramatic increase of H^+ current in the range of bodily temperature.

Conductance is also heat-sensitive. Q_{10} of H^+ conductance is estimated to be about 2.8 (at $>20^\circ\text{C}$) and 5.3 (at $<20^\circ\text{C}$) using the excised patch clamp recording (DeCoursey and Cherny 1998). This is consistent with the results of the pioneering study of Hv current in snail neurons ($Q_{10} = 2.1$) (Byerly and Suen 1989). Another study reported that Q_{10} of H^+ conductance of Hv in rat microglial cell line was estimated to be about 2.8 (at 5°C) and 2.2 (at 45°C) under the temperature-jump control system (Kuno et al. 2009). This setup enabled to completely separate temperature dependence of conductance from that of gating. It is interesting that these values are larger than the value of 1.17 for H^+ conductivity in bulk water (Stokes and Robinson 1948), suggesting that Q_{10} of H^+ conductance includes component of Q_{10} of the channel protein and of access resistance from the bulk water. It is also interesting that Q_{10} was not the same over the temperature range as found in an earlier study (DeCoursey and Cherny 1998), suggesting that multiple processes underlie temperature sensitivity of H^+ conduction. It is difficult to measure the single channel current of Hv because of the small conductance. The unitary conductance of H^+ analyzed using the fluctuation analysis for the macroscopic current is reported to be 38 fS at pH 6.5 (Cherny et al. 2003). Although transfer rate of H^+ through Hv is also the maximum among proton transporting proteins, it is about only one thousandth smaller than that of other ion channels. H^+ selectivity is highest among proton transporting proteins, and Asp in S1 (including Arg in S4) in the voltage-sensor domain is considered to contribute to the selectivity (Musset et al. 2011) (Berger and Isacoff 2011). These suggest that permeation mechanisms are complex and H^+

conduction of Hv does not occur as simple diffusion in bulk solution; the mechanism of heat-sensitivity of H^+ conductance is a subject for future analysis.

Various blocker of Hv have been reported. Zinc is the most commonly used inhibitor of Hv and is supposed to inhibit the motion of voltage sensor activation (Cherny and DeCoursey 1999; DeCoursey 2003). Release of the zinc block is reported to be a signal to initiate sperm movement (Lishko et al. 2010). Hanatoxin, a spider toxin, can also suppress Hv activities (Alabi et al. 2007). 2GBI, a guanidium-related reagent, was shown to be open channel blocker from intracellular side (Hong et al. 2013). Relationships between these blockers' effects and the temperature sensitivity of Hv have not been elucidated.

8.3 Physiological Roles of Hv

Native Hv currents have been described in blood cells, microglia, alveolar cell, airway epithelium, osteoclast, cardiac fibroblast, and human sperm.

Neutrophils of HVCN1-deficient mice exhibit reduced production of superoxide anions (Okochi et al. 2009) (Fig. 8.1). Membrane potential is more depolarized in neutrophils derived from the HVCN1 gene-deficient mice than in those from the wild-type mice (El Chemaly et al. 2010). Neutrophils of HVCN1 gene-deficient mice exhibit lower motility than normal neutrophils, reflecting the low calcium influx due to less driving force (El Chemaly et al. 2010). It is also interesting that Hv inhibits neutrophil granule release via the compensating charge imbalance due to the activation of NADPH oxidase (Okochi et al. 2016).

Activities of B-lymphocytes are also regulated by Hv. Studies of knockout mice of HVCN1 suggest that Hv plays a role in antibody production through regulating intracellular signaling with protein phosphorylation (Capasso et al. 2010). Coimmunoprecipitation studies show that Hv is located close to B-cell receptor (BCR) and facilitates activity of NADPH oxidase activity, shutting down phosphatase activity of SHP, leading to sustaining the level of phosphorylated Syk (Capasso et al. 2010).

T-lymphocyte derived from HVCN1-deficient mice exhibits reduced production of superoxide anion, and HVCN1-deficient mice exhibit frequent nephritis: the characteristic of autoimmune disorders (Sasaki et al. 2013). T-lymphocytes show two phases of O_2^- production upon stimulation by PMA of which the latter phase is completely lacking in HVCN1-deficient mice (Sasaki et al. 2013).

Mast cell and eosinophils are blood cells that secrete histamine and heparin in response to IgE binding. Hv current has been reported in these cells (Kuno et al. 1997) (Musset et al. 2008). In eosinophil, Hv plays a role in regulating NADPH oxidase activity (DeCoursey et al. 2001) (Petheo et al. 2003). In mast cell, degranulation by IgE has been shown to be dependent on Hv (Musset et al. 2008). In basophil, Hv plays a role in regulating IgE-mediated release of histamine (Musset et al. 2008).

In microglia, brain phagocyte, ROS production depends on Hv (Kawai et al. 2017), and this is in particular important for neuronal cell death following brain ischemia. Knockout mice of HVCN1 exhibit milder neuronal cell death following brain ischemia (Wu et al. 2012).

Human sperm has robust Hv in the membrane of the principal piece, and Hv has been reported to regulate initiation of motility (Lishko et al. 2010). In epididymis, sperm is exposed to zinc ions at the order of mM, which inhibits activities of Hv leading to prevention of sperm motility (Lishko et al. 2010). When sperm is ejaculated into female reproductive tract, which contains little zinc, Hv escapes from inhibition by zinc, and H^+ efflux through Hv causes intracellular alkalization. Intracellular alkalization in sperm activates CatSper calcium channel, and Ca^{2+} triggers sperm locomotion (Lishko et al. 2012).

8.4 Temperature-Dependent Activity of Blood Cells

As above mentioned, Hv is mainly expressed in blood cells whose function is known to be highly temperature-sensitive. Although relationships between temperature sensitivity of Hv and of blood cells have not been elucidated enough yet,

discussions cannot be avoided; points are listed in the following.

Neutrophils are the cells that primarily attack pathogen and kill it by phagocytosis. Changes due to temperature have been examined in several different aspects of neutrophil function. In vitro, bacterial exclusion by neutrophil has been reported to be optimal at 37 °C. At temperature higher than 37 °C, killing efficiency increases toward *Pneumococcus* but decreases toward *Escherichia coli* (Sebag et al. 1977). Production of reactive oxygen species (ROS) in neutrophil, which is critical for pathogen exclusion, is also temperature-dependent and it is commonly optimal at 37 °C. ROS production is reduced in fever, and its temperature dependence is varied among ROS species: O_2^- is mildly reduced at 42 °C (70%), whereas HOCl is remarkably reduced (13–15%) (Severns et al. 1986). HOCl production in neutrophil depends on the activity of myeloperoxidase of which activities are optimized at body temperature (Severns et al. 1986). Neutrophil life is ephemeral and it undergoes apoptosis, which is reported to be temperature-dependent. At temperature higher than 37 °C, more apoptosis is facilitated by heat-activated translocation of Bax to mitochondria (Pryde et al. 2000) or by stopping TNF- α -dependent NF- κ B signaling (Salanova et al. 2005). Chemotaxis, known as the capture behavior of phagocytes of neutrophil, is also temperature-dependent: formylmethionyl-leucyl-phenylalanine, a chemoattractant, is known to induce chemotaxis in a temperature-dependent manner (Chodniewicz et al. 2004). Proliferation of neutrophil is also temperature-dependent: recovery of blood neutrophil after X-ray treatment is more rapid when body temperature is kept at over 37 °C (Capitano et al. 2012).

Eosinophil is a prominent component of allergic and hypersensitive disorders and thus an important factor in the pathogenesis of these diseases. Electron currents were measured to quantify NADPH oxidase activity in eosinophil by whole cell patch recording, and NADPH oxidase activity is optimal at 37 °C (Morgan et al. 2003). Steady-state activity of electron current (i.e., oxidase activity) was sharply dependent on temperature ($Q_{10} = 4.2$) when temperature was slowly

changed in a range between 20 °C and 30 °C, which is considered to be caused by superimposition of temperature-dependent events of cells. This is also consistent with a finding of temperature-primed effect of human neutrophil: ROS production at 37 °C is enhanced when cells were preincubated shortly at 38 °C (Jozefowicz-Okonkwo and Nowak 2004).

Mast cell releases histamine in response to nerve stimuli or binding of antigen to IgE present on the cell surface. Release of histamine linearly increases with temperature from 15 °C to 27 °C (Lagunoff and Wan 1974).

Hv has been discovered to be expressed to high level in breast cancer, brain tumor, and colon cancer cells (Wang et al. 2012; Wang et al. 2013). Many cancer cells undergo anoxic metabolism with lactic acidosis and thus are exposed to local acidic condition. It is well-known that cancer cells are less tolerant to temperatures higher than normal body temperature. This provides the basis for cancer thermotherapy.

8.5 Molecular Structure of Hv

cDNA of Hv was first identified by bioinformatics approach (Sasaki et al. 2006; Ramsey et al. 2006) based on the sequence similarity to amino acid sequence of the voltage-gated ion channel superfamily. It has four transmembrane segments with N-terminal and C-terminal cytoplasmic stretches (Fig. 8.2a). C-terminus contains coiled-coil motif, which is required for dimerization of channel (Koch et al. 2008; Fujiwara et al. 2012). In the N-terminal cytoplasmic region, the length varies among animal species (Sasaki et al. 2006; Smith et al. 2011). The N-terminus region has been reported to be related to the activation kinetics, interacting to the third transmembrane region, S3 (Sakata et al. 2016). PKC-phosphorylation sites in the N-terminal cytoplasmic region have been suggested to be responsible for the activation (Decoursey 2003). The voltage-sensor domain of Hv harbors signature motif in the fourth transmembrane segment, S4, with a pattern of alignment of positively charged residues (Fig. 8.2b). Three Arg, R1, R2, and R3, are highly conserved among Hv ortholog proteins, and they

play roles in voltage-sensing (Gonzalez et al. 2013). Other three transmembrane segments, S1–S3, have acidic residues; some of them serve as counter charges for stabilizing positive charges of S4 within membrane or protein. Asp in S1 is reported to be critical for proton-selective permeation (Musset et al. 2011) and R3 reportedly forms a salt bridge with Asp of S1 in activation, which have been proposed to form the H⁺ permeation pathway (Berger and Isacoff 2011). Crystal structure of Hv is considered to be a resting form of channel (Takeshita et al. 2014), and the overall picture of the H⁺ permeation pathway has been unidentified yet.

Crystal structures of mouse Hv1 chimeric protein have been determined: the transmembrane channel domain and the cytoplasmic coiled-coil assembly domain. The transmembrane domain (S1–S4) forms the voltage-sensor structure like voltage-sensors of other voltage-gated ion channels. The structure shows that the four helices (S1–S4) are opened wider on the inner side, forming an inner vestibule and another short helix, S0, is present at the proximal end of S1, flanking the membrane (Fig. 8.2c). S4 stands straight in the membrane and is continuous with the cytoplasmic coiled-coil. The H⁺ permeation pore is occluded by hydrophobic residues, and therefore the structure is considered to be a closed state. The phenylalanine in S2 (F146 in mHv1, F150 in hHv1), corresponding to a hydrophobic plug of the voltage gated K⁺ channels, provides a charge transfer center for the motion of the voltage sensor (Tao et al. 2010). D108, which is critical for proton selectivity, is at about the same level as F146 (Fig. 8.2d). When S4 were compared with those of other voltage-gated channels, the arginines in mouse chimeric Hv1 were lower (closer to the cytoplasmic side) than in other voltage-sensor structure. Thus, the crystal structure is most likely a resting state of the voltage sensor. In addition, the crystal structure contains zinc ion, a physiological blocker of Hv, at outer side of the pore, which also suggests that the structure would be a resting state.

The crystal structure of the mouse chimeric Hv1 showed a trimer, which would be an unnatural form due to its chimeric construction of the cytoplasmic coiled-coil domain with the yeast

GCN4 coiled-coil (Takeshita et al. 2014). The unique structural property of Hv in its natural state is the dimeric structure. Western blot analysis of Hv using cross-linkers showed two bands, suggesting a dimer formation (Koch et al. 2008) (Lee et al. 2008), and the subunit counting experiment also demonstrated that Hv has two pores (Tombola et al. 2008). The C-terminal cytoplasmic domain consisting of ~55 residues is well conserved among species (Fig. 8.3a). In cross-linking assays with a C-terminus deletion mutant, dimer fraction was remarkably reduced, indicat-

ing the importance of the C-terminal region for dimeric assembly (Koch et al. 2008; Fujiwara et al. 2012). The data in analytical ultracentrifuge for the C-terminal domain protein was perfectly fit by a single species model having the molecular mass of a dimer (Fujiwara et al. 2012). The molecular architecture of the C-terminal domain was clarified by solving X-ray crystal structures. The structure of the C-terminal coiled-coil domain of the human and mouse Hv was determined (Fig. 8.3b) (Li et al. 2010) (Fujiwara et al. 2012). The crystal structure of mHv1 coiled-coil

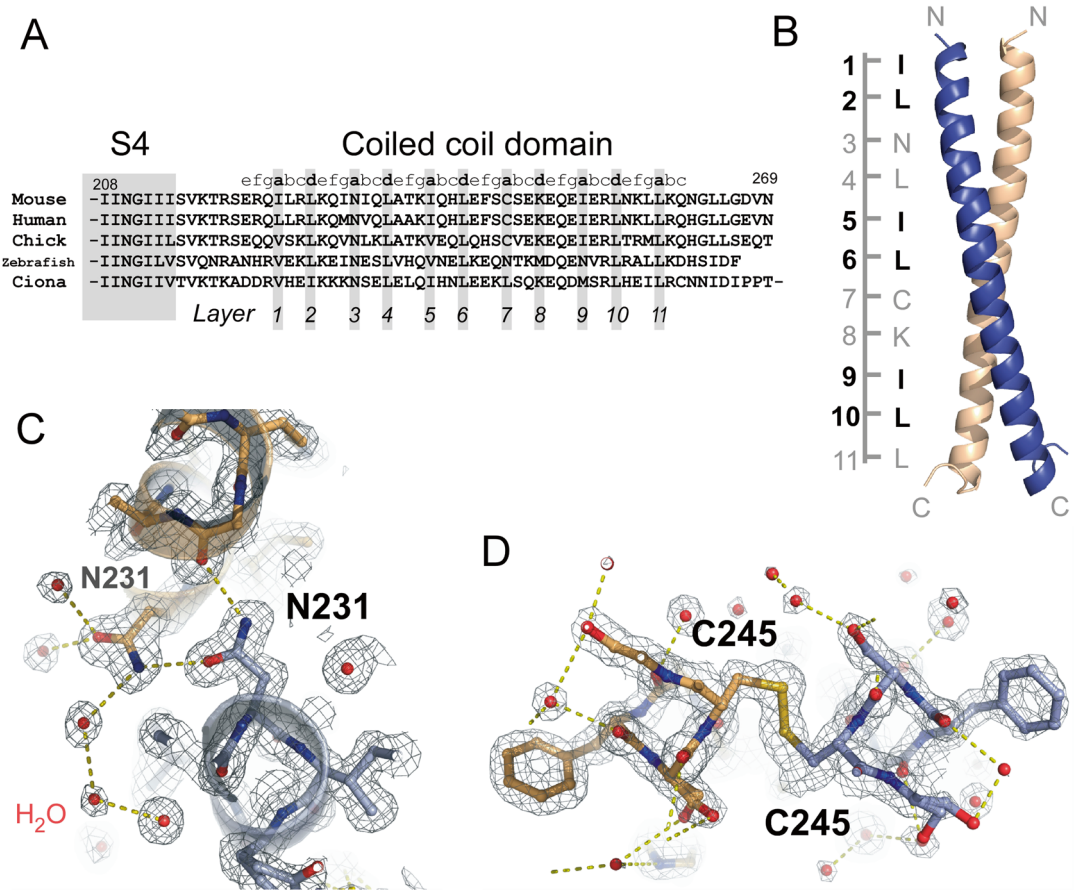


Fig. 8.3 The C-terminal region of Hv as the dimer assembly domain. (a) Sequence alignment of the C-terminal cytoplasmic coiled-coil domain from various species of Hv. The positions of the coiled-coil heptad repeat (abcdefg) are indicated above the alignment. Coiled-coil residues occupying hydrophobic “a” and “d” positions are highlighted. Layer positions for each residue in the structure are indicated below the alignment. (b) Crystal structure of the mouse Hv1 coiled-coil domain protein (PDB code: 3VMX). Layer numbers and the

amino acid residues are indicated. (c) Structure (stick models) and 2Fo-Fc composite-omit maps in layer-3. The cartoon helix models are also indicated to grasp the strand orientation. Red balls depict oxygen atoms of water molecules. Maps are contoured at 1.0 σ . Yellow dashed lines depict polar contacts. (d) Structure and 2Fo-Fc maps (1.0 σ) in layer-7 in the oxidized form (PDB code: 3VMZ) (Fujiwara et al. 2013b). Figures adapted from (Fujiwara et al. 2012)

indicated a parallel left-handed, two-stranded coiled-coil, highlighting the following features (Fujiwara et al. 2012).

- (1) Intersubunit salt bridges and hydrogen bonds on the outer wall of the coiled-coil are missing in the coiled-coil. These are observed in most of coiled-coils and are reported to increase the thermal stability of the coiled-coil assembly (Acharya et al. 2006; Acharya et al. 2002; Howard et al. 2007).
- (2) Hydrophilic Asn residues (N231) instead of hydrophobic residues comprise the core at the layer-3 (Fig. 8.3c). They are asymmetrically packed and form hydrogen bonds with water molecules and a carbonyl oxygen in a rational fashion (Fig. 8.3c). N231 is conserved among species, and its neighboring residues are hydrophobic (I230 and I232) in Hv (Fig. 8.3a). In fact, such a pattern is uncommon for coiled-coil motif. The well-ordered symmetric architecture of the hydrophobic residues in the core layers is expected to stabilize the coiled-coil assembly (Akey et al. 2001; Harbury et al. 1993), and therefore this asymmetric packing at the layer-3 could be a weakness of the thermal stability of coiled-coil.
- (3) Cys residues are present in the layer-7 (Fig. 8.3d). Crystal structures of the coiled-coil domain in reduced, oxidized, and mutated (C245S) states showed that the structure could be changed in response to redox state (Fujiwara et al. 2013b). Such redox sensitivity of the coiled-coil could affect its thermal stability and might have some physiological impact on cellular functions such as phagocytosis in neutrophils or signals of antibody production in B-lymphocytes.
- (4) An I/L core packing pattern is periodically present along the length of the coiled-coil (layers 1/2, 5/6 and 9/10 in Fig. 8.3b). Studies of artificially mutated GCN4 coiled-coils suggest that this I/L core alignment prefers dimerization over other stoichiometries (Harbury et al. 1993). Alteration of such

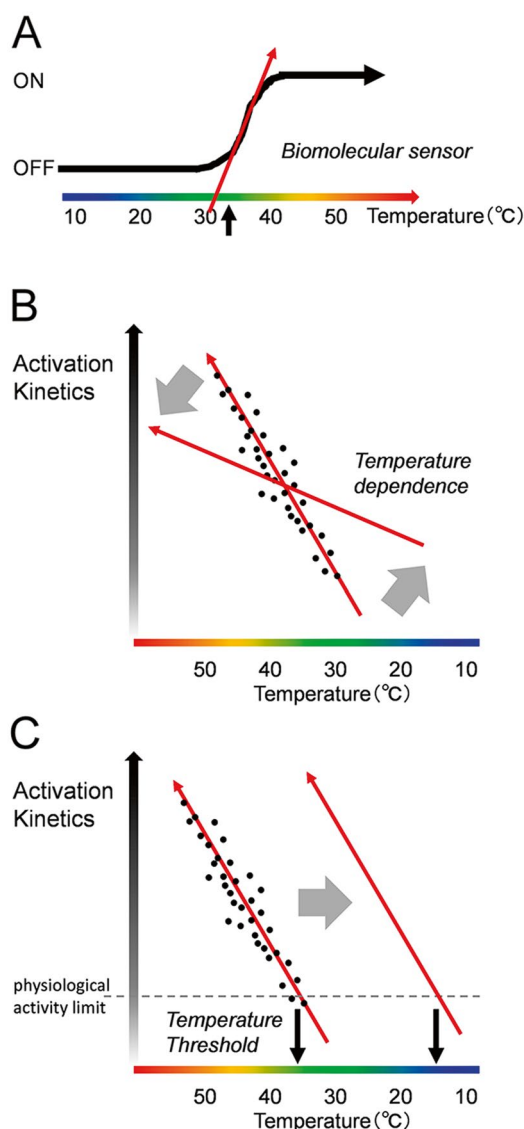
packing patterns resulted in distinct oligomeric states of the full-length channel: the channels harboring the I/I and L/L core packing patterns are expressed as trimers, whereas the channel harboring the L/I pattern was a tetramer (Fujiwara et al. 2013a). The Hv coiled-coil, thus, is optimally designed for assembling dimer units.

These structural features of the coiled-coil assembly well reflect the thermosensitivity of the channel function, which is explained in the following part.

8.6 Interpretation of the Temperature-Sensitive Gating

This paragraph will discuss biophysical aspects of temperature sensitivity of the protein activity. Biopolymers such as proteins and nucleic acids are generally temperature-sensitive, but in order to function as a biomolecule sensor, they have a “temperature range value” adjusted to physiological temperature and a “strong temperature dependence” that responds sharply to temperature. They correspond to the temperature-axis intercept and slope of the Arrhenius plot, respectively (Fig. 8.4). Industrial products such as thermistors are highly temperature-dependent (with almost upright slopes) and clearly switch on and off in response to changes in temperature, and therefore the temperature threshold is also clearly defined. However, the structure of biomolecules, which are macromolecules, is highly flexible, and their functions are also flexible, so the Arrhenius plot presents a milder slope. In this way, it is necessary to consider the temperature threshold and the temperature dependence separately. If a change in the tilt angle is observed in an experiment using a mutant, it means that the temperature dependence has changed (Fig. 8.4b). In this case, even a small temperature change causes a rapid increase in the channel current. If the plot moves parallel to the left and right, it means that the temperature threshold has changed

Fig. 8.4 Scheme of the temperature-sensitive change of the Arrhenius plot. **(a)** Switching of biological reactions by bio-thermalsensors. **(b)** A change in the tilt angle means that the temperature dependence has changed. The slope indicates how much the activity changes as the temperature changes by how many degrees. **(c)** A parallel shift of the plot means that the temperature threshold has changed. The temperature at the physiological minimum limit of the activation kinetics that can cause a biological reaction represents the temperature range value



(Fig. 8.4c). The Hv channel exhibits a strong temperature dependence ($\Delta H = \sim 40$ kcal/mol and $Q_{10} = \sim 10$) (DeCoursey and Cherny 1998) and activates from temperatures close to body temperature (Kuno et al. 1997). The molecular structure basis that determines the high temperature dependence of its channel activity is not completely understood and is still being elucidated using various methods. The next paragraph presents our efforts to elucidate the temperature sensitivity of Hv channels through structural, functional, and proteinochemical approaches.

8.7 Thermal Stability of the Coiled-Coil Domain and the Thermal Sensitivity of Hv

Circular dichroism (CD) spectroscopy demonstrates that the mouse Hv1 coiled-coil domain is unfolded in the range of body temperature (Fujiwara et al. 2012). The melting temperature (for unfolding), T_m , was 41 °C and the slope for melting transition was steeper than those of coiled-coils from other proteins that have over

60 °C as T_m (Deng et al. 2006; Hoshino et al. 1997; Liu et al. 2007; Tsuruda et al. 2006). The unfolding of the coiled-coil of mHv1 was irreversible (Fig. 8.5a, b). These indicate that the Hv assembles into a dimer based on a thermally labile coiled-coil.

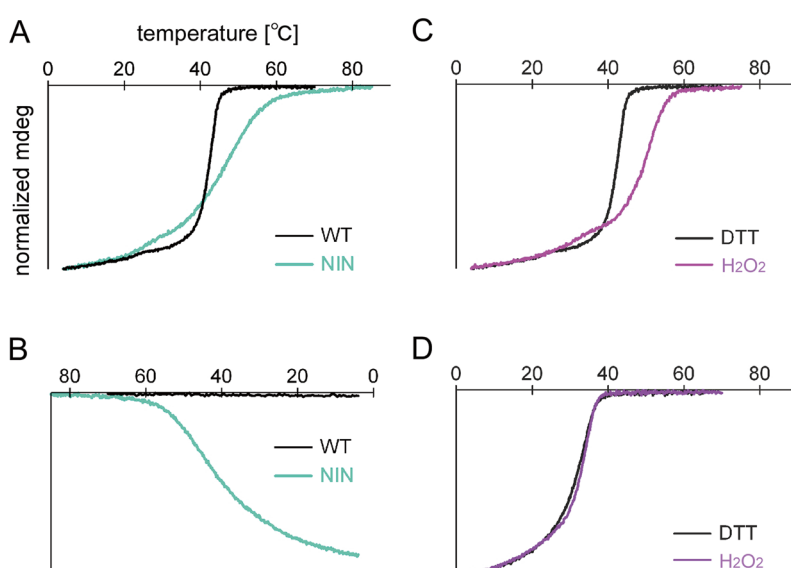
The crystal structure showed that the coiled-coil domain twists and bends at the layer-3, suggesting the weakness of the assembly (Fujiwara et al. 2012). Introducing an inverse sequence, NIN, instead of native INI sequence at the layer 3, increased the thermal stability of the coiled-coil region: melting temperature, T_m , was increased to 45 °C in NIN. In the NIN mutant, the coiled-coil folding is now reversible (Fig. 8.5a, b) and the steepness of the temperature dependence is shallower than the coiled-coil polypeptide with the wild-type sequence.

Thermal stability of the Hv1 coiled-coil also depends on the redox condition (Fujiwara et al. 2013b). The melting temperature is shifted to a higher temperature (apparent T_m = 48–49 °C) by oxidation with H_2O_2 (Fig. 8.5c). The C245S mutation at the layer-7 shows a lower T_m (30–31 °C) than that of WT with DTT, and does not show the T_m shift by oxidation (31–32 °C) (Fig. 8.5d). Thus, the intersubunit disulfide bond formed by oxidation increases the thermal stability of the coiled-coil assembly. It should be noted

that Hv is abundantly expressed in neutrophils or macrophage in which intracellular environment becomes acutely oxidized upon production of reactive oxygen species. Role of redox-dependent regulation of coiled-coil region of Hv in cellular functions still remains elusive.

As described above, the Hv channel activity is known to exhibit high temperature dependence (DeCoursey and Cherny 1998; Kuno et al. 1997) and is expressed in phagocytes and contributes to cellular events relevant to immune responses under conditions of local and whole-body fever (Capasso et al. 2010; Morgan et al. 2009; Okochi et al. 2009). The activation kinetics of Hv1 evoked by depolarization was highly sensitive to the temperature (Fig. 8.6a). The activation kinetics was further accelerated by disruption of the channel dimerization by deletion of the C-terminal coiled-coil domain (Fig. 8.6a) (Fujiwara et al. 2012; Koch et al. 2008). Activation kinetics reflects the transition between the open and closed states of the channel. Given that the assembly of the Hv1 coiled-coil was thermally labile (Fig. 8.5), the relationship between temperature and activation kinetics was studied in detail by whole-cell patch clamp recordings (Fujiwara et al. 2012). The activation kinetics of the full-length (WT) channel at 40 °C was similar to that of ΔC at 25 °C, while activation of WT

Fig. 8.5 Thermal stability of the coiled-coil domain protein of mHv1. Melting temperature of the coiled-coil proteins, WT (black, **a**), NIN mutant (blue, **a**), WT (reduced/oxidized, **c**), and the C245S mutant (reduced/oxidized, **d**) were analyzed by circular dichroism (CD) spectrometry. (**b**) The recoveries after the melting were also analyzed in WT (black) and NIN mutant (blue). Data are taken from the papers (Fujiwara et al. 2012, 2013b)



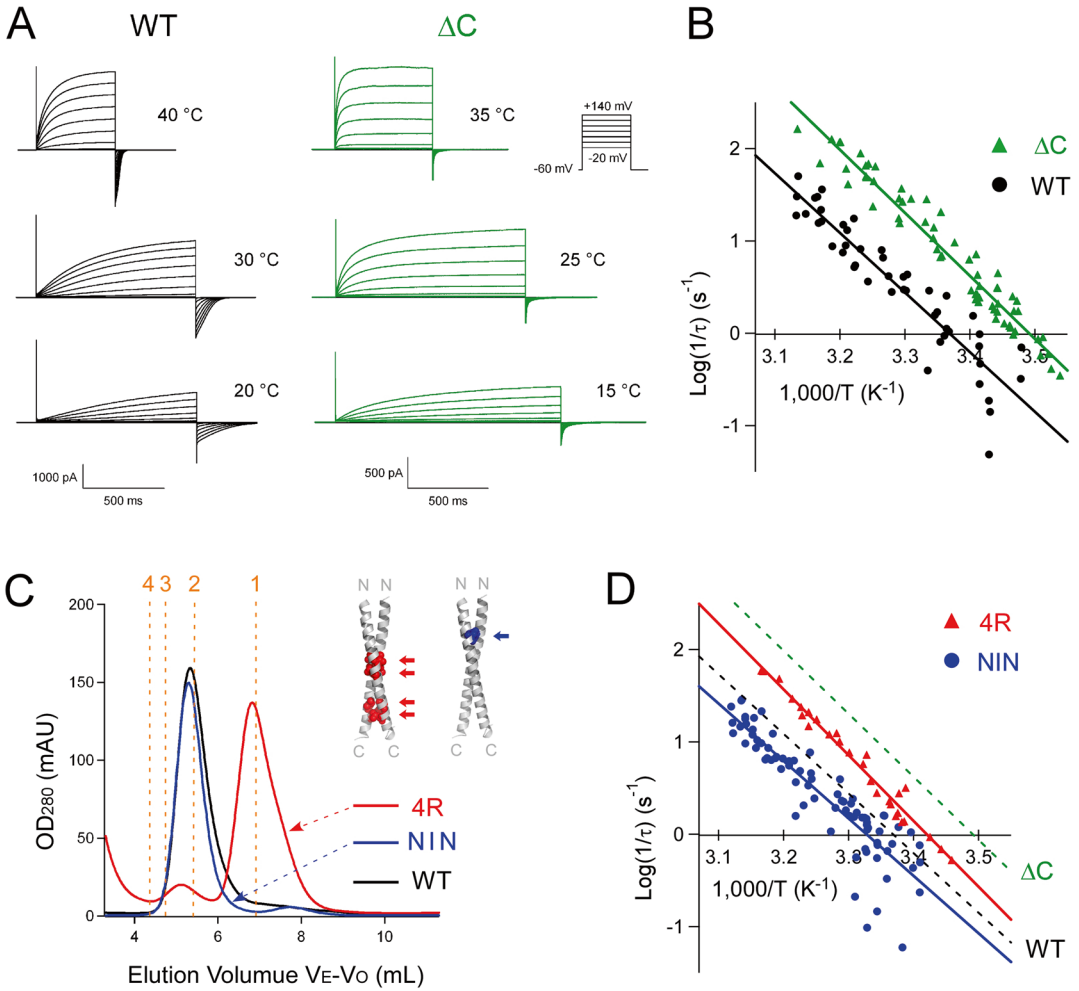


Fig. 8.6 Effect on the temperature-sensitive activation kinetics by the coiled-coil domain. **(a)** Representative currents through WT (black) and ΔC (green) Hv1 recorded at the indicated temperatures from HEK293 cells (Fujiwara et al. 2012). The pulse protocol is indicated in the right. **(b)** Accumulated data from WT (black) and ΔC (green) channels and the linear regression lines showing the correlation of the activation kinetics with temperature. **(c)** Coiled-coil stoichiometry determined by size exclusion chromatography monitored at 280 nm. For clear size separation we used HMT-tagged proteins in

these experiments. Structure models are indicated to grasp positions of the mutations: 4R (red) and NIN (blue). Dotted lines indicate the predicted elution volumes for tetrameric, trimeric, dimeric, and monomeric proteins. Oligomeric state of the main component was 2.29 for WT and 1.15 for 4R. **(d)** Accumulated data from 4R (red) and NIN (blue) mutant channels recorded as in **(a)** and the linear regression lines. Dotted lines depict the linear regression lines for the data from WT (black) and ΔC (green) channels. Figures are adapted from the paper (Fujiwara et al. 2012)

channel at 30 °C was similar to that of ΔC channel at 15 °C (Fig. 8.6a). This means that the deletion of the coiled-coil domain shifted the thermosensitivity relationship by about 15 °C without changing the slope (Fig. 8.6b).

Experiments of deleting the whole C-terminal segment cannot completely neglect the possibility whether not just disruption of dimerization but other effects of the deletion of the C-terminus might have altered channel properties. This point

was also fully examined (Fujiwara et al. 2012). Bulky hydrophilic Arg residues were introduced into the coiled-coil core layers so that the dimeric assembly is weakened (layers 5, 6, 9 and 10 in Fig. 8.3a, b). Size exclusion chromatography showed that dimeric assembly of the coiled-coil was suppressed by this “4R” mutation (Fig. 8.6c). The 4R mutation accelerated the activation kinetics, and shifted the relationship between activation speed and temperature by $\sim 8^\circ\text{C}$ in the direction of the ΔC phenotype (Fig. 8.6d). The NIN mutant was also analyzed as the construct with more stabilized assembly of the coiled-coil. The NIN mutant showed a slower activation and shifted the activation-temperature relationship by $\sim 5^\circ\text{C}$ away from the WT phenotype (Fig. 8.6d), which was consistent with the result of the CD spectrum (Fig. 8.5a). Thus, the stability of the C-terminal domain determined the channel activation kinetics in a temperature-dependent manner and coiled-coil dimer assembly inhibited channel activation. Interestingly, the N231 is conserved among all species of Hv so far identified (Fig. 8.3a), suggesting a unique role in optimizing the stability within the coiled-coil domain for regulation of channel activation.

These experiments clarified two features of the temperature-dependent activation of Hv. (1) The temperature dependence for activation (slope of the relationship between activation time constant and temperature) of the coiled-coil domain deletion/mutation channels is practically identical to those of WT channels. (2) The temperature dependence of the unfolding of the coiled-coil domain is much steeper (Fig. 8.5a) and occurs over a different temperature range than in the gating of the channel (Fig. 8.6d). These imply that the coiled-coil domain does not determine the slope of temperature dependence but affects the energy of activation, which is critical for the rate of channel activation over a range of temperature. In other words, the higher energetic impact (the higher temperature) is required for transition to the channel open state in the wild-type channel due to the restriction by the coiled-coil dimeric assembly. The dimeric coiled-coil assembly regulates the channel activation kinetics in a temperature-dependent manner, such that kinet-

ics of depolarization-induced increase in H^+ conductance is optimized in the range of body temperature (cf. WT and ΔC in Fig. 8.6a, b). This is one of the functional significances of the dimeric molecular design of the Hv. If transition between dimer and monomer occurs in native cells, the thermal stability of the coiled-coil domain may have physiological impact on cellular function.

How is the thermosensor coiled-coil coupled to the transmembrane gating? The role of the linker between the transmembrane S4 and the coiled-coil was also addressed with the mutants (Fujiwara et al. 2012). The Gly-Gly-Gly mutant is expected to confer more flexibility to the linker region, while the Ala-Ala-Ala mutant is expected to have the nature of forming α -helix thus keeping the stability of the linker region. The thermosensitivity plot (Fig. 8.7a) of the GGG mutant was similar to that of the ΔC channel (monomer type), while that of the AAA mutant was similar to that of WT channel (dimer type). This suggests that linker flexibility/stability plays an important role in channel gating. These findings indicate that the mere close positioning of two channel subunits bound by the coiled-coil domain is not sufficient to acquire the thermal stability of the channel gating, but the structural nature, most likely flexibility/stability, in the linker between the transmembrane region and the coiled-coil domain is important. Further, the electrophysiological analysis of mutant channels harboring the various lengths of the S4-coiled-coil linker demonstrated that the functional interaction between the coiled-coil and the transmembrane domains occurs in the periodicity of α -helix (Fujiwara et al. 2014). The crystal structure of chimeric mouse Hv1 channel showed the continuous α -helical structure along S4 and the coiled-coil (Takeshita et al. 2014). These results also support the idea that the rigid structure of the α -helical linker plays a role in coupling between the coiled-coil domain and the transmembrane segment.

It has been established that gating of the Hv is cooperative: the gating of one protomer channel affects the gating of the other protomer within the dimeric unit (Gonzalez et al. 2010; Musset et al. 2010; Tombola et al. 2010). As cooperative gat-

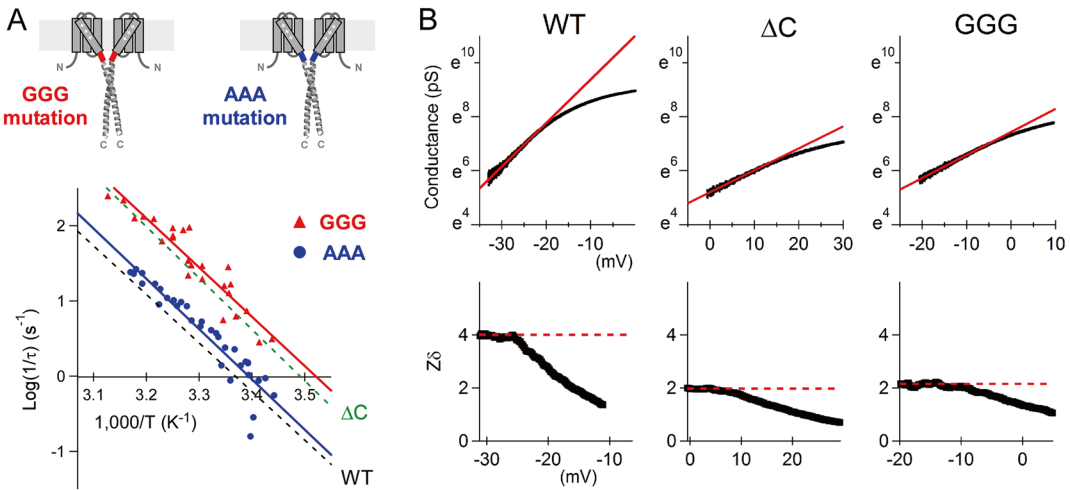


Fig. 8.7 Temperature-dependent activation of linker mutant channels of mHv1. **(a)** Accumulated data from the GGG (red) and AAA (blue) mutant channels recorded as in Fig. 8.6a and their linear regression lines; the dotted lines depict the linear regression lines for the data from WT (black) and ΔC (green) channels. **(b)** Representative

chord conductance of WT and the mutants (top) and fittings of the slope factor $Z\delta$ for different voltages (bottom). The current amplitude evoked by the slow ramp pulse, and the slope factors were fitted linearly in the marginal conductance range (top and bottom). Figures are adapted from the paper (Fujiwara et al. 2012)

ing requires channel dimerization by the coiled-coil assembly, the relationship between the thermosensitive gating and the cooperative channel gating was examined by the limiting slope measurement using whole-cell patch clamp recordings (Fig. 8.7b) (Fujiwara et al. 2012). The values of the effective gating charge ($Z\delta$) were calculated from the slope of the conductance elicited during a ramp pulse. $Z\delta$ in the WT channel was 4.1, and it was 2.0 in ΔC channel (Fig. 8.7b). This $Z\delta$ value in the WT channel, twofold that with of the monomeric ΔC channel, suggests that two protomers gate in a cooperative manner within dimer. Introduction of the GGG mutation into the linker segment decreases $Z\delta$ to half (2.2), while the AAA mutation does not affect the $Z\delta$ value (4.1). 4R mutant exhibits unstable thermostability of dimerization and reduced value of $Z\delta$ (2.5), while the NIN mutation showed an intact gating cooperativity ($Z\delta = 4.2$). The results of the effective charge from the GGG/AAA linker mutant and 4R mutant well matched to those in the analysis on the temperature-dependent activation (Figs. 8.6 and 8.7), suggesting that the modulation of the temperature-dependent activation by the coiled-

coil assembly lies in the same biophysical principle to the cooperative gating of the channel. Thus, the gating cooperativity of the Hv channel depends on the structural base consisting of the coiled-coil domain and the linker domain, and the coiled-coil assembly adjusts the range of temperature by elevating the energy of activation for the channel opening.

8.8 The Model of the Thermosensitive Channel Gating

Schematic illustrations of the gating property of the WT and the mutant channels are shown in Fig. 8.8. The stabilization of the temperature-dependent activation by the coiled-coil is observed in the WT channel (Fig. 8.8a, blue arrow), while it is not present and the temperature threshold of the activation kinetics is set lower in the 4R and GGG channels (Fig. 8.8b, c, red broken arrow). The gating cooperativity is also lost in the 4R and GGG mutation, where the 4R destabilized coiled-coil assembly and GGG mutation loosened connection between S4 and

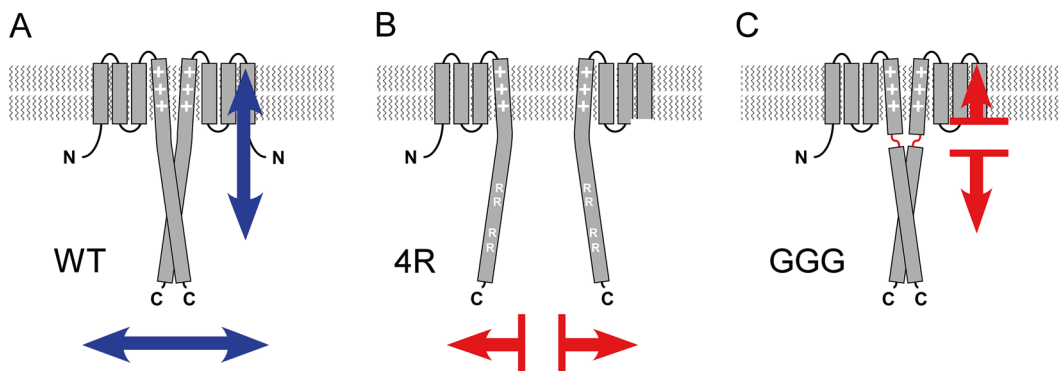


Fig. 8.8 Schemes of the thermosensitive gating of Hv. (a) Schematic drawings of temperature dependent gating in the WT (or the AAA mutant) channel. Both the interaction between coiled-coil region and voltage-sensing transmembrane segment through rigid linker structure (blue arrow) and the dimerization by coiled-coil (blue arrow) are important for the thermostability of channel activation and the cooperative gating. (b) Model of the 4R mutant channel. The channel dimerization is loosened due to the

instability of the coiled-coil assembly (broken red arrow). This results in instability and shifted temperature dependence of activation gating. (c) Model of the GGG mutant channel. Although the channel is dimerized, the thermostability of channel activation is not mediated by the coiled-coil domain (red broken arrow) due to the loose connection by GGG. Figures are adapted from the paper (Fujiwara et al. 2012)

the coiled-coil domain (Fig. 8.8b, c). Phenotypes of the temperature-dependent activation and the cooperative gating in the AAA channel are the same as those of the WT channel. The ΔC channel does not have a coiled-coil domain and the gating partner, and therefore regulation of the temperature-dependent activation is destabilized and the cooperative gating disappears. Thus, rigid structure of the linker between S4 and coiled-coil mediates the stability of the coiled-coil to the transmembrane domain, thus playing a critical role in achieving the cooperative gating within the dimer. The linker structure is supposed to be α -helix based on the results of the CD spectrometry (Fujiwara et al. 2012), our systematic studies of mutagenesis (Fujiwara et al. 2014), and crystal structure of the chimeric mouse Hv1 channel (Takeshita et al. 2014). In Hv, the coiled-coil structure is a cytoplasmic machinery that accomplishes both subunit assembly and gating modulation by temperature.

8.9 Future Challenges

In this chapter, I focused on temperature-dependent mechanisms of Hv with relevance to its cellular physiology, and discussed them based

on various data of structures and functions from a biophysical point of view. Hv has a “temperature range value” adjusted to physiological temperature and a “strong temperature dependence” that responds sharply to temperature. They correspond to the temperature-axis intercept and slope of the Arrhenius plot, respectively. The mutations to the cytoplasmic coiled-coil domain shifted the thermosensitivity relationship without changing the slope of the Arrhenius plot, suggesting that the coiled-coil domain determines the temperature range value. In the future, the challenge of clarifying the structural basis that determines the temperature dependence is desired. As shown in Fig. 8.6a, b, the temperature dependence as in WT still remains in the coiled-coil deleted monomeric mutant. The interaction between the channel protein and the membrane lipid during the structural change should also be considered. This will require efforts to solve not only the closed structure but also the open structure. Comprehensive thermodynamic studies, including membrane biochemistry and computational chemistry, are expected. Furthermore, it is expected that the role of temperature sensitivity of Hv in living organisms can be explained from the microscopic behavior of the channel molecule.

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Sensory Processing of Cutaneous Temperature in the Peripheral and Central Nervous System

9

Kei Eto, Dennis Lawrence Cheung,
and Junichi Nabekura

Abstract

Thermal perception is critical for sensing environmental temperature, keeping body temperature consistent, and avoiding thermal danger. Central to thermal perception is the detection of cutaneous (skin) temperature information by the peripheral nerves and its transmission to the spinal cord, thalamus, and downstream cortical areas including the insular cortex, primary somatosensory cortex, and secondary somatosensory cortex. Although

much is still unknown about this process, advances in technology have enabled significant progress to be made in recent years.

This chapter summarizes our current understanding of how the peripheral nerves, spinal cord, and brain process cutaneous temperature information to give rise to conscious thermal perception.

Keywords

Temperature · Peripheral nerve · Spinal cord · Thalamus · Cortex

K. Eto (✉)

Division of Homeostatic Development, National Institute for Physiological Sciences, Okazaki, Japan

Department of Physiology, School of Allied Health Sciences, Kitasato University, Tokyo, Japan
e-mail: keieto@kitasato-u.ac.jp

D. L. Cheung

Division of Homeostatic Development, National Institute for Physiological Sciences, Okazaki, Japan

Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama, Japan

J. Nabekura

Division of Homeostatic Development, National Institute for Physiological Sciences, Okazaki, Japan

Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama, Japan

Department of Physiological Sciences, The Graduate School for Advanced Study, Okazaki, Japan

9.1 Main

Thermal perception is fundamental to our body's ability to sense environmental temperature. This is critical for survival, given that our body's life-sustaining biochemical reactions can only operate within a narrow temperature range and that body temperature can be drastically affected by changes in environmental temperature. For example, in cold environments, our body's temperature would quickly drop if it failed to engage in adaptive physiological responses such as increasing its basal metabolic rate, and behaviors such as seeking a warmer environment. Furthermore, extreme changes in temperature can cause direct harm to our body such as when we accidentally touch a hot frying pan.

Thermal perception encompasses both the reflexive detection and response to temperature changes as well as the conscious realization of these temperature changes. Reflex thermal perception is self-evidently critical for survival, such as increased metabolic rate in cold weather or hand withdrawal from a hot frying pan. Conscious thermal perception is also important for survival as it allows us to engage in complex behavioral responses that enable us to adapt to changes in temperature, such as shelter seeking in cold weather or learning to avoid touching hot objects.

Temperature information about the surrounding environment is detected by thermosensitive receptors located in the skin and peripheral nerves and transmitted to the brain via the spinal cord (Bokinić et al. 2018). While much is still unknown about how temperature information is processed, significant progress has been made in recent years. In this chapter, we summarize our current understanding of how cutaneous temperature information is processed in the peripheral nerves, spinal cord, and brain to give rise to conscious thermal perception.

9.2 Processing Cutaneous Temperature in the Peripheral Nerve

Environmental temperature is broadly perceived as being either cold, cool, warm, or hot based on its relative difference with body temperature and absolute temperature. In humans, temperature changes are sensed at the skin through spots that have perceptual thresholds of less than 0.5 °C (Frenzel et al. 2012; Stevens and Choo 1998) and which are exclusively sensitive to either cold temperature (cold spot) or warm temperature (warm spot) (Green et al. 2008). The temperature sensitivity of these spots is derived from their innervation by specialized peripheral nerve endings, which detect skin temperature by transducing changes in the local thermal energy into electrical signals. These same nerves then transmit these electrical signals to temperature information processing centers within the spinal cord.

Peripheral nerves that are capable of temperature detection derive this ability from the expression of ion channels, which show temperature-dependent activation. The overwhelming majority of these temperature-sensitive ion channels belong to the transient receptor potential (TRP) ion channel family (Palkar et al. 2015; Vriens et al. 2014; Hoffstaetter et al. 2018). Importantly, the different TRP channel subtypes possess different temperature response ranges and thus their collective temperature detection repertoire enables the distinction between noxious cold, innocuous cold (cool), innocuous warm, and noxious warm (hot) temperatures (Vriens et al. 2014).

Cold sensation transduction is underpinned by the TRPM8 (Bautista et al. 2007; Colburn et al. 2007) and TRPA1 (Kwan et al. 2006) subtypes, which mediate innocuous cold and noxious cold sensation, respectively. TRPM8 is activated by temperatures between 15–28 °C and TRPM8 knockout mice show an impaired ability in distinguishing between warm and cold environments (Dhaka et al. 2007). TRPA1 is activated by temperatures below 17 °C and TRPA1 knockout mice are insensitive to cold-induced pain (Karashima et al. 2009). The expression patterns for these two TRP subtypes show some divergence. TRPM8 is expressed in nonmyelinated C-fibers and myelinated A-fibers (Kobayashi et al. 2005) with direct anatomical evidence indicating that both project to the superficial layers of the spinal cord (Takashima et al. 2010). Notably, TRPM8-positive DRG neurons do not co-express TRPA1 or TRPV1 (a TRP channel subtype classically associated with nociception). In contrast, TRPA1-positive DRG neurons form a subset of TRPV1-expressing DRG neurons (Kobayashi et al. 2005). Taken together, this anatomical data suggests that the transmission pathway for innocuous cold sensation is different from the transmission pathway for noxious cold sensation, which overlaps with general nociception.

Warm sensation transduction is underpinned by a greater number of TRP channels and cell types. Innocuous warm sensation is mediated by TRPM2, TRPV3, and TRPV4 whereas noxious

warm (hot) sensation is mediated by TRPV1. TRPM2, TRPV3, and TRPV4 are activated by moderately warm temperatures ($\approx 35\text{--}43^\circ\text{C}$) (Togashi et al. 2006) and in knockout mouse studies, TRPM2 knockout mice lose their natural preference for the cooler range of the innocuous warm temperature spectrum (Tan and McNaughton 2016) while TRPV3 knockout mice show a more generalized loss of sensitivity to warm stimuli (Moqrich et al. 2005). TRPV1 is activated at temperatures greater than 43°C (Caterina et al. 1997) and numerous studies have demonstrated that loss of TRPV1 activity through either genetic knockout or pharmacological blockade renders insensitivity to heat-induced pain and nociception more generally (Premkumar and Sikand 2008). The expression patterns for these four TRP subtypes show significant divergence. TRPM2 is expressed in some peripheral nerve fibers and stimulation of these fibers with moderately warm temperatures induces Ca^{2+} responses (a proxy of underlying action potential firing). TRPV3 and TRPV4 are both expressed in keratinocytes (Chung et al. 2004) and stimulation of these keratinocytes with moderately warm temperatures induces the secretion of ATP, with the resultant increase in the local extracellular ATP concentration stimulating DRG peripheral nerves (Mandadi et al. 2009). TRPV1 is expressed in DRG C-fibers and DRG A δ -fibers that project to the superficial layers of the spinal cord and which are classically associated with nociception (Caterina et al. 1997; Premkumar and Sikand 2008). It is also expressed in the trigeminal nerve, although, somewhat surprisingly, loss of TRPV1 expression here impairs discrimination between different innocuous warm temperatures (Yarmolinsky et al. 2016). The underlying mechanism for this particular case of TRPV1 function is unclear and further studies are necessary for clarification.

The investigation of neuronal activity in peripheral nerves has traditionally relied on unit recordings. Such studies are able to confirm whether a given thermal stimulus evokes a response in a single or a small number of nerve fibers. However, this falls short of the spatial

scale and resolution that is required when determining how multiple peripheral neurons respond to changes in cutaneous temperature, process the information, and transmit it to the spinal cord. In recent years, this methodological deficiency has been largely overcome, particularly with the advent of in vivo 2-photon microscopy. Here, the activity of multiple neurons is most often observed by recording the changes in their Ca^{2+} activity, which is a proxy for underlying action potential firing.

Given that the cell bodies of sensory peripheral nerve fibers are located within the dorsal root ganglia (DRG), application of in vivo 2-photon microscopy to this area enables efficient population level observation of sensory peripheral nerve activity. Nevertheless, the number of studies employing this method is still fairly small and the results to date are somewhat conflicting (Yarmolinsky et al. 2016; Chisholm et al. 2018; Emery et al. 2016; Wang et al. 2018). For example, Emery et al. 2016 reported that for the vast majority of DRG neurons, Ca^{2+} responses were only elicited in a given neuron by one particular mode (mechanical, coldness or warmth) of sensory stimuli (Emery et al. 2016). Thus, they concluded that DRG neurons strictly code in a modality-specific manner. This is essentially a “labeled line system,” wherein the nerve fibers that transmit temperature information are distinct from those which transmit touch or pain information (Craig 2003; Knowlton et al. 2013; Mishra and Hoon 2010). However, this paradigm is not supported by the results of other studies (Chisholm et al. 2018; Wang et al. 2018). In particular, Wang et al. 2018 reported that a large proportion of DRG neurons responded to several modes (mechanical, coldness, or warmth) of sensory stimuli and furthermore the encoding strategies for cold and warm sensation were different (Wang et al. 2018). When warm stimuli were presented, both the number of active neurons within the warm-responding neuron population and their firing rate increased as the degree of warmth was increased. In contrast, when cold stimuli were presented, different subgroups of neurons within the cold-responding neuron population

were only active at precise subranges of the cold temperature spectrum. Thus, warm sensation is encoded in a graded fashion by warm-responding DRG neurons, while cold sensation is encoded in a combinatorial fashion by cold-responding DRG neurons (Wang et al. 2018).

A large subset of sensory peripheral nerve fibers that do not pass through the dorsal root ganglia are those located within the cranial nerves. Here, the processing of cutaneous temperature has been best characterized for nerve fibers within the trigeminal nerve through observation of the trigeminal ganglion (Yarmolinsky et al. 2016; Poulos and Lende 1970). Most recently, Yarmolinsky et al. 2016 used in vivo 2-photon microscopy to characterize the activity of trigeminal neurons in response to warm and cold stimulation of the oral cavity (Yarmolinsky et al. 2016). Most trigeminal neurons observed exclusively responded to either warm or cold stimuli although a small subpopulation did respond to both. Cold sensation was mediated by TRPM8-positive neurons, similar to other sensory peripheral nerves, and these could be subdivided into three categories based on their activity response profiles. Discrimination between different innocuous warm stimuli was dependent on TRPV1-positive neurons. This is somewhat surprising as TRPV1 knockout in the dorsal root ganglion has little impact on the detection or discrimination of warm innocuous stimuli at the corresponding skin regions (Paricio-Montesinos et al. 2020).

9.3 Spinal Cord

The spinal cord is the first integration site for temperature information collected by all peripheral nerves (Fig. 9.1). The cranial nerves are a notable exception as these project directly to the ventral surface of the brain. Neurons in the superficial and deep layers of the dorsal horn of the spinal cord receive inputs from sensory neurons and subsequently project to various brain regions. It should also be noted that both innocuous warm and noxious warm (hot) stimuli have been directly observed to evoke some activity in neu-

rons within the deep layers (V-VI) of the spinal cord (Coghill et al. 1993), although the precise identity of these neurons and their functional relevance is still unknown.

Animal studies suggest that peripheral nerves that transmit temperature information mainly project to layers I and II of the spinal cord. In monkeys, layer I of the spinal cord is responsive to both noxious and innocuous cold stimuli (Dostrovsky and Craig 1996). In cats, individual layer I neurons respond to both innocuous cold and innocuous warm stimuli (Christensen and Perl 1970; Craig and Dostrovsky 2001; Burton 1975). In rodents, there are subpopulations of layer I neurons that respond either exclusively or to both cold and warm temperatures—roughly a third of neurons that are strongly responsive to noxious warm (hot) stimuli are also moderately responsive to noxious cold stimuli (Bester et al. 2000; Hellon and Misra 1973a). In addition, anatomical studies have found that both layers I and II of the spinal cord receive projections from DRG peripheral neurons that express either TRPM8 (Takashima et al. 2010) or both TRPV1 and TRPA1 (Kobayashi et al. 2005).

The importance of layers I and II of the spinal cord for temperature information processing is further suggested by their projection patterns to the brain. The neuronal axons originating from both layers travel along the spinothalamic tract (STT) to reach the brain, and injury here results in impaired thermal perception (Craig 1991; Ralston 3rd and Ralston 1992). Moreover, layer I neurons project to the ventromedial posterior nucleus (VMpo) in the brain, which is well characterized in terms of its involvement in temperature information processing (Dostrovsky and Craig 1996).

To date, the study by Ran et al. (2016) is one of the most in-depth investigations on how temperature information is preliminarily processed in the superficial layers of the spinal cord (Ran et al. 2016). Here, laminectomies were performed in anesthetized mice in order to facilitate in vivo 2-photon calcium imaging while thermal stimuli ranging from 5 °C to 50 °C were applied to the hind paw. At each specific temperature, subpopulations of neurons became activated as indicated

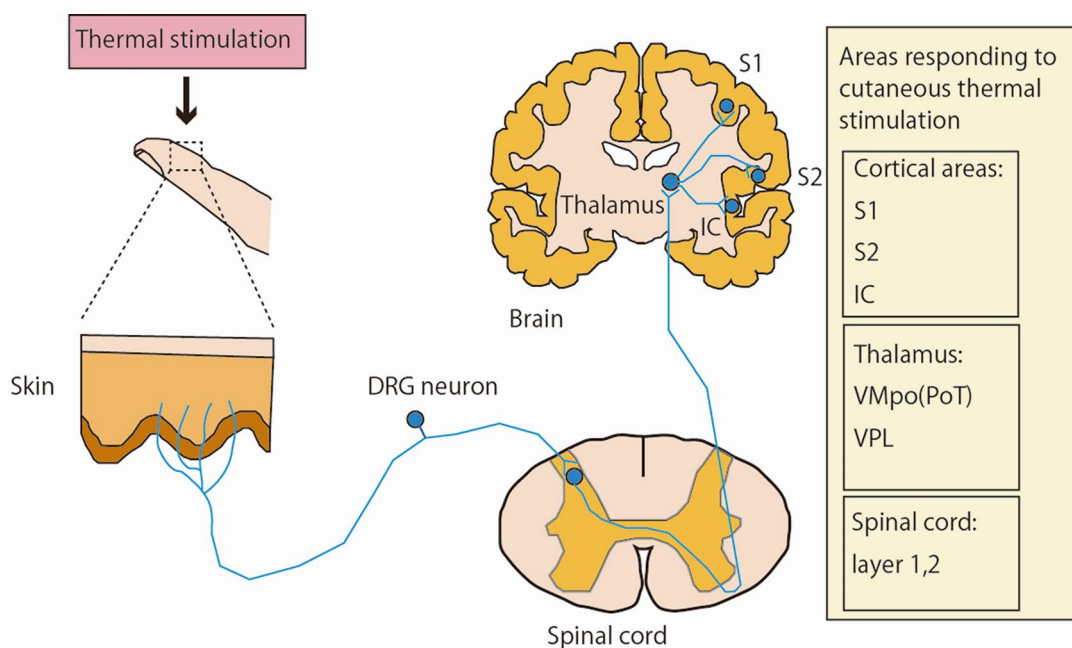


Fig. 9.1 Diagram showing the thermosensory pathway. First, thermal stimulation of the skin activates afferent fibers (Aδ and C fibers) originating from dorsal root ganglion (DRG). These fibers send thermal information to the spinal cord dorsal horn, especially to layers 1 and 2. Next, spinal cord neurons send this thermal information to the

thalamus, especially to the VMpo and VPL. Finally, thalamic neurons send this thermal information to various cortical areas, including the primary somatosensory cortex (S1), secondary somatosensory cortex (S2), and insular cortex (IC). The thermal information undergoes some processing by neural circuits at all stages of this relay

by increases in Ca^{2+} activity. Notably, while the response amplitudes of warm-responding neurons correlated with the absolute temperature, the response amplitudes of cold-responding neurons correlated with the degree of temperature change irrespective of the absolute temperature. Ablation of TRPV1 impaired but did not completely abolish warm-responsiveness presumably due to the remaining action of other TRP channels. Ablation of TRPM8 greatly impaired innocuous cold-responsiveness but had little effect on noxious cold-responsiveness presumably due to the remaining action of TRPA1.

One caveat with this and most other similar studies is that the experiments were performed under anesthesia. This is because anesthetics alter neuronal activity directly (Goltstein et al. 2015). This uncertainty would be circumvented if experiments were performed on fully awake subjects. One way for achieving this is to implant robust anchors and transparent windows into the spinal cord bone (Farrar et al. 2012). Under this

approach, spinal cord dorsal horn neurons are still accessible to *in vivo* 2-photon microscopy even without anesthetics and moreover the same neurons can be repeatedly observed over different successive time-points (Farrar et al. 2012). This capability will be especially important when studying how the activity of spinal cord neurons in response to cutaneous thermal stimuli might change during learning or under disease conditions such as chronic pain.

9.4 Thalamus

The thalamus brain region is a major receiver site for many modes of spinal cord input, including temperature information. It performs preliminary processing on this input before relaying it to higher brain regions, such as the cortical areas (Fig. 9.1). In humans, both cold and warm stimulation evoke increases in thalamic neuron activity as detected by unit recordings (Lenz et al. 1993;

Lenz and Dougherty 1998), fMRI imaging (Davis et al. 1998; Mazzola et al. 2012), and PET imaging (Casey et al. 1996; Egan et al. 2005) with this trend similarly observed in other animal species (Poulos and Benjamin 1968; Craig et al. 1994; Burton et al. 1970; Auen et al. 1980; Landgren 1960; Martin 3rd and Manning 1971). Several studies focusing on two thalamic subregions, the ventromedial posterolateral nucleus (VMpo) and ventral posterolateral nucleus (VPL), have gleaned significant insights into the schemes that they use to encode temperature information.

The VMpo receives inputs from layer I of the spinal cord (Craig et al. 1994). A human subject study by Davis et al. (1999) revealed the importance of the VMpo in processing cold sensation. In humans, stimulation of the VMpo causes subjects to experience a “cold feeling.” Furthermore, the VMpo appears to topographically map the location of cold sensation. Localized stimulation of the VMpo using a stimulating-recording electrode results in a “cold feeling” confined to a specific body region and, reciprocally, cold temperature stimulation of that specific body region results in increased neuronal activity that is localized to that stimulating-recording electrode (Davis et al. 1999). Animal studies examining the VMpo suggest that its involvement in temperature information processing is not limited to cold sensation. In a monkey study by Craig et al. (1994), the application of thermal stimuli to either the tongue or hand induced changes in the activity of unique VMpo neuron subpopulations. For one subpopulation, the application of cold stimuli to the tongue evoked a sustained increase in activity while the application of innocuous warm stimuli evoked a sustained suppression in activity. For another subpopulation, the application of noxious thermal stimuli to the hand evoked increases in activity, which were graded in proportion to the degree of heating (Craig et al. 1994). In a rodent study by Gauriau et al. (2004), the application of noxious warm (hot) stimuli to the hind paw evoked increased activity in specific neurons within the PoT, a structure analogous to the primate VMpo (Gauriau and Bernard 2004). Taken together, both human and animal studies suggest that the VMpo is responsive to thermal

stimuli across the entire temperature spectrum and has critical roles in the conscious perception of skin temperature.

The VPL receives input from the superficial layers of the spinal cord (Zhang et al. 2006). In humans, innocuous warm and cold sensations have been correlated with increased blood flow to the VPL as detected via fMRI (Davis et al. 1998). In monkeys and rats, thermal stimuli applied to the skin elicit changes in the activity of the thalamic ventrobasal complex, which encompasses the VPL (Burton et al. 1970; Hellon and Misra 1973b; Hellon and Mitchell 1975; Schingnitz and Werner 1980). The changes in VPL activity in response to thermal stimuli are underpinned by altered activity levels of neuronal subpopulations, which are defined by their temperature preferences. A study by Zhang et al. (2006), has identified three subpopulations of VPL neurons as defined by stimulus preference with each subpopulation located in different areas of the VPL (Zhang et al. 2006). The first subpopulation was responsive to painful stimuli. The second subpopulation showed peak levels of activity in response to noxious cold and noxious warm (hot) stimuli and linearly graded activity within the innocuous cold temperature range. The third subpopulation was exclusively responsive to both noxious cold and noxious warm (hot) stimuli. Thus, spinal cord neurons transmit a wide range of thermal information directly to distinct regions of the VPL.

9.5 Cortical Areas

Activity in the cortical brain regions is modulated by thermal stimuli (Landgren 1957; Kreisman and Zimmerman 1973), and lesions here result in impaired thermal perception (Adams and Burke 1989; Porter et al. 1993). It is likely that for temperature information processing, most of the input to the cortical areas is preprocessed by the thalamus given the anatomical connection between these two brain regions. The cortical subdivisions believed to have significant involvement in temperature information processing are the insular cortex (IC), primary somatosensory

cortex (S1), and secondary somatosensory cortex (S2) (Fig. 9.1).

The anterior region of the insular cortex (IC) receives projections from the VMpo (Craig et al. 1994). Its activity is modulated by the application of thermal stimuli to both the skin (Davis et al. 1998; Egan et al. 2005; Craig et al. 2000; Peltz et al. 2011) and oral cavity (Guest et al. 2007). Consistently, electrical stimulation of the IC provokes thermal sensation in humans (Mazzola et al. 2012; Penfield and Faulk 1955) and lesions here result in reduced sensitivity to thermal stimuli applied to the skin (Birklein et al. 2005; Veldhuijzen et al. 2010). However, the precise role that the IC plays in temperature information processing and its encoding scheme is still poorly characterized.

The primary somatosensory cortex (S1) is classically associated with the conscious perception of touch, pain, and temperature (Kenshalo Jr and Isensee 1983). In humans, S1 activity is modulated in response to innocuous warm and cold stimulation as observed via electroencephalography (EEG) (Chatt and Kenshalo 1977), fMRI imaging (Rolls et al. 2008; Moulton et al. 2012), and PET imaging (Egan et al. 2005). Consistently, stimulation of the S1 provokes the feeling of a cold sensation on the body (Penfield and Boldrey 1937) and lesioning of this area eliminates thermal perception (Adams and Burke 1989). S1 activity is similarly modulated in response to thermal stimulation of the skin in other animals including cats (Tsuboi et al. 1993; Iwata et al. 1990), monkeys (Vierck et al. 2013), rats (Hellon et al. 1973), and mice (Milenkovic et al. 2014). For example, some S1 neurons have been observed to increase their action potential firing in response to warm stimulation of the scrotum (Hellon et al. 1973) or cold stimulation of the tongue (Yamamoto et al. 1981).

To date, the study by Milenkovic et al. (2014) is one of the most in-depth investigations on how the S1 processes temperature sensation. Here, mice were trained to execute licking behavior to obtain rewards upon the cue of an innocuous cold stimuli applied to the fore paw. Patch clamp electrophysiological monitoring of S1 activity throughout this task identified neurons that fired

action potentials in response to cold stimulation of the fore paw. Subsequent blockade of S1 neuronal activity by either tetrodotoxin (voltage-gated sodium channel blocker) or muscimol (GABA agonist) impaired task execution. In addition, TRPM8 knockout mice were unable to learn this behavior task and innocuous cold stimulation of the forepaw no longer evoked action potentials in any S1 neurons. Taken together, this indicates that the S1 plays a critical role in the conscious recognition of cold sensation (Milenkovic et al. 2014). Currently, a similar level of understanding about how the S1 specifically processes innocuous warm sensation is lacking. While both warm and cold stimuli evoke activation of the same areas within S1 (Egan et al. 2005), further studies with single-cell resolution are required to clarify whether this is underpinned by separate or overlapping neuronal subpopulations.

Individual S1 neurons can be responsive to different modes of sensory information. In the previously mentioned study by Milenkovic et al. (2014), both innocuous cold and tactile stimulation of the fore paw in awake mice increased blood flow to the same area of S1 cortex as measured by the intrinsic optical signal. Consistently, random sampling of neurons in that activated S1 area in awake mice found that innocuous cold and tactile stimulation evoked subthreshold responses in 13/17 and 7/10 neurons, respectively, and action potential firing in 6/17 and 5/10 neurons, respectively. Furthermore, 9/10 innocuous cold-responsive neurons also responded to tactile stimulation in anesthetized mice. Notably, greater numbers of action potentials tended to be evoked by tactile rather than cold stimulation in awake mice (Milenkovic et al. 2014). Taken together this suggests that neurons in the S1 are multimodal for touch and innocuous cold modes of sensory input. On the other hand, innocuous warm and noxious warm (hot) stimuli evoke maximal responses in the monkey S1 3b and S1 3a, respectively, which are spatially separated S1 subdivisions (Vierck et al. 2013). Thus, the rules that determine whether a given temperature-responding S1 neuron responds exclusively to one sensory mode or is multimodal have yet to be

fully clarified but temperature preference may be an important factor.

The involvement of the secondary somatosensory cortex (S2) in temperature information processing is under-explored. A number of studies report that the S2 is activated by thermal stimulation (Moulton et al. 2012; Craig et al. 1996). However, MRI imaging raises the possibility that this may be more relevant to nociception as S2 activity is modulated by innocuous tactile, noxious cold, and noxious warm (hot) stimulation but not innocuous cold or innocuous warm stimulation (Davis et al. 1998). On the other hand, changes in S2 activity appear to better correlate with subjective rankings of heat intensity rather than those for pain intensity (Moulton et al. 2012). Further studies on the S2 are required to clarify its role and encoding scheme for temperature information processing.

9.6 Conclusion and Future Direction

In this chapter, we have summarized the current understanding of the mechanisms by which thermal stimuli is detected and processed by the peripheral and central nervous systems to give rise to conscious thermal perception. We have also highlighted how cutting-edge technologies, such as 2-photon microscopy (Yarmolinsky et al. 2016; Chisholm et al. 2018; Emery et al. 2016; Wang et al. 2018; Ran et al. 2016), have been critical for many of the insights into the encoding schemes used by neurons when processing temperature information. Furthermore, while previous animal studies only associated changes in neural activity with thermal perception, the use of behavior tasks in more recent studies (Paricio-Montesinos et al. 2020; Milenkovic et al. 2014) has enabled specific aspects of thermal perception to be investigated separately and in detail.

One aspect of temperature information processing that future studies should seek to address is the contribution of inhibitory neurons. All of the studies examining temperature information processing with single-cell resolution discussed here have exclusively focused on the activity of

excitatory neurons. It is highly unlikely that thermal stimuli do not modulate the activity of inhibitory neurons or that such an effect is unimportant for thermal perception. In *Drosophila*, cold-responding inhibitory neurons directly inhibit the activity of warm-responding excitatory neurons and thus sharpen temperature discrimination sensitivity (Liu et al. 2015). With recent advances in *in vivo* imaging technologies, we are now able to directly observe excitatory and inhibitory neuronal activities simultaneously in higher animals (Eto et al. 2012; Khan et al. 2018) and we expect that this will allow for the important role of inhibitory neurons in temperature information processing to be elucidated.

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Part II

Temperature-Dependent Physiological Responses

Central Mechanisms of Thermoregulation and Fever in Mammals

10

Kazuhiro Nakamura

Abstract

Thermoregulation is a fundamental homeostatic function in mammals mediated by the central nervous system. The framework of the central circuitry for thermoregulation lies in the hypothalamus and brainstem. The preoptic area (POA) of the hypothalamus integrates cutaneous and central thermosensory information into efferent control signals that regulate excitatory descending pathways through the dorsomedial hypothalamus (DMH) and rostral medullary raphe region (rMR). The cutaneous thermosensory feedforward signals are delivered to the POA by afferent pathways through the lateral parabrachial nucleus, while the central monitoring of body core temperature is primarily mediated by warm-sensitive neurons in the POA for negative feedback regulation. Prostaglandin E₂, a pyrogenic mediator produced in response to infection, acts on the POA to trigger fever. Recent studies have revealed that this circuitry also functions for physiological responses to psychological stress and starvation. Master psychological stress signaling from the medial prefrontal cortex to the DMH has been discovered to drive a variety of physiological responses for

stress coping, including hyperthermia. During starvation, hunger signaling from the hypothalamus was found to activate medullary reticular neurons, which then suppress thermogenic sympathetic outflows from the rMR for energy saving. This thermoregulatory circuit represents a fundamental mechanism of the central regulation for homeostasis.

Keywords

Body temperature · Brown adipose tissue · Central nervous system · Fever · Homeostasis · Metabolism · Neural circuit · Psychosomatic · Stress · Thermoregulation

Body temperature regulation is an essential function for animals to sustain life. In particular, mammals, most of which are homeothermic and endothermic, have involuntary (autonomous) and voluntary (behavioral) mechanisms to maintain internal body temperature within a narrow range under changing external temperature. This homeothermic ability is important for mammals to inhabit diverse thermal environments on earth. Autonomous thermoregulatory responses in humans include sympathetic non-shivering thermogenesis in brown adipose tissue (BAT), somatic motor-driven shivering thermogenesis in skeletal muscles, sympathetic constriction of skin blood vessels, and sympathetically mediated sweat secre-

K. Nakamura (✉)

Department of Integrative Physiology, Nagoya University Graduate School of Medicine, Nagoya, Japan
e-mail: kazu@med.nagoya-u.ac.jp

tion from eccrine glands (Fig. 10.1). These autonomous responses are often accompanied by thermoregulatory behaviors, such as warm-seeking behavior in a cold environment. Thermoregulatory behavior is an innate behavior to enhance the efficacy of involuntary thermoregulatory responses. All these thermoregulatory responses are controlled by the central nervous system.

Several pioneering scientists have proposed the central circuit mechanisms of autonomous thermoregulatory responses based on *in vivo* physiological and neuroanatomical findings (Nagashima et al. 2000; Nakamura 2011; Morrison and Nakamura 2011). This central circuit model for thermoregulation has been refined and established by recent studies that employed state-of-the-art methodology to manipulate activities of a specific group of neurons, such as optogenetic and chemogenetic techniques (reviewed in Tan and Knight 2018; Morrison and Nakamura 2019; Nakamura et al. 2022a). The central circuitry that regulates efferent outflows to the sympathetic nervous system and the somatic motor system for autonomous thermoregulatory responses involves the preoptic area (POA), dorsomedial hypothalamus (DMH), and

rostral medullary raphe region (rMR) (Fig. 10.2). The hypothalamomedullary pathways through the DMH and rMR constitute excitatory “trunk pathways” that separately exist for efferent outflows to BAT, skeletal muscles, skin blood vessels, and the heart to drive thermogenic and cardiovascular responses (Fig. 10.2). The excitatory pathways through the DMH and rMR are controlled principally by tonic inhibitory signaling from the POA, which is altered by the level of body core (brain) temperature and by thermosensory signal inputs from the skin to the POA (Fig. 10.2).

Recent studies have revealed that this fundamental circuitry not only regulates basal body temperature, but also functions for the development of fever in response to infection. Furthermore, this circuit also drives physiological responses to psychological stress and regulates metabolic balance under starved and sated conditions. Therefore, this circuit is relevant to the mechanisms by which mammals defend homeostasis and life from various environmental stressors. This chapter first describes the fundamental central circuit mechanisms of autonomous responses for basal thermoregulation and

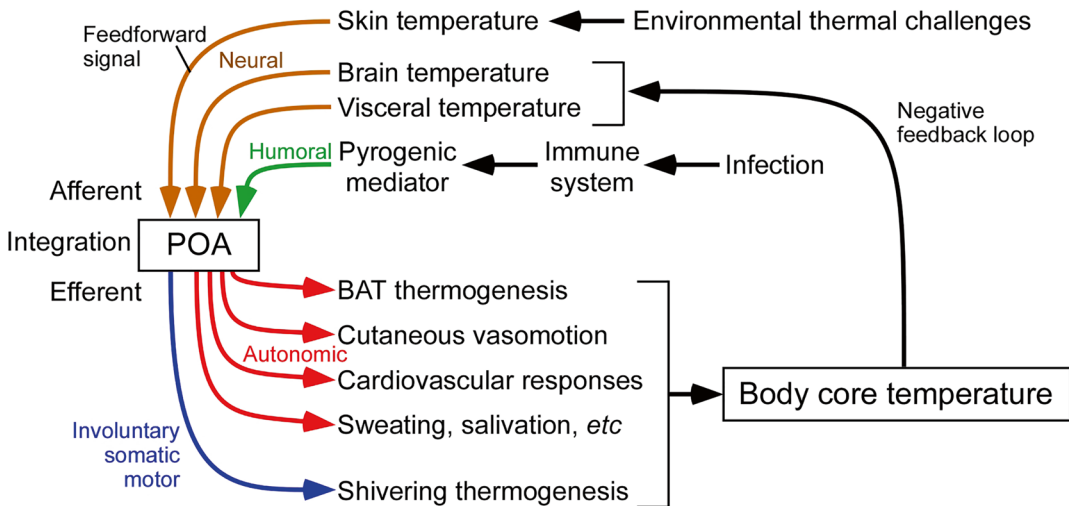


Fig. 10.1 A conceptual diagram of the thermoregulatory system. The system has negative feedback and feedforward mechanisms. The POA receives and integrates neural (thermosensory information) and humoral afferent

signals to provide efferent signals for the regulations of effector response pathways through the autonomic and involuntary somatic motor systems. See main text for details

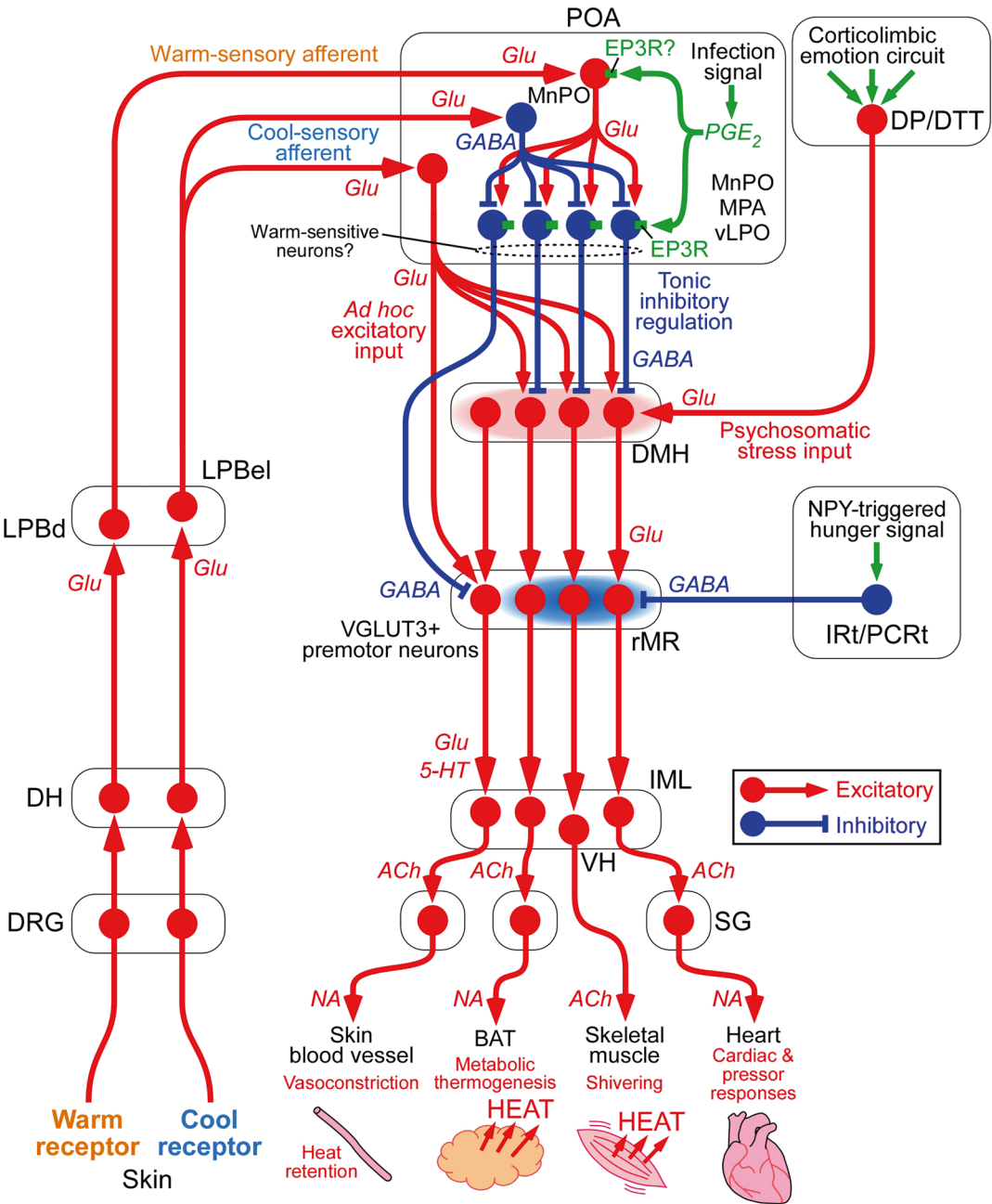


Fig. 10.2 A schematic model of the neural circuit network for thermoregulation, fever, and psychological stress and hunger responses. This circuit diagram illustrates separate but parallel efferent pathways from the POA that control skin vasoconstriction, BAT thermogenesis, shivering thermogenesis (skeletal muscles), and cardiac functions. In response to environmental thermal (heat and

cold) challenges, cutaneous warm-sensory and cool-sensory afferent signals are delivered to interneurons in the POA to activate and inhibit, respectively, GABAergic efferent neurons descending from the POA. These descending GABAergic neurons, which express EP3R, are also inhibited by PGE_2 produced in response to infection. These neurons, which may be warm-sensitive, pro-

(continued)

fever in mammals, which have been studied mostly in rodents, and then summarizes recent findings on the contribution of this circuitry to the defense of homeostasis from psychological stressors (e.g., natural enemies) and starvation.

10.1 Thermoregulatory Responses

In thermoneutral environments, regulation of radiative heat loss from the body surface, which is mediated primarily by regulation of skin blood flow, is sufficient for mammals to maintain their body core temperatures within normal homeostatic ranges. Skin blood flow is primarily controlled by sympathetic innervation of skin blood vessels. An increase in the sympathetic nerve activity leads to vasoconstriction, which reduces skin blood flow to decrease heat dissipation, while a decrease in the sympathetic activity facilitates heat dissipation through skin vasodilation. In response to an increase in environmental temperature, the sympathetic activity is decreased, thereby increasing skin blood flow to prevent hyperthermia. However, the defense of body core temperature in environmental temperatures above the thermoneutral range requires a more effective mechanism for heat loss—evaporation of water. The best-known physiological response to increase evaporative heat loss is sweating in humans and horses. Sweat secretion from eccrine glands is primarily stimulated by cholinergic sympathetic nerves (Shibasaki et al. 2006). Because laboratory mammals widely used in physiological experiments, such as rats, mice,

cats, and dogs, do not sweat for heat defense, the central regulation of sweating has been poorly understood. Instead, many nonhuman mammals have other mechanisms to increase evaporative heat loss for heat defense, such as saliva spreading in rats and mice and panting in dogs.

In response to lowered environmental temperatures, skin blood flow is reduced to prevent hypothermia. To defend body core temperature in environmental temperatures below the thermoneutral range, adaptive thermogenesis is elicited. Adaptive thermogenesis includes sympathetic (metabolic) thermogenesis in BAT and involuntary somatic motor-driven shivering thermogenesis in skeletal muscles. The threshold of ambient temperature to turn on BAT thermogenesis is higher than that for shivering (McAllen et al. 2010). These thermogenic responses are often accompanied by an increase in heart rate, resulting in increased cardiac output. This tachycardic response boosts blood flow to the thermogenic organs to supply more fuel and oxygen for thermogenesis. The augmented cardiac pumping also increases heat production in the heart, which has been proposed as “cardiac thermogenesis” (Morrison and Nakamura 2011).

10.2 Negative Feedback and Feedforward Regulation

The thermoregulatory system has two mechanisms for homeothermic temperature regulation: negative feedback mechanism and feedforward mechanism (Fig. 10.1). The negative feedback mechanism functions to correct deviations in

Fig. 10.2 (continued) vide tonic inhibitory inputs to the DMH and rMR to regulate the descending excitatory pathways to the effectors. Increased cool-sensory inputs, reduced body core temperature, or the action of PGE₂ reduces the tonic inhibition from the POA to disinhibit the descending excitatory pathways to the effectors. In addition to this withdrawal of the tonic inhibition, a group of glutamatergic POA neurons, which may be activated by cutaneous cool-sensory inputs, provide ad hoc excitatory inputs to the excitatory descending neurons in the DMH and rMR to drive the sympathetic and somatic motor

responses. Psychological stress activates psychosomatic transmission from the DP/DTT to the DMH, which stimulates the excitatory DMH neuron subgroups (on pink shaded area) to drive the autonomous effector responses. Under starvation, a GABAergic input from the IRt/PCrt to the rMR inhibits subgroups of premotor neurons (on blue shaded area) to inhibit thermogenic and cardiac activities. See main text for details. ACh acetylcholine, DH dorsal horn, DRG dorsal root ganglion, Glu glutamate, IML intermediolateral nucleus, NA noradrenaline, SG sympathetic ganglion, VH ventral horn

body core temperature from a defended control range. Body core temperature is primarily monitored by warm-sensitive neurons in the POA, whose firing rates are increased by elevation of local brain tissue temperature (Nakayama et al. 1961; Boulant and Dean 1986). Because the POA is positioned in the ventral portion of the brain and receives an abundant blood supply from body core structures, tissue temperature in the POA well represents body core temperature. Local cooling of the POA elicits BAT thermogenesis and shivering (Hammel et al. 1960; Imai-Matsumura et al. 1984), while local warming of this site evokes heat-defensive responses, such as skin vasodilation and saliva secretion (Kanosue et al. 1990, 1994). Therefore, warm-sensitive neurons in the POA are considered to monitor deviations in body core temperature for the negative feedback mechanism of thermoregulation. It is proposed that the temperature-dependent changes in the firing activity of warm-sensitive POA neurons alter the efferent controls of thermoregulatory effectors from the POA to correct deviations in body core temperature (Nakamura 2011). Although TRPM2, a member of the transient receptor potential (TRP) family of cation channels, has been proposed to be involved in the temperature sensation of warm-sensitive POA neurons, TRPM2 is broadly expressed in the hypothalamus, and mice with genetic ablation of this channel can regulate their body temperature as normal (Song et al. 2016). Thus, other molecular mechanisms are likely to play a more critical role in the central sensation of warmth.

Although the negative feedback mechanism is an important component of the thermoregulatory system, a problem is that the feedback regulation becomes active after core body temperature deviates from a control range. Feedforward regulation, on the other hand, is a mechanism that elicits “preventive” thermoregulatory responses before body core temperature is affected by changes in environmental temperature. Environmental temperatures are sensed by cutaneous thermoreceptors. Although the cutaneous thermoreceptors responsible for body temperature regulation have not been definitively determined, TRPM8, a menthol receptor, is likely involved in cutaneous

cool sensation to elicit cold-defensive responses, and TRPM2 and TRPV1, a capsaicin receptor, may be involved in warm sensation to elicit heat-defensive responses (Nakamura 2018). When environmental temperature changes, the thermosensory information detected by cutaneous thermoreceptors is delivered to the POA via afferent neural pathways to rapidly elicit thermoregulatory responses for the defense of body core temperature from the environmental temperature change (Fig. 10.1). Because the skin is exposed to the outside of the body (i.e., environment), its temperature is more directly affected by changes in environmental temperature than are body core structures including the brain. When mammals move into a cold environment, their skin temperature rapidly drops, whereas brain and rectal temperatures are unaffected or even slightly increased (Forster and Ferguson 1952; Hellstrom and Hammel 1967; Bratincsák and Palkovits 2005). Therefore, the feedforward regulation triggered by cutaneous thermosensation is important for stable regulation of body core temperature under rapidly changing environmental temperature. The feedback regulation mediated by central thermosensation is expected to play roles in (1) setting the basal tone of thermoregulatory efferent outflows, (2) enhancing thermoregulatory responses in extreme thermal environments where feedforward responses have proven inadequate to prevent changes in body core temperature, and (3) responding to challenges that directly affect internal body temperature, such as exercise, ingestion of hot and cold fluids, and hemorrhage.

10.3 Thermosensory Afferent Pathways to the POA

Sensory information on environmental temperatures, detected by cool and warm receptors in the skin, is transmitted by primary somatosensory nerves to the spinal and medullary (trigeminal) dorsal horns. The cool- and warm-sensory signals are then separately transmitted by secondary somatosensory neurons from the dorsal horns to the thalamus and/or the lateral parabrachial

nucleus (LPB) partly via axon collaterals (Hylden et al. 1989; Craig et al. 2001; Andrew and Craig 2001; Li et al. 2006) (Fig. 10.2). Thermosensory signals delivered to the thalamus are then relayed to the primary somatosensory cortex with somatotopic organization and processed for conscious perception and discrimination of skin temperature (Craig et al. 1994; Craig 2002). Interestingly, disruption of the thalamic nuclei mediating the sensory relay affects neither autonomic nor behavioral thermoregulation (Nakamura and Morrison 2008a; Yahiro et al. 2017). This finding indicates that the spinothalamocortical pathway is not required for thermoregulation and that the thermosensory afferent circuit mechanism for thermal homeostasis, even for thermoregulatory behavior, is distinct from that for conscious thermal perception.

Studies have demonstrated that the cutaneous thermosensory afferent signaling for involuntary and behavioral thermoregulation is mediated by the LPB (Fig. 10.2). Cutaneous cool-sensory and warm-sensory inputs from the dorsal horns, respectively, activate two segregated groups of neurons in the external lateral part (LPBel) and the dorsal part (LPBd) of the LPB (Nakamura and Morrison 2008a, 2010). Both groups of the thermoresponsive LPB neurons provide glutamatergic projections to the midline part of the POA including the median preoptic nucleus (MnPO) (Nakamura and Morrison 2008a, 2010; Geerling et al. 2016) (Fig. 10.2). Inactivation of LPB neurons with bilateral nanoinjections of muscimol, a GABA_A receptor agonist widely used as a neuronal inhibitor, eliminates BAT thermogenic, shivering and tachycardic responses to skin cooling as well as skin vasodilation induced by skin warming (Nakamura and Morrison 2008a, 2010). Stimulation of LPBel and LPBd neurons recapitulates the cold-defensive and heat-defensive responses (Nakamura and Morrison 2008a, 2010). Furthermore, genetic inactivation of LPB→MnPO neuronal transmission by expression of tetanus toxin light chain eliminates BAT thermogenic, metabolic, and tachycardic responses to skin cooling, and causes failure of heat and cold defense, developing hyperthermia in a hot environment and lowering body tempera-

ture in a cold environment (Yahiro et al. 2023). These findings indicate that LPBel and LPBd neurons, respectively, mediate cutaneous cool-sensory and warm-sensory ascending signaling to the POA that is required to elicit the feedforward cold-defensive and heat-defensive responses to environmental thermal challenges. Interestingly, inactivation of LPB neurons also abolishes thermoregulatory behaviors, such as cold avoidance and heat avoidance behaviors (Yahiro et al. 2017). More recently, it has been shown that LPB→MnPO transmission mediates heat avoidance behavior, whereas LPB→amygdala transmission mediates cold avoidance behavior (Yahiro et al. 2023). These LPB-mediated thermosensory signals are likely to generate the thermally evoked emotions of comfort and discomfort that drive the thermoregulatory behaviors, and further studies are awaited to elucidate the circuit mechanism.

10.4 Pyrogenic Signaling from the Immune System to the POA

The POA serves not only as a thermoregulatory center, but also as a febrile center when animals are infected. The mechanism by which infection signals are transmitted from the immune system to the POA for fever development has been a focus of research. A number of studies have demonstrated that prostaglandin E₂ (PGE₂) is a key mediator of the immune signaling that triggers the febrile neural circuit mechanism. Systemic administration of lipopolysaccharide (LPS) to rodents, which has been widely used as an experimental model of bacterial infection, induces the expression of enzymes for PGE₂ biosynthesis in brain endothelial cells, such as cyclooxygenase-2 (COX-2) and microsomal PGE synthase (mPGES) (Matsumura et al. 1998; Yamagata et al. 2001). These gene inductions are mostly stimulated by actions on endothelial cells of inflammatory cytokines released from activated immune cells, in addition to the direct action of LPS (Yamagata et al. 2001). Deletion of either COX-2 or mPGES gene in brain endothelial cells

diminishes LPS-induced fever (Wilhelms et al. 2014). These findings indicate that PGE₂ produced in brain endothelial cells stimulated by immune signals mediates a major part of LPS-induced fever. It has also been proposed that another source of PGE₂ to trigger fever is macrophages in the lungs and liver, and that the released PGE₂ circulates through the brain blood vessels and diffuses into the POA to develop an early phase of LPS-induced fever (Ivanov et al. 2002; Steiner et al. 2006; Ootsuka et al. 2008b).

PGE₂ triggers the febrile circuit mechanism by acting on prostaglandin EP3 receptors (EP3Rs) expressed on POA neurons (Fig. 10.2). EP3Rs are localized to cell bodies and dendritic fibers in the MnPO and medial preoptic area (MPA) (Nakamura et al. 1999, 2000). Injection of PGE₂ into these POA subregions induces intense fever by eliciting BAT and shivering thermogenesis, skin vasoconstriction, tachycardia, and pressor responses (Scammell et al. 1996; Nakamura et al. 2002; Nakamura and Morrison 2011; Tanaka et al. 2013). Systemic or POA-selective ablation of the EP3R gene abolishes PGE₂- and LPS-induced fever (Ushikubi et al. 1998; Lazarus et al. 2007). EP3Rs are primarily coupled to inhibitory G_i proteins in cultured cells (Narumiya et al. 1999), and PGE₂ has recently been shown to inhibit the activities of EP3R-expressing POA neurons to trigger febrile responses (Nakamura et al. 2022b) (Fig. 10.2).

10.5 Afferent Signal Processing in POA Local Circuitry for Efferent Regulation

The cutaneous thermosensory inputs to the POA are integrated with the central thermosensory information on body core temperature monitored by warm-sensitive neurons. The thermosensitivity of warm-sensitive POA neurons to local tissue temperature is affected by the level of skin temperature: for example, the tonic discharge of warm-sensitive POA neurons is reduced by skin cooling (Boulant and Hardy 1974). Furthermore, the PGE₂-mediated infection signal from the immune system is also likely integrated with the

central thermosensory signal by impinging on the discharge activity of warm-sensitive POA neurons. Although it is unknown whether warm-sensitive POA neurons express the EP3R, heat exposure of rats activates EP3R-expressing POA neurons and cold exposure inhibits them (Nakamura et al. 2022b), indicating that thermosensory signals alter the activity of EP3R-expressing POA neurons. PGE₂ reduces the tonic discharge of many warm-sensitive POA neurons (Schoener and Wang 1976; Gordon and Heath 1980; Rannels and Griffin 2003). These findings are consistent with the signal integration model (Fig. 10.2) in which cutaneous thermosensory inputs and immune signal inputs to the POA affect the thermosensitivity and tonic discharge of warm-sensitive POA neurons, which may express EP3Rs, to alter the regulatory signaling from the POA to the DMH→rMR excitatory efferent pathway.

Many lines of evidence support the view that the excitatory efferent outflows from the DMH are regulated by tonic GABAergic inhibitory signaling from the POA (Nakamura 2011; Nakamura et al. 2022b) (Fig. 10.2). Consistent with this view, an acute transection immediately caudal to the POA causes lethal hyperthermia by inducing intense BAT thermogenesis and cutaneous vasoconstriction (Chen et al. 1998; Rathner et al. 2008). Although the projection sites of warm-sensitive POA neurons have yet to be determined, many warm-sensitive POA neurons are GABAergic (Tabarean et al. 2005; Lundius et al. 2010). Therefore, warm-sensitive POA neurons may provide the tonic inhibitory signals to regulate the DMH→rMR efferent drives to thermoregulatory effectors. The EP3R-expressing group of POA neurons provides direct projections to the DMH and rMR (Nakamura et al. 2002, 2005b, 2009), and finally, it has recently been demonstrated that EP3R-expressing POA neurons provide the tonic GABAergic inhibitory signal to regulate the excitatory efferent pathways through the DMH and rMR (Nakamura et al. 2022b) (Fig. 10.2). EP3R-expressing POA neurons predominantly release GABA from their axon terminals in the DMH, and chemogenetic stimulation of EP3R-expressing POA neurons reduces body

core temperature via skin vasodilation, whereas inhibition of them increases body temperature by eliciting BAT thermogenesis (Nakamura et al. 2022b). Local chemogenetic inhibition of transmitter release from DMH axon terminals of EP3R-expressing POA neurons also elicits BAT thermogenesis (Nakamura et al. 2022b). These findings indicate that EP3R-expressing POA neurons serve as a “master controller” of body temperature for thermal homeostasis and fever by providing tonic GABAergic inputs to control the DMH→rMR sympathoexcitatory pathways that innervate multiple thermoregulatory effectors (Fig. 10.2).

A recent molecular biological study also strongly supports the notion that the POA provides tonic GABAergic signaling to the DMH for thermoregulation. Skin warming activates POA neurons co-expressing pituitary adenylate cyclase-activating polypeptide (PACAP) and brain-derived neurotrophic factor (BDNF), which provide predominantly GABAergic projections to the DMH, and optogenetic stimulation of their DMH axon terminals inhibits BAT thermogenesis (Tan et al. 2016). Optogenetic stimulation of cell bodies of PACAP/BDNF neurons in the POA reduces body temperature by eliciting skin vasodilation and inhibiting BAT thermogenesis (Tan et al. 2016). Although PACAP/BDNF neurons in the POA do not possess warm sensitivity (Tan et al. 2016), they might express EP3Rs. These findings further support the circuit model (Fig. 10.2) in which GABAergic projection neurons, mostly distributed in the MnPO and MPA, mediate the tonic inhibitory regulation of the excitatory efferent pathways through the DMH and rMR to thermoregulatory organs. An increase in cutaneous warm-sensory inputs to the POA or an elevation of body core (brain tissue) temperature increases the tonic activity of the GABAergic projection neurons to augment the inhibition of the excitatory drives from the DMH, resulting in suppressed thermogenesis and increased skin blood flow (heat loss) through skin vasodilation. On the other hand, an increase in cutaneous cool-sensory inputs to the POA, a reduction of body core temperature, or an action of PGE₂ on EP3R-expressing POA neurons dur-

ing infection reduces the tonic activity of the GABAergic projection neurons to disinhibit the excitatory pathways, resulting in stimulation of BAT and shivering thermogenesis and a reduction of skin blood flow by skin vasoconstriction.

Another group of GABAergic neurons that play a similar role in thermoregulation is distributed in the ventral part of the lateral preoptic area (vLPO). These neurons, which may express galanin, are activated in response to skin warming to elicit heat-defensive responses (Zhao et al. 2017; Kroeger et al. 2018; Conceição et al. 2019; Ma et al. 2019). These GABAergic neurons in the vLPO inhibit thermogenesis probably by inhibiting the rMR, but not the DMH (Conceição et al. 2019).

The POA local circuit mechanism by which cutaneous thermosensory afferent signals are processed to influence the tonic activity of the GABAergic neurons in the MnPO, MPA, and vLPO has yet to be determined. However, there is some evidence to propose a local circuit model (Fig. 10.2). Cutaneous cool-sensory and warm-sensory glutamatergic inputs from the LPB are independently delivered to the MnPO and probably received by separate groups of neurons. These thermosensory inputs to the MnPO are required to elicit feedforward cold-defensive and heat-defensive responses, because antagonizing glutamate receptors or inhibiting neurons in the MnPO blocks skin cooling-induced BAT and shivering thermogenesis as well as skin warming-induced skin vasodilation (Nakamura and Morrison 2008b, 2010, 2011). Pharmacological stimulation of MnPO neurons elicits cold-defensive responses including BAT and shivering thermogenesis, tachycardia, and skin vasoconstriction, mimicking skin cooling (Nakamura and Morrison 2008b, 2011; Tanaka et al. 2011). These evoked cold-defensive responses are inhibited by subsequent stimulation of MPA neurons, likely due to stimulation of the GABAergic projection neurons that tonically regulate the excitatory efferent pathways (Nakamura and Morrison 2008b, 2011; Tanaka et al. 2011). These findings support the POA local circuit model (Fig. 10.2), in which cutaneous cool-sensory inputs from the LPB elicit inhibitory, perhaps GABAergic,

local neurons in the MnPO, which then inhibit the GABAergic inhibitory projection neurons that tonically control the DMH and rMR, leading to increased sympathetic and somatic motor outflows to thermoregulatory effectors to drive cold-defensive responses.

On the other hand, optogenetic or chemogenetic stimulation of glutamatergic neurons in the MnPO induces hypothermia by eliciting heat-defensive responses in mice placed at room temperature (Yu et al. 2016; Song et al. 2016; Abbott and Saper 2017; Harding et al. 2018). It is likely that cutaneous warm-sensory glutamatergic inputs to the POA from the LPBd activate glutamatergic, perhaps NOS1-positive, excitatory interneurons in the MnPO, which then stimulate the GABAergic projection neurons that tonically control the DMH, leading to heat-defensive responses by suppressing sympathetic and somatic motor outflows to thermoregulatory effectors (Nakamura 2011; Harding et al. 2018) (Fig. 10.2). However, a part of the hypothermic responses evoked by stimulation of glutamatergic MnPO neurons may not recapitulate the homeostatic thermoregulatory responses in hot environments, but may, in fact, result from stimulation of a “non-homeostatic” circuit mechanism inherent in rodents. The POA has been shown to contain a predominantly glutamatergic group of neurons that are activated by cold-sensory inputs and thereby, induce severe hypothermia similar to torpor or hibernation (Takahashi et al. 2020; Hrvatin et al. 2020). Inhibition of these POA neurons has no effect on basal body temperature regulation (Takahashi et al. 2020; Hrvatin et al. 2020), unlike EP3R-expressing GABAergic POA neurons, whose inhibition raises body temperature like fever (Nakamura et al. 2022b). These findings indicate that these glutamatergic POA neurons induce the non-homeostatic response, cold-induced hypothermia, only when animals need to enter torpor or hibernation. This is an important caveat to the interpretation of the hypothermic responses evoked by stimulation of glutamatergic MnPO neurons, particularly because this evoked hypothermia has been used as the basis for proposing a contribution of glutamatergic descending projection POA neurons to the

tonic regulation of sympathetic outflow for thermal homeostasis (Yu et al. 2016; Song et al. 2016; Abbott and Saper 2017; Harding et al. 2018). These studies may, in fact, have observed hypothermia related to torpor or hibernation, but not a heat-defense response for thermal homeostasis.

Nonetheless, another group of glutamatergic neurons in the MnPO likely contributes to the drive of cold defense responses for thermal homeostasis. These neurons are presumably activated by cutaneous cool inputs and provide an ad hoc excitatory input to the DMH, which drives thermogenic and vasoconstrictor responses for cold defense, together with the withdrawal of the tonic GABAergic inhibition from the POA (Fig. 10.2). In support of this view, the MnPO contains glutamatergic neurons that are activated by skin cooling and project to the DMH (Dimitrov et al. 2011; da Conceição et al. 2020; Piñol et al. 2021), and antagonizing glutamate receptors in the DMH blocks BAT thermogenesis evoked by PGE₂ injection into the POA (Madden and Morrison 2004). Although the POA local circuit mechanism of the cooling-induced activation of these glutamatergic MnPO neurons is unknown, these neurons may directly receive cutaneous cool-sensory glutamatergic inputs from the LPBel (Morrison and Nakamura 2019) or controlled by local inhibitory inputs from warm-sensitive GABAergic POA neurons (da Conceição et al. 2020) to provide the DMH with thermogenic excitatory command signals dependent on the intensity of body cooling.

The EP3R-expressing group of POA neurons predominantly express vesicular GABA transporter proteins, a marker for GABAergic neurons, and indeed release GABA from their axons in the DMH, but more than half of their cell bodies also express mRNA for vesicular glutamate transporter (VGLUT) 2, a marker for glutamatergic neurons (Nakamura et al. 2022b). Mice with global deletion of the EP3R gene in VGLUT2-expressing cells exhibited a blunted febrile response to LPS injection, although the specificity of the gene deletion in the targeted cell population was not confirmed (Machado et al. 2020). This result may suggest that EP3Rs in glutamatergic POA neurons play a role in LPS-induced

fever. However, some neuronal groups in the POA expressing VGLUT2 also express glutamic acid decarboxylase, a GABAergic marker (Moffitt et al. 2018), and which transmitter is released from these neuronal groups is unknown. It is also important to note that mRNA levels determined by such transcriptomic analyses do not necessarily predict the abundance of their proteins, and therefore, undetectable levels of mRNAs may produce their proteins at levels sufficient to determine the neuronal properties (Vogel and Marcotte 2012). These facts indicate that VGLUT2 mRNA is not a reliable marker for predicting glutamatergic phenotype, especially in the POA. Nonetheless, it is possible that EP3Rs are expressed not only in the GABAergic POA neurons projecting to the DMH or rMR (Nakamura et al. 2002, 2005b, 2009, 2022b), but also in the glutamatergic MnPO interneurons that are activated by warm-sensory inputs to excite the GABAergic projection neurons (Fig. 10.2).

10.6 Hypothalamomedullary Efferent Excitatory Pathways

The DMH, which consists of the dorsomedial hypothalamic nucleus and dorsal hypothalamic area, contains abundant glutamatergic neurons that project to sympathetic premotor neurons in the rMR, and optogenetic activation of these DMH→rMR projection neurons elicits BAT thermogenesis and cardiovascular responses (Kataoka et al. 2014). DMH→rMR neurons form a cluster in the dorsal part of the DMH (Hosoya et al. 1987; Cao et al. 2004; Samuels et al. 2004; Nakamura et al. 2005b) and receive GABAergic inputs from EP3R-expressing POA neurons (Nakamura et al. 2022b), and some of these DMH neurons express the leptin receptor (Rezai-Zadeh et al. 2014), bombesin-like receptor 3 (Piñol et al. 2018), and/or melanocortin-4 receptor (Oya et al. 2024). DMH→rMR neurons are activated by cold and psychological stress and pyrogenic stimulation (Sarkar et al. 2007; Yoshida et al. 2009; Kataoka et al. 2020). DMH neurons mediate BAT thermogenic, shivering, tachycardic, and pressor responses to cold and pyrogenic stimuli

(Zaretskaia et al. 2003; Nakamura et al. 2005b; Nakamura and Morrison 2007). Intriguingly, DMH neurons do not mediate skin vasoconstriction stimulated by body cooling or pyrogenic stimulation (Rathner et al. 2008), but do mediate psychological stress-evoked skin vasoconstriction (Kataoka et al. 2020). Skin vasoconstrictors are likely regulated by a tonic GABAergic inhibitory signaling pathway from the POA to the rMR bypassing the DMH, and their constriction for cold defense and fever development is driven by both withdrawal of the tonic inhibition and an ad hoc glutamatergic input from the MnPO to the rMR (Nakamura 2011; Tanaka et al. 2011) (Fig. 10.2). Nonetheless, the DMH contains a group of neurons that drive skin vasoconstriction (Rathner et al. 2008), and a psychological stress-driven, glutamatergic excitatory input from the corticolimbic circuit to the DMH (see below) stimulates these neurons to drive skin vasoconstrictor response to stress through DMH→rMR transmission (Kataoka et al. 2020) (Fig. 10.2). It is also noted that the pressor response accompanying the cold-defensive and febrile responses is mediated not only by the DMH→rMR pathway, but also in part by a direct or indirect pathway from the DMH to the rostral ventrolateral medulla (RVLM), which contains a group of sympathetic premotor neurons that control visceral blood vessels and the heart to regulate basal blood pressure (Horiuchi et al. 2004).

The rMR harbors groups of sympathetic premotor neurons that drive BAT thermogenesis, tachycardia, and skin vasoconstriction. Somatic premotor neurons for shivering are also distributed in the rMR (Nakamura and Morrison 2011). Sympathetic premotor neurons are defined as excitatory neurons that directly innervate sympathetic preganglionic neurons in the thoracolumbar spinal cord, and the sympathetic premotor neurons in the rMR that control BAT and skin blood vessels express VGLUT3, a putative glutamatergic neuronal marker (Nakamura et al. 2004a) (Fig. 10.2). Some of VGLUT3-expressing neurons in the rMR are also serotonergic (Nakamura et al. 2004a; Stornetta et al. 2005), and sympathetic preganglionic neurons in the spinal cord are contacted by many VGLUT3-

containing synaptic terminals derived from the rMR, 6–22% of which contain serotonin (Nakamura et al. 2004a, b; Stornetta et al. 2005). BAT thermogenesis driven by premotor neurons in the rMR is blocked by antagonizing glutamate receptors in the spinal sites where sympathetic preganglionic neurons are localized (Nakamura et al. 2004a). Therefore, the sympathetic premotor drives from the rMR to the spinal cord are primarily mediated by glutamatergic transmission. The co-transmitter serotonin released from the nerve terminals in the spinal cord seems to potentiate the glutamatergic excitatory transmission to sympathetic preganglionic neurons (Madden and Morrison 2006). This serotonergic modulation may be important to boost thermogenesis to meet the heat demand for thermoregulation under severely cold environments (Hodges et al. 2008).

Stimulation of premotor neurons in the rMR elicits intense BAT thermogenesis, shivering thermogenesis, skin vasoconstriction, and tachycardia (Morrison et al. 1999; Blessing and Nalivaiko 2001; Cao and Morrison 2003; Nason and Mason 2004; Nakamura and Morrison 2011). Also, chemogenetic stimulation of VGLUT3-expressing neurons in the rMR evokes BAT thermogenesis (Zeng et al. 2019). On the other hand, inactivation of rMR neurons eliminates BAT thermogenic, shivering, skin vasoconstrictor, and tachycardic responses to skin cooling or pyrogenic stimulation (Nakamura et al. 2002; Morrison 2003; Nakamura and Morrison 2007; Nakamura and Morrison 2011). These findings indicate that premotor neurons in the rMR mediate sympathetic and somatic motor (shivering) outflows to drive thermal and cardiovascular responses to cold and pyrogenic stimuli (Fig. 10.2).

Because the activity of premotor neurons in the rMR critically determines the intensities of the physiological responses, it is important to understand how the premotor neurons are regulated. VGLUT3-expressing premotor neurons in the rMR are contacted by VGLUT2-containing glutamatergic nerve terminals from the DMH (Kataoka et al. 2014). BAT thermogenic and cardiac responses elicited by optogenetic stimulation of DMH→rMR transmission are dependent

on glutamatergic synaptic transmission in the rMR (Kataoka et al. 2014). Antagonizing glutamate receptors in the rMR blocks BAT thermogenic response to skin cooling or pyrogenic stimulation (Madden and Morrison 2003; Nakamura and Morrison 2007). Therefore, physiological excitation of premotor neurons in the rMR is caused by glutamatergic inputs from the DMH that are provided in an ad hoc manner when heat is demanded for cold defense, fever development, or other physiological responses (see also discussion on psychological stress-induced hyperthermia below) (Fig. 10.2). Recently, it has been shown that sympathetic premotor neurons in the rMR are also activated by oxytocin released from axons projecting from the paraventricular hypothalamic nucleus (PVH) to drive BAT thermogenesis and a cardiac response (Fukushima et al. 2022), which may be related to emotional responses. Oxytocin released in the rMR also potentiates the glutamatergic excitatory action on sympathetic premotor neurons, enhancing BAT thermogenic and cardiac responses (Fukushima et al. 2022).

In addition to the excitatory inputs, premotor neurons in the rMR are under the control of tonic GABAergic inhibitory inputs. Antagonizing GABA_A receptors in the rMR evokes intense BAT thermogenesis and skin vasoconstriction (Morrison et al. 1999; Blessing and Nalivaiko 2001; Cao and Morrison 2003), and VGLUT3-expressing neurons in the rMR are contacted by numerous GABAergic nerve terminals (Nakamura et al. 2005a). The tonic GABAergic inputs to premotor neurons in the rMR are likely provided by POA neurons including EP3R-expressing neurons (Nakamura et al. 2002, 2022b) (Fig. 10.2). EP3R-expressing POA neurons have two separate subgroups that provide projections to the DMH and to the rMR, which may control thermogenesis and skin vasoconstriction, respectively (Nakamura et al. 2009). In support of this view, it has been proposed that tonic GABAergic control through a POA→rMR projection is important for the regulation of skin vasoconstrictors (Tanaka et al. 2011). It is also possible that premotor neurons in the rMR receive additional tonic GABAergic inputs from

as yet unidentified, other brain areas. Taken together, the premotor drives from the rMR are stimulated both by the ad hoc glutamatergic excitatory inputs from the DMH and by the withdrawal of tonic GABAergic inhibitory inputs from the POA and other sites, if any. In addition, there is also an ad hoc GABAergic inhibitory input to the sympathetic premotor neurons to reduce metabolic rates during starvation (see below).

10.7 Central Circuit Mechanism for Psychological Stress Responses

The central circuitry that has been discussed in this chapter functions not only for basal thermoregulation and fever development, but also for coping with psychological stressors and starvation stress. In response to psychological stress, mammals exhibit a variety of physiological responses, including hyperthermia and tachycardia. These physiological responses to stress boost physical performances of animals by warming the body and increasing the supplies of fuels and oxygen to the CNS and skeletal muscles—beneficial for wild animals when they encounter their enemies. However, chronic stress causes psychosomatic problems, such as development of psychogenic fever (Oka 2015). Psychogenic fever is associated with fever-like symptoms including fatigue and flushed skin in addition to elevated body temperature, but is resistant to antipyretics that block PGE₂ biosynthesis, indicating that the etiology of psychogenic fever is different from that of infection-induced fever (Lkhagvasuren et al. 2011; Oka 2015; Vinkers et al. 2009). Recently, remarkable progress has been made in understanding the central circuit mechanism of physiological responses to psychological stress.

The elevation of body temperature by psychological stress is due to increased BAT thermogenesis and intensified skin vasoconstriction (Lkhagvasuren et al. 2011; Ootsuka et al. 2008a). The stress-induced BAT thermogenesis and hyperthermia are mediated by sympathetic premotor neurons in the rMR that are activated by

psychological stress (Lkhagvasuren et al. 2011; Kataoka et al. 2014). The activation of sympathetic premotor neurons in the rMR is driven by a stress-driven glutamatergic excitatory input from the DMH, and optogenetic stimulation of the DMH→rMR transmission elicits BAT thermogenic, tachycardic, and pressor responses mimicking stress responses (Kataoka et al. 2014). DMH→rMR neurons, which cluster in a dorsal part of the DMH, are activated by psychological stress (Sarkar et al. 2007; Kataoka et al. 2014). Inactivation of DMH neurons eliminates BAT thermogenic, hyperthermic, tachycardic, and pressor responses to psychological stress (Kataoka et al. 2014, 2020). These findings demonstrate that the DMH→rMR hypothalamomedullary pathway mediates the glutamatergic excitatory transmission that drives sympathetic responses to psychological stress (Fig. 10.2).

The psychosomatic neural connection from the corticolimbic circuit processing stress and emotion to the hypothalamic circuit for homeostatic regulation has long been a subject of stress research (Nakamura 2015). A recent study discovered a novel neural pathway from the ventral medial prefrontal cortex to the DMH that transmits stress signals to drive the physiological stress responses (Kataoka et al. 2020). A group of pyramidal neurons in the dorsal peduncular cortex and dorsal tenia tecta (DP/DTT), located at the ventral limit of the ventral medial prefrontal cortex, is activated by psychological stress and provides VGLUT1-positive glutamatergic projections to the DMH (Kataoka et al. 2020). Optogenetic stimulation of the DP/DTT→DMH pathway activates the DMH→rMR pathway and elicits BAT thermogenic, tachycardic, and pressor responses mimicking stress responses, via glutamatergic neurotransmission at the DMH (Kataoka et al. 2020). Selective lesions of DP/DTT→DMH projection neurons eliminate stress-induced BAT thermogenesis and hyperthermia (Kataoka et al. 2020). Furthermore, optogenetic inhibition of DP/DTT→DMH transmission abolishes BAT thermogenic, skin vasoconstrictor, hyperthermic, tachycardic, and pressor responses to psychological stress (Kataoka et al. 2020). Intriguingly, optogenetic inhibition of the DP/

DTT→DMH pathway suppresses avoidance of stressors (Kataoka et al. 2020). These results indicate that the DP/DTT→DMH pathway mediates master psychosomatic signaling for various sympathetic and behavioral responses to psychological stress. Because the DP/DTT receives stress-related inputs from multiple corticolimbic sites (Kataoka et al. 2020), psychological stress and emotion signals are likely integrated in the DP/DTT and then, the integrated psychogenic signals are transmitted to the DMH to influence the circuit system for homeostatic regulation (Fig. 10.2). This central psychosomatic connection is not involved in basal homeostasis (Kataoka et al. 2020). Therefore, the DP/DTT→DMH pathway can be a potential target for treating aberrant physiological responses in various stress-related disorders, such as panic disorder and post-traumatic stress disorder.

10.8 Central Circuit Mechanism for Hunger Responses

Mammals under starvation exhibit hypothermia even in thermoneutral environments. This is a physiological response of reducing energy expenditure to survive starvation as long as possible. Starved mammals also exhibit increased appetite to facilitate energy intake and can start mastication immediately after food is placed in the mouth. These hunger responses are driven by the central circuit mechanism, and a recent study identified a group of medullary neurons that play a pivotal role in driving the hunger responses (Nakamura et al. 2017).

Negative energy balance, which means less energy intake than expenditure, is sensed by several mechanisms, among which a hypothalamic circuit mechanism involving ghrelin–neuropeptide Y (NPY) signaling is well known. The peptide hormone ghrelin is released from the stomach under starved conditions and delivered to the arcuate nucleus of the hypothalamus to activate a group of neurons expressing NPY (Kohno et al. 2003; Kojima and Kangawa 2005). These neurons innervate the PVH and an action of NPY released from their axons on PVH neurons trig-

gers the hunger responses: reduced energy expenditure and increased appetite (Stanley and Leibowitz 1985; Walker and Romsos 1993; Atasoy et al. 2012). The NPY action reduces energy expenditure in excess of basal metabolic rate, which most likely includes expenditure for adaptive thermogenesis in cold environments (Székely et al. 2005). Consistent with this view, NPY action in the PVH leads to inhibition of adaptive thermogenesis in BAT (Egawa et al. 1991; Nakamura et al. 2017).

The NPY-induced inhibition of BAT thermogenesis is mediated by GABAergic inhibition of sympathetic premotor neurons in the rMR (Nakamura et al. 2017). Neural tracing studies discovered a group of medullary GABAergic neurons that directly innervate VGLUT3-expressing premotor neurons in the rMR, and these GABAergic neurons are distributed in the intermediate and parvocellular reticular nuclei (IRt/PCRt) of the medulla oblongata (Nakamura et al. 2017). In vivo electrophysiological unit recordings combined with post-recording *in situ* hybridization identified rMR-projecting GABAergic neurons in the IRt/PCRt that are activated by a hypothalamic action of NPY (Nakamura et al. 2017). Chemogenetic stimulation of GABAergic IRt/PCRt neurons suppresses cooling-evoked BAT thermogenesis, whereas inactivation of IRt/PCRt neurons abolishes NPY-induced inhibition of BAT thermogenesis (Nakamura et al. 2017). These results demonstrate that NPY-triggered hunger signaling from the hypothalamus activates GABAergic IRt/PCRt neurons, which then inhibit the thermogenic sympathetic premotor drive from the rMR to BAT (Fig. 10.2).

Intriguingly, pharmacological stimulation of neurons in the IRt/PCRt not only suppresses BAT thermogenesis, but also evokes mastication under both anesthetized and free-moving conditions (Nakamura et al. 2017). The same stimulation also increases food intake in free-moving rats (Nakamura et al. 2017). Inactivation of IRt/PCRt neurons diminishes hypothalamic NPY-induced mastication and hyperphasia (Travers et al. 2010). Therefore, the IRt/PCRt mediates both NPY-triggered hunger responses: reduction of

adaptive thermogenesis (for energy saving) and facilitation of food intake (for energy intake). Anatomical evidence suggests that GABAergic IRt/PCRt neurons play a pivotal role in both hunger responses: many GABAergic IRt/PCRt neurons were found to innervate both rMR and motor trigeminal nucleus (Mo5) (Nakamura et al. 2017), the latter of which contains masticatory motor neurons. These GABAergic neurons, together with glutamatergic neurons, likely constitute the local central pattern generator circuit in the IRt/PCRt that generates the rhythmic activity for masticatory movements of jaw muscles (Moore et al. 2014; Stanek et al. 2014). Consistently, optogenetic stimulation or inhibition of GABAergic neurons in the IRt/PCRt inhibits or increases masseter muscle activity, respectively (Han et al. 2017), and rMR-projecting GABAergic neurons in the IRt/PCRt that are activated following hypothalamic NPY injection exhibit phasic bursting patterns similar to masticatory rhythms (Nakamura et al. 2017). The rhythmic premotor input from the IRt/PCRt to the Mo5 under starved conditions likely primes masticatory motor neurons to initiate mastication immediately after food is placed in the mouth.

These findings indicate that upon receiving hunger signals from the hypothalamus, the IRt/PCRt takes simultaneous control of both sympathetic nervous system and masticatory motor system to command hunger responses for surviving starvation longer. The circuit mechanism that mediates the hunger signaling from the hypothalamus to the IRt/PCRt remains unknown. However, it is presumed that NPY-triggered excitatory signaling from the PVH is mediated by the nucleus tractus solitarius to activate GABAergic IRt/PCRt neurons (Nakamura et al. 2017; Nakamura and Nakamura 2018a, b).

10.9 Conclusion

Body core temperature of many mammals is precisely regulated by both negative feedback and feedforward mechanisms (Fig. 10.1). The negative feedback mechanism regulates body core temperature within a defended control range and

functions primarily dependent on the levels of body core (brain tissue) temperature monitored by warm-sensitive neurons in the POA. The feedforward mechanism is essential for eliciting “preventive” thermoregulatory responses and behaviors before body core temperature is affected by environmental thermal challenges and is activated by afferent inputs to the POA of cutaneous cool- and warm-sensory signals, which are separately delivered by spinal-LPB-POA pathways. The POA continuously integrates the central and peripheral thermosensory information and provides tonic control signals to regulate excitatory efferent neurons in the DMH and rMR for thermal homeostasis.

The efferent control signals from the POA to the DMH and rMR consist of tonic GABAergic inhibition and ad hoc glutamatergic excitation (Fig. 10.2). The tonic inhibitory signals, which are principally provided by EP3R-expressing POA neurons, continuously regulate the excitatory efferent pathways through the DMH and rMR to thermoregulatory effectors and are intensified in response to elevation of body temperature or increased cutaneous warm-sensory inputs to the POA, leading to inhibition of the sympathetic and somatic motor outflows to facilitate heat loss through skin vasodilation. On the other hand, a decrease in body core temperature or increased cutaneous cool inputs to the POA attenuate the tonic inhibition from the POA to the DMH and rMR to disinhibit (activate) the excitatory efferent neurons. At the same time, the cold signals also likely activate glutamatergic transmission from the POA to the DMH and rMR in an ad hoc manner to provide excitation to the excitatory efferent neurons. The excited efferent neurons in the DMH and rMR drive sympathetic and somatic motor outflows to elicit cold-defensive responses including BAT and shivering thermogenesis, skin vasoconstriction, and tachycardia.

When animals are infected, immune signals are delivered to the parenchyma of the POA via production of PGE₂. The action of PGE₂ on EP3R-expressing neurons in the POA reduces the tonic inhibition, as well as perhaps stimulates the excitatory transmission, from the POA to the

DMH and rMR to activate the excitatory efferent pathways to drive thermogenic, vasoconstrictor, and cardiac responses to develop fever (Fig. 10.2). The tone of the inhibitory signaling from EP3R-expressing POA neurons to the DMH and rMR, which strongly influences the levels of the excitatory efferent outputs to thermoregulatory effectors, is important in determining the control range (set-point) of body core temperature. However, because other inputs to the DMH and rMR, which include stress and hunger signals, also alter the levels of the excitatory outputs to effectors strongly enough to shift the defended control range of body core temperature, the POA is not the sole brain site that determines the set-point of body temperature.

The central circuit mechanisms discussed in this chapter (Fig. 10.2) are relevant to understanding the entire central neural network that maintains systemic homeostasis. As discussed, this central circuitry for basal body temperature regulation also functions for the development of fever in response to systemic infection. Furthermore, this circuitry has also been found to develop physiological responses to psychological stress and starvation stress. Psychological stress signals in the corticolimbic emotion circuit are integrated and transmitted from the DP/DTT to the DMH to drive a variety of involuntary and behavioral stress responses. The DP/DTT→DMH pathway represents a central “mind–body” connection through which psychological stress and emotion affect the homeostatic circuit system, and therefore, can be a potential target for the treatment of aberrant sympathetic responses in stress-related disorders. During starvation, hunger signaling from the hypothalamus activates a GABAergic population of neurons in the IRt/PCRt, which then inhibit BAT sympathetic premotor neurons in the rMR to suppress metabolic thermogenesis for energy saving. These IRt/PCRt neurons also contribute to the generation of masticatory rhythm to prime trigeminal motor neurons to initiate mastication immediately after food becomes available. Because unbalanced regulation of the IRt/PCRt neurons may cause obesity or anorexia, elucidating the circuit mechanisms that mediate

hunger and satiety signaling from the hypothalamus to the IRt/PCRt may be important for understanding the etiologies of these disorders.

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Inter-Organ Communication Involved in Brown Adipose Tissue Thermogenesis

11

Kei Takahashi, Tetsuya Yamada,
and Hideki Katagiri

Abstract

Brown and beige adipocytes produce heat from substrates such as fatty acids and glucose. Such heat productions occur in response to various stimuli and are called adaptive non-shivering thermogenesis. This review introduces mechanisms known to regulate brown and beige adipocyte thermogenesis. Leptin and fibroblast growth factor 21 (FGF21) are examples of periphery-derived humoral factors that act on the central nervous system (CNS) and increase brown adipose tissue (BAT) thermogenesis. Additionally, neuronal signals such as those induced by intestinal cholecystokinin and hepatic peroxisome proliferator-activated receptor γ travel through vagal afferent-CNS-sympathetic efferent-BAT pathways and increase BAT thermogenesis. By contrast, some periphery-derived humoral factors (ghrelin, adiponectin, plasminogen activator inhibitor-1, and soluble leptin recep-

tor) act also on CNS but inhibit BAT thermogenesis. Neuronal signals also reduce BAT sympathetic activities and BAT thermogenesis, one such example being signals derived by hepatic glucokinase activation. Beige adipocytes can be induced by myokines (interleukin 6, irisin, and β -aminoisobutyric acid), hepatokines (FGF21), and cardiac-secreted factors (brain natriuretic peptide). Cold temperature and leptin also stimulate beige adipocytes via sympathetic activation. Further investigation on inter-organ communication involving adipocyte thermogenesis may lead to the elucidation of how body temperature is regulated and, moreover, to the development of novel strategies to treat metabolic disorders.

Keywords

Inter-organ communication · Brown adipose tissue · Beige adipocyte · Uncoupling protein-1 · Leptin · Plasminogen activator inhibitor-1 · Vagal nerve · Liver · Peroxisome proliferator-activated receptor γ · Glucokinase

K. Takahashi · H. Katagiri
Department of Diabetes, Metabolism and
Endocrinology, Tohoku University Graduate School
of Medicine, Sendai, Japan

T. Yamada (✉)
Department of Molecular Endocrinology and
Metabolism, Graduate School of Medical and Dental
Sciences, Tokyo Medical and Dental University,
Tokyo, Japan
e-mail: tyamada.mem@tmd.ac.jp

11.1 Introduction

Body weight is determined by the balance of energy intake and energy expenditure. In recent years, thermogenic adipose tissues involved in

systemic energy expenditure have gained attention. Two types of thermogenic adipose tissues exist, classical brown adipose tissues (BATs) and white adipose tissues (WATs) containing beige adipocytes. The primary cellular function of both brown and beige adipocytes is thermogenesis. Both cell types are characterized by the expression of uncoupling protein-1 (UCP1). Recent studies demonstrate that beige adipocytes are present in adult humans and can be induced by environmental factors such as cold stimulation. One reason that adipose tissue thermogenesis has attracted attention is that it may provide a new therapeutic target to treat obesity. This review discusses mechanisms underlying body weight regulation, mainly focusing on inter-organ/inter-cellular communication that regulates BAT thermogenesis and beige adipocyte induction.

11.2 Brown Adipose Tissue

BAT primarily drives thermogenesis. Brown adipocytes are characterized by the expression of UCP-1. ATP is produced in mitochondria by ATP synthase, a mitochondrial enzyme driven by a proton gradient across mitochondrial inner membranes. UCP1 is a proton transporter protein localized in the mitochondrial inner membrane and uncouples electron transport from ATP synthesis by eliminating membrane potential. In

turn, this action dissipates energy as heat. BAT thermogenesis is primarily regulated by sympathetic nerve stimulation and thyroid hormones. Sympathetic activity is crucial for this regulation because sympathetic nerve stimulation enhances type-2 iodothyronine deiodinase activity and consequently induces BAT thermogenesis. The characteristics of BAT and WAT are presented in Fig. 11.1.

11.3 Central Nervous System (CNS) Input to Metabolic Regulation of BAT Thermogenesis

Blood-borne and neuronal inputs are major pathways with which the brain receives metabolic information from peripheral tissues regarding energy metabolism (Fig. 11.2). For example, leptin, an adipokine secreted from WAT (described in more detail in the following sections), circulates to the hypothalamus and directly activates hypothalamic neurons. In turn, sympathetic nerves controlling BAT are activated. On the contrary, afferent nerve pathways are also important. These pathways transmit metabolic information from intraabdominal organs to the brain. Intraabdominal organs are innervated by the vagus and splanchnic nerves. Afferent nerve fibers constitute approximately 75–90% of vagus and 50% of splanchnic nerves.

Fig. 11.1 Biochemical and functional characteristics of brown and white adipocytes

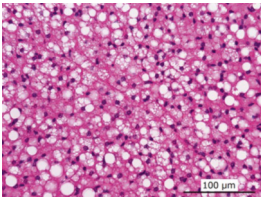
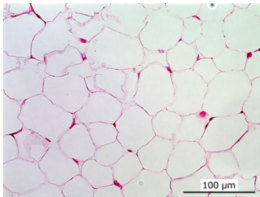
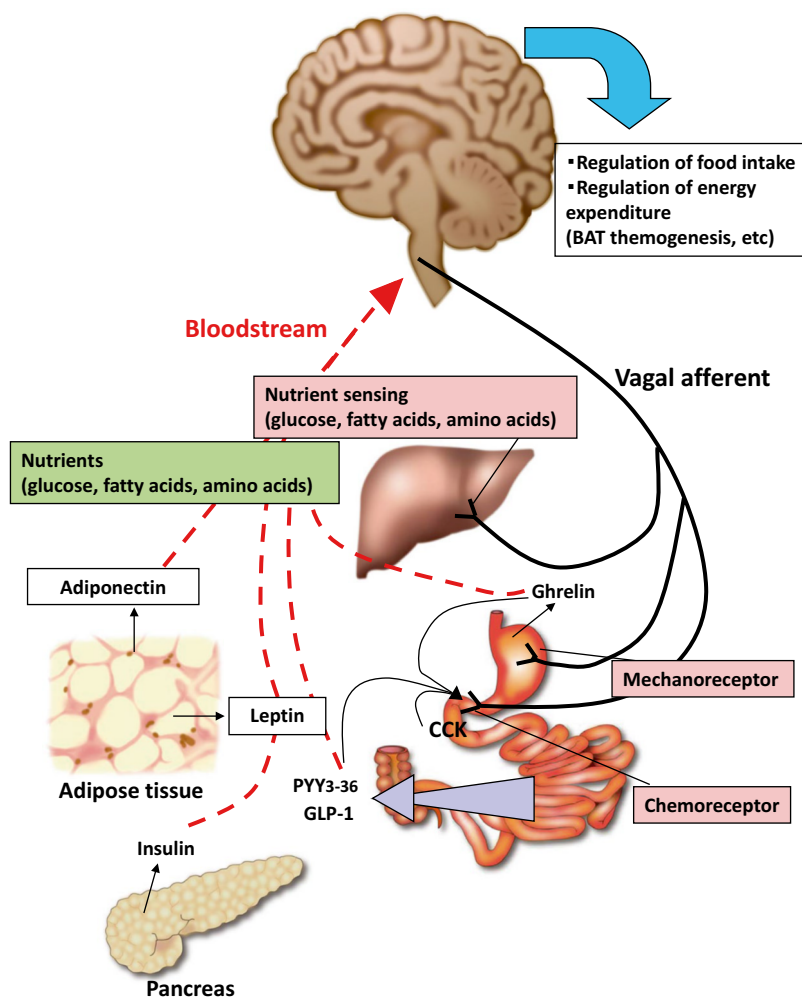
	Brown Adipose Tissue: BAT	White Adipose Tissue: WAT
		
<u>Locations</u>	Inter-scapular regions	Subcutaneous /visceral regions
<u>Morphology</u>	Small multilocular lipid droplets Abundant mitochondria High vascularization	Large unilocular lipid droplet Flattened peripheral nucleus
<u>Function</u>	Dissipation of energy as heat (cold induced thermogenesis, diet induced thermogenesis)	Storage of excess energy as triglyceride Secretion of adipocytokines (Leptin, Adiponectin, TNFα, etc)

Fig. 11.2 Brain input of metabolic information regarding energy metabolism. (Modified from reference (Yamada et al. 2008))



11.4 BAT and Adaptive Thermogenesis

Individuals who eat more and move less typically gain body weight. However, weight gain is not necessarily equal to the difference between energy intake and energy consumed through physical activity. A substantial amount of “non-volitional energy expenditure” is derived from basal metabolism and adaptive thermogenesis, which also contribute to systemic energy expenditure. That is, an individual’s weight is regulated by the balance between energy intake (diet) and energy expenditure (basal metabolism

[60%] + adaptive thermogenesis [10%] + physical activity [30%]). Adaptive thermogenesis can further be divided into cold-induced thermogenesis and diet-induced thermogenesis. Cold-induced thermogenesis helps maintain body temperature in response to low environmental temperature through “shivering thermogenesis” by skeletal muscles and “non-shivering thermogenesis” by BAT. In addition, BAT is responsible for diet-induced thermogenesis that occurs in response to energy intake (Tseng et al. 2010). Sympathetic nerve stimulation is crucial for the induction of BAT thermogenesis. As shown in Fig. 11.2, the brain (primarily the hypothalamus)

regulates autonomic nerve activity, including sympathetic nerve activity, which controls BAT in response to various metabolic signals reflecting energy metabolism in other tissues.

11.5 Inter-Organ Communication Leading to Enhanced BAT Thermogenesis

Organs communicate via humoral factors and neural signals to maintain constant body weight and prevent excessive weight gain (obesity).

11.5.1 Humoral Factors

11.5.1.1 Leptin

Leptin, which is an adipokine primarily secreted from WAT, circulates and acts directly on the brain (mainly on hypothalamic neurons). Leptin suppresses food intake and increases diet-induced BAT thermogenesis via sympathetic nerve activation. Leptin is produced in proportion to WAT accumulation (which reflects the amount of systemic energy storage) and therefore serves as a negative feedback mechanism to suppress body weight gain and thus prevent excessive energy storage. Consistently, mouse strains that harbor a homozygous leptin gene mutation (*ob/ob* mice) become substantially obese but become leaner when leptin is injected. In *ob/ob* mice, leptin suppresses appetite and induces BAT thermogenesis, with the former and latter contributing 65% and 35% to body weight reduction, respectively. Mechanistically, leptin binds to the leptin receptor expressed in the neurons of hypothalamic nuclei, such as the arcuate nucleus, paraventricular nucleus, and dorsomedial hypothalamic nucleus. These neurons transmit neuronal signals to various secondary neurons, which leads to the activation of the raphe nucleus in the medulla oblongata, the sympathetic premotor region that regulates BAT via sympathetic nerve activity.

11.5.1.2 FGF21 (Fibroblast Growth Factor 21)

FGF21 is produced in the liver, BAT, and WAT, but the majority of circulating FGF21 is derived from the liver (Hsuehou et al. 2007). Hepatic FGF21 production is enhanced by the activation of the transcription factor, peroxisome proliferator-activated receptor alpha (PPAR α) (Inagaki et al. 2007). Circulating FGF21 crosses the blood–brain barrier and directly acts on neurons in the CNS (Hsuehou et al. 2007). FGF receptor complexes with β -Klotho protein (β -Klotho-FGF receptor 1 complex), which is essential for FGF21 action. Although FGF receptors are expressed in various regions of the CNS, β -Klotho is expressed only in regions such as the suprachiasmatic nucleus and paraventricular nucleus of the hypothalamus (Bookout et al. 2013; Liang et al. 2014). Hypothalamic β -Klotho is crucial for FGF21-induced energy expenditure and BAT thermogenesis (Owen et al. 2014). Thus, the CNS appears to play an important role in FGF21-induced activation of BAT.

11.5.1.3 Thyroid Hormone

Thyroxine (T₄) is produced in the thyroid gland and is converted into triiodothyronine (T₃) by iodothyronine deiodinases (particularly D2) expressed in peripheral tissues, including WAT and BAT (Obregon 2014). T₃ exerts its effects by binding to the thyroid hormone receptor (TR), which then binds to thyroid response elements to regulate target gene expression (Obregon 2014). All known isoforms of TRs are present in WAT and BAT. T₃ treatment increased UCP1 expression both in primary cultures of brown adipocytes (Hernandez and Obregon 2000) and in rats (Bianco and Silva 1988). The relevance of thyroid hormones in BAT is supported by a human case study (Skarulis et al. 2010). Treatment with levothyroxine for 14 days resulted in increased fluorodeoxyglucose (FDG) uptake in the lower neck, suprascapular, mediastinal, and thoracic paravertebral regions. Tissues in all these regions

were found to be adipose by computed tomography (CT) scanning. Biopsy samples were obtained from the suprascapular region from both the case subject and a control subject, and quantitative polymerase chain reaction analysis revealed that UCP1 expression was increased in the case subject.

11.5.2 Neural Signals

11.5.2.1 Neural Signals from the Stomach and Intestinal Tract

Mechanisms underlying appetite regulation have been elucidated in numerous studies. These studies demonstrate that neural signals are transmitted from the stomach and intestinal tracts to the brain and affect appetite. Neural signals are in part induced by extension of the intestinal tract and by various peptides secreted from the intestinal tract (Fig. 11.2). Cholecystokinin (CCK), a peptide secreted from I cells in the duodenum and jejunum, activates the vagal afferent pathway. These signals are then transmitted to the CNS, thereby inducing suppression of food intake (Smith et al. 1981). In addition, glucagon-like peptide-1 (Abbott et al. 2005) and PYY3–36 (Koda et al. 2005), both peptides secreted from intestinal L-cells, induce similar action via the vagal afferent pathway. Furthermore, ghrelin, primarily secreted from gastric cells, also acts via the vagal afferent pathway but acts to enhance food intake (Date et al. 2002).

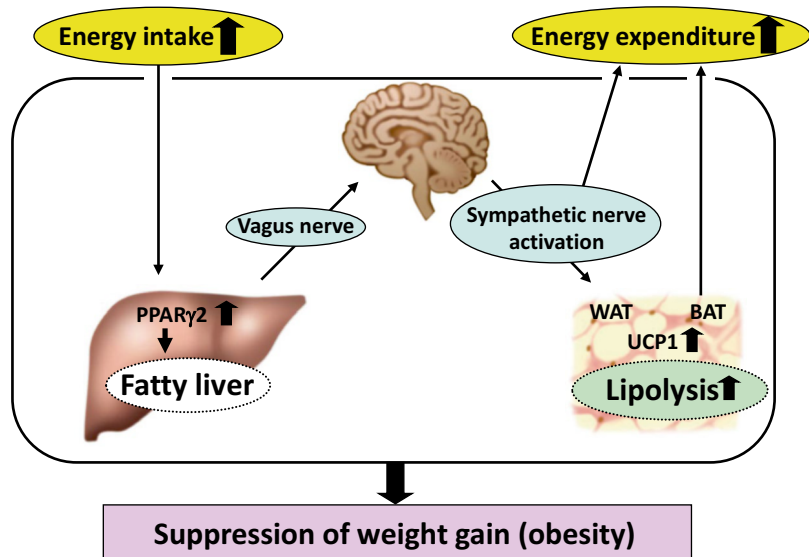
Details of the regulation of BAT thermogenesis via neural signals from the stomach and intestinal tract have long remained unclear. Recently, signals transmitted to the brain via the vagus nerve were shown to regulate BAT thermogenesis (Blouet and Schwartz 2012). Direct administration of lipids into the duodenum increased BAT thermogenesis. Blockade of intestinal vagal sensory fibers decreased BAT temperature, demonstrating that the vagal afferent pathway from the intestinal tract plays a major role in lipid-induced

BAT thermogenesis. More recently, studies in rats showed that dietary fat-induced BAT thermogenesis was mediated by CCK (Wang et al. 2019). However, additional work is needed to identify specific molecules in orally administered fat that induce BAT thermogenesis, and more importantly, how this induction occurs.

11.5.2.2 Neural Signals from the Liver

Excess energy due to overeating is primarily accommodated in WAT in the form of lipids. However, prolonged overeating may lead to an increased influx of free fatty acids even into the hepatocytes and cause non-alcoholic fatty liver disease (NAFLD). Peroxisome proliferator-activated receptor γ (PPAR γ) is a transcription factor that is primarily expressed in adipose tissues in a non-obese state, but its expression is also enhanced in livers of patients with NAFLD. Interestingly, liver-specific PPAR γ deficiency suppressed the development of fatty liver and resulted in increased peripheral fat, increased insulin resistance, and impaired glucose tolerance, suggesting an important role of hepatic PPAR γ in inter-organ interactions for energy metabolism. Our previous work identified the underlying mechanisms of this inter-organ communication. Overexpression of PPAR γ 2 in the liver of mice (PPAR γ 2 mice) was found to increase BAT thermogenesis via sympathetic nerve activation that led to suppression of weight gain. Additionally, increased BAT thermogenesis was abolished when the hepatic branch of the vagus nerve was selectively dissected in PPAR γ 2 mice, indicating that this nerve fiber conveyed signals from the liver to the brain. Based on these findings, a novel inter-organ communication system was found to transmit metabolic signals that reflect excessive energy accumulation in the liver. These inter-organ communications link the liver and brain via the vagal afferent pathway and, consequently, activate sympathetic activity to increase BAT thermogenesis. Thus, the system serves as negative feedback in energy accumulation (Uno et al. 2006) (Fig. 11.3).

Fig. 11.3 Inter-organ communication mediating induction of liver PPAR γ 2 and enhancement of BAT thermogenesis via neural signals. (Modified from reference (Yamada 2013))



11.6 Inter-Organ Communication Leading to Reduced BAT Thermogenesis

Individuals always store adequate amounts of energy within the body, typically as adipose tissue, to compensate for periods of inadequate access to food. The ideal way to maintain energy stores is to maximize energy storage as fat or to minimize the loss of fat during periods of inadequate food access.

11.6.1 Humoral Factors

11.6.1.1 Ghrelin

Ghrelin is secreted from gastric X/A-like cells during fasting. Ghrelin reportedly activated NPY/agouti-related peptide (AgRP)-containing neurons in the hypothalamic arcuate nucleus, which then inhibit BAT sympathetic activity and attenuate BAT thermogenesis (Nakamura and Nakamura 2018; Nakamura et al. 2017). Moreover, genetic suppression of ghrelin receptors activated BAT thermogenesis and decreased fat storage in high-fat diet (HFD)-induced obese rats (Mano-Otagiri et al. 2010). These findings further support the role of ghrelin in inhibiting BAT thermogenesis. Also, ghrelin stimulates appetite, therefore countering negative energy balance induced by a deficit of food intake.

11.6.1.2 Adiponectin

Adiponectin is an adipokine secreted by adipocytes. Plasma adiponectin levels decrease in obesity and increase in response to weight loss. Recent studies showed that adiponectin administration reduced BAT UCP1 expression and suppressed energy expenditure in mice, and adiponectin deletion enhanced BAT UCP1 expression (Kubota et al. 2007; Qiao et al. 2014). Adiponectin inhibited UCP1 expression in brown adipocytes by suppressing β 3-adrenergic receptor expression (Qiao et al. 2014). These findings suggest that adiponectin reduces BAT thermogenesis and energy expenditure and helps minimize fat loss when the energy balance is negative.

11.6.1.3 Leptin Resistance

Leptin acts on hypothalamic neurons and activates sympathetic nerves controlling BAT. Obesity is associated with leptin resistance, a state in which elevated circulating leptin and exogenously delivered leptin are both less effective in creating satiety and suppressing food intake (Liu et al. 2015). Various mechanisms may be involved in leptin resistance. Increased circulating leptin due to excess food intake activates hypothalamic leptin receptors and induces SOCS-3 expression, a signal transduction inhibitory protein. Expression of SOCS-3 in turn suppresses intracellular leptin signaling (Mori et al. 2004).

Additionally, obesity leads to decreased leptin permeability across the blood–brain barrier. As a result, cerebrospinal leptin concentration is not increased despite hyperleptinemia, thus limiting leptin action within the CNS. Furthermore, several studies reported that HFD feeding caused hypothalamic inflammation and endoplasmic reticulum stress. These disorders may also contribute to the development of leptin resistance (De Souza et al. 2005; Kleinridders et al. 2009; Zhang et al. 2008; Banno et al. 2010). In obesity-prone mice (mice prone to obesity during HFD feeding), HFD feeding suppresses BAT UCP1 expression (Watson et al. 2000; Prpic et al. 2002). This suppression may be due to decreased hypothalamic leptin sensitivity. This mechanism helps individuals increase energy stores during times when food is plentiful.

11.6.1.4 Plasminogen Activator Inhibitor-1 (PAI-1)

PAI-1 is a serine protease inhibitor, which is primarily produced by endothelial cells and functions as a physiological inhibitor of tissue-type and urokinase-type plasminogen activators (Vousden et al. 2019). On the contrary, in states of obesity, PAI-1 is produced also by adipocytes at high levels (Bodary 2007). In addition, body weight gain in response to HFD feeding was unexpectedly attenuated in PAI-1 knockout mice (Ma et al. 2004). Therefore, it has been proposed that PAI-1 might participate in inter-organ tissue communications regulating body weight. We discovered that both food intake and body weight gain were significantly attenuated in PAI-1 knockout mice under HFD feeding conditions compared with control mice, although both mice groups similarly ate and gained body weight under chow feeding conditions (Hosaka et al. 2020). Additionally, in HFD-fed wild-type mice, oral treatment with a PAI-1 inhibitor markedly suppressed body weight gain, in a PAI-1-dependent manner. Pharmacological PAI-1 inhibition also alleviated HFD-induced systemic and hypothalamic leptin resistance, prior to suppression of body weight gain. Moreover, the improvement of leptin sensitivity in response to pharmacological PAI-1 inhibition was accompanied by increased BAT thermogenesis. These results suggest that under HFD-fed conditions,

PAI-1 reduces hypothalamic leptin sensitivities, thereby decreasing BAT thermogenesis (Hosaka et al. 2020).

11.6.1.5 Soluble Leptin Receptor (sLepR)

sLepR is a leptin receptor produced and released primarily from hepatocytes into the circulation (Schaab and Kratzsch 2015). As ligand-binding domains are present in sLepR, circulating liver-derived sLepR binds to plasma leptin, thereby inhibiting leptin from acting on hypothalamic neurons (which also expresses LepR on their cell surfaces) (Yamada et al. 2019). Recently, we discovered an inter-organ communication in which decreased hepatic insulin action increases liver-derived sLepR and suppresses hypothalamic leptin action (Takahashi et al. 2023). In this study, we generated inducible liver insulin receptor knockout (iLIRKO) mice and unexpectedly found that body weight gain was increased in iLIRKO mice. We further discovered that in iLIRKO mice, plasma levels of liver-derived sLepR were increased, accompanied by a series of decreased hypothalamic leptin action, increased food intake, decreased BAT sympathetic activities and BAT thermogenesis, and reduced systemic energy expenditure. When liver-derived sLepR was depleted from iLIRKO mice, all of these metabolic phenotypes were reversed, indicating that decreased hepatic insulin action increases liver-derived sLepR and suppresses hypothalamic leptin action. Interestingly, even in humans, hepatic LepR expressions were inversely correlated with hepatic insulin action. Moreover, in individuals with diabetes mellitus, plasma sLepR levels were positively correlated with fasting blood glucose levels, a biomarker of reduced hepatic insulin action in humans. These results indicate that reduced hepatic insulin action increases sLepR also in humans.

We further studied the physiological role of sLepR elevations triggered by decreased hepatic insulin action. To elucidate this, we subjected wild-type mice to food restriction, a physiological setting of decreased hepatic insulin action. In food-restricted mice, plasma sLepR levels were increased, and BAT thermogenesis and systemic energy expenditure were both suppressed. In line with this, liver-derived sLepR depletion, caused

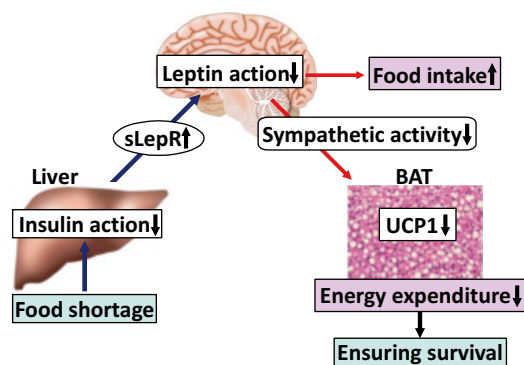


Fig. 11.4 Schematic model illustrating how decreased hepatic insulin action regulates systemic energy metabolism via sLepR. (Modified from reference (Takahashi et al. 2023))

by an inducible liver leptin receptor knockout (iLLeprKO), insufficiently suppressed BAT thermogenesis and systemic energy expenditure even though mice were restricted from foods. Strikingly, mortality rates were increased in food-restricted iLLeprKO mice.

When energy intake is deprived for long periods, individuals are known to decrease energy expenditure to maintain survival. Increased mortality rates in food-restricted iLLeprKO mice suggest that during prolonged food shortages, liver-derived sLepR reduces energy expenditure to protect individuals from systemic energy loss. In other words, the liver converts metabolic response reflecting energy deficiency (decreased hepatic insulin action) into a suppressor of systemic energy dissipation (liver-derived sLepR) to ensure whole-body survival, once food becomes scarce in nature (Fig. 11.4) (Takahashi et al. 2023).

11.6.2 Neural Signals

11.6.2.1 Neural Signals from the Stomach and Intestinal Tract

Ghrelin's actions on suppression of BAT sympathetic activities are derived not only from the direct action of circulating ghrelin on hypothalamic neurons but also from neuronal signals transmitted from ghrelin receptor-expressing vagal afferent neurons in the stomach. The latter was demonstrated by vagotomy experiments.

Vagal nerve dissection abolished ghrelin-induced suppression of BAT sympathetic activity (Mano-Otagiri et al. 2009). Therefore, two pathways allow ghrelin to suppress BAT thermogenesis, but the relative physiological significance of these pathways remains unclear.

11.6.2.2 Neural Signals from the Liver

Previous work in our laboratory demonstrated the inter-organ neural network triggered by enhanced glucokinase (GK) expression in the liver that contributes to the efficient storage of ingested energy. We first found that HFD feeding rapidly enhances hepatic GK expression. Such GK expression preceded body weight gain. Furthermore, transgenic mice that overexpress GK in the liver reportedly gained more body weight (Ferre et al. 2003), prompting the question of how increased hepatic GK expression triggers body weight gain. We therefore overexpressed GK in the livers of mice and explored inter-organ communication. We found that the expression of thermogenic molecules, such as UCP1, was decreased in BAT, which was accompanied by decreased thermogenesis. Additionally, we demonstrated that metabolic signals triggered by hepatic GK induction were transmitted to the brain via the afferent vagal nerve, resulting in the inactivation of sympathetic nerve input to BAT, and suppression of BAT thermogenesis (Fig. 11.5) (Tsukita et al. 2012).

We further explored whether this novel inter-organ communication might explain predisposition to obesity. The degree of weight gain markedly differs among individuals, even if they are similarly hyperphagic. In humans, genetic susceptibility is considered to be an important risk factor for obesity. Mouse strains with different genetic backgrounds are useful to demonstrate susceptibility to particular diseases. As the degree of HFD-induced weight gain also differs among mouse strains, different levels of diet-induced thermogenesis among mouse strains are proposed as a cause of varying predisposition to obesity (Prpic et al. 2002; Surwit et al. 1998), but the precise mechanism has long been unknown. To address this, we compared endogenous hepatic GK expression among HFD-fed obesity-prone and obesity-resistant mice. Hepatic GK expression was markedly

higher in obesity-prone than in obesity-resistant mouse strains. Furthermore, in these mouse strains, BAT UCP1 expression was inversely correlated with hepatic GK expression, suggesting a cause-effect relationship between hepatic GK expression and predisposition to obesity. To test this hypothesis, we overexpressed hepatic GK in obesity-resistant mice. BAT thermogenesis was diminished in these mice, and mice gained weight. In contrast, when we knocked

down hepatic GK in obesity-prone mice, mice presented with increased BAT thermogenesis and gained less body weight. Differences in this system among murine strains might explain differences in predisposition to obesity (Tsukita et al. 2012). A recent study revealed that the body weight of patients with GK mutation was significantly lower than that of healthy controls (Steele et al. 2014), suggesting that hepatic GK activity is involved in a “tendency toward obesity” in humans as well.

We recently reported another inter-organ network mechanism that minimizes the depletion of fat stores during periods of negative energy balance. We investigated the effects of an anti-diabetic drug sodium glucose transporter 2 (SGLT2) inhibitor on BAT thermogenesis and systemic energy expenditure in mice. SGLT2 inhibitor treatment promotes urinary glucose excretion, mimicking a state of negative energy balance. We found out that SGLT2 inhibitor treatment decreased energy expenditure in mice, which involved reduced BAT sympathetic activity and thermogenesis. Furthermore, common hepatic branch vagotomy abolished phenotypes seen in BAT. Thus, a neural network consisting of the hepatic vagal branch and sympathetic nerves plays a major role in this inter-organ communication system (Fig. 11.6) (Chiba et al. 2016).

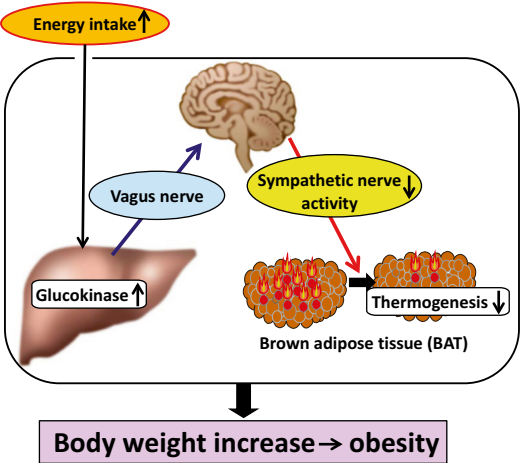
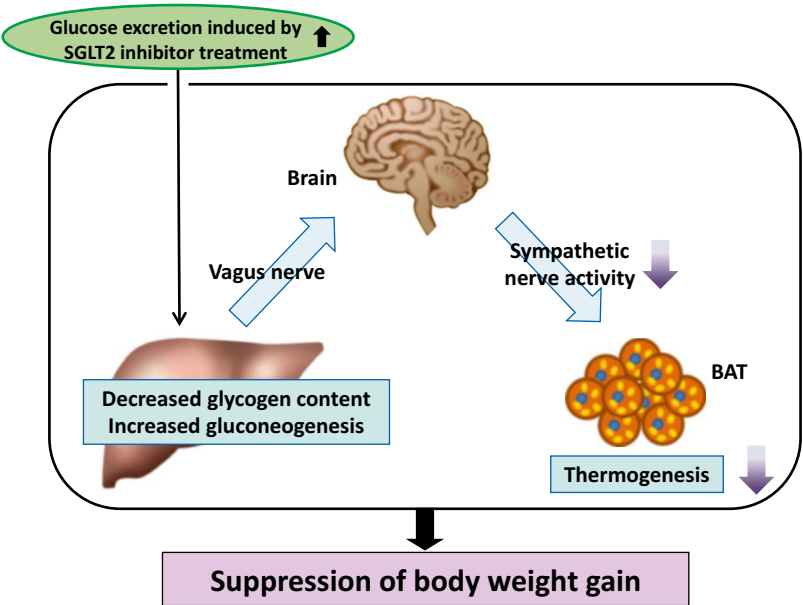


Fig. 11.5 Inter-organ communication mediating induction of liver GK and suppression of BAT thermogenesis via neural signals. (Modified from reference (Uno et al. 2006))

Fig. 11.6 Inter-organ communication mediating liver and suppression of BAT thermogenesis via neural signals during SGLT2 inhibitor treatment. (Modified from reference (Chiba et al. 2016))



The inter-organ communication discussed in this section may have been adaptive for individuals during periods of frequent starvation. However, in modern society where food is typically plentiful, this system might make it difficult for individuals to lose weight or prevent obesity (Yamada 2013; Yamada et al. 2013).

11.7 Beige Adipocytes

Like brown adipocytes, beige adipocytes express abundant UCP1 and thus possess thermogenic properties. However, brown adipocytes and beige adipocytes have features that are distinct in many aspects, such as anatomical localization, development, and differentiation mechanisms (Table 11.1). Brown adipocytes are characterized by multilocular lipid droplets and are located in interscapular regions, the axilla, and around the kidney. They differentiate during embryonic development from Myf5-positive dermomyotomal precursor cells, a subset of cells that are also capable of differentiating into skeletal muscle cells. In contrast, beige adipocytes sporadically reside within WAT depots, such as inguinal and anterior subcutaneous WAT in mice and cervical, supraclavicular, axillary, and paravertebral regions in adult humans (Sidossis and Kajimura

2015; Ikeda et al. 2018) (Fig. 11.7). Beige adipocytes also form multilocular lipid droplets, but unlike brown adipocytes, they differentiate from pre-adipocytes that are Myf5-negative but PDGFR α positive. Beige adipocytes are strongly induced in response to environmental conditions and external cues, including chronic cold acclimation, exercise, and long-term treatment with PPAR γ agonists or β 3-adrenergic receptor agonists. This phenomenon is called the “browning/beiging” of white fats.

In humans, brown adipocytes have long been thought to be present only in neonates or patients with certain diseases, such as catecholamine-producing tumors. Brown adipocytes were thought to disappear and play no significant physiological role in adult humans. However, brown adipocytes are detectable and can be evaluated in adult humans by combining positron emission tomography (PET), a functional imaging technique using the glucose analog FDG as a tracer to detect regional glucose uptake, and X-ray CT. This method is called FDG-PET/CT (Cypess et al. 2009; Saito et al. 2009). This imaging revealed that brown fat mass was inversely correlated with white fat mass or HbA1c, an index that reflects blood glucose levels in humans (Cypess et al. 2009; Ouellet et al. 2011). Negative correlations were also seen between body fat

Fig. 11.7 Anatomical distribution and histology of brown adipocytes/beige adipocytes in mice and humans. (Modified from reference (Ikeda et al. 2018))

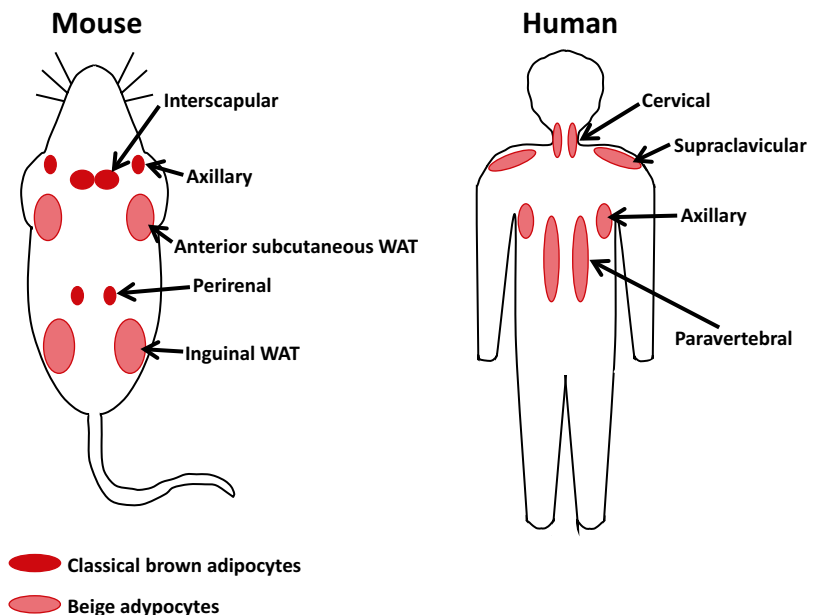


Table 11.1 Biochemical and functional characteristics of brown adipocytes and white adipocytes. (Modified from reference (Ikeda and Yamada 2020))

	Classical brown adipocytes	Beige adipocytes
Locations in mice	Interscapular, axillary, perirenal	Inguinal WAT, anterior subcutaneous WAT
Locations in human	Interscapular	Cervical, supraclavicular, axillary, paravertebral
Lineage	Myogenic lineage (<i>Myf5</i> ⁺)	Multiple lineages (<i>Myf5</i> ⁻ , <i>SMA</i> ⁺ , <i>PDGFRα</i> ⁺)
Cell induction mechanisms	Pre-existing	Inducible
Thermogenic mechanisms	UCP-1 dependent Creatine cycling Ca ²⁺ cycling	UCP-1 dependent UCP-1 independent (creatine cycling, Ca ²⁺ cycling)

mass and brown fat mass (Yoneshiro et al. 2013). These results suggest that if energy expenditure by beige adipocytes can be enhanced, it may improve obese and/or hyperglycemic states. Roughly 50 g of brown fat that is maximally activated by cold stimulation is estimated to expend about 20% of basal metabolism, approximately 200–400 kcal.

11.8 Inter-Organ Communication Leading to Beige Adipose Tissue Induction

Many transcription factors that participate in the regulation of brown adipocyte differentiation are also reportedly involved in the differentiation of beige adipocytes. For example, PRDM16, a transcriptional regulatory factor that strongly induces differentiation of classic brown adipocytes, is also essential for the differentiation of beige adipocytes (Seale et al. 2008; Kajimura et al. 2009). Several other intracellular factors essential for the induction of beige adipocytes have also been clarified, but in this section, we will focus on extracellular stimulations, that is, inter-organ signals that regulate the induction of beige adipocytes.

11.8.1 Humoral Factors

11.8.1.1 Inter-Organ Communication from Skeletal Muscles

Various humoral factors secreted from skeletal muscles are involved in the induction of beige adipocytes. IL-6 secretion is enhanced by con-

traction of skeletal muscles, affecting glycolipid metabolism (Febbraio and Pedersen 2002). Exercise training induces beige adipocyte induction in mice. IL-6 was recently reported to be required for such induction, partially through a mechanism involving PGC-1 α activation (Knudsen et al. 2014). Skeletal muscle contractions also enhance the secretion of irisin, another humoral factor, which also triggers the induction of beige adipocytes (Bostrom et al. 2012). Irisin is an extracellular domain of FNDC5, a transmembrane protein of skeletal muscle cells, and is secreted into the bloodstream probably in response to muscle contraction (Bostrom et al. 2012). Plasma concentrations of IL-6 and irisin were reportedly elevated in humans after exercising, but further research is needed to elucidate their roles in beige cell induction.

β -Aminoisobutyric acid (BAIBA) is another myokine identified as a regulator of browning of white fat (Stanford and Goodyear 2018). In humans, plasma BAIBA concentrations showed a trend toward an inverse association with body mass index (Roberts et al. 2014). Treatment with BAIBA increased UCP1 expression in white adipocytes in mice (Roberts et al. 2014), in cultured adipocytes (Colitti et al. 2018), and in human iPS cells differentiated into mature white adipocytes (Roberts et al. 2014). Browning may be mediated through a PPAR α -dependent mechanism because BAIBA-induced increase in thermogenic gene expression was lost in PPAR α null mice and in primary adipocytes treated with a PPAR α antagonist (Roberts et al. 2014).

Muscle-specific overexpression of a splice form of PGC-1 α in mice induced secretion of the myokine, meteorin-like (Metrl) (Rao et al.

2014). Treatment of mice with *Metnrl* increased UCP1 expression in WAT and also enhanced whole-body energy expenditure (Rao et al. 2014). Browning is not the consequence of a direct effect of *Metnrl*, as treatment with *Metnrl* did not increase the expression of UCP1 in cultured adipocytes (Rao et al. 2014). Expression of UCP1 was significantly inhibited in mice with the *Gata1* promoter mutation that lacks eosinophils. Thus, eosinophils may be required for *Metnrl*-mediated thermogenic gene responses (Rao et al. 2014).

11.8.1.2 Inter-Organ Communication from the Liver

FGF21 is a circulating hormone that is derived mostly from the liver (Markan et al. 2014). In mice, FGF21 induces thermogenesis both in BAT and in beige adipocytes (Fisher et al. 2012). These effects are largely mediated by FGF's effect on the brain, leading to increased sympathetic nervous system output (Phillips 2019).

11.8.1.3 Inter-Organ Communication from the Heart

Brain natriuretic peptide (BNP) is a humoral factor secreted from the heart. Reportedly, BNP treatment in mice induced beige adipocytes with enhanced PGC-1 α and UCP1 expression and increased systemic energy expenditure (Bordicchia et al. 2012).

11.8.2 Neural Signals: Activation of Sympathetic Nerves

Chronic cold acclimation activates the sympathetic nervous system, which leads to the induction of beige adipocytes. Noradrenaline released by sympathetic neurons activates the β 3-adrenergic receptor-PKA-PGC-1 α pathway in white adipocytes, leading to mitochondrial biogenesis and enhanced UCP1 expression (Harms and Seale 2013; Jiang et al. 2017). Activation of the sympathetic nervous system by leptin was also reported to stimulate the induction of beige adipocytes (Plum et al. 2007). Leptin action on hypothalamic POMC neurons that express leptin receptors is essential in this process, but precise neural circuitry that links the hypothalamus and

the sympathetic nervous system to induce beige adipocytes has not been elucidated (Dodd et al. 2015).

11.8.3 Gut Microbiota

Recent studies showed a relationship between gut microbiota and BAT (Park et al. 2019). Cold exposure altered the gut microbial community characterized by increased levels of microbiota associated with leanness and decreased levels of microbiota associated with obesity (Zietak et al. 2016). Mice receiving transplanted microbiota from cold-exposed mice had significantly higher BAT UCP1 expression (Zietak et al. 2016) and WAT UCP1 expression (Chevalier et al. 2015) compared with mice transplanted with microbiota from mice maintained at higher temperatures. In addition to cold exposure, intermittent fasting also led to both changes in gut microbiota composition and significant stimulation of beiging of WAT (Zhou et al. 2019). Microbiota-depleted mice were resistant to intermittent fasting-induced beiging, but transplantation of the microbiota from intermittently fasted mice to microbiota-depleted mice activated beiging (Li et al. 2017). These findings indicate a gut microbiota-BAT/WAT communication that regulates adipose tissue thermogenesis.

11.8.4 Mother–Infant Communication

In addition to intra-individual inter-organ communications, emerging studies suggest the possibility of inter-individual inter-organ communication in the regulation of adipose tissue thermogenesis. The World Health Organization (WHO) recommends that all infants be exclusively breastfed from birth until 6 months of age, partly because mounting evidence suggests that breastfeeding has long-term benefits, including a reduced risk of obesity in childhood and adolescence (WHO 2023). The onset of obesity may be associated with the alteration of BAT because early weaning reduced BAT UCP1 expression in rats (Peixoto et al. 2020). A recent study provides

some insight (Yu et al. 2019). Alkylglycerols comprise a lipid group found almost exclusively in breast milk, and alkylglycerol treatment was found to increase beige cells in neonatal mice. Moreover, abundant UCP1 expression was seen in adipose tissue of breastfed human infants, but not in formula-fed infants, based on analysis of biopsy samples obtained from human infants. Molecular studies revealed that adipose tissue macrophages metabolized alkylglycerols into a substance that activated adipocyte IL-6/STAT3 signaling and promoted beige cell development. Thus, novel mother–infant communication involving lipid signals may also be important for the early formation of beige cells.

11.9 Conclusion

In this review, we highlight important inter-organ communication pathways implicated in the activation of BAT thermogenesis and beige adipocyte induction. Elucidating mechanisms for the regulation of thermogenic adipose tissue and how adipose tissue thermogenesis affects body weight will lead to an understanding of how body temperature is physiologically adjusted to maintain homeostasis. Such understanding may be translated into strategies for the treatment of diseases such as obesity, diabetes, and metabolic syndromes. Future research focusing on physiological roles and clinical applications of thermogenesis in adipocytes will be of great interest.

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Circadian Clock and Body Temperature

12

Takahito Miyake, Yuichi Inoue, Yota Maekawa,
and Masao Doi

Abstract

The circadian fluctuation of body temperature is one of the most prominent and stable outputs of the circadian clock and plays an important role in maintaining optimal day–night energy homeostasis. The body temperature of homothermic animals is not strictly constant, but it shows daily oscillation within a range of 1–3 °C, which is sufficient to synchronize the clocks of peripheral tissues throughout the body. The thermal entrainment mechanisms of the clock are partly mediated by the action of the heat shock transcription factor and cold-inducible RNA-binding protein—both have the ability to affect clock gene expression. Body temperature in the poikilotherms is not completely passive to the ambient temperature change; they can travel to the place of preferred temperature in a manner depending on the time of their endogenous clock. Based on this behavior-level thermoregulation, flies exhibit a clear body temperature cycle. Noticeably, flies and mice share the same molecular circuit for the controlled body temperature; in both species, the calcitonin receptors participate in the formation of body

temperature rhythms during the active phase and exhibit rather specific expression in subsets of clock neurons in the brain. We summarize knowledge on mutual relationships between body temperature regulation and the circadian clock.

Keywords

Circadian clock · Body temperature · Clock genes · SCN · Calcr

12.1 Introduction

The 24-h rotation of Earth leads to the creation of temporal changes in the environment, such as cycles of light and darkness and temperature fluctuations, forcing the organisms to acquire the ability to adapt to recurrent, thus anticipatable environmental changes (Bollinger and Schibler 2014). A variety of biological functions, such as sleep/wake cycle, thermogenesis, endocrine system, metabolism, immune function, reproduction, and stem cell development have been identified to exhibit daily variation (Logan and McClung 2019). Among those, the circadian rhythm of body temperature is one of the most conspicuous outputs of the circadian clock in animals. As its name suggests, homeothermic animals maintain high body temperatures in the range of 36–42 °C (Ivanov 2006), but this does

T. Miyake · Y. Inoue · Y. Maekawa · M. Doi (✉)
Department of Systems Biology, Graduate School of
Pharmaceutical Sciences, Kyoto University,
Kyoto, Japan
e-mail: doimasao@pharm.kyoto-u.ac.jp

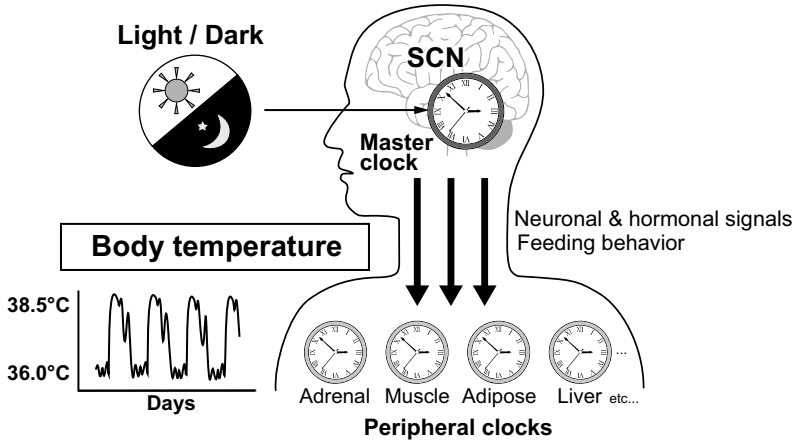


Fig. 12.1 Hierarchical organization of the mammalian circadian clock. The master clock resides in the hypothalamic SCN and controls peripheral clocks in many tissues (i.e., the adrenal glands, muscle, adipose tissue, and liver) by orchestrating body temperature rhythm as well as other

physiological organismal circadian rhythms including neuronal activity, hormone release, behavior, and feeding behavior. Light is a dominant time cue for the master clock

not mean that their body temperatures are constant throughout the day. The body temperatures of birds and mammals undergo a daily regular cycle with a difference between the highest and lowest values of 1–3 °C, depending on the species (Refinetti 1996). Physiologically, circadian clock-controlled body temperature plays a key role in maintaining circadian metabolic homeostasis and in synchronizing numerous tissue clocks distributed across the body. In mammals, the central circadian clock pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Stephan and Zucker 1972), while innumerable autonomous cellular clocks operate throughout the body. As such, in the body, the SCN and peripheral tissue clocks constitute a hierarchical multi-oscillator network, in which the SCN serves as the chief orchestrator dominating the rhythms in the peripheral clocks (Fig. 12.1) (Mohawk et al. 2012; Schibler and Sassone-Corsi 2002). As a requirement of this network model, there exist systemic signals through which peripheral clocks adjust their phase and period according to the rhythm imposed by the SCN (Bollinger and Schibler 2014). Importantly, body temperature plays this part. In addition to body temperature, a number of different parallel signals from the SCN, either neuronal or hormonal, are involved in the entrain-

ment of tissue clocks. Yet, body temperature is quite unique in that it can globally and simultaneously affect numerous tissue clocks throughout the body (Buhr et al. 2010). In this chapter, we provide an overview on how the master clock in the brain regulates body temperature, and we also illustrate the current knowledge of the molecular mechanism of thermal entrainment of peripheral clocks. In addition, in this chapter, we also describe how the body temperature rhythms of cold-blooded animals are regulated. Interestingly, despite the fundamental difference between flies and mice in thermoregulation (poikilotherms vs. homeotherms), the evolutionarily conserved calcitonin receptor (Calcr) has been shown to be important for both flies and mice to maintain normal circadian fluctuation of body temperature. In this review, we summarize the interrelationship between body temperature and circadian clock.

12.2 Molecular Machinery of Circadian Clock in Mammals

Genetic and molecular biological studies have identified self-sustained molecular oscillators called “clock genes” (Fig. 12.2). Clock genes generate circadian rhythms by regulating their

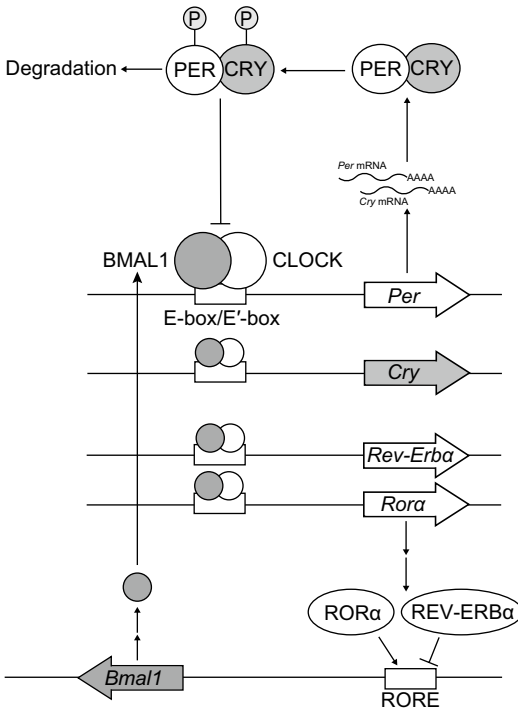


Fig. 12.2 Schematic diagram of circadian feedback loops of the molecular circadian clock. BMAL1 and CLOCK form heterodimers and initiate transcription of *Per* and *Cry* by binding to their E-box/E'-box enhancer sequences. Then PER and CRY form heterodimers that translocate to the nucleus and repress the transcription of themselves by binding to BMAL1:CLOCK heterodimers. The activity and quantity of PER and CRY heterodimers are regulated at a post-transcriptional level via phosphorylation and following ubiquitinylation. RORα and REV-ERBα whose expression is also regulated by CLOCK and BMAL1 heterodimers promote and inhibit the transcription of *Bmal1*, respectively. P, phosphorylation

own transcription (transcriptional–posttranscriptional feedback loop, TTFL) (Masri and Sassone-Corsi 2013; Takahashi 2017; Rosbash et al. 2007). In mammals, the expression of core clock genes *Period* (*Per1*, *Per2*, and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) is regulated by transcription factors BMAL1 and CLOCK. BMAL1 and CLOCK form heterodimers and initiate transcription of *Per* and *Cry* and other target genes by binding to their E'-box/E-box *cis*-regulatory enhancer sequences (Hao et al. 1997; Doi et al. 2019; Gekakis et al. 1998; Ueda et al. 2005). Then, phosphorylated PER and CRY heterodimers translocate to the nucleus and repress the transcription of them-

selves by binding to BMAL1:CLOCK heterodimers (Cao et al. 2013). Consequently, *Per* and *Cry* transcript levels decrease, followed by a progressive decline in PER and CRY proteins through proteasomal degradation, resulting in restart of the cycle (Zhou et al. 2015; Reischl et al. 2007; Fustin et al. 2018; Lamia et al. 2009; Miyake and Doi 2019). This basic, intracellular transcription-based oscillation is considered to be the origin of all seen circadian rhythms expressed by organisms, including those in body temperature and locomotor activity. Particularly, studies in mutant mice carrying a mutation only at the E'-box *cis*-element in the promoter of the core oscillatory gene *Per2* demonstrate that the circadian *cis*-regulatory element-mediated transcriptional loop of *Per2* is indispensable for maintaining proper circadian rhythms in body temperature and locomotor activity (Doi et al. 2019). These results constitute the first genetic evidence that non-coding *cis*-element-based clock gene transcription is essential for maintaining organismal circadian behavior and body temperature rhythmicity (Nakagawa et al. 2020).

Besides the *Per2* loop, the expression of *Bmal1* is also regulated by another loop. In this loop, BMAL1:CLOCK heterodimers activate the transcription of *Rev-Erbα* and retinoic acid-related orphan nuclear receptor α (RORα) by binding to their E-box enhancer sequences (Sato et al. 2004). Then, REV-ERBα and RORα bind to retinoic acid-related orphan receptor response elements (ROREs) in the *Bmal1* promoter region to activate and repress the expression of *Bmal1* mRNAs, respectively. Thus, REV-ERBα and RORα indirectly regulate the expression of *Per2* and other clock genes by regulating the transcription of *Bmal1*. These RORE-based interlocking loops are thought to enhance the stability of the *Per2* core loop.

12.3 Body Temperature Control by the Circadian Clock

Circadian control of body temperature is fundamental to maintaining optimal homeostasis in metabolism and sleep as well as to entrain peripheral clocks in the body (Refinetti and Menaker

1992; Gilbert et al. 2004; Krauchi 2007a, b; Buhr et al. 2010; Morf and Schibler 2013). In humans, body temperature increases during wakefulness and decreases during sleep (Duffy et al. 1998). Diurnal changes in body temperature are not a simple consequence of behavioral activity rhythm. Thermographic video imaging revealed that mice exhibit non-locomotor activities (i.e., activities that do not involve movement from place to place) such as grooming and postural adjustments, which alone induce considerable elevation of body temperature (Shimatani et al. 2021). Crucially, these subtle movements always preceded the initiation of locomotor activity, indicating that non-locomotor activity-associated body temperature elevation is necessary for mice to initiate active locomotion. Nevertheless, non-negligible body temperature variations were also displayed by mice during rest, without detectable non-locomotor activity, demonstrating the presence of movement-independent body temperature regulation (Shimatani et al. 2021). There is evidence that body temperature fluctuates even when locomotor activity is restricted in humans (Smith 1969; Gander et al. 1986). The phase of the body temperature rhythm can be dissociated from that of behavioral rhythm, a phenomenon known as spontaneous internal desynchronization (Lavie 2001). These reports strongly suggest that the body temperature rhythm is likely controlled separately from behavioral activity.

Not surprisingly, at the molecular level, core clock genes are essential to generate body temperature rhythm in humans. In humans, a mutation in *CRY1* causes impaired diurnal body temperature rhythm in familial delayed sleep phase syndrome (Patke et al. 2017). At the anatomical level, the master clock SCN is considered to be crucial for the generation of body temperature rhythmicity because it cannot be sustained when the SCN is surgically destroyed in rodents (Eastman et al. 1984). More than 70% of nerve projections from the SCN end in the subparaventricular zone (SPZ) located dorsocaudal to the SCN. Damage to the dorsal region of the SPZ (dSPZ) markedly decreases the magnitude of temperature fluctuation, while damage to the ventral region of the SPZ (vSPZ) impairs activity and sleep–wake rhythms

but does not affect body temperature, indicating that dSPZ plays an important role in relaying signals from the SCN to control diurnal changes in body temperature (Lu et al. 2001; Saper et al. 2005). Thus, the SCN likely controls body temperature via nerves in a manner that is distinct from how it controls the locomotor and sleep–wake rhythms (Fig. 12.3) (Hsiao and Doi 2024). It has also been reported that the body temperature and locomotor activity rhythms are simultaneously impaired by severing the nerve that connects the SCN to the arcuate nucleus (ARC), which is the region known to be regulated by peripheral hormones such as leptin and insulin, as well as nutrients such as glucose, amino acids, and fatty acids, and crucial for normal energy homeostasis (Belgardt et al. 2009; Buijs et al. 2017).

Pineal gland, which is a small endocrine gland that is located under the back part of the corpus callosum, is one of the candidates which regulates physiological daily thermoregulation in non-mammalian vertebrates (Bell-Pedersen et al. 2005). The pineal gland predominantly secretes a hormone melatonin in a circadian manner (Dibner et al. 2010). Pinealectomy abolishes the normal circadian rhythm of body temperature as well as the behavior of the house sparrow in constant darkness and significantly alters the amplitude of body temperature rhythms even under normal 12-h light/12-h dark cycle conditions (Binkley et al. 1971). In Japanese quails, continuous melatonin administration caused arrhythmicity or period alteration in the body temperature as well as locomotor activity rhythm by raising the level of the nocturnal body temperature (Underwood and Edmonds 1995). In mammals, melatonin also appears to have a role in thermoregulation. The body temperature rhythm is disrupted in streptozotocin-induced diabetes model rats, which is restored by co-treatment with insulin and melatonin (Ramos-Lobo et al. 2015). In humans, a recent study has demonstrated that light stimuli followed by melatonin administration increase body temperature (Lok et al. 2019). As melatonin receptor MT2 is expressed in the SCN (Dibner et al. 2010), melatonin may contribute to the regulation of body temperature rhythm through the SCN.

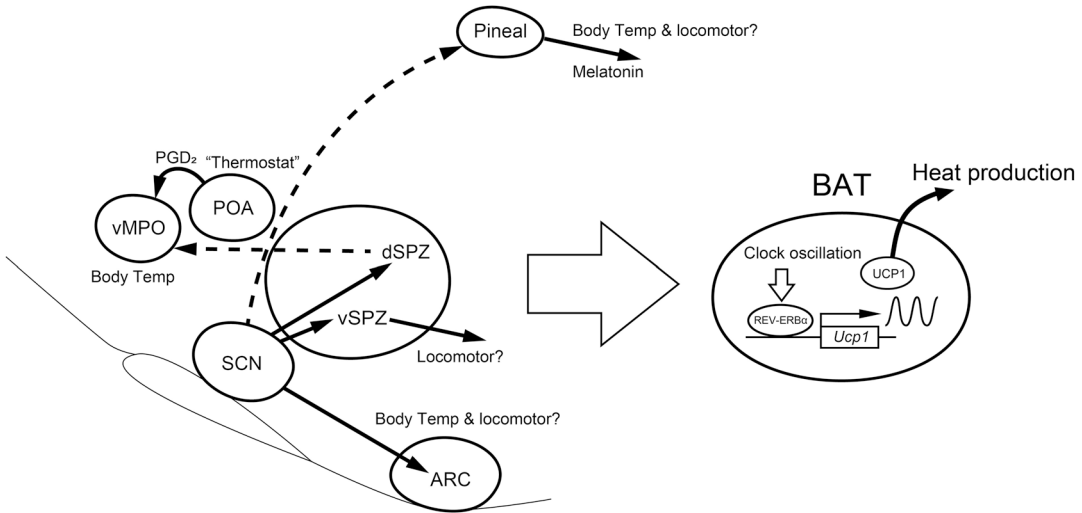


Fig. 12.3 SCN-mediated output pathways from the SCN that generate circadian control of body temperature rhythm. More than 70% of nerve projections from the SCN end in the SPZ. While the dSPZ contributes to controlling the body temperature rhythm, the vSPZ contributes to controlling the rhythm of locomotor activity. POA acts as a “thermostat” because the POA neurons are acti-

vated by a body temperature increase and suppress the activity of vMPO neurons by releasing PGD₂. ARC and the pineal gland are also considered to contribute to controlling the body temperature-controlling signal rhythm. In addition, circadian regulation of BAT activity is essentially reported to underlie the controlled body temperature cycle

Although many aspects of the SCN outputs and associated molecular mechanisms remain unclear, a recent study has shown that the *Calcr*-mediated pathway is one of the underlying mechanisms (Goda et al. 2018). *Calcr* is a G-protein coupled receptor for a peptide hormone calcitonin. As an additional feature of *Calcr*, *Calcr* forms a complex with a single-pass transmembrane protein receptor activity-modifying protein (RAMP) and acts as a receptor for amylin, which is another peptide hormone that is produced by the β cells of the pancreas (Hay et al. 2018). Intense expression of *Calcr* can be seen in several specific regions in the brain, including the circadian center SCN (Goda et al. 2018; Becskei et al. 2004; Nakamoto et al. 2000). From a viewpoint of locomotor activity rhythm, *Calcr* knockout (KO) mice are normal and indistinguishable from wild-type mice. However, the body temperature rhythm of *Calcr* KO mice is significantly different from that of wild-type mice. The body temperature rhythm of wild-type animals normally displays bimodal two peaks, one in the early night and one at dawn, with a deep trough at midnight that corresponds to “siesta,” but the body

temperature rhythm of *Calcr* KO mice lost this characteristic dip at midnight and remained relatively unchanged throughout the night. These data indicate that *Calcr* contributes to the output signal which regulates specifically body temperature during siesta. Interestingly, body temperature in humans also drops during the active phase (at around 3:00 pm) (Patke et al. 2017), implying that the human SCN also possesses a similar molecular mechanism to control body temperature.

In mammals, body temperature is regulated by the preoptic area (POA) of the anterior hypothalamus. POA contains a fraction of neurons that are sensitive to brain temperature change (Boulant and Dean 1986) (see other chapters for the illustration of POA). Recent single-cell RNA-seq combined with whole-cell patch clamp recording revealed that prostaglandin-D synthase (PTGDS)-expressing neurons in the medial POA are thermosensitive and act as a “thermostat” (Wang et al. 2019). As body temperature raises, the PTGDS-expressing POA neurons are activated independent of synaptic transmission, secrete prostaglandin D₂ (PGD₂), and activate PGD₂

receptor (DP1)-expressing cells in the ventromedial posterior nucleus, which results in a body temperature decrease. Notably, the magnitude of the PTGDS-expressing POA neurons-mediated body temperature control varies depending on the time of day, indicating its potential contribution to regulating circadian body temperature rhythm (Fig. 12.3). In addition, circadian regulation of brown adipose tissue (BAT) activity is reported to underlie controlled body temperature (see other chapters for the illustration of BAT). In mice, genetic depletion of *Rev-Erba* reduces the magnitude of diurnal fluctuation in body temperature, which is accompanied by abnormal *Ucp1* expression in BAT (Fig. 12.3) (Gerhart-Hines et al. 2013).

12.4 Thermal Controls over the Circadian Clocks in Mammals

Physiological body temperature changes are thought to serve as an internal entrainment signal for the peripheral clocks. Although subtle, variations in body temperature within a physiologic range in vivo (36–38.5 °C in mice) appear to have a significant impact on the clocks in tissues (Buhr

et al. 2010; Shimatani et al. 2021). In accordance with this notion, externally applied temperature cycles that mimic body temperature rhythms can sustain rhythmic clock gene expression in cultured Rat-1 fibroblasts and primary glial cells (Brown et al. 2002). At the organismal level, liver transcripts encoding PER2, heat shock proteins (HSPs), and cold-inducible RNA-binding proteins continue to show robust circadian oscillation irrespective of whether the molecular clock inside the liver is intact or not. In contrast, in ex vivo cultured liver explants, circadian cycles of PER2 could only be observed when the oscillators in hepatocytes are operational. These results highlight the importance of systemic time cues, perhaps including body temperature rhythm, for keeping the entrainment of the peripheral clock (Saini et al. 2011; Kornmann et al. 2007). As described below, subsequent studies revealed that heat shock factor 1 (HSF1) and cold-inducible RNA binding protein (CIRBP), which are responsive to heat shock and cold stimulation, respectively, are both involved in the thermal entrainment mechanism of the peripheral clock (Fig. 12.4) (Reinke and Carthew 2008; Saini et al. 2012; Morf et al. 2012; Buhr et al. 2010).

HSF1 is an evolutionarily conserved transcription factor that coordinates stress-induced

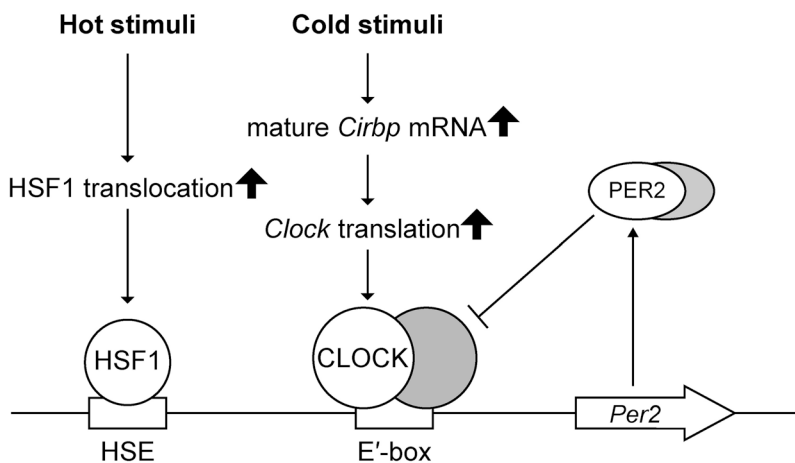


Fig. 12.4 A schematic drawing of the *Per2* promoter region where body temperature signals are input. When the body temperature is elevated during the active phase, HSF1 translocates to the nucleus and promotes *Per2* transcription by binding to the HSE which exists proximal to the E'-box

in the *Per2* promoter. On the contrary, when the body temperature is decreased, CIRBP increases the cytoplasmic accumulation of *Clock* mRNAs. This leads to an increase in *Per2* mRNA transcription, which is followed by the acceleration of upregulated *Clock* protein translation

transcription and directs versatile physiological processes mainly through its strong transcriptional effect on the genes encoding the HSPs. Yet, recent genomewide target surveys have provided additional evidence to support the idea that HSF1 is capable of reprogramming transcription more extensively than previously assumed, extending its contribution to a range of fundamental physiologies such as metabolism, gametogenesis, cancer progression, and aging (Vihervaara and Sistonen 2014) (see other chapters for the details). Electrophoretic mobility shift assays with mouse liver nuclear extracts revealed the interaction of HSF1 with its binding site to be highly rhythmic. Conceivably, upon circadian elevation of body temperature, HSF1 translocates to the nucleus and binds to its target sequence referred to as the heat shock element (HSE) (Reinke et al. 2008). Importantly, cells that are cultured in vitro preserve the ability to be entrained to circadian cycles of temperature (Saini et al. 2012). Notably, smooth temperature oscillations with an amplitude as small as 1 °C are able to synchronize the clocks in cells. Temperature rhythms also synchronize clock gene expression to various periods different than 24 h, ranging from 10 to 30 h (Saini et al. 2012). Although it may not be considered physiological, an acute exposure to heat stress, or heat shock, has the ability to reset circadian *Per2* expression in cells. This reset mechanism involves the HSF1 binding to the HSE sequence in the *Per2* promoter, indicating that HSF1 is involved in the heat stress-induced phase resetting of the circadian clock (Tamaru et al. 2011). Nevertheless, pharmacological inhibition of HSP induction cannot completely inhibit cellular temperature entrainment (Buhr et al. 2010). Moreover, temperature cycles are still capable of entraining the diurnal rhythm of *Bmal1* expression in HSF1-deficient cells, indicating that yet-unknown molecule mechanism(s) contribute to this synchrony (Saini et al. 2012).

CIRBP is an 18 kD protein that consists of an amino-terminal RNA binding domain and a carboxyl-terminal glycine-rich domain, and its expression can be induced by mild cold shock (Nishiyama et al. 1997). Under constant temperature culture conditions, *Cirbp* does not show cir-

cadian expression. However, at the organismal level, the level of *Cirbp* mRNA in the liver in mice undergoes drastic circadian variations in accordance with the rhythms in body temperature. The expression level of *Cirbp* mRNA seems to be regulated at the posttranscriptional level because the efficiency of *Cirbp* pre-mRNA splicing is temperature-dependent (Schibler et al. 2015). CIRBP knockdown leads to lowered amplitude of circadian gene expression of *Bmal1* and *Dbp* in a cell type-specific manner (Schibler et al. 2015). CIRBP-knockdown cells can readjust their clocks faster than control siRNA transfected cells after being exposed to a mimic of jet lag induced by changing the temperature cycles. Such accelerated adaptation is predicted theoretically for low-amplitude oscillators. In addition, further investigation revealed that CIRBP enhances the amplitude by increasing the cytoplasmic accumulation of *Clock* mRNAs, which is followed by the acceleration of their translation (Morf et al. 2012).

12.5 Diurnal Body Temperature Control in Cold-Blooded Animals

Ambient temperature changes highly influence body temperature in cold-blooded animals. However, this does not necessarily mean that these animals passively accept changes in ambient temperature. Cold-blooded animals prefer higher temperatures to increase their metabolism in the active phase and lower temperatures to reduce their metabolism in the resting phase. Through this temperature-selected, activity-based rhythm, cold-blooded animals can produce an optimal body temperature rhythm. It has been documented that freshwater turtles control their body temperature through frequent sunbathing around midday, but not in the morning or evening (Clavijo-Baquet and Magnone 2017). The zebrafish and lizards also alter their temperature preference in a manner synchronized with the light–dark cycle, thereby achieving an optimal regulation of daily body temperature rhythm (Ellis et al. 2009; Lopez-Olmeda and Sanchez-Vazquez 2009).

Interestingly, the duration of the daytime (i.e., light phase) is also reported to be important to determine the temperature preference of lizards that exhibit seasonal variation in body temperature rhythm, indicating that the photoperiod is a prominent time cue for seasonal temperature preference and acclimatization of this animal (Rismiller and Heldmaier 1988).

In certain species of cold-blooded animals, including lizards, salamanders, frogs, and some kinds of fish, the parietal eye (pineal eye) is important for keeping normal daily locomotor activity and body temperature rhythmicity. These animals possess the parietal eye at the top of their head as a part of the epithalamus associated with the pineal gland. This organ is photoreceptive and participates in the daily regulation of body temperature. For example, the lizards that underwent surgical removal of the parietal eye select significantly higher temperatures than controls at all hours of the day except from 8:00 to 12:00 in the light–dark cycle (Hutchison and Kosh 1974). Another study indicates that parietectomy temporarily abolishes the body temperature rhythm while it has no effect on the behavior rhythm in lizards (Innocenti et al. 1993). In iguanas, although parietectomy has slight effects on the circadian rhythm of physiology, subsequent pinealectomy completely abolishes the rhythm of body temperature and blood melatonin concentration but not the rhythm of locomotor, demonstrating that the pineal complex has a central role in the generation and control of the body temperature rhythm (Tosini and Menaker 1998). Other studies also indicate that melatonin has an instrumental role in daily regulated body temperature in cold-blooded vertebrates. The intraperitoneal administration of melatonin lowers the average body temperature over the circadian cycle and enhances the amplitude, or max to min difference, in body temperature in a Transvaal girdled lizard (Skinner 1991). Daily melatonin administration also enhances the body temperature rhythm in iguanas (Cothran and Hutchison 1979). On the contrary, body temperature rhythm in a totally aquatic salamander is disrupted when melatonin is administered intraperitoneally (Wurtman and Axelrod 1966). These species and

context-dependent differences may result from differential effects of melatonin injection between seasons (Bertolucci and Foa 1998). Interestingly, the melatonin rhythm varies with season in lizards (Bertolucci et al. 2002), and the melatonin rhythm is also influenced by ambient temperature in lizards and frogs (Underwood 1985; Delgado and Vivienroels 1989), indicating that the cold-blooded vertebrates may adapt to the seasonal changes by harmonizing body temperature rhythm and melatonin rhythm.

12.6 Diurnal Body Temperature Control in Invertebrates

Circadian control of body temperature is also achieved by behavioral level regulation in invertebrates. For example, temperature preference rhythms of fruit flies can be observed under laboratory conditions. When fruit flies are released over a metal plate with a temperature gradient, they land at random positions on the metal plate at first but then gradually move towards their preferred temperature; by 30 min after release, they gather around an area with a certain temperature. There is a clear rhythm in their selection of a preferred temperature; flies prefer higher temperatures in the daytime and lower temperatures in the nighttime, which is similar to the body temperature rhythm in humans (Kaneko et al. 2012).

Similar to the SCN in the mammalian clock system, fruit flies possess a set of central circadian clock neurons in the brain. These are composed of ~150 neurons and can be separated into different groups, containing three groups of dorsal neurons (DN1, 2, and 3) and four groups of lateral neurons (ILNv, sLNv, LNd, and LPN) (Dubowy and Sehgal 2017). The rhythm of body temperature in fruit flies is mainly controlled by a DN2-based pacemaker circuit in the brain (Kaneko et al. 2012). Other studies show that neurons in DN2, LNv, and DN1 mediate entrainment to the thermal cycle (Busza et al. 2007; Zhang et al. 2010; Picot et al. 2009). A thermo-sensitive ion channel *trpA1* has been shown to be involved in this thermal entrainment mechanism. The *trpA1*-positive neurons in LPN and those in LNv, DN1, and DN2 are thought

to be responsible for temperature sensing for the entrainment (Lee and Montell 2013), while the peripheral thermosensory neurons also contribute to temperature entrainment of the central clock neurons (Sehadova et al. 2009; Glaser and Stanewsky 2005). Among the potential routes connecting the peripheral sensory neurons to the central clock neurons, the posterior DN1 neurons act as the major gateway for temperature sensation into the circadian neural network, which continuously integrates temperature changes to coordinate the timing of sleep and activity (Yadlapalli et al. 2018). The diuretic hormone 31 receptor (DH31R), which is expressed in DN1, DN3, and LNV neurons, has been shown to be indispensable for optimal diurnal rhythm of body temperature, while this receptor does not contribute to locomotor activity control (Goda et al. 2018). Most interestingly, the mammalian homolog of DH31R is the Calcr, which controls body temperature rhythms but not behavioral rhythms during the active phase in mice as explained above (Fig. 12.5). Therefore, it is highly likely that signaling through calcitonin-related receptors in circadian pacemaker neurons conducts a phylogenetically conserved role in governing the shapes of body temperature rhythms, which are the consequence of ambient temperature selection in flies and hardwired SCN outputs in mammals.

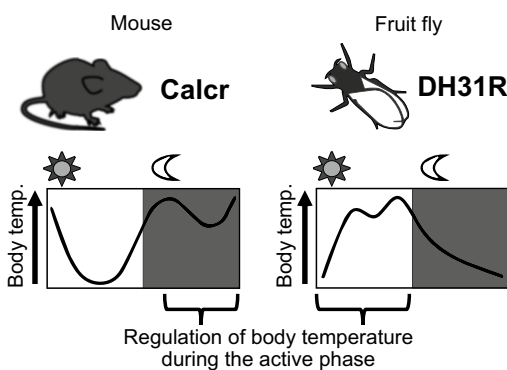


Fig. 12.5 Evolutionally conserved common receptor is responsible for the diurnal control of body temperature. The body temperature rhythms of both mice and flies display two bimodal peaks with a deep trough during the active phase corresponding to the “siesta.” Both mouse Calcr and its homolog, *Drosophila* DH31R, play a central role in the regulation of the body temperature rhythm during the siesta

12.7 Future Perspective

In mammals, it is evident that the SCN has a central role in governing daily body temperature fluctuation. To date, however, less is known about how and which SCN neurons contribute to the circadian body temperature setting. Calcr is expressed in the dorsomedial area of the SCN, and approximately 84% of Calcr-positive cells express arginine vasopressin (AVP), while they do not show expression of vasoactive intestinal peptide (VIP) (Goda et al. 2018). In the SCN, VIP has a pivotal role in maintaining the circadian oscillation of the master clock, while AVP acts principally as a phase-reentrainment signal (Yamaguchi et al. 2013) and/or the output signal from the SCN (Herzog et al. 2017). Thus, it is interesting to compare the Calcr-positive and AVP-positive neurons (~65% of AVP-positive neurons) and Calcr-negative and AVP-positive neurons to identify the neuronal circuits that play a specific role in controlling body temperature rhythmicity.

The precise mechanism of how the body temperature rhythm entrains the peripheral clocks is still unclear. Previous studies show the contribution of HSF1 and CIRBP to the mechanism of thermal clock entrainment, but their inhibition cannot completely inhibit the entrainment. Among the core circadian genes, the oscillation of *Per2* can be created by systemic signals even when the circadian clock within the tissue is not operative (Kornmann et al. 2007), implying that *Per2* may provide a hub to convert the circadian systemic signal, including body temperature, into cues for entrainment. Consistently, it is reported that feeding-regulated hormones insulin and insulin-like growth factor 1 (IGF-1) entrain circadian clocks via inducing *Per2* protein synthesis (Crosby et al. 2019), indicating that *Per2* provides a nodal point for feeding-induced entrainment. Further investigation is required to understand how temperature change controls the core clock genes. Especially, as body temperature changes gradually over the course of the day, it remains to be clarified whether and how such a gradual (or parametric) temperature change entrains the core clock machinery (Miyake et al. 2023; Shao et al. 2024).

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Experimental Approaches Used to Investigate Voluntary Behavioral Thermoregulatory Responses

13

Airi Ido, Minami Kanai, Natsuko Hitora-Imamura, Hiroshi Nomura, and Masabumi Minami

Abstract

A new behavioral test was developed to investigate the neural mechanisms of voluntary, behavioral thermoregulatory responses. The apparatus used in this test consisted of a thermostatic chamber that maintained the ambient temperature at a chosen level and two side-by-side floor plates that were placed in the thermostatic chamber and could be set to different temperatures. As the three temperatures, ambient temperature and two plate temperatures, can be controlled independently, we term this behavioral test the three-temperature (3 T) test. When the ambient temperature was 28 °C with floor plate temperatures of 25 °C and 35 °C, mice showed preference to the warm plate over the cool one. By contrast, when the ambient temperature was 40 °C, the mice showed preference to the cool plate, that is, a cool-seeking behavior. Detailed analyses of the time courses of the plate preference and core body temperature revealed that this cool-seeking behavior contributed to the regulation of body temperature. By using the 3 T test in combination with the latest in vivo imaging

techniques for real-time measurement of neuronal activities and neurotransmitter releases in the brain of freely-moving animals, the neural mechanisms of voluntary, behavioral thermoregulatory responses could be elucidated in the near future.

Keywords

Behavioral thermoregulatory responses · Cool-seeking behavior · In vivo calcium imaging · Paraventricular thalamic nucleus · Three-temperature behavioral test · Warm-seeking behavior

13.1 Introduction

Temperature affects various physiological functions and is a key factor in maintaining homeostasis in living bodies (Tominaga 2017). Thermoregulatory responses in homeothermic animals, including humans, are divided into involuntary, autonomous thermoregulatory responses (e.g., sweating, skin vasodilation, and shivering) and voluntary, behavioral thermoregulatory responses (e.g., cool- and warm-seeking behaviors in hot and cold environments, respectively) (Morrison and Nakamura 2011). In terms of autonomic thermoregulatory responses, a number of studies have addressed the mechanisms of thermoreception (Caterina et al. 1997), transmission

A. Ido · M. Kanai · N. Hitora-Imamura · H. Nomura · M. Minami (✉)
Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan
e-mail: mminami@pharm.hokudai.ac.jp

of sensory information from the periphery to the brain, and body heat production/loss (Morrison and Nakamura 2011). However, the neuronal mechanisms underlying behavioral thermoregulatory responses remain poorly understood.

Temperature selection behaviors have been examined using a floor plate featuring a temperature gradient (Lee et al. 2005; Tan et al. 2016) or two side-by-side floor plates set at different temperatures (Yahiro et al. 2017) (Fig. 13.1). Specifically, Lee et al. (2005) reported that TRPV4-knockout mice selected warmer floor temperatures than did wild-type littermates when offered a temperature gradient. Yahiro et al. (2017) used two side-by-side floor plates, one of which was set to 28 °C and the other was set to 38 °C or 15 °C, and demonstrated that the hot (38 °C)- and cold (15 °C)-avoidance behaviors of rats were eliminated by inactivation of neurons in the lateral parabrachial nucleus, but not by lesions in the ventral posteromedial and ventral posterolateral thalamic nuclei. The various reports have thus clearly described the molecules and brain regions involved in temperature selection behaviors. However, it is not clear whether the behaviors observed in such studies were based on voluntary, behavioral thermoregulatory responses or preference/aversion to the floor temperatures that the body surfaces of the animals (e.g., the paws) were experiencing. Furthermore, the ambient temperature is what makes us feel hot or cold in daily life, not the surface temperature of something that we might be touching. To address this

issue, we developed a novel behavioral test to explore whether mice perceive the ambient temperature as hot or not and whether their temperature selection behaviors contribute to the regulation of body temperature.

13.2 Development of a Novel Behavioral Test to Investigate Voluntary, Behavioral Thermoregulatory Responses

The apparatus used in this test consisted of a thermostatic chamber (KCL-2000 W; Tokyo Rikakikai Co., Ltd., Tokyo, Japan) that maintained the ambient temperature at a chosen level and two side-by-side floor plates that could be set to different temperatures (Versatile Cold/Hot Plate AHP-1200, DCP, IL, USA). As the floor plates were placed in the thermostatic chamber, it was possible to control three temperatures (the ambient temperature and the two floor plate temperatures). Thus, we term this behavioral test the three-temperature (3 T) test. When the ambient temperature was 28 °C with floor plate temperatures of 25 °C and 35 °C (Fig. 13.2a), mice showed preference to the warm plate (35 °C) over the cool one (25 °C). Specifically, the animals were allowed to explore the two plates for 30 min, their movements were recorded and analyzed using a video-tracking system (ANY-maze, Muromachi Kikai Co., Ltd., Tokyo, Japan), and the times spent on each floor plate were calculated. When the ambient temperature was 28 °C, the animals spent more time on the warm plate (64.3 ± 3.0% of the exploration period) (Fig. 13.2b). By contrast, when the ambient temperature was 40 °C, the mice showed preference to the cool plate over the warm one. The animals spent more time on the cool plate (61.1 ± 3.3% of the exploration period) (Fig. 13.2c). Collectively, the behavior observed was cool-seeking behavior in a hot environment.

Next, we examined whether the cool-seeking behavior observed in the 3 T test contributed to the regulation of body temperature. To monitor

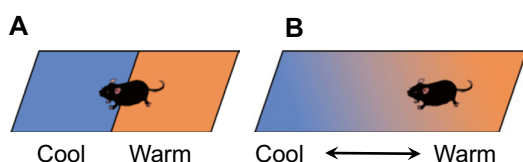


Fig. 13.1 Temperature preference tests. (a) Two-temperature choice test. Animals are allowed to move freely between two adjacent plates maintained at different temperatures. The times spent on each plate are used to assess temperature preference. (b) Temperature gradient test. Animals are allowed to move freely along a linear or circular path featuring a temperature gradient. The temperature of the chosen zone is used to evaluate temperature preference

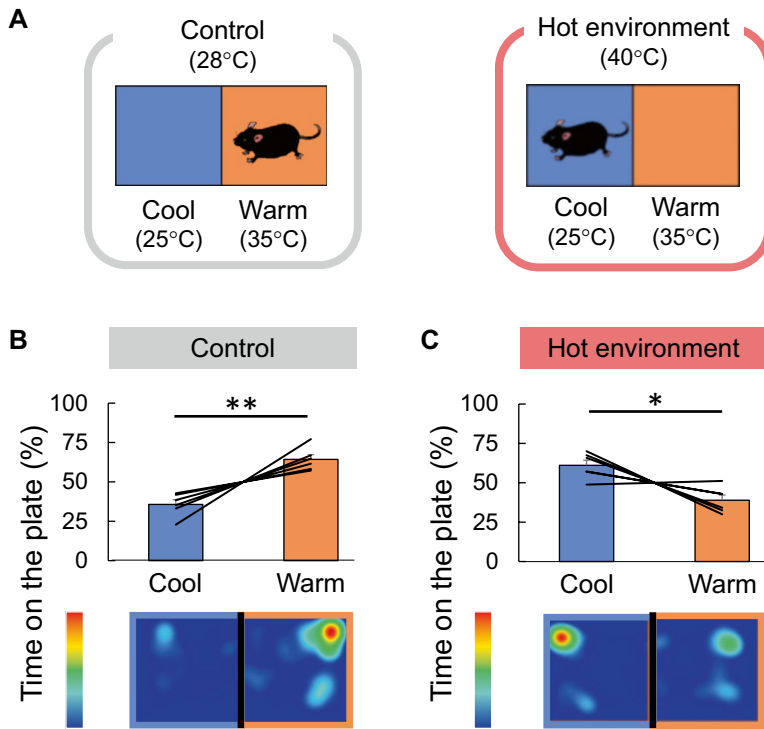


Fig. 13.2 Three-temperature test for investigating voluntary, behavioral thermoregulatory responses. (a) Schematics of the 3 T test. Mice were allowed to explore two floor plates set at 25 °C and 35 °C for 30 min when the ambient temperatures were 28 °C (left) and 40 °C (right). Their movements were recorded and analyzed using a video-tracking system. (b) When the ambient temperature was 28 °C, mice stayed longer on the warm plate. The blue and orange bars show the proportions of time spent on plates at 25 °C and 35 °C, respectively (** $P < 0.01$, mean $\pm$ SEM) (top). Black lines show the

time proportions for individual animals. A representative heat map showing where a mouse traveled during the exploration period is shown (bottom). (c) When the ambient temperature was 40 °C (hot environment), mice stayed longer on the cool plate. The blue and orange bars show the proportions of time spent on plates at 25 °C and 35 °C, respectively (* $P < 0.05$, mean $\pm$ SEM) (top). Black lines show the time proportions for individual animals. A representative heat map showing where a mouse traveled during the exploration period is shown (bottom)

core body temperature, each mouse was intraperitoneally implanted with a device that measured temperature and activity (nano tagTM; Kissei Comtec Co., Ltd., Matsumoto, Japan). After a recovery period of 3 days, cool-seeking behaviors were examined using the 3 T test with monitoring of core body temperature. The ambient temperature was set to 40 °C, and the floor plate temperatures were 25 °C and 35 °C. Figure 13.3a shows the changes in the core body temperature and floor plate preference over time. Floor plate preference was shown as the percentages of time spent on the cool and warm plates every 2 min. During the first 4 min, the animals showed preference to the warm plate.

During this period, the core body temperature increased from 36.5 ± 0.1 to 37.6 ± 0.2 °C. As the body temperature increased, the preference for the cool floor plate also increased. At 4 min from the start of the 3 T test, the animals continued to spend more time on the cool plate. As the time spent on the cool plate increased, the rate of body temperature increase slowed. Specifically, the core body temperature increased to 37.9 ± 0.2 °C 6 min after the start of the test, then plateaued for 6–8 min, gradually decreased, and fell to 37.1 ± 0.3 °C by the end of the test. These data suggest that the cool-seeking behavior observed in the 3 T test contributed to the regulation of body temperature.

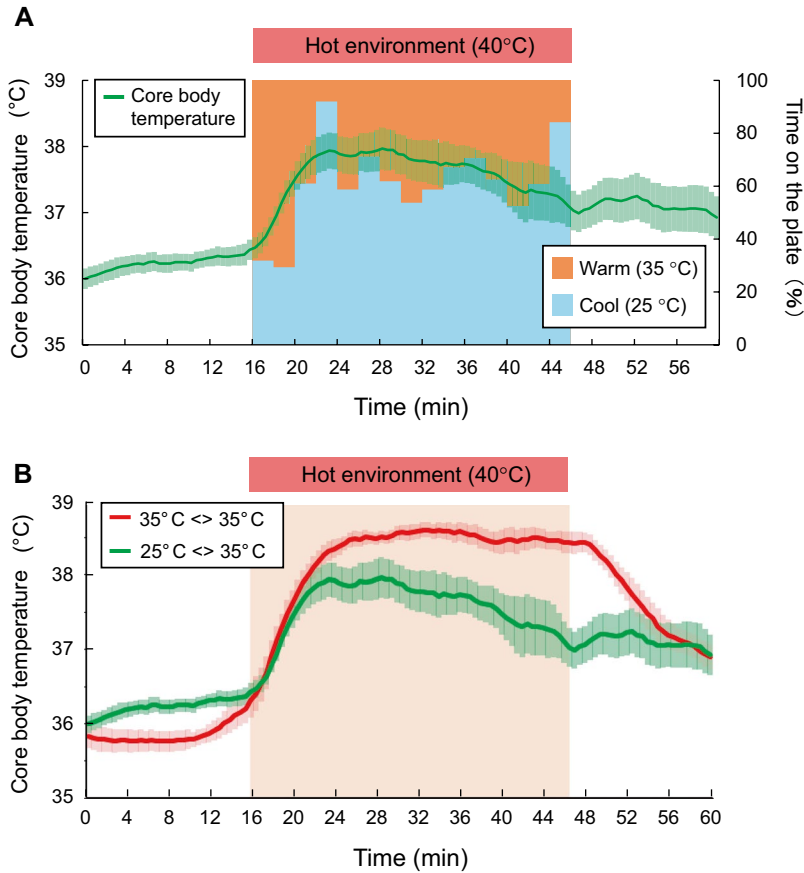


Fig. 13.3 Changes in core body temperature during the 3 T test. **(a)** Time courses of changes in core body temperature and floor plate preference during the 3 T test performed in a hot environment (40 °C). Mice were exposed to 40 °C from 16 to 46 min. The line graph (green) shows the core body temperature ($n = 6$, means $\pm$ SEMs). The bar graphs show the percentages of time spent on cool (blue) and warm (orange) plates for all 2-min intervals. **(b)** Changes in core body temperature during exposure to

a hot environment (40 °C) with or without a choice of floor plate temperature. When one plate was at 25 °C and the other at 35 °C, the core body temperature, which was initially elevated, gradually fell as the time spent on the cold plate increased (green line). When both plates were at 35 °C, the core body temperature remained elevated until the end of the test (red line) ($n = 6$ for each group, means $\pm$ SEMs)

We also examined changes in the core body temperature when the animals could not engage in cool-seeking behavior (Fig. 13.3b). In this case, the ambient temperature was 40 °C and both floor plate temperatures were 35 °C. The core body temperature of these mice rapidly increased to 38.4 ± 0.8 °C and did not decrease until the animals were freed from the hot environment. This result confirms that the cool-seeking behavior observed in the 3 T test contributed to the regulation of body temperature.

13.3 In Vivo Imaging of Neuronal Activity during Voluntary, Behavioral Thermoregulatory Responses

To identify the brain regions activated in a hot ambient temperature, we performed immunostaining for the c-Fos protein and compared the expression levels between animals exposed to room temperature (28 °C) and hot (38 °C) envi-

ronments. Consistent with the previous report (Bratincsák and Palkovits 2004), we found that the number of c-Fos-immunoreactive cells in various brain regions increased upon exposure to a hot environment (Table 13.1). However, analyses that employ c-Fos expression as an indicator of neuronal activity have serious limitations. As the time resolution is low, the detailed time courses of changes in neuronal activity caused by exposure to a hot/cold environment cannot be analyzed. In addition, histochemical analyses such as immunostaining and in situ hybridization can yield data at only one time point from any animal because the analyses are destructive. Therefore, it is difficult to determine whether the same or different neurons respond to various temperature stimuli using only histochemical analyses.

Recently, in vivo calcium imaging has rendered it possible to measure neuronal activity at much higher time resolutions than histochemical analyses, in a non-destructive manner. In vivo calcium imaging exploits Ca^{2+} -sensitive fluorescent proteins such as GCaMP, which is a

well-known Ca^{2+} indicator created by adding calmodulin (CaM) and the myosin light chain M13 fragment (M13) to the N- and C-terminals of green fluorescent protein (GFP), respectively (Nakai et al. 2001; Grienberger and Konnerth 2012). When Ca^{2+} binds to CaM, the Ca^{2+} /CaM complex interacts with M13 to change the GFP conformation, thereby increasing the fluorescence intensity. This renders it possible to measure changes in intracellular calcium concentrations because these affect the GCaMP fluorescence intensity. In vivo calcium imaging techniques using fiber photometry (Cui et al. 2013; Gunaydin et al. 2014; Kim et al. 2016) or a microendoscope (Ghosh et al. 2011; Resendez et al. 2016) are employed for real-time measurements of neuronal activities in the mouse brain during various behavioral tests. Fiber photometry employs an optical fiber implanted into the brain. Through the optical fiber, excitation light is delivered to a specific brain region, and the fluorescent emission is measured (Fig. 13.4a). As the optical fiber is thin (200–400 μm in diameter), it is possible to

Table 13.1 c-Fos positive cells in various brain regions after exposure to control (28 °C) and hot (38 °C) environments. Rats were placed in their home cages at 28 °C and 38 °C for 2.5 h. Food and water were freely available. Then the animals were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally) and sacrificed for c-Fos immunostaining. c-Fos-positive neurons in the regions of interest were counted in various brain regions

Brain region	Subarea	28 °C	38 °C	Brain region	Subarea	28 °C	38 °C
Nucleus accumbens	Shell	–	++	Central amygdala	Lateral	–	++
	Core	–	++		Medial	+	+
Bed nucleus of the stria terminalis	Oval	–	++	Basolateral amygdala		–	++
	Dorsal	–	+				
	Ventral	–	++	Raphe nucleus	Dorsal	+	+++
Preoptic area	Dorsomedial	+	+++		Ventral	–	++
	Dorsolateral	+	+++	Lateral parabrachial nucleus	Medial	+	++
	Ventromedial	+	+++		Central	–	++++
	Ventrolateral	+	+++		Lateral	+	+++
Medial habenula		–	+		Superior	+	+++
Lateral habenula	Lateral	+	++	Medial parabrachial nucleus		–	++
	Medial	+	++++	Periaqueductal gray	Dorsomedial	++	++
Paraventricular hypothalamic nucleus		+	++++		Dorsolateral	+	+
Paraventricular thalamic nucleus	Anterior	++	+++		Lateral	+	++
	Middle	++	++++		Ventrolateral	+	+++
	Posterior	++	++++	Locus coeruleus		+	+++

–: 0 nuclei/mm²; +: 1–24 nuclei/mm²; ++: 25–99 nuclei/mm²; +++: 100–249 nuclei/mm²; ++++: > 250 nuclei/mm²

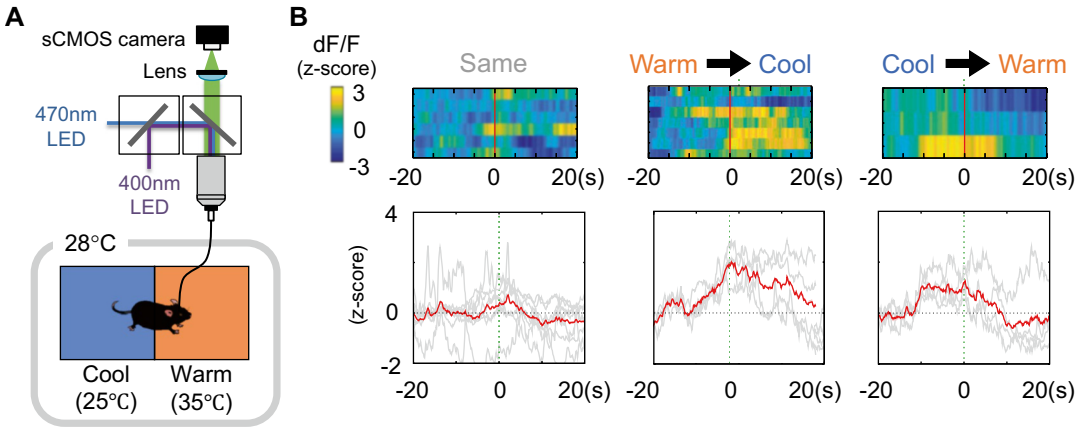


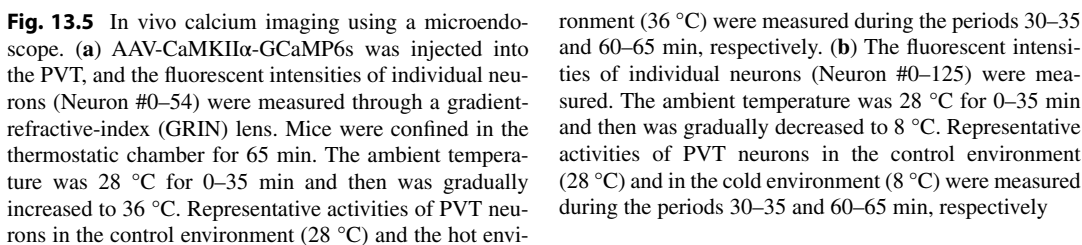
Fig. 13.4 Measurement of neuronal activity via fiber photometry during the 3 T test. (a) Schematic of neuronal activity measurements obtained via fiber photometry during the 3 T test. GCaMP-expressing neurons were excited with light of 470 and 400 nm via a 400-μm-diameter optical fiber implanted into the brain, and Ca^{2+} -dependent and Ca^{2+} -independent fluorescence, respectively, was measured using the same fiber and an sCMOS camera. (b) mPFC neurons projecting to the PAG were labeled with GCaMP by injecting AAVdj-DIO-GCaMP6m and AAV2retro-Cre into the mPFC and PAG, respectively. Fiber photometry

was used to measure the activities of PAG-projecting mPFC neurons over 20 min of the 3 T test. The ambient temperature was 28 °C, and the floor plate temperatures were 25 °C and 35 °C. In the control experiment, both floor plates were at 30 °C. We calculated relative fluorescence intensities (Z scores) over the 20 s periods before and after each movement of a mouse from one plate to the other. Same (left): Z scores when the mice moved from 30 °C to 30 °C. Warm > Cool (middle): Z scores when the mice moved from 35 °C to 25 °C. Cool > Warm (right): Z scores when the mice moved from 25 °C and 35 °C

implant several fibers and simultaneously acquire fluorescent outputs from multiple brain regions. However, it is difficult to measure the fluorescence of individual neuronal cells. On the contrary, calcium imaging using a microendoscope, where a gradient-refractive-index (GRIN) lens is implanted in the brain and application of the excitation light and measurement of fluorescent emission is performed via this lens, can measure the neuronal activity of individual neuronal cells (Fig. 13.5).

In vivo calcium imaging enables high time-resolution analyses of neuronal activity in the brain during voluntary thermoregulatory behaviors and may thus clarify the neuronal mechanisms of behavioral thermoregulatory responses. We measured neuronal activity during the 3 T test using fiber photometry. GCaMP was expressed in neurons of the medial prefrontal

cortex (mPFC) that projected to the periaqueductal gray (PAG) by injecting an adeno-associated virus (AAV) vector conveying the GCaMP gene, which is expressed in a Cre-dependent manner, into the mPFC and an AAV vector, which delivers Cre in a retrograde manner, into the PAG. After the period required for GCaMP expression (>8 weeks), we performed fiber photometry for 20 min during 3 T tests. The ambient temperature was 28 °C and the temperatures of both floor plates were 30 °C on day 1; the temperature of one plate was 25 °C and that of the other was 35 °C on day 2. Figure 13.4b shows the changes in relative fluorescence intensities (the Z scores) over the 20 s before and after the animals moved from one plate to the other. The intensities increased over the 5 s before and after such movement, when the temperatures of the two plates were 25 °C and 35 °C. On the con-



We sought to clarify whether the same or different neurons respond to hot and cold environments in the paraventricular thalamic (PVT) nucleus, the brain region that has been implicated in unpleasant emotions. For this purpose, we performed in vivo calcium imaging using a microendoscope. GCaMP was expressed in neuronal cells of the PVT and the fluorescence intensities of individual neurons were measured. Mice were confined in the thermostatic chamber, the ambient temperature of which was initially 28 °C but then was gradually raised to 36 °C (Fig. 13.5a). Some neurons exhibited increased average fluorescence intensities at

36 °C. In a similar experiment with a gradual change in ambient temperature from 28 °C to 8 °C, instead of that from 28 °C to 36 °C, some neurons exhibited increased average fluorescence intensities at 8 °C (Fig. 13.5b). To examine whether the same or different neurons respond to hot (36 °C) and cold (8 °C) temperature exposure, the same animals experienced a cold environment then, after a recovery period, a hot environment. The changes in average fluorescence intensities from 28 °C to 36 °C and from 28 °C to 8 °C were calculated for individual neurons (Fig. 13.6a). We found no correlation between the fluorescence changes at 36 °C and 8 °C, suggesting that different neurons were activated at hot and cold ambient temperatures (Fig. 13.6b). On the contrary, when the mice were exposed to the 36 °C ambient temperature twice (Fig. 13.7a), the changes in fluorescence of individual neurons were similar between the

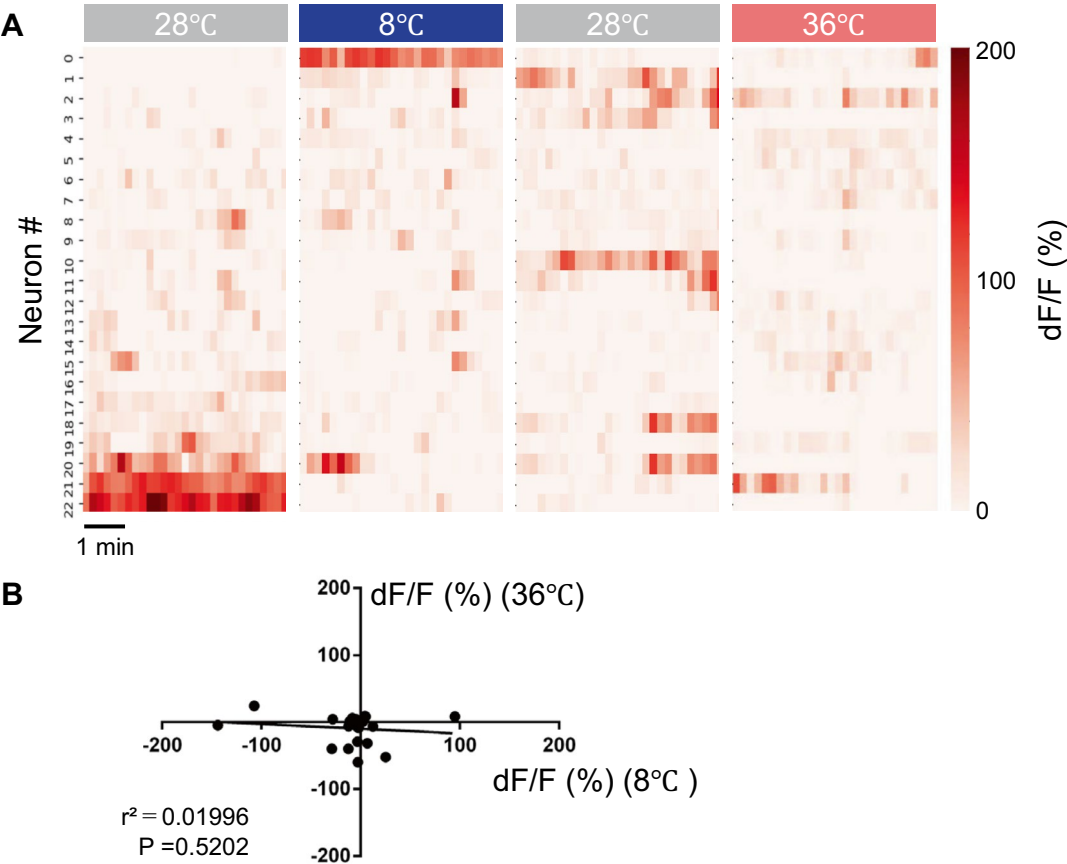


Fig. 13.6 In vivo calcium imaging using a microendoscope: The different groups of PVT neurons respond to hot and cold environments, respectively. **(a)** Representative activities of PVT neurons at ambient temperatures of 28, 8, and 36 °C. **(b)** Changes in the average fluorescence

(dF/F) from 28 °C to 8 °C (cold) and from 28 °C to 36 °C (hot) were calculated for individual neurons (Neurons #0–22). dF/F values in the cold and hot environments are shown on the horizontal and vertical axes, respectively

first and second exposures (Fig. 13.7b), and significant correlation was observed between the fluorescence changes at the first and second exposures (Fig. 13.7c), suggesting that the same population of neurons responds to repeated exposure to a hot environment.

Although further studies are needed to elucidate the details of the neuronal mechanisms involved in voluntary, behavioral thermoregulatory responses, we have established the methods required to measure neuronal activities during temperature choice behaviors.

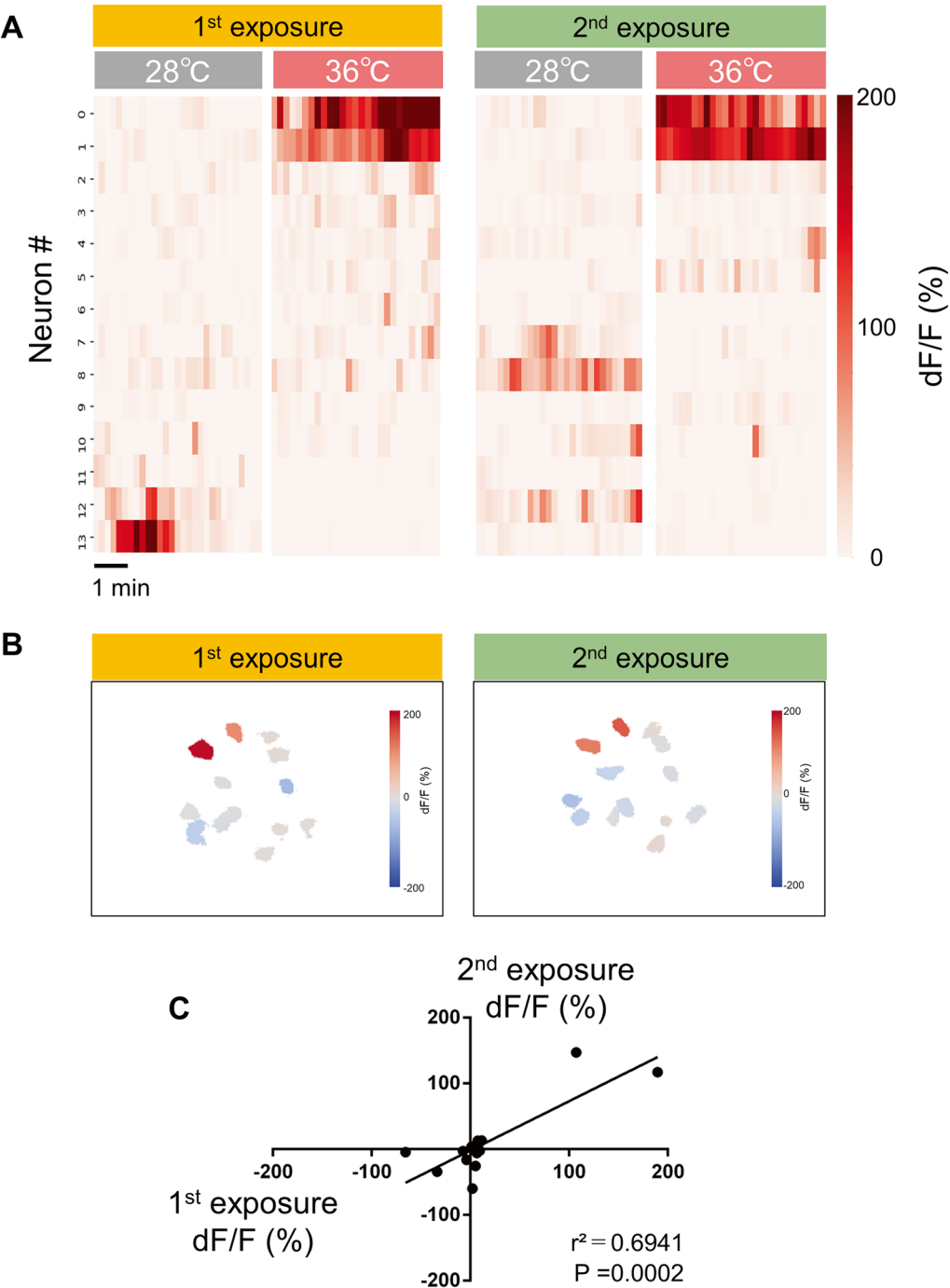


Fig. 13.7 In vivo calcium imaging using a microendoscope: The same population of PVT neurons responds to repeated exposures to a hot environment on different days. (a) Representative activities of PVT neurons at ambient temperatures of 28 °C and 36 °C when mice underwent the 36 °C exposure twice (first and second exposures). (b) The changes in the average fluorescence

(dF/F) from 28 °C to 36 °C (hot) were calculated for individual neurons (Neurons #0–13). The localizations and dF/F values of the individual neurons during the first (left) and second (right) exposures to 36 °C. (c) dF/F values during the first and second exposures to 36 °C are shown on the horizontal and vertical axes, respectively

13.4 Neurotransmitter Release in Hot and Cold Environments

It is important to measure the extracellular levels of various neurotransmitters during temperature selection to clarify the neuronal mechanisms involved in voluntary, behavioral thermoregulatory responses. We earlier reported enhanced noradrenaline release in the bed nucleus of the stria terminalis (BNST) of rats exposed to hot and cold environments (Minami et al. 2018). In vivo microdialysis experiments revealed that exposure to hot (38 °C) and cold (8 °C) environments significantly increased noradrenaline release in the BNST. However, when seeking to acquire data linking neurotransmitter releases to neuronal activities, in vivo microdialysis does not yield neurotransmitter release data of adequate time resolution. Thus, this technique cannot be employed to measure rapid changes in neurotransmitter release or to correlate such release with behavioral and neuronal activities that are measured in seconds. Very recently, methods that measure neurotransmitter release in the brains of live (awake) animals at high time resolution have been developed. Patriarchi et al. (2020) described a molecular tool that measured extracellular dopamine levels in the brain. As in the case of calcium sensors such as GCaMP, the new sensor is a GFP-based sensor engineered using a GFP variant and a dopamine receptor. The GFP fluoresces when dopamine binds to the receptor. Changes in fluorescence intensities in the brains of freely moving animals can be measured using various methods including the fiber photometry described above. Similar molecular tools have been developed to measure other neurotransmitters including noradrenaline. Combinations of these advanced neurotransmitter release measurement techniques with the 3 T test may clarify the neuronal mechanisms involved in voluntary, behavioral thermoregulatory responses.

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Regulation of Neural Functions by Brain Temperature and Thermo-TRP Channels

14

Koji Shibasaki

Abstract

Body temperature is an important determinant in regulating the activities of animals. In humans, a mild 0.5 °C hyperthermia can cause headaches, demonstrating that the maintenance of normal body temperature is a key for our health. In a more extreme example, accidental acute hypothermia can lead to severe shivering, loss of consciousness, or death, although the details of these mechanisms are poorly understood. We previously found that the TRPV4 ion channel is constitutively activated by normal body temperature. The activation threshold of TRPV4 is >34 °C in the brain, which enables TRPV4 to convert thermal information into cellular signaling. Here we review the data that describe how the deletion of TRPV4 evokes abnormal behavior in mice. These studies demonstrate that the maintenance of body temperature and the sensory system for detecting body temperature, such as via TRPV4, are critical components for normal cellular function. Moreover, abnormal TRPV4 activation exacerbates cell death, epilepsy, stroke, or brain edema. Notably, TRPV4 can detect mechanical stimuli and

contributes to various neural functions similar to the mechanosensitive characteristics of TRPV2. In this review, I summarize the findings related to TRPV2/TRPV4 and neural functions.

Keywords

TRPV2 · TRPV4 · Body temperature · Brain · Epilepsy · Neuron · Glia · Astrocyte · Retina

14.1 Introduction

The transient receptor potential ion channel was originally identified in mutant *Drosophila* photoreceptors (Montell and Rubin 1989). In the *Drosophila trp* mutant, responses to light were acute and transient, and Ca²⁺ influx was significantly reduced. Hence, the ion channel responsible for these changes was termed “transient receptor potential.” Later, it was revealed that the TRP protein has six transmembrane domains and acts as an ion channel with high Ca²⁺ permeability. The TRP channels consist of six subfamilies including TRPC, TRPV, TRPM, TRPML, TRPP, and TRPA in mammals. Among these, 11 TRP channels are classified as thermosensitive TRP channels, which means that they can be activated by various temperature ranges. TRPV2 is activated by high temperatures with a threshold of >52 °C. TRPV4 is activated by non-noxious heat

K. Shibasaki (✉)
Laboratory of Neurochemistry, Department of
Nutrition Science, University of Nagasaki, Nagasaki,
Japan
e-mail: kshibasaki@sun.ac.jp

stimuli (27–34 °C). Both channels can also act as mechanosensitive ion channels and are related to various neural functions. In this review, I summarize the important physiological roles of TRPV2 and TRPV4 as a sensor of body temperature and/or mechanical stimuli and discuss the relationships between both channel activities and neural functions.

14.2 TRPV2 Ion Channel

The first identified member of the TRPV family was osmotic avoidance abnormal family member 9 (OSM-9) from *Caenorhabditis elegans* (Peralvarez-Marín et al. 2013; Colbert et al. 1997). This protein was shown to have specific functions for mechanosensation, olfaction, and olfactory adaptation. *C. elegans* has five TRPV channels including OSM-9. In *Drosophila*, the TRPV channels Nanchung and Inactive are involved in sensory perception (Kim et al. 2003). In 1997, TRPV1 was the first TRPV family member identified in mammals using a Ca^{2+} -imaging-based expression cloning method (Caterina et al. 1997). TRPV1 was shown to be highly expressed in sensory neurons, and pungent compounds such as capsaicin could evoke TRPV1 activation (Caterina et al. 1997; Tominaga et al. 1998). Based on these studies, it was hypothesized that TRPV1 was related to pain sensation, and indeed, TRPV1KO mice showed impaired pain sensation (Caterina et al. 2000). TRPV1 is highly expressed in sensory neurons with small diameters which give rise to unmyelinated C-fibers. Given the burning qualities of capsaicin-induced pain, it suggested that capsaicin and heat may evoke painful responses through a common molecular mechanism. As hypothesized, TRPV1 can be activated by heat at >43 °C, a temperature threshold that is consistent with that of heat-evoked pain (Caterina et al. 1997).

Another molecule with 49% shared identity to TRPV1 was also isolated and later named TRPV2 (Caterina et al. 1999; Tominaga and Caterina 2004; Kanzaki et al. 1999). In contrast to TRPV1, TRPV2 is not activated by vanilloids such as capsaicin, protons, or thermal stimuli near 43 °C;

this is likely attributed to the structure of the ankyrin repeat domains of TRPV2 which are clearly different from those of TRPV1 (Jin et al. 2006). However, TRPV2 can be activated by higher temperatures with a threshold of >52 °C. TRPV2 currents showed similar properties to TRPV1 such as an outwardly rectifying I–V relationship and relatively high Ca^{2+} permeability ($P_{\text{Ca}}/P_{\text{Na}} = 2.9$). While translocation of TRPV2 from the cytosol to the plasma membrane by IGF-1 stimulation has been reported (Kanzaki et al. 1999), this phenomenon is under debate (Cohen et al. 2013). In contrast to the expression of TRPV1, TRPV2 is expressed in medium- to large-diameter neurons that give rise to myelinated A δ - or A β -fibers in the DRG (Caterina et al. 1999). The temperatures at which TRPV2 is activated are more noxious than those that activate TRPV1. This may explain the expression of TRPV2 in myelinated fibers. However, TRPV2KO mice did not display phenotypes which would suggest altered heat sensation (Park et al. 2011). Therefore, further research is required to determine the importance of TRPV2 in the detection of noxious heat stimuli using TRPV2KO mice. TRPV2 expression was not only found in sensory neurons but also in motor neurons and in many non-neuronal tissues (Peralvarez-Marín et al. 2013; Shibasaki et al. 2010; Mihara et al. 2010; Shibasaki et al. 2013). These observations indicate that TRPV2 has numerous physiological roles in addition to nociception. For example, we demonstrated that TRPV2 activation regulates the formation of neural circuits in a membrane stretch-dependent manner (Shibasaki et al. 2010).

14.3 Regulation of Axonal Outgrowth by TRPV2 Mechanosensitivity

We have previously demonstrated that TRPV2 expression begins at embryonic day 10 (E10) in mouse DRG sensory and motor neurons (Shibasaki et al. 2010). Given that mouse embryos are maintained in a constant temperature environment, we hypothesized that TRPV2 has other functions dur-

ing the developmental process. Therefore, we examined embryonic stage-specific events that might involve TRPV2, such as cell proliferation, apoptosis, cell migration, and axon outgrowth. While TRPV2 was not found to be related to cell proliferation, apoptosis, or cell migration, we did identify a role for TRPV2 in the enhancement of axon outgrowth in developing neurons (Shibasaki et al. 2010). We further sought to identify the activators of TRPV2 within developing neurons. As it was suggested that TRPV2 can be activated by membrane stretch in cardiomyocytes (Muraki et al. 2003), we hypothesized that membrane stretch resulting from axon outgrowth might endogenously activate TRPV2. To examine this hypothesis, we prepared HEK293T cells expressing mouse TRPV2 and applied artificial membrane stretch (+3%) to the cells using a pulse-motor-driven stretch machine called STREX (Mochizuki et al. 2009) during Ca^{2+} -imaging experiments (Fig. 14.1). The membrane stretch-

evoked increase in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) was observed only in transfected (TRPV2-expressing) cells, indicating that TRPV2 can be activated by weak membrane stretch (Fig. 14.1). Consistent with the HEK293T results, most embryonic DRG and motor neurons showed a stretch-evoked $[\text{Ca}^{2+}]_i$ increase, whereas dominant-negative TRPV2 significantly inhibited this increase (Shibasaki et al. 2010). Thus, we demonstrated that developing neurons can sense membrane stretch via TRPV2, and the subsequent activation enhances axonal outgrowth. Moreover, we showed that Ca^{2+} influx through TRPV2 is necessary for enhancing axonal outgrowth. We also demonstrated the regulation of axonal outgrowth by TRPV2 in vivo using chick embryos (Fig. 14.2) (Shibasaki et al. 2010). More recently, it has been reported that phosphorylation of TRPV2 by NGF-ERK signaling resulted in the activation of intracellular, but not membranous, TRPV2 as well as enhanced axonal outgrowth (Cohen et al. 2015).

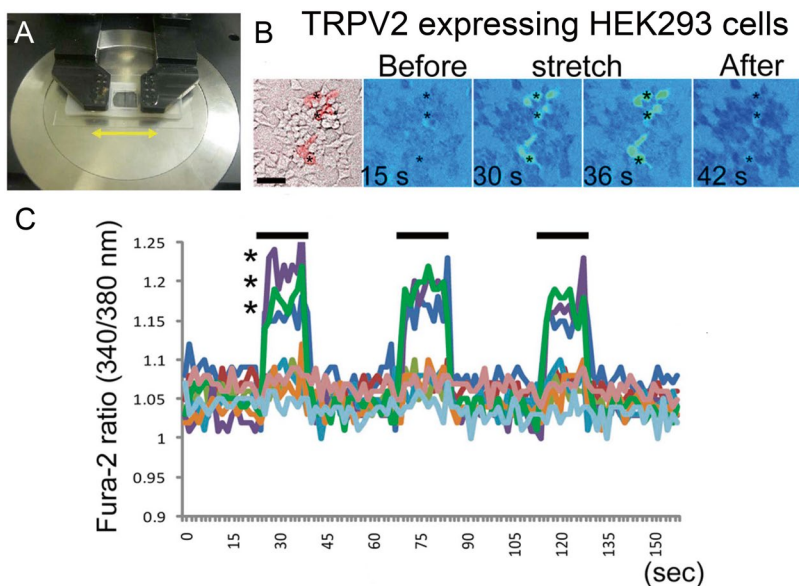


Fig. 14.1 TRPV2 is activated by membrane stretch. (a) After 48 h of cell culture, the silicone chamber is set in two arms of the extension device on the Ca^{2+} -imaging microscope. An arrow in *C* indicates the direction of extension. (b) HEK293 cells expressing TRPV2 were exposed to membrane stretch (102.8% extension) for 15 s by the STREX machine during Ca^{2+} imaging. The red signals in the left-most picture represent the TRPV2 trans-

fected cells revealed by Ds-Red co-expression. Fura-2 ratio traces by symbols are from the cells indicated by the same symbols in the pseudocolor image. Ca^{2+} influx was observed only in the transfected cells (red cells) by 102.8% stretch. (c) The representative traces are shown in the graph (both transfected and non-transfected cells). These figures are cited and modified by a previous report (Shibasaki et al. 2010)

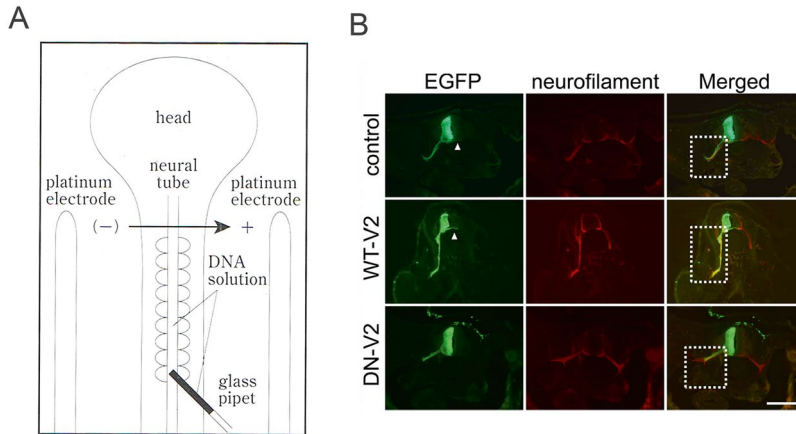


Fig. 14.2 TRPV2 regulates axonal outgrowth through its activation by membrane stretch. **(a)** Schematic drawing of *in ovo* electroporation method. Egg shells were broken by scissors, and the chick embryos were visualized. DNA solution (5 mg/mL) was injected into the neural tube by glass pipette (the author uses mouth glass pipet like ES cell injection for KO mice generation). After DNA injection (you can check by the color of Fastgreen), platinum electrodes were placed on both sides of the embryo. Then, five square pulses (33 mV) of 50-ms duration with 950-ms intervals were applied by a pulse generator, ECM830 (BTX). The egg shells were sealed by tapes, and the eggs were incubated for 24 h at 38.5 °C. **(b)** Representative images of motor neurons,

which were identified by neurofilament expression (red) in chick embryos; a control spinal cord tissue expressing EGFP, a tissue expressing wild type TRPV2 (WT-V2), and a tissue expressing dominant negative TRPV2 (DN-V2). Arrowheads indicate commissural axons. All plasmid DNAs were incorporated by electroporation *in ovo* at HH 10–14 stages. After 1 day, chick embryos were fixed, and tissue sections were prepared. WT-V2 expression significantly enhanced axon outgrowth compared with EGFP (dashed square), but DN-TRPV2 expression significantly reduced axon outgrowth compared with EGFP (dashed square). Scale bar, 1 mm. These figures are cited and modified by a previous report (Shibasaki et al. 2010)

14.4 Mechanosensitivity of TRPV2 Regulates Intestinal Motility

We also analyzed the expression of TRPV2 in the mouse intestine (Mihara et al. 2010). TRPV2-immunoreactive (IR) cells were co-stained with the neuronal marker PGP9.5. However, TRPV2-IR was not observed in glial fibrillary acidic protein (GFAP)-IR enteroglia or KIT-IR interstitial cells of Cajal (ICCs). These results indicated that TRPV2 was expressed in neurons, but not in enteroglia or ICCs. About half of the PGP9.5-IR sensory neurons were TRPV2-positive. Furthermore, most of the neuronal nitric oxide synthase (nNOS)-IR neurons were TRPV2 positive. It has been reported that nNOS-IR neurons are inhibitory circular muscle motor neurons, inhibitory longitudinal muscle motor neurons, and descending interneurons. In the

inhibitory motor neurons, even slight pressure could evoke TRPV2-mediated current responses, suggesting that enteric neurons expressing TRPV2 might be responsive to weak intestinal distortion. These results strongly suggested that intestinal TRPV2 (Mihara et al. 2010) is also mechanosensitive, similar to its role in the DRG and spinal cord (Shibasaki et al. 2010). We examined nitric oxide (NO) production in specimens from the small intestine that were stimulated by a TRPV2 agonist using an enzymatic Griess reaction and confirmed that TRPV2 activation induces NO release from inhibitory motor neurons which relaxes intestinal tone (Mihara et al. 2010). Furthermore, we also revealed that TRPV2 contributes to transit within the gastrointestinal tract *in vivo*. Thus, TRPV2 might be a good molecular target for developing a cure for irritable bowel syndrome.

14.5 TRPV2 in Glial and Immune Cells

In addition to neurons, glial cells are also important for the maintenance of brain function. Astrocytes provide metabolic support and eliminate waste products, such as neurotransmitters, from the extracellular synaptic space (Kettenmann and Ransom 2005). These glia also regulate blood flow depending on neuronal activity (Takano et al. 2006; Mulligan and MacVicar 2004). Astrocytes are also essential for bidirectional communication with neurons and thus can modulate neuronal activity (Piet and Jahr 2007; Sakatani et al. 2008; Shibasaki et al. 2014, 2015). We have shown that astrocytes in the brain express functional TRPV2 (Shibasaki et al. 2013), and astrocytic TRPV2 is activated by lysophosphatidylcholine (LPC), a known endogenous lipid ligand of TRPV2. In a separate study, we demonstrated that another thermo-TRP member, TRPV4, was only expressed in a specific subtype of astrocytes (Shibasaki et al. 2014, 2007). This astrocytic TRPV4 responded to arachidonic acid (AA) and modulated neuronal excitability through the release of gliotransmitters (Shibasaki et al. 2014). During the generation of AA at postsynaptic sites, LPC is simultaneously generated which could affect the excitation of astrocytes through TRPV2 activation. Therefore, astrocytic TRPV2 might be activated in response to increased lipid metabolism at synaptic sites.

Microglial cells also express TRPV2 (Hassan et al. 2014). Microglia are present throughout the central nervous system (CNS) and are activated by numerous pathogens. The characteristics of microglial cells are very similar to those of macrophages. Using TRPV2KO mice, it was clearly shown that TRPV2 is involved in the phagocytosis of macrophages (Link et al. 2010). It has also been reported that an inflammatory peptide, fMetLeuPhe, could induce membrane-translocation of TRPV2, and TRPV2 is expressed in immune-related tissues such as the spleen (Peralvarez-Marín et al. 2013; Link et al. 2010; Nagasawa et al. 2007). TRPV2 expression is also found in mast cells and lymphocytes (Zhang

et al. 2012; Stokes et al. 2004; Saunders et al. 2007). One study of mast cells revealed that physical stimuli that lead to cellular degranulation are associated with TRPV2 activation and Ca^{2+} entry (Stokes et al. 2004). Thus, these studies highlight the importance of TRPV2 function in numerous immune cell types.

14.6 The TRPV4 Ion Channel

TRPV4 was identified as an osmotically activated channel (Strotmann et al. 2000; Liedtke et al. 2000). Since the original discovery of TRPV4 as a stretch-activated channel (Lacampagne et al. 1994), TRPV4 has also been shown to be activated by hypotonic stimuli (Nilius et al. 2001; Goldenberg et al. 2015). Cellular swelling activates phospholipase A2 (PLA2) (Goldenberg et al. 2015; Ryskamp et al. 2014), and PLA2 produces AA by hydrolysis of membrane lipids. AA is metabolized to epoxyeicosatrienoic acid (EET), which directly activates the TRPV4 channel by cytochrome P450 (Ryskamp et al. 2014; Watanabe et al. 2003). TRPV4 is also a thermosensitive channel activated by warm stimuli (Watanabe et al. 2002; Guler et al. 2002). Both our lab and another group reported that TRPV4 is activated by physiological temperatures ($>34^\circ\text{C}$) in naïve cells (Chung et al. 2003; Shibasaki et al. 2007). This heat-activation of TRPV4 is required for endogenous signaling as suggested by the failure of TRPV4 heat activation in inside-out membrane patches (Watanabe et al. 2002).

14.7 Relationship Between Brain Function and TRPV4 as a Thermosensitive Ion Channel

Clinical evidence suggests that brain temperature is a critical determinant of cognitive function. It has also been reported that therapeutic hypothermia leads to cognitive dysfunction in heart disease patients (Torgersen et al. 2010), and cooling of the prefrontal cortex in monkeys causes per-

formance impairment during a delayed matching-to-sample task (Fuster and Bauer 1974). In rodents, brain cooling significantly impairs spatial learning (Moser and Andersen 1994). These reports indicate that the maintenance of brain temperature is important for healthy brain function. However, the underlying molecular mechanisms for this regulation remain unknown.

We previously reported that physiological temperature activates TRPV4 (Fig. 14.3) and enhances neuronal excitability through TRPV4 activation in dissociated hippocampal neurons (Shibasaki et al. 2007). Furthermore, we revealed that brain temperature constitutively activates

TRPV4 and enhances hippocampal activity in vivo (Shibasaki et al. 2015). This was demonstrated in TRPV4-deficient mice which displayed a significant decrease in social and depressive-like behaviors (Shibasaki et al. 2015). These results indicate that constitutive activation of TRPV4 by physiological brain temperature is necessary for normal brain function (Fig. 14.4). Indeed, it was reported that in a study involving whole-genome sequencing of 85 quartet families with autism, frame-shift mutations in TRPV4 were found in the patients with autism (Yuen et al. 2015). Furthermore, genome-wide association studies in control subjects and those with major depressive disorder revealed that TRPV4 mutations are a risk factor for depression (Wong et al. 2017). These studies demonstrate that disruption of TRPV4 function might lead to psychiatric symptoms.

Very recently, we revealed that brain ischemia evokes hyperthermia (an increase of approximately 2 °C) in ischemic regions (Hoshi et al.

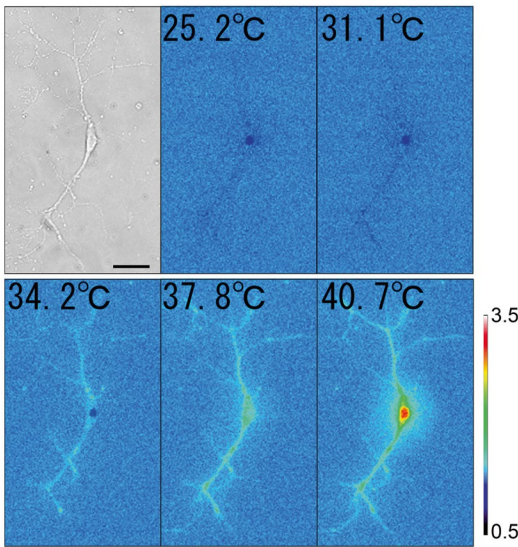


Fig. 14.3 TRPV4-mediated heat-evoked response is observed in a hippocampal pyramidal neuron. The pictures show heat-evoked responses in a dissociated hippocampal pyramidal neuron. The hippocampal neuron (top-left picture was the bright field image) was prepared from wild-type neonatal mice at P0 and cultured for 3 weeks. Fura2 fluorescence was measured upon application of the heat ramp (from room temperature to 45 °C). The 340:380 nm ratio pseudocolor expression was shown. An increase in cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) was observed at ~33 °C. Increased $[\text{Ca}^{2+}]_i$ was not observed in the absence of extracellular Ca^{2+} or in TRPV4-deficient neurons, suggesting that functional TRPV4 was expressed in the hippocampal pyramidal neurons. TRPV4 is a thermosensitive ion channel activated at temperatures approaching the physiological core body temperature (>34 °C); therefore, TRPV4 was thought to be an important molecule to regulate neural excitability in the hippocampus at body temperature (Shibasaki et al. 2007). Scale bar: 50 μm

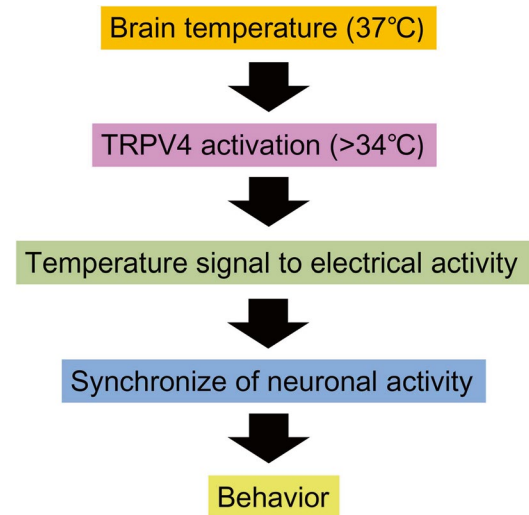


Fig. 14.4 TRPV4 regulates brain activity and behavior through its body temperature-induced activation. TRPV4 can be activated by normal brain temperature which leads to the enhancement of electrical activity. Thermal signal is converted to electrical activity. This results in the synchronization of neuronal activity in the brain and contributes to normal behavior. TRPV4KO mice lack this mechanism, lose synchronization, and display altered behaviors such as reduced depression-like behaviors and social behaviors (similar to psychiatric diseases)

2018). This hyperthermia caused abnormal hyperactivation of TRPV4 and exacerbated brain edema (Hoshi et al. 2018). Hence, a TRPV4 inhibitor may effectively suppress ischemia-induced brain edema. Hyperthermia also negatively affects neural development during fetal stages, and TRPV4 is involved in maternal “fever-associated” neural birth defects (Hutson et al. 2017). TRPV4 is also expressed in endothelial cells and controls cerebral blood flow via activation of the Gq protein-coupled receptor (Harraz OF et al. 2018). Moreover, brain temperature and hyperthermia might affect blood flow through the activation of TRPV4. In addition, TRPV4 plays an essential role in thermal hyperalgesia in sensory nerves (Todaka et al. 2004).

In sensory neurons, it has also been reported that TRPV4 is involved in chemotherapy-induced neuropathic pain (Alessandri-Haber et al. 2004). Hence, the abnormal activation of TRPV4 under pathological conditions is strongly related to pain sensation. Specifically, many TRPV4 mutations cause peripheral neuropathy. Mutations in TRPV4 are associated with many severe diseases such as scapuloperoneal spinal muscular atrophy, Charcot-Marie-Tooth disease type 2C, and skeletal dysplasia (Nilius and Owsianik 2010; Rock et al. 2008). TRPV4 gain-of-function mutations are responsible for Charcot-Marie-Tooth disease type 2C (Nilius and Owsianik 2010), in which

mutated TRPV4 becomes sensitized at body temperature. Skin keratinocytes strongly express TRPV4 which is activated by body temperature causing Ca^{2+} influx. This leads to the enhancement of barrier function through increased E-cadherin expression and re-organization of the actin cytoskeleton (Sokabe et al. 2010). During the winter season, skin dehydration is a common occurrence, and cold winter temperatures are thought to inhibit constitutive TRPV4 activation (by skin temperature) (Kida et al. 2012). Recently, it has also been reported that TRPV4 expressed in skin keratinocytes causes Ca^{2+} influx in response to histaminergic pruritogens, leading to itch (Chen et al. 2016). Hence, TRPV4 is a pruriceptor acting on skin keratinocytes. TRPV4 is also expressed in esophageal keratinocytes and is associated with the exacerbation of *Helicobacter pylori* infection (Mihara et al. 2017).

In another study, we found that TRPV4 expression was restricted to ~30% of the specific astrocyte population, indicating that astrocytic subtypes can be classified on the basis of their expression patterns (Shibasaki et al. 2014). When TRPV4-positive astrocytes are activated by endogenous ligands such as AA, the activation propagates to neighboring astrocytes through gap junctions and by ATP release from the TRPV4-positive astrocytes (Fig. 14.5). Following activation, both TRPV4-positive and TRPV4-negative

TRPV4-positive astrocyte regulates synaptic transmission

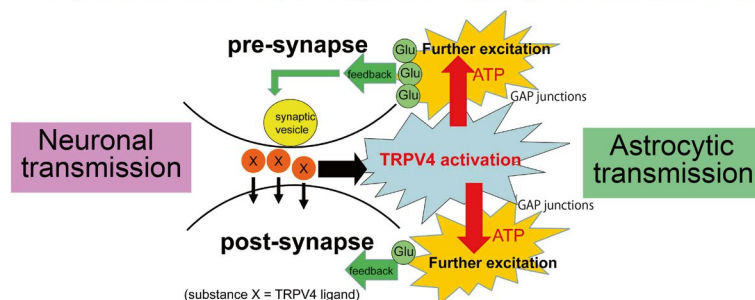


Fig. 14.5 Specific TRPV4-positive astrocyte populations enhance synaptic transmission. Schematic representation summarizes our findings (Shibasaki et al. 2014). TRPV4-positive astrocytes, shown by the blue color, are specifically localized in the brain; activation of TRPV4 in these astrocytes causes excitation in neighbor-

ing astrocytes through gap junctions and ATP release, shown as red arrows. These cells form a unit of excitatory astrocytes that release glutamate, shown as green arrows. The glutamate that is released signals through type 1 mGluRs at presynaptic sites to enhance neurotransmitter release

astrocytes release glutamate to increase synaptic transmission through group 1 mGluR (Fig. 14.5) (Shibasaki et al. 2014). These results indicate that TRPV4-positive astrocytes constitute a novel subtype of the population and are solely responsible for initiating excitatory gliotransmitter release to enhance synaptic transmission (Fig. 14.5). In addition to the astrocytic expression of TRPV4 (Shibasaki et al. 2013), we also found that microglial cells express the TRPV4 in brain (Konno et al. 2012). Robust microglial activation after an injection of lipopolysaccharide (LPS) into the mouse cerebral ventricle was suppressed by the activation of TRPV4 (Konno et al. 2012). The release of tumor necrosis factor- α (TNF- α) and expression of galectin-3 were both increased by LPS. However, these increases were significantly suppressed by the activation of TRPV4. The amplitude of voltage-dependent K⁺ current was also suppressed by the activation of TRPV4. Opening of TRPV4 channels induced membrane depolarization mainly by increasing Na⁺ influx in microglia (Konno et al. 2012). These results indicate that TRPV4 activation in microglia has a protective effect to suppress the inflammation state of the cells.

14.8 Physiological Roles of TRPV4 in Cerebrospinal Fluid Production

The highest level of TRPV4 expression is observed in the choroid plexus in the brain (Liedtke et al. 2000; Takayama et al. 2014). It has been reported that TRPV4 in choroid plexus epithelial cells (CPECs) plays a pivotal role in the calcium homeostasis of cerebrospinal fluid (CSF) through α -klotho type 1. The calcium concentration of CSF is reduced in α -klotho type 1 KO mice, and α -klotho type 1 release from CPECs is enhanced by activation of TRPV4 (Imura et al. 2007), although the physiological significance of TRPV4 in CPECs remains unclear. The choroid plexus is involved in maintaining the brain environment in the lateral, third, and fourth ventricles and forms the blood–CSF barrier. The most important function of CPECs is CSF pro-

duction. The generation of CSF is dependent on electrolyte transport from the basolateral to apical membranes of the CPECs and involves many ion transport proteins (Takayama et al. 2014). We found that TRPV4 in CPECs is activated by body temperature, and CPEC movement further activates TRPV4 through the production of EETs. TRPV4 activation leads to the activation of anoctamin 1 (ANO1), a Ca²⁺-activated chloride channel, and causes Cl⁻ efflux (Takayama et al. 2014). This Cl⁻ efflux can be a specific driving force for water efflux from CPECs. Hence, the choroid plexus can effectively generate a large amount of CSF without ineffectual energy loss. Another study revealed a different function of TRPV4 in the choroid plexus; they found that the activation of TRPV4 induced a marked decrease in filamentous actin, disintegrated the cell junctions, and affected basolateral-to-apical transport in the choroid plexus (Narita et al. 2015). Taken together, these studies suggest that TRPV4 generates the specific driving force for CSF production.

14.9 Neural Function and TRPV4 as a Mechanosensitive Ion Channel

TRPV4 can functionally act as an osmoreceptor (O'Neil and Heller 2005). Many cells such as vascular aortic endothelial cells, vascular smooth muscle cells, renal collecting duct cells, and lung epithelial cells, all of which endogenously express TRPV4, display hypotonicity-induced Ca²⁺ influx (O'Neil and Heller 2005; Sidhaye et al. 2006). The mechanism by which hypotonicity induces cellular swelling to activate TRPV4 seems to involve the production of lipid metabolites as described above (Goldenberg et al. 2015; Ryskamp et al. 2014). Furthermore, studies with TRPV4KO mice revealed that these animals have a reduced ability to regulate osmolality and show impaired osmotically induced release of antidiuretic hormone (Mizuno et al. 2003; Liedtke and Friedman 2003).

As TRPV4 is a mechanosensitive channel, TRPV4 can act as a sensor of shear stress or fluid

flow. TRPV4 is highly expressed in aortic endothelial cells, microvascular endothelial cells, distal nephrons, and collecting duct epithelial cells, where it contributes to shear stress/fluid flow-dependent Ca^{2+} influx (O'Neil and Heller 2005). Furthermore, mechanical stimuli-induced TRPV4 activation significantly enhanced TGF- β 1-evoked α -SMA fiber formation in cardiac fibroblasts (Adapala et al. 2013). These results suggest that mechanical stress and TGF- β 1-mediated signaling synergistically promote myofibroblast differentiation through TRPV4 activation.

In the eye, it has recently been reported that trabecular meshwork cells express TRPV4 (Ryskamp et al. 2016). In glaucoma, the elevation of intraocular pressure is harmful to the eye, and anti-glaucoma treatments aim to reduce intraocular pressure by decreasing the production of aqueous fluid in the anterior eye (inflow) or increasing its drainage through outflow pathways. The trabecular meshwork forms part of the outflow pathway toward Schlemm's canal. In a glaucomatous eye model, intraocular pressure was significantly elevated, and TRPV4 inhibition restored intraocular pressure to normal levels (Ryskamp et al. 2016). These results suggest that TRPV4 regulates aqueous fluid outflow and intraocular pressure within the trabecular meshwork. However, the detailed mechanisms of how TRPV4 decreases intraocular pressure in glaucoma remain unknown; thus, future studies are warranted.

14.10 Synergistic Activation of TRPV4 by Heat and Mechanical Stimuli Exacerbates Several Diseases

As described above, TRPV4 can act as heat and/or stretch sensors. Notably, the weak artificial mechanical stimuli used in our protocol did not activate any mechanosensing activity in TRPV4-expressing HEK293 cells. In contrast, we observed significant mechanosensitive activity of TRPV4 in retinal Müller glia (Matsumoto et al. 2018). These results indicate that the Müller glia

possess specific characteristics promoting the mechanosensitive properties of TRPV4. Based on our previous findings that TRPV4 is activated at temperatures above 34 °C (Shibasaki et al. 2007) and that various ligands combined with increased temperature had synergistic-potential effects on TRPV4 activation (Gao et al. 2003), we hypothesized that body temperature significantly reduces the TRPV4-activation threshold against mechanical stimuli. Hence, we performed whole-cell patch clamp recordings by application of computer-programmed mechanical stimuli at different temperatures. We applied minimal artificial membrane stretch (10 mmHg) to Müller glial cells at 25 or 37 °C and recorded the mechanical stimuli-evoked TRPV4 currents at each temperature. Compared with 25 °C conditions, physiological temperature (37 °C) significantly potentiated the mechanosensing activity of TRPV4 (Matsumoto et al. 2018). These results indicate that body temperature significantly elevates TRPV4 sensitivity to membrane stretch as a result of the synergistic effect of temperature and mechanical stimuli. In retinal detachment, significant Müller glial swelling (three times the normal cell volume) has been observed among reactive glia (Matsumoto et al. 2018; Machemer 1968; Machemer and Norton 1969). Taken together, this suggests that retinal detachment-induced Müller glial swelling significantly activates TRPV4 channels. Furthermore, we revealed that this swelling-induced TRPV4 activation triggered Ca^{2+} influx and evoked MCP-1 release in Müller glia. Excessive amounts of MCP-1 recruit numerous macrophages near the retina, which target and kill photoreceptors (Fig. 14.6) (Matsumoto et al. 2018). In addition to hypoxia, this is the critical mechanism by which photoreceptor death is exacerbated in retinal detachment (Fig. 14.6). Thus, TRPV4 inhibitors could suppress photoreceptor death in retinal detachment, and targeting TRPV4 in Müller glial might represent a novel therapeutic target for the prevention of photoreceptor cell death after retinal detachment.

We have also reported that body temperature constitutively activates neural TRPV4 and regulates brain activity, as described above (Shibasaki et al. 2015, 2007; Shibasaki 2016). In another

TRPV4 and photoreceptor death in retinal detachment

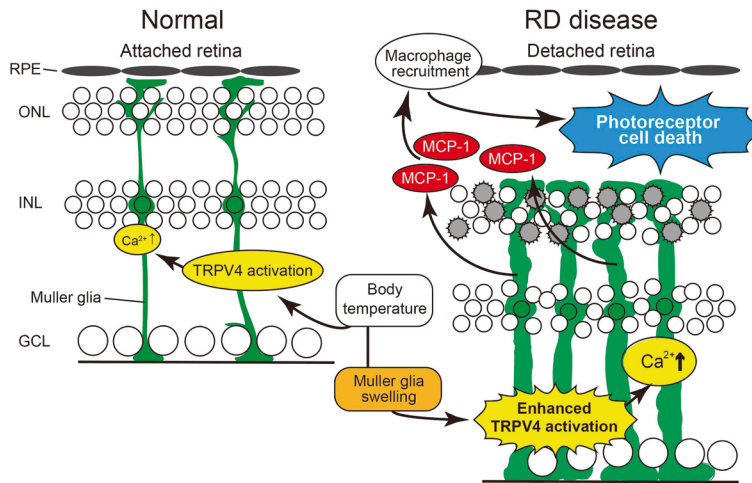


Fig. 14.6 Pathological conditions in RD potentiate glial TRPV4 activation and exacerbate photoreceptor cell death. Green cells represent the morphology of Müller glia in normal and RD retinas. In the normal retina, TRPV4 is activated by body temperature and contributes to the homeostatic function of Müller glial cells. In contrast, RD induces significant Müller glial swelling, causing further activation of TRPV4 by membrane stretch in addition to the activation induced by body temperature.

TRPV4 activation induces Ca^{2+} influx and evokes MCP-1 release from Müller glial cells. MCP-1 recruits numerous macrophages, which attack and kill photoreceptors (shown in gray). Thus, RD adversely impacts photoreceptor viability via TRPV4-dependent MCP-1 release from Müller glial cells. RPE: retinal pigment epithelium; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. This figure is modified from my previous publication (Matsumoto et al. 2018)

study, we revealed that the synergistic effects of temperature and mechanical stimuli on TRPV4 expressed in Müller glial cells are specifically required to regulate synaptic transmission in normal retina (manuscript in preparation). Based on these findings, our observations suggest that the contribution of Müller glial TRPV4 to synaptic transmission by retinal neurons through the effects of body temperature might apply to homeothermic animals in general (Hanaoka et al. 2013). Compared with cold-blooded animals, birds and mammals must perform advanced behaviors that require a finely tuned visual transmission system. Thus, homeostatic constitutive TRPV4 activation could contribute to this visual fine-tuning by taking advantage of a constant body temperature. However, temperature-dependent TRPV4 activation can negatively affect membrane stretch-dependent activation of TRPV4 in the presence of pathological cellular swelling.

14.11 Disease and Body Temperature

As described above, brain temperature is a critical determinant in the regulation of neuronal excitability (Shibasaki et al. 2015). Hence, acute hypothermia (such as in hypoglycemia, alcohol addiction, or cerebrovascular disorders, among others) causes severe shivering, lethargy, and reduction of respiration/heart function. The mechanisms by which hypothermia exerts its effects include: (i) reduction of cellular function and oxygen consumption; (ii) reduction of energy production and organ function; and (iii) peripheral circulatory disturbance. In acute hypothermia, both active external warming and core rewarming are required to restore physiological functions. It is well known that therapeutic hypothermia is effective for several diseases such as ischemic stroke, traumatic brain injury, or cardiac arrest,

due to the ability of therapeutic hypothermia to reduce oxygen consumption, cellular inflammation, and blood flow. In contrast to hypothermia, hyperthermia induces severe cell death over 43 °C. Based on this property, therapeutic hyperthermia is one strategy used in cancer treatment, although the mechanisms are poorly understood. Very recently, we reported that the temperature of epileptogenic foci was elevated by around 1 °C. This 1 °C hyperthermia caused the over-activation of TRPV4, triggering abnormal brain activity and exacerbating epileptogenic discharge (Shibasaki et al. 2020).

14.12 Conclusion

The maintenance of body temperature is very important for our health. TRPV4 senses this body temperature and, more specifically, TRPV4 channels detect dynamic local temperature changes in the brain. Very recently, we revealed for the first time that the range of local brain temperature can change by approximately 0.5 °C (Shibasaki et al. 2020), and this small change exerts pivotal physiological effects depending on the tissue. These findings suggest that TRPV4 might represent a novel therapeutic target for preventing the progression of several diseases.

Conflict of Interest The author declares no conflicts of interest.

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β -Adrenergic Signal and Epigenomic Regulatory Process for Adaptive Thermogenesis

Yoshihiro Matsumura, Timothy F. Osborne,
Ryo Ito, Hiroki Takahashi, and Juro Sakai

Abstract

Activation of β -adrenergic (β -AR) signaling induces fight-or-flight stress responses which include enhancement of cardiopulmonary function, metabolic regulation, and muscle contraction. Classical dogma for β -AR signaling has dictated that the receptor-mediated response

results in an acute and transient signal. However, more recent studies support more wide-ranging roles for β -AR signaling with long-term effects including cell differentiation that requires precisely timed and coordinated integration of many signaling pathways that culminate in precise epigenomic chromatin modifications. In this chapter, we discuss cold stress/ β -AR signaling-induced epigenomic changes in adipose tissues that influence adaptive thermogenesis. We highlight recent studies showing dual roles for the histone demethylase JMJD1A as a mediator of both acute and chronic thermogenic responses to cold stress, in two distinct thermogenic tissues, and through two distinct molecular mechanisms. β -AR signaling not only functions through transient signals during acute stress responses but also relays a more sustained signal to long-term adaptation to environmental changes.

Y. Matsumura

Division of Molecular Physiology and Metabolism,
Tohoku University Graduate School of Medicine,
Sendai, Japan

Department of Biochemistry and Metabolic Science,
Akita University Graduate School of Medicine,
Akita, Japan

T. F. Osborne

Institute for Fundamental Biomedical Research
Division of Endocrinology, Diabetes and Metabolism
Johns Hopkins University School of Medicine, Johns
Hopkins All Children's Hospital,
St. Petersburg, FL, USA

R. Ito · H. Takahashi

Division of Molecular Physiology and Metabolism,
Tohoku University Graduate School of Medicine,
Sendai, Japan

J. Sakai (✉)

Division of Metabolic Medicine, Research Center for
Advanced Science and Technology, The University of
Tokyo, Tokyo, Japan

Division of Molecular Physiology and Metabolism,
Tohoku University Graduate School of Medicine,
Sendai, Japan

e-mail: juro.sakai.b6@tohoku.ac.jp

Keywords

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Abbreviations

ADRB	β -adrenergic receptors
BAT	Brown adipose tissue

cAMP	cyclic AMP
H3K27	Histone 3 at lysine 27
H3K4	Histone 3 at lysine 4
H3K9	Histone 3 at lysine 9
JMJD1A	Jumonji domain-containing 1A
PGC1 α	PPAR γ coactivator-1 α
PKA	Protein kinase A
PPAR γ	Peroxisome proliferator-activated receptor γ
PRDM16	PR/SET domain 16
scWAT	subcutaneous white adipose tissue
UCP1	Uncoupling protein 1
β -AR	β -adrenergic receptor

15.1 β -Adrenergic Signaling and Adaptive Response to Cold

When animals are exposed to exogenous stressors including environmental changes, they acutely respond to counter the immediate challenge, and upon prolonged exposure, they adapt to the new environment by fundamental stable long-term responses such as tissue remodeling. In response to cold stress, mammals utilize at least two distinct types of thermogenic adipocytes; pre-existing brown adipocytes established during development are rapidly engaged to generate heat, and newly generated beige (or brite) adipocytes that reside in white adipose depots mediate the more long-term adaptation (Kajimura et al. 2015). Upon acute cold stress, which can be life-threatening, the animals' "first-aid" response is to rapidly produce heat by the combined action of non-shivering thermogenesis in brown adipocytes in brown adipose tissue (BAT) (Box 15.1) and shivering thermogenesis in skeletal muscle myocytes to maintain body temperature. When cold stress persists, animals gradually adapt to the cold environment through epigenomic modifications (i.e., DNA methylation and histone post-translational modifications) (Box 15.2) that re-wire chromatin and remodel white adipose tissues. The β -adrenergic (β -AR) signaling pathway plays a central role in the mammalian adaptation to low-temperature exposure. When animals feel cold, the neurotransmitter noradrenaline is secreted from sympathetic neurons, and it

signals through β -AR receptors (ADRB) (Box 15.3) expressed at the cell membrane of brown adipocytes, signaling through the receptor activates adenylate cyclase which increases intracellular cyclic AMP (cAMP), which then activates protein kinase A (PKA) (Collins 2012). Activated PKA phosphorylates a series of substrates including metabolic enzymes and transcription factors to facilitate thermogenesis. Thus, ADRB/PKA signaling functions as an acute and transient signal in brown adipocytes to combat cold stress.

Box 15.1 Brown Adipose Tissue

Brown adipose tissue (BAT) is abundantly present in neonatal humans, small rodents, and hibernating animals. By using diet-derived energy, BAT produces heat (non-shivering thermogenesis) to maintain body temperature. The brown adipocyte-specific uncoupling protein 1 (UCP1) is uniquely expressed at the inner mitochondrial membrane of brown adipocytes and generates heat through dissipation of the hydrogen ion gradient generated by the electron transport chain instead of using it for ATP production. Before birth, neonatal humans reside in the temperature-controlled womb; however, soon after birth, they are exposed to an uncontrolled temperature environment and they need to maintain their body temperature by inducing their own thermogenic program. Many transcription factors (PPAR γ , C/EBP β , EBF2, IRF4, NF1, and KLF11) (Rosen et al. 1999; Kajimura et al. 2009; Rajakumari et al. 2013; Kong et al. 2014) (Hiraie et al. 2017; Loft et al. 2015), mediators (MED1) (Harms et al. 2015), coactivators (PGC1 α , PRDM16) (Puigserver et al. 1998; Uldry et al. 2006; Seale et al. 2007; Seale et al. 2008), and chromatin factors (EHMT1, LSD1, UTX, JMJD3, and MLL4) (Ohno et al. 2013; Hino et al. 2012; Sambeat et al. 2016; Zeng et al. 2016; Wang et al. 2012; Pan et al. 2015; Lai et al. 2017) are all involved in lineage commitment and differentiation of

brown adipocytes, and their roles are summarized in other reviews (Kajimura et al. 2015; Inagaki et al. 2016). PRDM16 is a transcription coregulator that facilitates the development of brown adipocytes in BAT. PRDM16 controls the cell fate between muscle and brown adipocytes. More recent studies show that PRDM16 is expressed in subcutaneous white adipose tissue and contributes to the development of beige adipocytes (Ohno et al. 2012; Abe et al. 2018) (also reviewed in Kajimura et al. (2015) and Inagaki et al. (2016)). The body temperature of animals is highest during waking and lowest during sleeping. Expression of *Ucp1* in brown adipocytes in mice is regulated by circadian rhythm and is highest just before sleeping and lowest just before waking (Gerhart-Hines et al. 2013). In hibernating animals, thermogenic activity in brown adipocytes is repressed to decrease body temperature during hibernation, while it is activated just before arousal to increase body temperature (Cannon and Nedergaard 2004). Brown adipocytes express *Ucp1* constitutively, but its expression and thermogenic activity are further induced upon exposure to cold stress.

Box 15.2 Epigenomic Modifications

The adaptation to chronic environmental stress often promotes a lasting adaptive phenotype that is associated with epigenomic modifications (i.e., DNA methylation and histone post-translational modifications). Among the known epigenomic modifications, histone acetylation accompanies rapid and transient phenotypic changes, while methylation of histones and DNA is associated with terminal cell differentiation and provides a semi-stable chromatin state that establishes a form of cellular “memory” and ensures stable gene expression patterns (Barth and Imhof 2010; Feil and Fraga 2012). Among the four core

histones (H2A, H2B, H3, and H4), modifications of histone H3 are more predictive of the state of gene expression (i.e., active, poised, or inactive), the type of chromatin (i.e., euchromatin or heterochromatin), and the regulatory element location (i.e., enhancer, promoter, and gene body) (Barski et al. 2007; Lee and Mahadevan 2009). Histone acetylation (e.g., acetylation of H3 lysine 27 (H3K27ac)) is an active epigenomic mark associated with euchromatin. Methylation of H3 lysine 4 (H3K4) is associated with activation where tri-methylation of H3K4 (H3K4me3) is enriched in promoters, while mono-methylation of H3K4 (H3K4me1) is enriched in enhancers. Methylation of both H3K9 and H3K27 (e.g., H3K9me2, H3K9me3, and H3K27me3) is associated with heterochromatin and gene repression. Environmental cues alter epigenomic modifications of specific genes via the regulation of epigenomic enzymes (Matsumura et al. 2022); however, the underlying mechanisms are not fully understood.

Box 15.3 β -Adrenergic Receptors (ADRB) in Adipocytes

Three subtypes of ADRB (ADRB1, ADRB2, and ADRB3) are expressed in brown adipocytes (Collins and Surwit 2001). Deletion of all three subtypes of ADRBs in mice impairs cold tolerance and results in an obese phenotype (Bachman et al. 2002; Jimenez et al. 2002). Expression of *Adrb3* mRNA is the highest among the three subtypes in brown adipocytes, and it has been postulated that ADRB3 has a major role in thermogenesis in brown adipocytes (Zhao et al. 1998; Cannon and Nedergaard 2004). Deletion of ADRB3 alone in mice impairs β -AR-induced lipolysis in white adipocytes and causes a moderately obese phenotype (Susulic et al. 1995; Revelli et al. 1997); however,

ADRB3 knockout mice exhibit no defect in cold-induced thermogenesis in brown adipocytes (Mattsson et al. 2011; Preite et al. 2016; de Jong et al. 2017). Upon cold exposure, ADRB3 mRNA and protein expressions are decreased; however, expression of ADRB1 is rapidly induced (Bengtsson et al. 1996, 2000; Abe et al. 2015). ADRB1 knockout mice are cold intolerant and are more susceptible to high-fat diet-induced obesity (Ueta et al. 2012). In addition, deletion of the histone demethylase JMJD1A, which regulates *Adrb1* mRNA expression in brown adipocytes, prevents *Adrb1* induction and impairs acute thermogenesis in brown adipocytes upon cold stress (Abe et al. 2015). The current most acceptable theory is that among three subtypes of ADRB, ADRB1 predominantly regulates acute thermogenesis in brown adipocytes upon cold stress.

Roles for ADRB subtypes in beige adipogenesis remain controversial. Beige adipogenesis requires activation of both β -AR signaling and the PPAR γ transcriptional cascade. The former is regulated by sympathetic tone (Zhu et al. 2016), while the latter is regulated by PPAR γ ligands such as thiazolidinedione TZD in the cell culture model (Ohno et al. 2012). Deletion of ADRB3, which is essential for β -AR-induced lipolysis in white adipocytes, inhibits beige adipogenesis in C57BL/6J and 129/Sv mouse strains (Jimenez et al. 2003; Barbatelli et al. 2010), while its deletion in FVB/N mouse strain has no effects on induction of thermogenic genes in white adipose tissue (de Jong et al. 2017). A comparison of C57BL/6J, 129/Sv, and FVB/NJ mouse strains shows that FVB/NJ has a more robust beige adipogenic response and is therefore more tolerant to cold stress than the others (Ferrannini et al. 2016). This suggests that genetic background can influence the relative contribution of ADRB subtypes in beige adipogenesis upon chronic cold stress, and this warrants further investigation.

15.2 β -AR Signaling Facilitates Rapid Transcription of Thermogenic Genes in Brown Adipocytes

The mitochondrial uncoupling protein UCP1 is uniquely expressed in brown adipocytes to mediate non-shivering thermogenesis (reviewed in Porter et al. (2015)). Most studies to date have been primarily focused on the regulatory mechanisms that control brown adipocytes' specific expression of *Ucp1* mRNA (Kozak et al. 1994; Cao et al. 2004; Rim et al. 2004). The thermogenic transcription program also includes additional genes important for the uptake of energetic substrates including glucose and fatty acids, as well as genes that accelerate energy consumption by mitochondrial respiration.

Brown adipocytes constitutively express *Ucp1* mRNA and protein to maintain basal thermogenesis. The *Ucp1* locus in brown adipocytes has active epigenomic modifications characteristic of euchromatin (i.e., H3K4 methylation and H3K27 acetylation) and very low levels of repressive epigenomic modifications (i.e., H3K9 methylation and H3K27 methylation) (Fig. 15.1, left). Upon cold stress, brown adipocytes transcriptionally upregulate *Ucp1* to maximize their thermogenic capacity. For example, when cultured brown adipocytes are treated with isoproterenol, a non-selective ADRB agonist, the expression of *Ucp1* mRNA in brown adipocytes is induced by more than 10-fold in 60 min (Abe et al. 2015). The rapid induction of *Ucp1* expression is mediated by the phosphorylation of transcription factors and coactivators in β -AR signaling pathways. PKA directly phosphorylates the transcription factor CREB (cAMP response elements binding-protein) (Michael et al. 2000) (also reviewed in Altarejos and Montminy (2011)). PKA also facilitates p38/MAPK-mediated phosphorylation of the transcription factor ATF2 (activating transcription factor 2) and the transcription coactivator PGC1 α (PPAR γ coactivator-1 α) (Cao et al. 2004) (Robidoux et al. 2005; also reviewed in Kajimura et al. (2015) and Inagaki et al. (2016)). PGC1 α regulates the expression of genes involved in mitochondrial biogenesis, energy consumption, and thermogenesis (e.g.,

Ucp1) as a transcriptional coactivator of PPAR γ (Puigserver et al. 1998; Uldry et al. 2006). Genome-wide analysis of mRNA expression revealed that ~70 genes related to thermogenesis are upregulated 60 min after activation of β -AR signaling in brown adipocytes (Abe et al. 2015). Thus, β -AR signaling and downstream transcription factors and co-factors mediate the rapid induction of thermogenic genes upon acute cold stress in brown adipocytes.

15.3 β -AR Signaling Facilitates Rapid Chromatin Looping in Brown Adipocytes

Three-dimensional (3D) chromatin organization (Box 15.4) plays an important role in the regulation of tissue-specific gene expression to complement simple changes of nucleosomal histone residues. Development of classical brown adipocytes occurs through two phases: lineage commitment of precursor cells, followed by the differentiation from brown preadipocytes to mature brown adipocytes. During the differentiation of brown preadipocytes, PRDM16 (see Box 15.1) binds to *Ucp1* and *Pgc1 α*

loci and recruits the mediator MED1 to facilitate promoter–enhancer interactions via chromatin looping (Harms et al. 2015). However, it was not clear whether β -AR signaling and a downstream phosphorylation cascade facilitate promoter–enhancer interaction of thermogenic genes via chromatin looping, in particular in response to acute cold stress.

Chromosome conformation capture (3C) is a method to determine 3D chromatin structures and interactions (e.g., the interaction between enhancers and a promoter) in vivo. By 3C analysis, it was recently revealed that β -AR signaling facilitates promoter–enhancer interaction of thermogenic genes via chromatin looping during acute cold stress in brown adipocytes (Abe et al. 2015). Upon activation of β -AR signaling downstream of sympathetic nerve stimulation, PKA phosphorylates histone demethylase JMJD1A (Jumonji domain-containing 1A, also known as KDM3A) at Ser265 in brown adipocytes (Abe et al. 2015). JMJD1A is a histone demethylase specific to lysine 9 of histone H3 (H3K9) and preferentially demethylates mono- and dimethylated H3K9 (H3K9me1 and H3K9me2). JMJD1A is involved in self-renewal, spermatogenesis, and embryonic development.

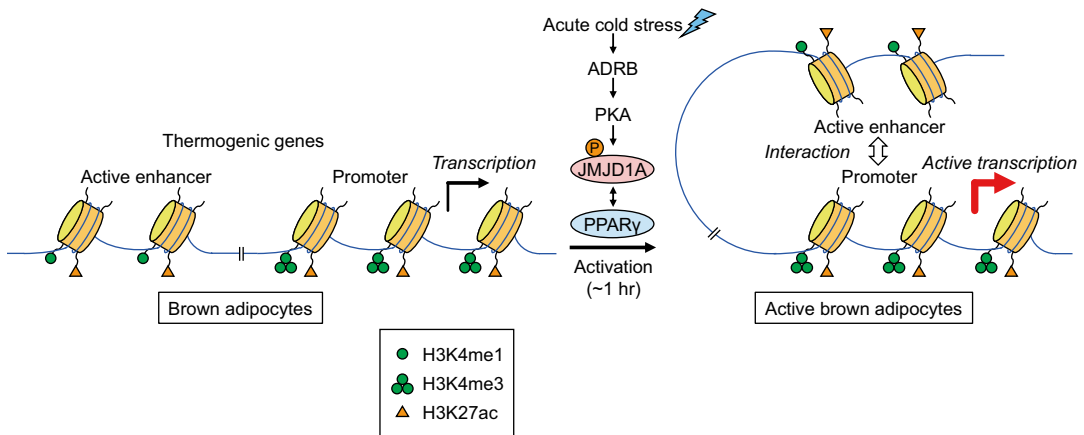


Fig. 15.1 Rapid chromatin conformation change of thermogenic genes in pre-existing brown adipocytes upon acute cold stress. Brown adipocytes constitutively express thermogenic genes, which have active histone modifications (left). Promoters of thermogenic genes have H3K4me3 and H3K27ac modifications, whereas enhancers have active H3K4me1 and H3K27ac modifications. Repressive H3K27me3 and H3K9me2 are scarcely observed on thermogenic genes in brown adipocytes (Abe et al. 2015, 2018). Upon acute cold stress, ADRB/PKA pathway phosphorylates

various substrates including metabolic enzymes, transcription factors, and chromatin factors. Among them, histone demethylase JMJD1A is directly phosphorylated at Ser265 by PKA. Phosphorylated JMJD1A interacts with PPAR γ and SWI/SNF chromatin remodeler complex (not shown in the figure) and facilitates enhancer–promoter interaction by chromatin looping within 1 h (Abe et al. 2015). This rapid chromatin conformation change allows the induction of thermogenic genes in pre-existing brown adipocytes upon acute cold stress (right)

genesis, sex determination, normal body weight control, thermogenesis, and so on. Phosphorylated JMJD1A recruits SWI/SNF chromatin remodeling factors together with PPAR γ and binds to PPAR response elements (PPRE) on thermogenic genes (e.g., *Ucp1* and *Adrb1*). The promoter–enhancer interaction via chromatin looping on *Adrb1* occurs within 15 min and peaks at 60 min after activation of β -AR signaling, and the interaction is correlated with the level of JMJD1A phosphorylation and *Adrb1* expression (Abe et al. 2015) (Fig. 15.1, right). Interestingly, the acute induction of thermogenic genes in brown adipocytes does not require histone demethylase activity by JMJD1A (Abe et al. 2015). Additionally, it was subsequently shown that mice harboring an S265A phospho-defective mutation of *Jmjd1a* failed to maintain body temperature during cold exposure (Abe et al. 2018).

These studies were in contrast to an earlier study by Tateishi et al. that concluded induction of *Ucp1* in brown adipocytes under activation of β -AR signaling required the histone demethylase activity of JMJD1A and removal of repressive H3K9me2 from the *Ucp1* locus (Tateishi et al. 2009). Thermogenic genes in brown adipocytes are constitutively expressed at relatively high levels beginning during the neonatal stage and their genomic loci have active histone modifications characteristic of euchromatin (i.e., H3K4 methylation and H3K27 acetylation) before cold stress (Fig. 15.1, left). In addition, repressive histone methylation at these genes (i.e., H3K9me2) is very low even before cold exposure (Fig. 15.1, left), and this is hardly affected by short time (several hours) activation of β -AR signaling (Abe et al. 2015, 2018). Because alterations in histone methylation are usually associated with more long-term lasting events such as terminal cell differentiation (Bintu et al. 2016) (also reviewed in Klose and Zhang (2007) and Barth and Imhof (2010)), animals could not effectively adapt to short-term cold exposure if epigenomic methylation alterations were required to rapidly regulate thermogenic genes. Therefore, upon acute cold stress, it is effective for animals to immediately enhance the thermogenic capacity of pre-existing brown adipocytes

via chromatin looping without epigenomic changes of histone methylation. In this context, JMJD1A protein functions as a sensing molecule of β -AR signaling to facilitate chromatin looping and maximize thermogenic activity in pre-existing brown adipocytes rather than as a histone demethylase.

Box 15.4 Three-Dimensional (3D) Chromatin Conformation

During development and cellular differentiation, 3D chromatin conformation changes are required for the regulation of tissue-specific gene expression in addition to post-translational epigenomic changes. 3D chromatin conformation regulates promoter–enhancer interactions via chromatin looping. Promoters are located near the transcription start sites of genes, and general transcription factors bind to promoters to initiate gene transcription. Enhancers are cis-regulatory elements that are often located up to several hundreds of megabases away from target genes and they interact with promoters via chromatin looping to enhance gene transcription (Heinz et al. 2015). The zinc finger protein CTCF (CCCTC-binding factor) (Ong and Corces 2014), cohesin complexes (Singh and Gerton 2015), and the Switch/sucrose non-fermentable (SWI/SNF) chromatin remodeling factors (Wilson and Roberts 2011) regulate chromatin conformation and looping. CTCF is a transcription factor whose primary role is in the regulation of chromatin 3D structure (Ong and Corces 2014). CTCF is also involved in part in establishing insulators, which are regulatory domains that block the interaction between enhancers and promoters. SWI/SNF is a chromatin remodeling protein complex that “loosens” the chromatin structure in an ATP-dependent manner. SWI/SNF also regulates promoter–enhancer interaction by facilitating chromatin looping (Wilson and Roberts 2011).

For example, an enhancer within an intron of *FTO* (fat mass and obesity-associated protein) regulates the expression of *IRX3* and *IRX4*, which encode homeobox transcription factors involved in the repression of beige adipocyte differentiation, via interactions that occur with regulatory elements located several megabases apart in the linear genome (Smemo et al. 2014; Herman and Rosen 2015). Single-nucleotide variants within the *FTO* intron influence beige adipocyte differentiation and are associated with an increased risk for obesity (Smemo et al. 2014; Herman and Rosen 2015). Hi-C is a method to analyze the genome-wide 3D structure by capturing interactions between all genomic regions, and promoter capture Hi-C is a derivative method of Hi-C to specifically analyze the interactions between promoters and all distal regions. Recent genome-wide approaches using Hi-C and promoter capture Hi-C analyses demonstrate tissue-specific and differentiation-specific 3D chromatin conformations that regulate lineage commitment and differentiation (Phillips-Cremins et al. 2013; Siersbaek et al. 2017).

Several recent studies show that 3D chromatin conformation also changes in much shorter-term biological responses than development or cellular differentiation. During the 24-h light–dark cycle, the transcription factor Rev-erb α rhythmically modulates chromatin looping to regulate circadian gene transcription in the liver by recruiting the NCoR-HDAC3 repressive complex and evicting looping factor MED1 (Kim et al. 2018). During fasting, corticosteroid hormone induces mRNA expression of histone methyltransferase SETDB2 in the liver, and then SETDB2 promotes 3D chromatin conformation changes within 2 h to facilitate gluconeogenesis (Roqueta-Rivera et al. 2016). A more rapid example is histone demethylase

JMJD1A, which facilitates promoter–enhancer interaction of thermogenic gene via chromatin looping within 1 h in brown adipocytes upon β -AR activation downstream of cold stress (Abe et al. 2015).

15.4 Chronic Thermogenic Response in Subcutaneous White Adipose Tissue Beige-ing

When cold stress persists, a fraction of subcutaneous white adipose tissues (scWAT), which do not normally express thermogenic genes before cold stress, are remodeled to induce thermogenic genes in order to adapt to chronic cold exposure. This process is called “browning” or “beige-ing,” and it is an adaptation in response to long-term cold exposure and is accompanied by de novo differentiation of preadipocytes to beige adipocytes and/or trans-conversion of white adipocytes to beige adipocytes (Wang and Seale 2016). When mice are kept at 4 °C for at least 1 week, UCP1-positive beige adipocytes are observed in scWAT. Differentiation of preadipocytes to beige adipocytes in cell culture requires activation of both β -AR signaling and PPAR γ transcriptional cascade for more than 1 week (Ohno et al. 2012; Abe et al. 2018). Activation of β -AR signaling by adipocyte-specific ectopic expression of forkhead transcription factor FOXC2 upregulates the RI α subunit of PKA to facilitate beige-ing in scWAT (Cederberg et al. 2001).

As mentioned in the previous section, thermogenic genes in brown adipocytes have active histone modifications (e.g., H3K4 methylation and H3K27 acetylation) characteristic of euchromatin under thermoneutral conditions, and this is essential to maintain high basal transcription for normal brown adipocyte function (Fig. 15.1, left) (Abe et al. 2015, 2018). In contrast, in preadipocytes and white adipocytes of scWAT, thermogenic genes have repressive histone modifications (H3K27 methylation and H3K9 methylation) characteristic of heterochromatin that silence

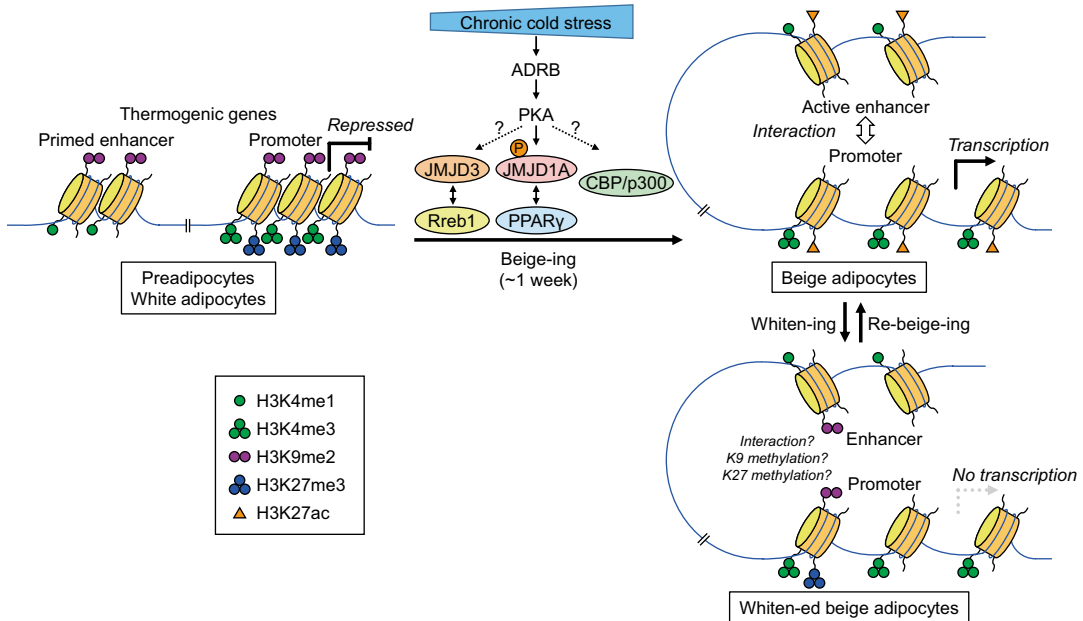


Fig. 15.2 Epigenomic alterations of thermogenic genes during beige-ing and whiten-ing of adipocytes under prolonged cold and warm environments. Thermogenic genes in preadipocytes of scWAT have repressive H3K9me2 and H3K27me3 on promoter and are not expressed (left) (Pan et al. 2015; Abe et al. 2018). Enhancers in preadipocytes have H3K4me1 but not H3K27ac and are in a primed state. Beige-ing takes chronic exposure of 1 week or longer in both mice and cell culture models. Among several chromatin factors involved in beige adipogenesis, only JMJD1A is known to be phosphorylated by PKA downstream from β -AR signaling. During beige-ing, JMJD3 interacts with transcription factor Rreb1 to facilitate the removal of repressive H3K27me3 from thermogenic genes (Bintu et al. 2016). Phosphorylated JMJD1A interacts with PPAR γ together with PGC1 α and PRDM16 (not shown in Figure) to facilitate the removal of repressive H3K9me2 from thermogenic genes (Abe et al. 2018). Histone acetyltransferase

CBP/p300 facilitates histone acetylation of H3K27. Enhancers in beige adipocytes have both H3K4me1 and H3K27ac and are in an active state. These epigenomic alterations are necessary for beige adipogenesis and activation of thermogenic genes (upper right). Enhancers and promoters of thermogenic genes should interact in beige adipocytes as observed in brown adipocytes although there are currently no direct data to show this. When the warm environment is prolonged, the H3K27ac mark on enhancers of thermogenic genes is erased, whereas H3K4me1 is retained during the whiten-ing of beige adipocytes (lower right) (Roh et al. 2018). It is unknown whether other epigenomic modifications (i.e., H3K27me3 and H3K9me2) are altered during the whiten-ing of beige adipocytes. It is also unknown whether promoters and enhancers of thermogenic genes interact in whitened beige adipocytes. Upon re-exposure to cold, the H3K27ac mark is re-introduced, and thermogenic genes are re-activated

transcription in these depots at thermoneutrality (Fig. 15.2, left) (Pan et al. 2015; Abe et al. 2018). Therefore, repressive histone modifications on thermogenic genes in scWAT need to be erased during the beige-ing process. However, it was enigmatic how β -AR signaling regulates both the short-term response in brown adipocytes (<1 h) and long-term adaptation accompanied by histone modification changes in beige-ing (>1 week) upon cold stress. A recent study by Abe et al. has resolved this enigma (Abe et al. 2018) at least in part as described below.

15.5 β -AR Signaling Facilitates Epigenomic Alteration during scWAT Beige-ing

Abe et al. showed that beige-ing of scWAT under chronic cold stress is severely attenuated in mice with a homozygous mutation (S265A) in *Jmjd1a* that prevents its phosphorylation downstream of β -AR signaling (Abe et al. 2018). They showed that during preadipocyte differentiation to beige adipocytes, persistent

activation of β -AR signaling facilitates JMJD1A phosphorylation mediated by PKA (Abe et al. 2018). Once phosphorylated, JMJD1A then forms a complex with PRDM16, PGC1 α , and PPAR γ , and this complex is recruited to distal enhancers of thermogenic genes (Abe et al. 2018). JMJD1A in turn removes repressive H3K9me2 and facilitates the induction of thermogenic gene expression (Fig. 15.2, upper right) (Abe et al. 2018; Takahashi et al. 2022; Matsumura et al. 2023). As covalent histone methylation is generally more stable than protein phosphorylation, it functions as a form of “epigenetic memory” which allows stable expression of thermogenic genes under a chronic cold environment. Thus, JMJD1A senses β -AR signaling and converts it to epigenetic memory in scWAT during chronic cold stress.

In addition to the demethylation of H3K9me2, the demethylation of H3K27me3 from thermogenic genes is another important step for gene induction during the differentiation of preadipocytes to beige adipocytes. H3K27me3 is also a repressive histone mark that silences transcription. Thermogenic genes in preadipocytes derived from scWAT have bivalent histone modifications that contain both repressive H3K27me3 and active H3K4me3 in the same nucleosome that are transcriptionally repressed but poised for activation. JMJD3, a histone H3K27 demethylase, facilitates the removal of this repressive mark from thermogenic genes to induce their expression during beige adipogenesis (Pan et al. 2015). Because the induction of thermogenic genes requires demethylase activity by JMJD3, epigenetic alterations during cellular differentiation are essential for chronic adaptation to cold. However, whether cold exposure/ β -AR signaling activation directly regulates JMJD3 leading to the demethylation of H3K27 remains to be determined. It is also still unclear how coordinate demethylation of H3K27 and H3K9 precisely alters chromatin structure to activate thermogenic genes during beige adipogenesis.

15.6 Reversible Enhancer Epigenomic Alteration in Beige-ing and Whiten-ing of scWAT

The histone modifications characteristic of enhancers are H3K4me1 and H3K27 acetylation (H3K27ac) (Calo and Wysocka 2013). Prior to activation, enhancers are thought to exist in a poised state as “primed enhancers” characterized by the presence of H3K4me1 (see Fig. 15.2, left) (Creyghton et al. 2010; Rada-Iglesias et al. 2011; Lee et al. 2013; Matsumura et al. 2021). To become “active enhancers,” primed enhancers are further acetylated by histone acetyltransferases (CBP/p300) to generate the combination of H3K4me1 and H3K27ac (see Fig. 15.2, upper right) (Zentner et al. 2011; Bonn et al. 2012; Lai et al. 2017; Matsumura et al. 2021). The presence of H3K27ac distinguishes enhancers in the active versus the primed state. Consequently, enrichment of H3K27ac at distal enhancers shows a high degree of cell-type specificity (reviewed in Calo and Wysocka (2013) and Heinz et al. (2015)).

Exposure of mice to cold for 1-week leads to the presence of beige adipocytes in scWAT, however, “re-warming” (i.e., housing mice back to the thermoneutral temperature from cold) results in morphological changes called “whiten-ing” that are accompanied by a reduction in expression of thermogenic genes in beige adipocytes. In beige adipocytes, enhancers of thermogenic genes are “active enhancers” having both H3K4me1 and H3K27ac. However, in whitened beige adipocytes, enhancers of thermogenic genes lose H3K27ac but still retain H3K4me1, indicating that those active enhancers changed back to primed enhancers (Fig. 15.2, lower right) (Roh et al. 2018). This finding implies that H3K27ac is reversible in response to shifts in ambient temperature to regulate thermogenic gene expression. Interestingly, however, H3K4me1 was not reversed. In this context, H3K4me1 functions as an example of epigenomic memory. This memory appears to provide a clear advantage as mice that experienced

one round of long-term cold stress are highly adaptive to a second bout of cold stress. During the second round of exposure to cold, they express thermogenic genes in the scWAT depot more rapidly and robustly to maintain body temperature (Roh et al. 2018). This cellular memory is a well-organized adaptive system for animals to prepare for future cold stress. Other than H3K27ac in distal enhancers, whether other histone modifications (e.g., H3K9me2 and H3K27me3) are altered upon re-warming of scWAT remains to be determined.

15.7 A Histone Demethylase JMJD1A Coordinates Both Acute and Chronic Adaptation to Cold Stress by Distinct Modes of Action

When animals are exposed to environmental changes, a quick response is essential for survival and if the stress persists, animals gradually adapt to maintain homeostasis. In the case of cold stress, non-shivering thermogenesis in BAT is a first-aid response, while beige adipogenesis in scWAT is a more lasting chronic response. Although temporal axes of acute response (<1 h) and chronic adaptation (>1 week) are different, the signal-sensing molecule JMJD1A plays essential roles in both responses. During the acute phase, JMJD1A senses β -AR signaling via its PKA-dependent S265 phosphorylation, which transduces rapid chromatin conformational changes of thermogenic genes for their rapid activation in brown adipocytes through chromatin looping (Abe et al. 2015; Ito et al. 2024). This acute response in brown adipocytes does not require histone modification changes of thermogenic genes because these genes in brown adipocytes are associated with euchromatin and are therefore already transcriptionally active, while, in scWAT, these genes are not constitutively expressed and they do not reside in active euchromatin. Thus, in the chronic phase, the persistent β -AR signaling facilitates the demethylation of histones that requires the demethylase activity of JMJD1A to stably change chromatin methylation

status (epigenomic memory) on thermogenic genes in scWAT. This enables sustained expression of thermogenic genes in beige adipocytes during long-term cold exposure (Abe et al. 2018; Ito et al. 2024). The acute and chronic roles of JMJD1A in BAT and scWAT provide a robust and complementary biological defense system for survival in response to both rapid and transient as well as more sustained periods of cold stress. Thus JMJD1A, sensing β -AR signaling, regulates a concerted thermogenic response in BAT and scWAT via two different mechanisms: one that is H3K9me2 independent and the other that is H3K9me2 dependent, respectively.

Many epigenomic enzymes assemble into heteromeric species with other chromatin modification proteins in complexes that range from relatively simple heterodimers such as between GLP1 and G9a (Tachibana et al. 2008) all the way to larger multiprotein complexes. While the biochemical or physiologic reasons for the evolution of these multi-subunit complexes are open to speculation, it is clear from the comparisons of complete gene knockout studies versus gene mutants engineered to express full-length catalytically dead variants that, in many cases, the catalytically dead versions are assembled into complexes that retain biological function whereas the gene knockouts do not. This is true for the function of JMJD1A in beige-ing discussed earlier, but it is also true for chromatin-dependent regulation by LSD1, GLP1, SETDB1, and SETDB2 where all are required for complex functions where their enzyme activity is dispensable (Tachibana et al. 2008; Roqueta-Rivera et al. 2016; Qin et al. 2021; Zhang et al. 2021).

15.8 Concluding Remarks and Future Perspectives

Two types of thermogenic adipocytes, pre-existing brown adipocytes and inducible beige adipocytes, protect animals from cold stress. β -AR signaling plays a central role in changes in gene expression required for cold-induced adaptive thermogenesis in both adipose depots; however, it regulates the activation of brown adipocytes and induction of

beige adipocytes in mechanistically distinct ways. Upon cold stress, β -AR signaling facilitates PKA-mediated phosphorylation of various proteins in brown adipocytes and induces thermogenic genes via rapid chromatin looping. In contrast, prolonged activation of β -AR signaling facilitates the remodeling of white adipose tissue where beige adipocytes are newly generated in scWAT by epigenomic changes and de novo differentiation. The studies discussed in this chapter highlight that β -AR signaling not only functions through transient phosphorylation signaling cascades required during acute stress responses but also relays a more sustained β -AR signal to increase expression of thermogenic genes that are maintained in repressed chromatin in scWAT depots by histone demethylation via the action of JMJD1A. Although recent studies have clarified the acute and chronic roles of β -AR signaling in cold adaptation, there still remain many unanswered questions. (1) Are there any other epigenomic enzymes that are regulated by phosphorylation by ADRB/PKA signaling? (2) Are the actions of JMJD1A and JMJD3 coordinated to facilitate the beige response? (3) Are there any other epigenomic modifications regulated by β -AR signaling during differentiation of beige adipocytes? (4) Is there any cross-talk between different types of histone methylations and acetylations to cooperatively regulate each other during beigeing and whitening of adipocytes? (5) How does β -AR signaling regulate genome-wide chromatin organization of adipocytes upon chronic cold stress? (6) Does β -AR signaling regulate epigenomic modifications and chronic adaptation in other types of cells that respond to β -AR stimulation (e.g., cardiomyocytes, skeletal myocytes, lung cells, cancer, and immune cells)? It would be also important to investigate a putative chronic role of β -AR signaling in other tissues responding to chronic stress because modulation of β -AR signaling by ADRB antagonists and agonists have been used in treatments for many human diseases (e.g., hypertension, heart failure, chronic obstructive pulmonary disease, cancer, and infectious diseases) (Wachter and Gilbert 2012; Baker and Wilcox 2017; Tang et al. 2013; Suzuki et al. 2016) and is related to cell differentiation (reviewed in Hajifathali et al. (2014)). Thus, further investiga-

tion of molecular mechanisms by which β -AR signaling regulates chronic adaptation through possible additional pathways influencing “epigenomic memory” will provide important insights into human health and strategies to prevent and treat lifestyle-related diseases.

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Mitophagy Responds to the Environmental Temperature and Regulates Mitochondrial Mass in Adipose Tissues

Shun-ichi Yamashita and Tomotake Kanki

Abstract

There are at least two types of adipose tissues in the body, defined as brown adipose tissues (BATs) and white adipose tissues (WATs). These tissues comprise brown and white adipocytes, respectively. The adipocytes are commonly endowed with mitochondria, but they have diverse characteristics and roles. Brown adipocytes have abundant mitochondria that contribute to the β -oxidation of fatty acids to produce chemical energy and the production of heat via uncoupling of the mitochondrial membrane potential from ATP synthesis. Alternatively, white adipocytes have fewer mitochondria that contribute to the generation of free fatty acids via lipogenesis by providing key intermediates. Besides the described types of adipocytes, brown-like adipocytes, termed beige adipocytes, are developed in WAT depots during cold exposure. Beige adipocytes also contribute to thermogenesis. Notably, beige adipocytes may transform into white-like adipocytes after the withdrawal of cold exposure. This process is marked by the elimination of mitochondria through the acti-

vation of mitochondria autophagy (mitophagy). This review aims to describe the mitophagy that occurs during the beige-to-white transition and discuss recent insights into the molecular mechanisms of this transformation. Additionally, we describe the mitophagy monitoring strategy in adipose tissues using three independent reporter systems and discuss the availabilities and limitations of the method.

Keywords

Mitochondria · Mitophagy · Autophagy · Beige adipocytes · Cold exposure · Thermogenesis · Beige-to-white transition

16.1 Introduction

Mitochondria play essential roles in the regulation of intracellular metabolism and cell function, including control over ATP synthesis, calcium buffering, apoptosis, β -oxidation of fatty acids, and thermogenesis (Brookes et al. 2004; Gamber and Ricquier 2007; McBride et al. 2006; Wallace 2005; Wanders et al. 2010). They are found in almost all cells in the body. Therefore, the maintenance of mitochondrial quality and quantity is important for normal tissue and organ functions (Nakada et al. 2001). Mitophagy is a specific intracellular degradation pathway that eliminates

S.-i. Yamashita (✉) · T. Kanki
Department of Cellular Physiology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
e-mail: yamashita.shunichi.029@m.kyushu-u.ac.jp;
kanki.tomotake.114@m.kyushu-u.ac.jp

excessive and dysfunctional mitochondria using autophagy (Kurihara et al. 2012). Thus, mitophagy is thought to be important for maintaining mitochondrial homeostasis (Kurihara et al. 2012).

There are two main types of adipose tissues in the body, including brown adipose tissue (BAT) and white adipose tissue (WAT) (Sanchez-Gurmaches et al. 2016). The primary role of BAT (brown adipocytes) is to produce heat through energy expenditure, whereas WAT (white adipocytes) stores excess energy in the form of free fatty acids (FFAs) as triglycerides (TGs). Brown adipocytes contain a lot of mitochondria that express high levels of uncoupling protein 1 (UCP1) in the inner mitochondrial membrane (IMM) (Cannon and Nedergaard 2004). UCP1 uncouples the mitochondrial membrane potential from the ATP synthesis to produce heat in a cold environment, thus maintaining the body temperature (Cannon and Nedergaard 2004). Alternatively, white adipocytes contain a smaller number of mitochondria which contribute to lipogenesis via the synthesis of its key intermediates and store TGs as energy reserve (Giralt and Villarroya 2013).

Besides these adipocytes, inducible brown-like adipocytes, called beige adipocytes, have been reported (Cousin et al. 1992; Young et al. 1984). Exposure to cold temperatures or treatment with β 3-AR agonist may transform some of

the WAT adipocytes that acquire brown adipocyte-like features (Kozak 2011; Nedergaard and Cannon 2014; Sidossis et al. 2015). Interestingly, beige adipocytes were shown to regain white adipocyte-like cell characteristics shortly after the withdrawal of cold exposure or β 3-AR agonists (Gospodarska et al. 2015; Rosenwald et al. 2013). The process was termed the beige-to-white transition and was marked by the elimination of mitochondria via mitophagy, contributing to body temperature control (Gospodarska et al. 2015; Rosenwald et al. 2013).

In this review, we summarize the mitochondrial function in different adipocytes, focusing on the regulation of mitochondrial mass by mitophagy during the beige-to-white transition. Furthermore, we discuss several mitophagy monitoring strategies in adipose tissues.

16.2 Mitochondrial Function in Adipose Tissues

Although all three types of adipocytes are commonly endowed with mitochondria, mitochondrial characteristics differ between brown/beige adipocytes and white adipocytes, resulting in adipocyte functional differences (Fig. 16.1) (Sanchez-Gurmaches et al. 2016). In this section,

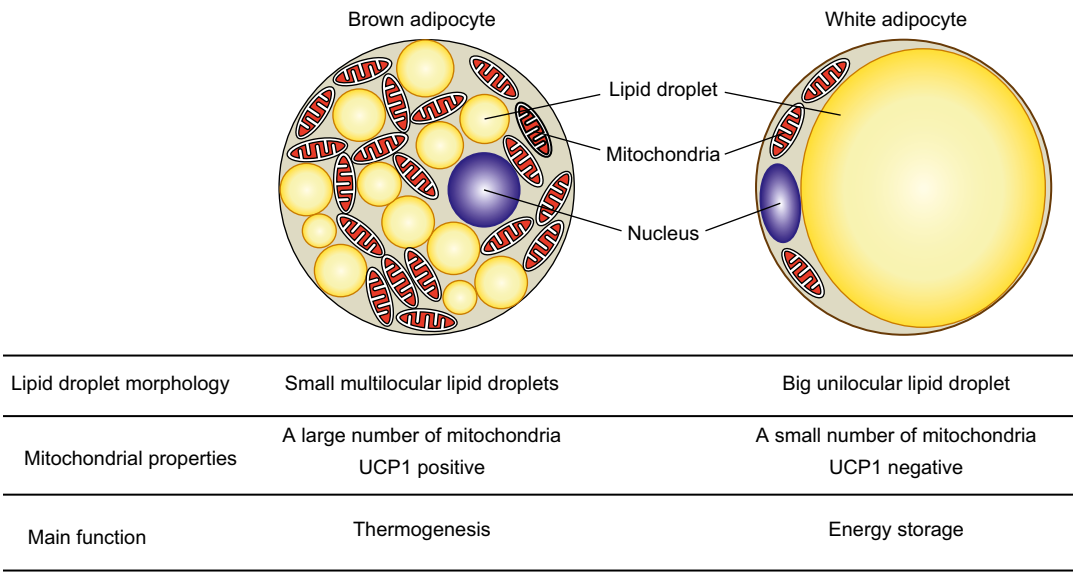


Fig. 16.1 Characteristics and functions of different types of adipocytes

we will discuss the role of mitochondria in each type of adipocyte and explain the association between mitochondrial differences and cell functions.

16.2.1 Brown Adipocyte Mitochondria

BAT depots are primarily found in interscapular, axillary, and cervical regions of the body. Brown adipocytes have distinct characteristics, including numerous mitochondria, small multilocular lipid droplets, and high expression of UCP1 in the IMM (Fig. 16.1a). In response to cold exposure, β_3 -AR is activated by norepinephrine (NE) which is released from sympathetic neurons. NE stimulates downstream cyclic AMP (cAMP)-protein kinase A (PKA) signaling pathway that upregulates the UCP1 expression and lipolysis. Produced FFAs during lipolysis are oxidized in mitochondria to generate chemical energy (Lehr et al. 2006; Nicholls 2006; Sluse et al. 2006). The FFAs are also utilized to activate UCP1 (Fedorenko et al. 2012). The activated UCP1 then uncouples the mitochondrial membrane potential to produce heat, which gives rise to non-shivering thermogenesis (NST). Furthermore, to sustain the NST and produce the chemical energy in mitochondria, brown adipocytes uptake extracellular glucose and FFAs (Bartelt et al. 2011; Labbe et al. 2015). The NST plays a vital role in the maintenance of the body temperature in hibernating animals, small rodents, and human infants. Notably, it has been widely believed that adult humans lack BAT depots. However, several recent reports demonstrated the existence of UCP1-positive BAT depots. The discovery was made using ^{18} fluoro-labeled 2-deoxyglucose positron emission tomography/computed tomography (^{18} FDG-PET-CT) and the biopsy analysis (Cypess et al. 2009; Nedergaard et al. 2007; Saito et al. 2009; van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009). The amount of human BAT inversely correlates with body mass index (BMI) and adiposity, suggesting the benefits of BAT activation as a therapeutic approach against obesity (Ouellet et al. 2012). Therefore, it is important to understand the mechanism that regulates

mitochondrial function, number, and thermogenesis (energy expenditure) in brown adipocytes.

16.2.2 White Adipocyte Mitochondria

Compared with brown adipocytes, white adipocytes have unilocular large lipid droplets and fewer UCP1-negative mitochondria (Fig. 16.1). The primary role of white adipocytes is to generate FFAs via de novo lipogenesis and to store them as TGs. During de novo lipogenesis, mitochondria-located reactions contribute to the content of intracellular acetyl-CoA, a key intermediate component required for lipogenesis (De Pauw et al. 2009). Furthermore, in white adipocytes, due to low level of acyl-CoA dehydrogenase and low activity of carnitine palmitoyl transferase 1, enzymes of fatty acid β -oxidation, FFAs are directed toward esterification, resulting in the generation of TGs and formation of lipid droplet (Kopecky et al. 2001; Yehuda-Shnaidman et al. 2010). However, under fasting conditions, white adipocytes may generate FFAs from TGs. The FFAs are released into the bloodstream as a source of energy for other tissues, including cardiac and skeletal muscle, and BAT (Lopez-Ibarra et al. 2015). Like brown adipocytes, the lipolysis is triggered by neurons of the sympathetic nerve system via activation of β_3 -AR and cAMP-PKA pathway. Insulin signaling and the PI3K-Akt pathway were shown to inhibit lipolysis (Collins et al. 2004; Holm et al. 2000).

16.2.3 Mechanisms of Beige Adipocyte Development

As described above, beige adipocytes demonstrated similar characteristics to brown adipocytes. However, beige adipocytes were shown to be developed in WAT depots upon cold exposure, and thus, they resemble white adipocytes under normal housing temperature conditions. Two hypotheses were proposed about the developmental origin of beige adipocytes. It has been demonstrated that beige adipocytes may arise from pre-existing fully differentiated white adi-

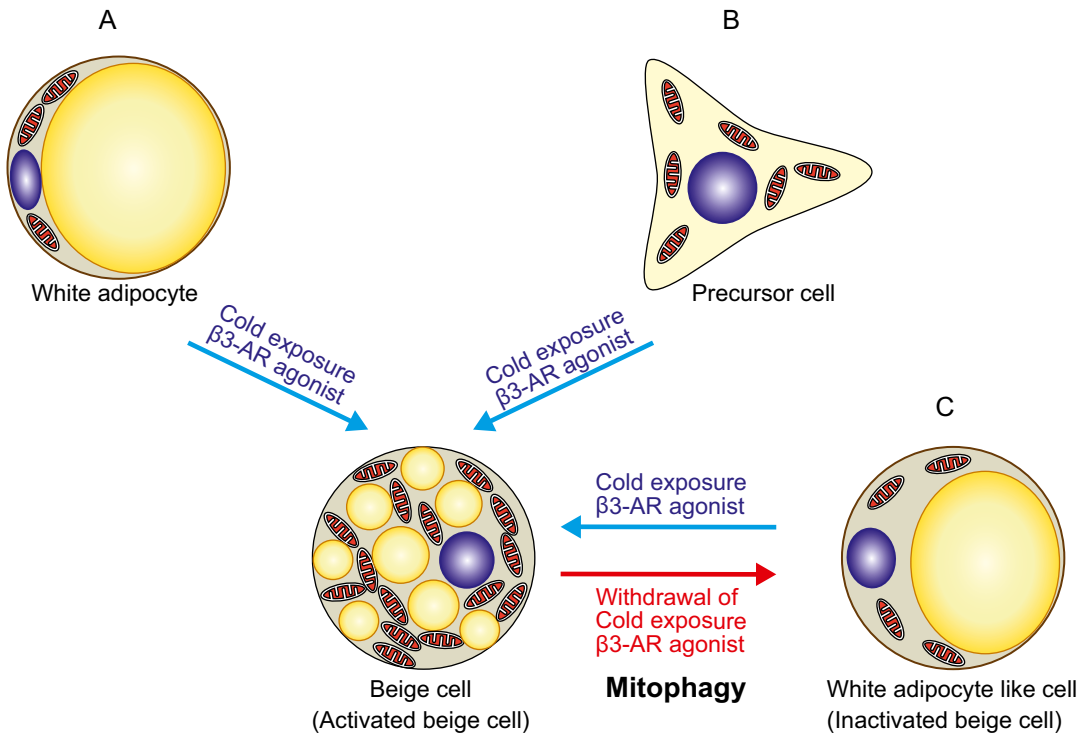


Fig. 16.2 Hypothetical origins of beige adipocytes. (a) Beige adipocyte originates from a pre-existing white adipocyte. (b) Beige adipocyte originates from a specific

precursor cell. (c) Beige adipocyte originates from its own inactive form during cold exposure

pocytes (trans-differentiation theory) (Fig. 16.2a) (Barbatelli et al. 2010; Himms-Hagen et al. 2000; Lee et al. 2015). Others found that beige adipocytes may be developed from specific precursor cells residing in WAT depots (Fig. 16.2b) (Wang et al. 2013). There are many reports for supporting both possibilities, and therefore, the origin of beige adipocytes remains debatable. Interestingly, it has been reported that beige adipocytes acquire a white-like phenotype when animals are withdrawn from cold temperatures (Rosenwald et al. 2013). However, the adipose cells in these animals start to re-express thermogenic genes again in response to another session of cold exposure (Rosenwald et al. 2013). These data suggest that if the mice have experienced a certain cold environment previously, some adipocytes in the WAT depot have already differentiated into beige adipocytes and regained white adipocyte characteristics at least once (Kajimura et al. 2015). Conclusively, the development of

beige adipocytes is likely the simple activation of the inactive form of these cells (Fig. 16.2c). To confirm the beige adipocyte origin and the differentiation process, it is required to examine the adipose tissues of mice bred at strictly controlled temperatures throughout their life.

16.3 Activation of Mitophagy in Adipose Tissue

As mentioned in the section above, mitochondrial biogenesis and thermogenic genes are increased in beige adipocytes in response to cold exposure. Interestingly, after withdrawal of cold or β3-AR agonist treatment, beige adipocytes lose the brown adipocyte-like properties and become white adipocyte-like cells. This transformation process is termed the beige-to-white transition. It has been reported that the beige-to-white transition occurs directly via the elimina-

tion of some mitochondria by mitophagy. This process does not require the involvement of an intermediate precursor cell (Altshuler-Keylin et al. 2016). However, the precise mechanism of mitophagy and its regulation are not fully understood. In this section, we describe the general molecular mechanisms of mammalian autophagy and mitophagy. Recent progress on the discovery of mitophagy in beige adipocytes will also be reviewed.

16.3.1 Molecular Mechanism of Autophagy in Mammalian Cells

Autophagy is an intracellular catabolic process induced by various environmental and internal factors, including nutrient starvation, hypoxia, and oxidative stress (Mizushima et al. 2011; Nakatogawa et al. 2009). During the initial step of autophagy induction, a membrane sac, termed the isolation membrane (also known as the phagophore), emerges at the autophagosome formation site. The sac then elongates and engulfs cargo molecules. Eventually, the edge of the isolation membrane is getting closed and a double-membrane vesicle, termed the autophagosome, is formed. The autophagosome then fuses with a lysosome to degrade the cargo molecules (Nakatogawa 2020; Soreng et al. 2018). Molecular mechanisms and the physiological role of autophagy have been extensively examined, leading to the identification of over 40 autophagy-related (ATG) proteins. ATG proteins are divided into several functional groups, including the ULK1 complex (ULK1/2, ATG13, FIP200, and ATG101), the class III phosphatidylinositol 3-kinase (PI3K) complex 1 (PI3KC3-C1) (VPS34, p150, ATG14, BECN1, and NRFB2), phosphatidylinositol 3-phosphate (PI3P)-binding proteins and their interaction partner proteins (WIPIs, ATG2, and ATG9), and two ubiquitin-like conjugation systems (ATG12, ATG7, ATG10, ATG5 and LC3/GABARPs, ATG7, ATG3). These core ATG proteins are highly conserved from yeast to mammals and essential for almost all autophagic pathways

(Mizushima et al. 2011; Nakatogawa et al. 2009; Melia et al. 2020) (Table 16.1).

Mammalian target of rapamycin (mTOR) complex 1 (mTORC1) was shown to repress autophagy via phosphorylation of ULK1 complex components under nutrient-rich conditions. During nutrient starvation, mTORC1 is inactivated, leading to activation of the ULK1 complex (Zachari and Ganley 2017) (Fig. 16.3a). The ULK1 complex and ATG9 vesicle are recruited to the autophagosome formation site, which is thought to be located close to the endoplasmic reticulum (ER) (Axe et al. 2008; Itakura et al. 2012; Karanasios et al. 2016). The activation of the ULK1 complex is followed by PI3KC3-C1, which in turn forms PI3P. PI3P-binding proteins (WIPIs) and their interaction partner, ATG2, are localized to the edge of the isolation membrane, where ATG2 tethers the edge to ER and transfers lipids to the isolation membrane from ER (Maeda et al. 2019; Osawa et al. 2019; Polson et al. 2010; Proikas-Cezanne et al. 2004; Valverde et al. 2019). ATG9 colocalizes with ATG2 and contributes to the elongation of the membrane employing activation of its lipid scramblase (Maeda et al. 2020; Matoba et al. 2020; Young et al. 2006). Ubiquitin-like protein, ATG12, is covalently conjugated to a lysine residue in ATG5. This reaction is supported by ATG7 (E1-like enzyme) and ATG10 (E2-like enzyme) proteins. ATG12-ATG5 conjugate interacts with ATG16L, resulting in the formation of the ATG16L-ATG12-ATG5 complex (Hanada et al. 2007; Otomo et al. 2013; Sakoh-Nakatogawa et al. 2013). ATG16L-ATG12-ATG5 complex is localized to the isolation membrane via binding of ATG16L to WIPI2 (Dooley et al. 2014; Fujita et al. 2008). Another type of ubiquitin-like ATG protein LC3/GABARAPs is expressed in its precursor form and processed by Atg4s, a cysteine protease in the cytoplasm. Processed LC3/GABARAPs are conjugated to PE in the isolation membrane. This reaction is supported by ATG7, ATG3 (E2-like enzyme), and ATG16L-ATG12-ATG5 complex (E3-like enzyme), which is required for the closure of the autophagosome (Fujita et al. 2008; Noda et al. 2009; Tsuboyama et al. 2016). Finally, the autophagosome fuses

Table 16.1 Core autophagy-related proteins in mammals

ULK1 complex	
ULK1/2	Serine/threonine protein kinase
FIP200	Component of ULK1 complex
ATG13	Component of ULK1 complex
ATG101	Component of ULK1 complex
Class III phosphatidyl inositol 3-kinase (PI3K) complex	
ATG14	Component of Class III PI3K complex
BECN1	Component of Class III PI3K complex
VPS34	Catalytic subunit of Class III PI3K complex
p150	Component of Class III PI3K complex
NRFB2	Component of Class III PI3K complex
Phosphatidyl inositol 3-phosphate (PI3P) binding protein and Atg9 vesicle cycling	
WIPI1/2/3/4	WD40 repeat protein, PI3P binding protein
ATG9	Transmembrane protein with lipid scramblase activity
ATG2	Lipid transfer protein
Atg12 conjugation system	
Atg12	Ubiquitin-like protein Conjugated to Atg5
Atg7	E1-like enzyme of LC3 family and Atg12
Atg10	E2-like enzyme of Atg12
Atg5	Conjugated by Atg12
Atg16L	Component of Atg12–Atg5–Atg16 complex
LC3 family protein conjugation system	
LC3A/B/C, GABARAP, GABARAPL1/2/3	Ubiquitin-like protein Conjugated to phosphatidylethanolamine
Atg4A/B/C/D	Cysteine protease for C-terminal region of LC3 family proteins
Atg7	E1-like enzyme of Atg8 family and Atg12
Atg3	E2-like enzyme of LC3 family proteins
Atg16L–Atg12–Atg5 complex	E3-like enzyme of LC3 family proteins

with a lysosome, generating the autolysosome, which initiates cargo degradation using lysosomal hydrolases (Fig. 16.3a).

Lysosomal biogenesis and some ATG proteins are regulated by microphthalmia/transcription factor E (Mi/TFE) family transcription factors, including microphthalmia-associated transcription factor (MITF), transcription factor EB (TFEB), and transcription factor binding to IGHM enhancer 3 (TFE3) (Martina et al. 2012; Roczniak-Ferguson et al. 2012; Settembre et al. 2012). As mentioned above, the β 3-AR-cAMP-PKA signaling pathway is important for UCP1 expression and lipolysis in brown and beige adipocytes during cold exposure (Nicholls 2006; Fedorenko et al. 2012). Furthermore, PKA inactivates the ULK1 complex and downregulates MITF and mitophagy-related genes (Altshuler-Keylin et al. 2016; Liu et al. 2016) (Fig. 16.3a).

Conclusively, the PKA signaling pathway contributes to the beige adipocyte development and maintenance of mitochondrial mass via inhibition of mitophagy.

16.3.1.1 Molecular Mechanisms of Mitophagy in Mammalian Cells

In mammalian cells, mitophagy is classified into two major pathways, including ubiquitin-mediated and receptor-mediated mitophagy (Hamacher-Brady and Brady 2016; Khaminets et al. 2016). One of the well-studied ubiquitin-mediated pathways involves the serine-threonine kinase, PTEN-induced putative kinase 1 (PINK1), and the E3 ubiquitin ligase, Parkin, which are the causative genes of familial Parkinson's disease (Narendra et al. 2008). PINK1 is stabilized on the outer mitochondrial

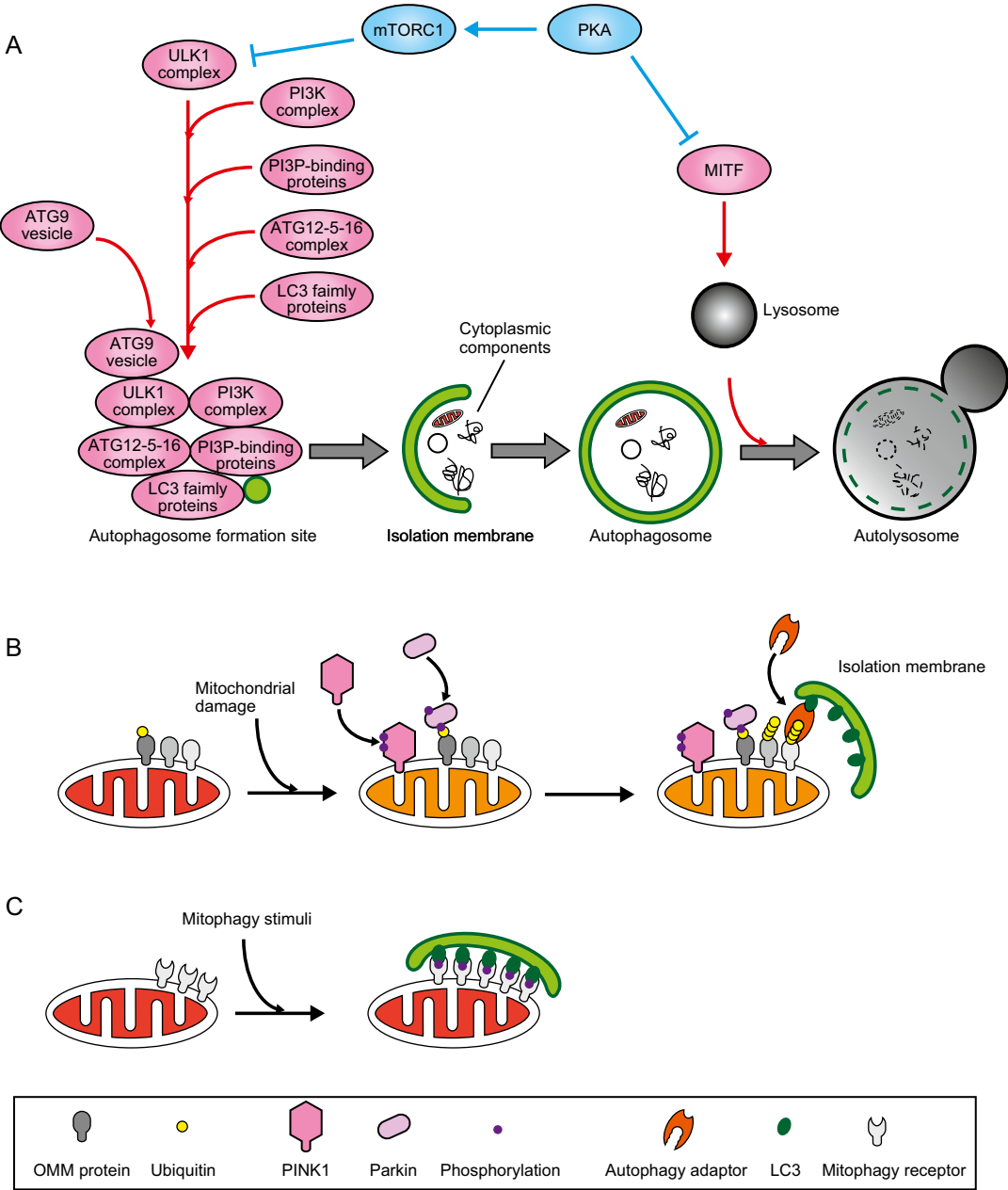


Fig. 16.3 Overview of general autophagy and mitophagy mechanisms. (a) Autophagy is mediated by ATG proteins. (b) Molecular mechanisms of Parkin-PINK1-mediated mitophagy. (c) Molecular mechanisms of receptor-mediated mitophagy

membrane (OMM) during dissipation of the mitochondrial membrane potential, where it phosphorylates pre-existing ubiquitin on the mitochondria (Matsuda et al. 2010; Narendra et al. 2010; Okatsu et al. 2015; Sekine and Youle 2018). Subsequently, cytoplasmic Parkin is

localized to the mitochondria via binding to phosphorylated ubiquitin. Parkin is then activated by PINK1-dependent phosphorylation, resulting in further ubiquitination of OMM proteins (Koyano et al. 2014; Lazarou et al. 2015). Autophagy adaptor proteins, including optineu-

rin, NDP52, and TAX1BP1, which have ubiquitin-binding domain (UBD) and LC3-interacting region (LIR), are localized to the ubiquitinated mitochondria via UBD. Subsequently, they bridge the mitochondria to the isolation membrane through binding to LC3 family proteins anchored to the isolation membrane, resulting in mitophagic degradation of the damaged mitochondria (Fig. 16.3b) (Lazarou et al. 2015; Heo et al. 2015). The stabilization of PINK1 is repressed by PKA through phosphorylation of IMM protein MIC60. Accordingly, the cAMP-PKA signaling pathway may block PINK1-mediated mitophagy upon cold exposure (Akabane et al. 2016). It has been found that *Parkin*^{-/-} mice do not develop Parkinson's disease but show higher energy expenditure than control mice. These mice are resistant to high-fat diet (HFD)-induced body weight gain, suggesting the importance of Parkin for body weight control (Goldberg et al. 2003; Kim et al. 2011; Palacino et al. 2004; Perez and Palmiter 2005; Taylor and Gottlieb 2017).

Mitochondria can be directly recognized by the isolation membrane through the interaction between the isolation membrane and the OMM proteins containing LIR, called the mitophagy receptors (Fig. 16.3c). Currently, five mitophagy receptors, including BNIP3, NIX, FUNDC1, BCL2L13, and FKBP8, have been reported to induce mitophagy through recruiting the isolation membrane to the mitochondria. This process is likely regulated by their phosphorylation at the site in the flanking region of the LIR (Bhujabal et al. 2017; Hanna et al. 2012; Liu et al. 2012; Murakawa et al. 2015; Novak et al. 2010). BNIP3, NIX, and FUNDC1 are involved in the mitophagy activated by several environmental factors, such as hypoxia, and iron depletion. However, the physiological role of the mitophagy mediated by these factors remains elusive (Diwan et al. 2007a; Hamacher-Brady et al. 2007; Tracy et al. 2007). *NIX*^{-/-} mice show inhibition of erythrocyte maturation and severe anemia, suggesting that NIX is involved in erythrocyte development through the elimination of mitochondria (Diwan et al. 2007b; Sandoval et al. 2008).

16.3.2 Activation of Mitophagy During Beige-to-white Adipocyte Transition

Beige adipocyte differentiation can be induced by cold exposure or β 3-AR stimulation of adipocytes in WAT depots located in the inguinal region (Gospodarska et al. 2015; Altshuler-Keylin et al. 2016). Altshuler-Keylin et al. demonstrated that beige adipocytes can be directly converted into white adipocytes. This conversion is mediated by mitophagy (Fig. 16.4). Furthermore, when mitophagy is inhibited in mice through either genetic ablation of ATG12 in UCP1-positive cells or administration of an autophagy inhibitor, the body weight loss, decreases in liver TGs and blood glucose were observed despite HFD feeding (Altshuler-Keylin et al. 2016). The data suggested that the functional presence of beige adipocytes protects mice from HFD-induced obesity and the development of insulin resistance.

Lu et al. reported that during the beige-to-white transition in *Parkin*^{-/-} mice, the mitochondrial elimination was reduced, and beige adipocytes were retained even after withdrawal of β 3-AR agonist, suggesting that Parkin-mediated mitophagy plays an important role in the beige-to-white transition. In cultured beige adipocytes (derived from wild-type mice), the Parkin recruitment to the mitochondria was strongly inhibited by NE treatment and activation of the PKA pathway, despite depolarization of the mitochondria by potent uncoupler (carbonyl cyanide m-chlorophenyl hydrazine (CCCP)) (Lu et al. 2018). These data are consistent with the previously reported findings that PKA inhibits the Parkin recruitment via phosphorylation of IMM protein MIC60 (Akabane et al. 2016). Conclusively, these data suggest that PKA-mediated repression of Parkin recruitment protects the mitochondria from degradation in beige adipocytes. Interestingly, in activated brown adipocytes, which have higher mitochondria numbers than normal brown adipocytes, mitochondrial elimination is usually induced even in the absence of Parkin. However, mitochondrial degradation is strongly repressed in beige adipocytes after ter-

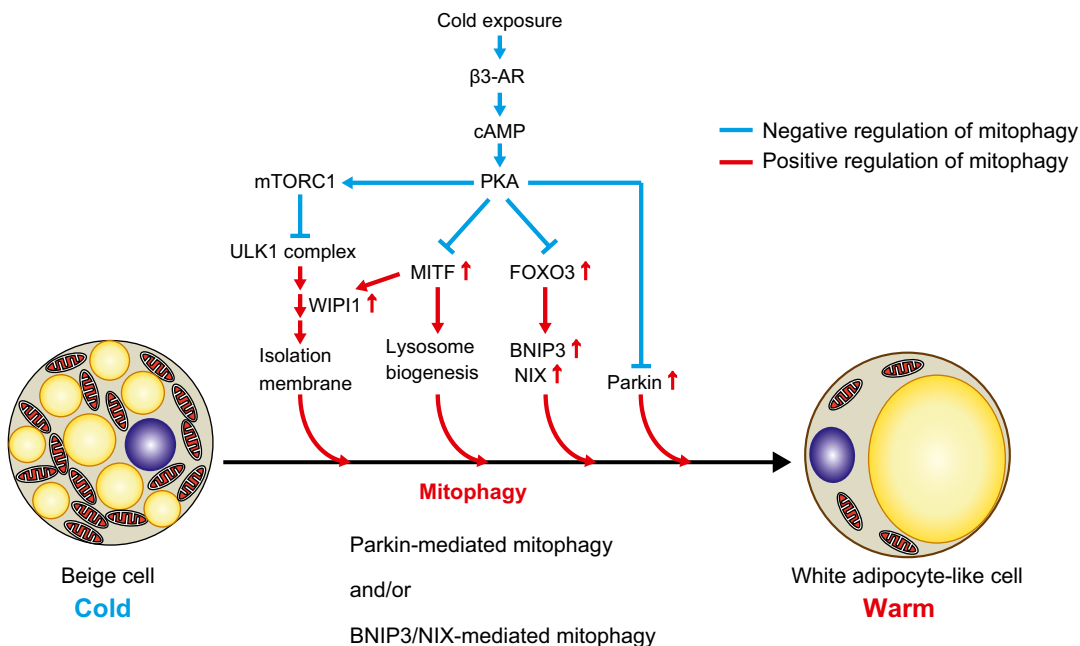


Fig. 16.4 Many mitochondria are eliminated by mitophagy during the beige-to-white transition

mination of β_3 -AR agonist use. The data suggest that the regulatory mechanism of mitochondrial elimination differs between beige and activated brown adipocytes (Lu et al. 2018). Moreover, Taylor and Gottlieb argued that beige adipocyte development at WAT depots is antagonized by Parkin expression because the expression level of Parkin is decreased under beige adipocyte induction conditions. Overexpression of Parkin also blocked mitochondrial remodeling and prevented the beige adipocyte development (Taylor and Gottlieb 2017). Altogether, these reports indicated that the expression level of Parkin and its recruitment to mitochondria are regulated in beige adipocytes to retain or eliminate the mitochondria through repression or activation of mitophagy.

Despite the recent research progress in mitophagy, the contribution of receptor-mediated mitophagy to the beige-to-white transition process is largely unknown. It has been reported that BNIP3 and NIX expression levels were increased during the beige-to-white transition and inhibited by activation of the cAMP-PKA pathway. Therefore, BNIP3- and/or NIX-mediated mitophagy may be involved in this process (Altshuler-Keylin et al. 2016). Further studies are warranted

to elucidate the contribution of PKA-associated pathways and the relationship between Parkin-mediated and BNIP3/NIX-mediated mitophagy during the beige-to-white transition. Compared with beige adipocytes located in inguinal WAT depots, in visceral WAT, including epididymal, and gonadal WAT, BNIP3 contributes to mitochondrial biogenesis through its transcriptional upregulation by peroxisome-proliferation activation receptor gamma (PPAR γ) (Choi et al. 2016). Thus, it is important to consider that BNIP3 may exert the opposite effects in different WAT depots.

16.4 Development of Methods to Assess Adipose Tissue Mitophagy

Currently, only a limited number of methods have been developed to assay mitophagy in vivo. A typical approach is to assess the reduction of mitochondrial mass, which is determined by measuring mitochondrial proteins or mitochondrial DNA. However, it is difficult to estimate precise mitophagy flux because mitochondrial mass is compensated by mitochondrial biogene-

sis. To overcome this problem, in the last 5 years, two reporter mice systems, mt-Keima mice and mito-QC mice, have been established to monitor mitophagy. These mice models lead to several remarkable discoveries, including the role of mitophagy in the beige-to-white transition (McWilliams et al. 2016; Sun et al. 2015). In this section, we will discuss the principles of these systems, their availability, and their limitations.

16.4.1 Mt-Keima Mitophagy Detection System

The Mt-Keima system had been originally established to monitor mitophagy in cultured cells (Katayama et al. 2011; Yamashita et al. 2016; Yamashita and Kanki 2018). Keima, which is a coral-derived and pH-sensitive fluorescent protein (FP), has two excitation peaks, depending on environmental pH (440 nm in neutral and 586 nm in acidic pH) (Katayama et al. 2011; Kogure et al. 2006). Mt-Keima, which is tagged with a mitochondria-targeting signal, is recruited in the mitochondrial matrix. As the mitochondrial matrix has a neutral pH, mt-Keima can be excited by a 458 nm laser, which corresponds to the mitochondria in the cytoplasm. Alternatively, when mt-Keima is recruited into lysosome with acidic pH, it can be excited by a 561 nm laser, indicating the autolysosome containing degraded mitochondria. Notably, Keima is resistant to lysosomal degradation. Therefore, autolysosome located in Keima can be detected cumulatively, leading to the sensitive measurement of mitophagy flux (Fig. 16.5a). Sun et al. generated mt-Keima-expressing mice and showed mitophagy signals in several tissues using an *in vivo* model (Sun et al. 2015). Lu et al. also successfully monitored the mitophagy in fresh unfixed beige adipocytes of mt-Keima mice by using FACS analysis (Lu et al. 2018). Mt-Keima mice system is a powerful tool for assessing *in vivo* mitophagy; however, there is one serious limitation regarding sample preparation. Utilization of unfixed tissues is required for mt-Keima analysis because mitophagic readouts of mt-Keima in autolysosome (excited by a 586 nm laser) cannot

be retained after tissue fixation due to the loss of pH gradient across the lysosomal membrane. Thus, it is impossible to visualize the mitophagic readouts in fixed mice tissue sections.

16.4.2 Mitochondria-Targeting Tandem Fluorescent Protein System

It has been shown that DsRed-derived red FPs, including mRFP1, DsRed, and mCherry, are resistant to lysosomal proteases and acidic pH. However, jellyfish-derived FPs including EGFP, ECFP, and EYFP can be easily degraded by lysosomal proteases. Taking advantage of their properties, the tandem FPs method has been developed to assay autophagy flux. When mRFP1-EGFP tandem FP is attached to the N-terminal of LC3 (autophagosome protein marker), the tandem FP-LC3 is recruited into lysosome and degraded during autophagy (Kimura et al. 2007). Therefore, when autophagy is induced in the mRFP1-EGFP-LC3-expressing cells, autolysosomes are visualized with mRFP1 alone. Alternatively, autophagosomes prior to fusion with lysosome and isolation membranes are visualized with both mRFP1 and EGFP. This allows to image autophagic flux using mRFP1 alone dots.

Using the tandem FP-based concept, McWilliams et al. generated mito-QC mice. The incorporate detection system contains mCherry-EGFP tandem FP, which is attached to the C-terminal region of Fis1, the C-tail anchored OMM protein. The system allows us to monitor the recruitment of mitochondria into lysosome and detect mitochondrial degradation by mitophagy (McWilliams et al. 2016). Unlike the mt-Keima system, mitophagy signals are retained in the fixed tissues, giving an advantage to visualize mitophagy in stored tissue sections. Using mito-QC mice, McWilliams et al. reported that basal mitophagy can be detected in several types of tissues and cells, including dopaminergic neurons, microglia, heart muscles, retina, and pancreas (McWilliams et al. 2016). The authors detected that the level of basal mitophagy in PINK1^{-/-} mice

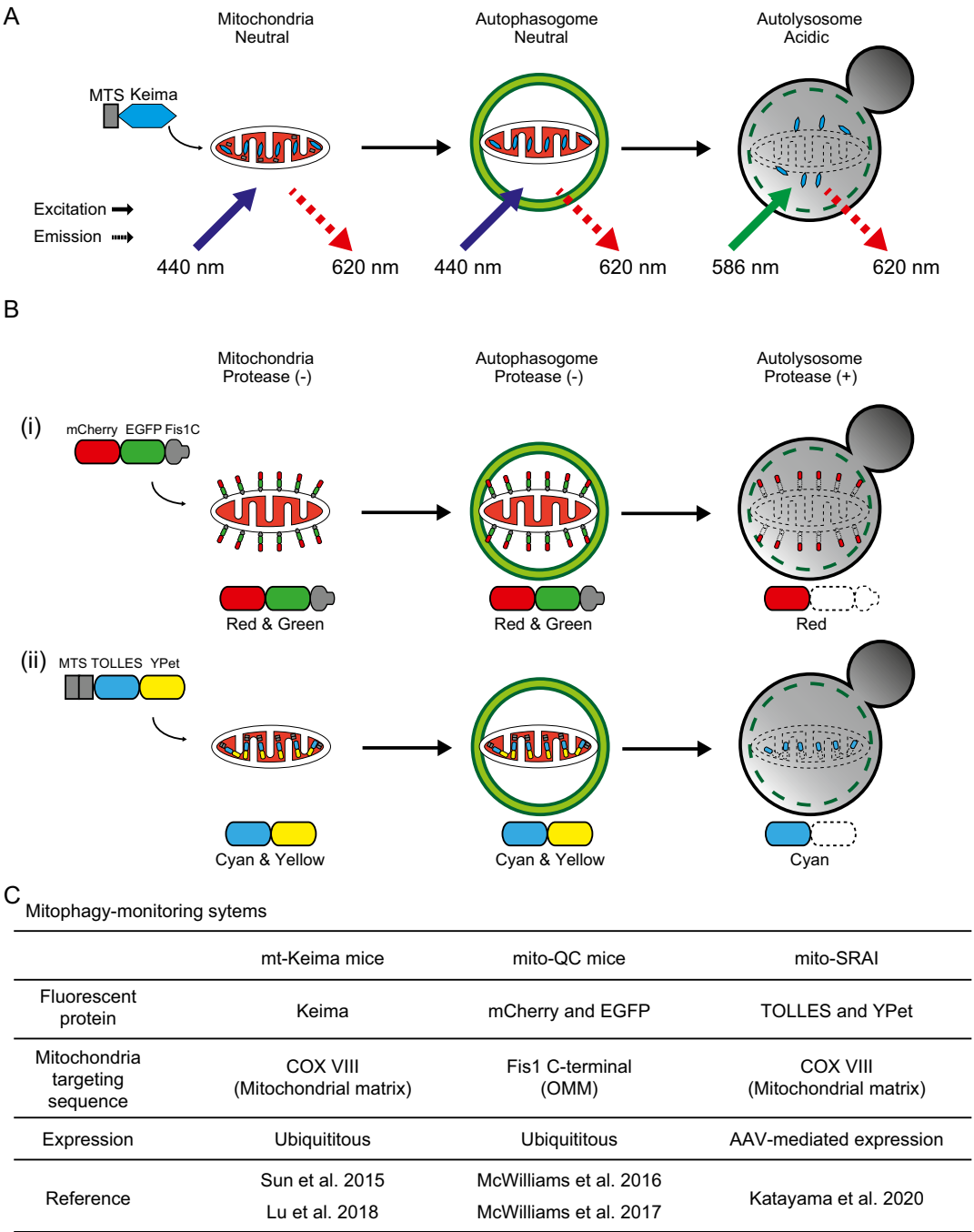


Fig. 16.5 Strategy to monitor mitophagy in vivo. (a) A strategy for mitophagy assessment using the mt-Keima system. (b) A strategy for mitophagy assessment using tandem FP systems, mito-QC (i) and mito-SRAI (ii). (c) Differences between two different mitophagy monitoring systems in mice

is comparable with that in wild-type mice. The data indicated that the basal mitophagy in various tissues in vivo is induced in a PINK1-independent manner; however, the mitophagy level and details of its regulation in adipose tissues remained unclarified (McWilliams et al. 2018).

Although the mito-QC system is a powerful tool for measuring *in vivo* mitophagy, there is one problematic issue with the localization of the FP-related reporter. Fis1 is found localized in not only mitochondria but also peroxisomes. The peroxisome localized reporter may respond to peroxisome autophagy (pexophagy). Although McWilliams et al. demonstrated that mCherry-EGFP-Fis1 does not visualize peroxisomes in heart sections and MEF derived from mito-QC mice, the system does not preclude the possibility that some portion of the reporter can be localized to peroxisomes at least partially in peroxisome abundant tissues, such as liver, brain, and adipose tissues (McWilliams et al. 2016). Furthermore, similarly to mitochondria, peroxisomal biogenesis-related genes were found significantly increased in response to cold exposure (Park et al. 2019). Peroxisomes are required for thermogenesis in brown and beige adipocytes, implying the regulation of peroxisomal mass via the elimination of peroxisomes by pexophagy during the beige-to-white adipocyte transition (Park et al. 2019). Thus, precise measurement of mitophagy in beige adipocytes using this system may be compromised by the potential for pexophagy.

Using tandem FP mitophagy reporter, Katayama et al. have recently reported another approach based on the application of mito-SRAI (Signal Retaining Autophagy Indicator), which is composed of a cyan FP, TOLerans of Lysosomal Environments (TOLLES), and a yellow FP, YFP for energy transfer (YPet) (Katayama et al. 2020). The authors suggested that the combination of mCherry and EGFP is not the best choice for tandem FP system because these FPs do not have extreme opposite sensitivities to lysosomal degradation. Therefore, the authors found the FP, which is extremely tolerant to lysosomal environment. The new FP is Anthozoan-derived cyan FP, TOLLES. Mito-SRAI indicator is targeted to the mitochondrial matrix using the mitochondria-targeting signal of COX VIII. Using mito-SRAI-expressed cultured cells, the authors discovered the mitophagy-inducing agent. Using AAV-mediated mito-SRAI expression, the authors

measured the mitophagy in the mouse brain (Katayama et al. 2020). However, the generation of systemically expressing mito-SRAI mice has not been reported.

Conclusively, there are two important points to note when measuring *in vivo* mitophagy using reporter-expressing mice: (1) the reporter signals should be retained after fixation and (2) the localization of the reporter should be strictly regulated and validated in mitochondria. To solve these issues, the generation of mice with reporter-expressing, mitochondrial matrix-targeted tandem FP is warranted.

16.5 Perspective

In this review, we described the role of mitochondrial degradation in adipose tissue, focusing on beige adipocytes. Upon exposure to lower temperatures, the number of mitochondria is increased via both induction of mitochondrial biogenesis and repression of mitophagy. The regulation of mitochondrial number is important during NST in brown and beige adipocytes. Excess mitochondria are removed via mitophagy upon withdrawal of cold temperature, triggering the transformation of beige adipocytes into white adipocyte-like cells, thus contributing to body temperature control. During the beige-to-white adipocyte transition, the degradation of mitochondria heavily relies on Parkin-dependent mitophagy, but the exact mechanism of this transition remains elusive (Lu et al. 2018). Mitochondrial targeting of Parkin is regulated by PINK1, which is post-translationally stabilized on the depolarized mitochondria. To elucidate the mechanism of this process, the regulation of PINK1 in beige adipocytes should be further addressed. For instance, investigation of the mitophagy using PINK1^{-/-} mice is warranted. Furthermore, the contribution of BNIP3 and NIX to the activation of mitophagy in beige adipocytes has not been investigated in full detail. Using BNIP3^{-/-} or NIX^{-/-} mice, future studies should investigate the mitophagy mechanism in beige adipocytes.

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Role of TRPA1 in Painful Cold Hypersensitivity

17

Takayuki Nakagawa and Shuji Kaneko

Abstract

Transient receptor potential ankyrin 1 (TRPA1) is a polymodal cation channel that plays a pivotal role in pain generation after exposure to irritant chemicals and is involved in the sensation of a wide variety of pathological pain. TRPA1 was first reported to be sensitive to noxious cold, but its intrinsic cold sensitivity still remains under debate. To address this issue, we focused on cold hypersensitivity induced by oxaliplatin, a platinum-based chemotherapeutic drug, as a peculiar adverse symptom of acute peripheral neuropathy. We and other groups have shown that oxaliplatin enhances TRPA1 sensitivity to its chemical agonists and reactive oxygen species (ROS). Our in vitro and animal model studies revealed that oxaliplatin, or its metabolite oxalate, inhibits hydroxylation of a proline residue within the N-terminus of human TRPA1 (hTRPA1) via inhibition of prolyl hydroxylase domain-containing protein (PHD), which

induces TRPA1 sensitization to ROS. Although hTRPA1 is insensitive to cold, PHD inhibition endows hTRPA1 with cold sensitivity through sensing the small amount of ROS produced after exposure to cold. Hence, we propose that PHD inhibition can unveil the cold sensitivity of hTRPA1 by converting ROS signaling into cold sensitivity. Furthermore, in this review, we summarize the role of TRPA1 in painful cold hypersensitivity during peripheral vascular impairment.

Keywords

TRPA1 · Pain · Cold hypersensitivity · Oxaliplatin · Prolyl hydroxylase domain-containing protein · Reactive oxygen species · Peripheral neuropathy

T. Nakagawa (✉)
Department of Clinical Pharmacology and
Pharmacotherapy, Wakayama Medical University,
Wakayama, Japan
e-mail: tnakaga@kuhp.kyoto-u.ac.jp

S. Kaneko
Department of Molecular Pharmacology, Graduate
School of Pharmaceutical Sciences, Kyoto University,
Kyoto, Japan

17.1 Introduction

Animals possess diverse sensors to detect ambient physical and chemical stimuli in the environment. In particular, the sensors for latent dangers play a pivotal role as an alarm system that warns of and helps to protect from tissue damage. Most of the molecules for the pain sensors are ion channels, which include the transient receptor potential (TRP) channel family (Dubin and Patapoutian 2010). Some TRP channels are temperature-sensitive and play an important role

as pain sensors for noxious heat or cold stimuli, although they are also activated by chemicals and other physical stimuli (Nilius and Szallasi 2014; Hilton et al. 2015; Jardín et al. 2017). For example, TRP melastatin 8 (TRPM8) is activated in response to innocuous cooling as well as noxious cold, and menthol, a chemical constituent of peppermint (Mckemy et al. 2002; Mckemy 2005). TRP ankyrin 1 (TRPA1) is another well-studied polymodal nociceptive sensor. As extensively described in other reviews (Zygmunt and Högestätt 2014; Viana 2016; Kádková et al. 2017), TRPA1 undoubtedly acts as a polymodal pain sensor for diverse physical and chemical stimuli of extracellular or intracellular origin. Its temperature sensitivity, especially intrinsic sensitivity to cold, has been extensively discussed but remains under debate (Voets 2014; Señarís et al. 2018; Talavera et al. 2020).

17.2 Temperature Sensitivity of TRPA1

TRPA1 is named for its 14 (mouse) or 16 (human) ankyrin repeats located in its long N-terminus, and its structure was resolved using cryoelectron microscopy (Paulsen et al. 2015; Meents et al. 2019). TRPA1 is opened by covalent or oxidative modification of multiple cysteine residues in the N-terminal region. Hence, TRPA1 can respond to a wide variety of natural products and chemical compounds, including pungent chemicals [allyl-isothiocyanate (AITC), cinnamaldehyde, and allicin], environmental toxins, irritant pharmaceutical agents, and oxidative stimuli, such as reactive oxygen species (ROS) and reactive nitrogen species (Story et al. 2003; Bandell et al. 2004; Takahashi et al. 2008, 2011; Meents et al. 2019). Because TRPA1 oxidation sensitivity is very high, it can even be activated by hyperoxia through glutathione-sensitive oxidation of some cysteine residues (Takahashi et al. 2011). TRPA1 was first identified as a sensor of noxious cold ($\leq 17^\circ\text{C}$) (Story et al. 2003), and subsequent studies showed the role of TRPA1 in cold nociception *in vitro* and *in vivo* (Bandell et al. 2004; Kwan et al. 2006; Karashima et al. 2009).

However, the cold sensitivity of TRPA1 is immediately controversial by other studies showing that TRPA1 is not activated directly by cold, and deletion of the TRPA1 gene fails to affect cold sensitivity (Jordt et al. 2004; Bautista et al. 2006; Zurborg et al. 2007; Zhou et al. 2013).

Increasing evidence suggests that the temperature sensitivity of TRPA1 is species-specific, although its chemical sensitivity to pungent compounds such as AITC and cinnamaldehyde is generally conserved (Laursen et al. 2015; Saito and Tominaga 2017). TRPA1 in mice and rats is activated in response to cold, but TRPA1 in humans and rhesus monkeys is cold-insensitive (Chen et al. 2013). By contrast, TRPA1 in chickens, reptiles, and frogs is activated in response to heat stimulation (Gracheva et al. 2010; Saito et al. 2012, 2014). In addition, zebrafish TRPA1 is activated in response to both heat and cold (Prober et al. 2008). Therefore, it is possible that TRPA1 activation in response to temperature has diverged evolutionarily and differs among animal species in adaptation to their specific living environments (Laursen et al. 2015; Saito and Tominaga 2017). However, human TRPA1 (hTRPA1) that is purified and reconstituted into an artificial lipid bilayer induces a response to cold (Moparthi et al. 2014). Hence, the intrinsic cold sensitivity of TRPA1 may depend on the species, the experimental conditions, and its interaction with other regulatory molecules (Talavera et al. 2020).

17.3 Role of TRPA1 in Noxious Cold Hypersensitivity

A growing body of literature demonstrates the involvement of TRPA1 in pain sensation in a wide variety of pain animal models, including inflammatory pain, cancer pain, post-operative pain, peripheral nerve injury-induced neuropathic pain, painful diabetic neuropathy, post-ischemic pain, osteoarthritis, migraines, and chemotherapy-induced peripheral neuropathy (CIPN) (Nassini et al. 2014; Nakagawa and Kaneko 2017; Antoniazzi et al. 2019; Souza Monteiro de Araujo et al. 2020). These studies have shown that pharmacological inhibition of

TRPA1 by antagonists or deletion of the TRPA1 gene attenuates noxious cold hypersensitivity, as well as mechanical and thermal hypersensitivity in animal models. Hence, TRPA1 mediates various sensory modalities of pain other than cold hypersensitivity, which leads to the hypothesis that diverse exogenous noxious stimuli not only activate TRPA1 directly but also activate it indirectly through endogenous molecules.

TRPA1 is expected as a potential therapeutic target for the treatment of pain, even though the evidence in noxious cold responses in humans is limited. Genetic mapping of familial episodic pain syndrome (FEPS), which is a rare autosomal-dominant disorder characterized by episodes of debilitating upper body pain that is triggered by fasting and physical stress (including noxious cold), identified a gain-of-function missense mutation in hTRPA1 (Kremeyer et al. 2010). Furthermore, the flare reaction and secondary hyperalgesia are increased in FEPS patients after TRPA1 stimulation. TRPA1 channelopathy suggests that the enhanced sensitivity of mutated hTRPA1 contributes to pain symptoms including noxious cold hypersensitivity.

17.4 TRPA1 in Acute Cold Hypersensitivity Induced by Oxaliplatin

Oxaliplatin is a platinum-based chemotherapeutic drug widely used to treat colorectal, gastric, and pancreatic cancers. However, it rapidly induces a peculiar acute peripheral neuropathy with typical symptoms, such as cold hypersensitivity, within hours after its infusion in 85–95% of patients (Colvin 2019; Kang et al. 2020). Oxaliplatin-specific acute peripheral neuropathy is a channelopathy caused by the oxaliplatin metabolite oxalate. Because it is often triggered or exacerbated after exposure to cold, the involvement of cold-sensitive TRP channels is suggested (Kang et al. 2020). Oxaliplatin-induced cold hypersensitivity observed several days or weeks after single or repeated administration of oxaliplatin is accompanied by increases in mRNA expressions of TRPA1 and TRPM8 in the dorsal

root ganglion (DRG) and is abolished by antagonists for or deletion of TRPA1 or TRPM8 (Gauchan et al. 2009; Descœur et al. 2011; Nassini et al. 2011). We focused on the acute phase of cold hypersensitivity and showed that a single administration of oxaliplatin, but not cisplatin or paclitaxel (CIPN-inducing chemotherapeutic drugs), produces rapid-onset cold hypersensitivity even 2 h after the injection in mice (Zhao et al. 2012, 2014), which is consistent with the clinical observation of oxaliplatin-induced acute peripheral neuropathy. Similarly, the rapid-onset cold hypersensitivity is abolished by an antagonist for or deletion of TRPA1. By contrast, our *in vivo* and *in vitro* experiments revealed that the enhanced sensitivity of TRPA1 function, rather than increased protein expression, induced by oxaliplatin or its metabolite oxalate, is responsible for the cold hypersensitivity (Zhao et al. 2012).

TRPA1 can be activated by two different mechanisms: one is mediated through covalent or oxidative modification of cysteine residues in the N-terminal region, as described above, and the other is mediated through prolyl hydroxylase domain-containing protein (PHD)-dependent hydroxylation of a proline residue. In the latter, a decrease in oxygen concentration diminishes the enzymatic activity of PHDs. This inhibition relieves hTRPA1 from the PHD-dependent hydroxylation of a proline residue located within the N-terminal ankyrin repeat domain (Pro³⁹⁴ in hTRPA1), leading to hypoxia-induced opening of TRPA1 (Takahashi et al. 2011). Because the chemical structure of oxalate resembles a PHD co-substrate and the artificial PHD inhibitor dimethyloxallylglycine (Schofield and Ratcliffe 2004), we hypothesized that PHD inhibition-mediated TRPA1 activation or sensitization by oxalate is responsible for oxaliplatin-induced rapid-onset cold hypersensitivity. In this context, we found that oxaliplatin, or a membrane-permeable oxalate analog, increases the amount of hypoxia-inducible factor-1 α , suggesting that PHD activity is suppressed. Furthermore, oxaliplatin or oxalate, as well as the PHD inhibitor, enhances the sensitivity of TRPA1 to the typical ROS hydrogen peroxide (H₂O₂), but sensitization

is absent in mutant TRPA1 lacking a hydroxylation-susceptible Pro³⁹⁴ residue. These findings suggest that oxaliplatin induces TRPA1 sensitization to ROS by relieving inhibition that is generated by PHD-mediated hydroxylation of the proline residue (Miyake et al. 2016; Nakagawa and Kaneko 2017). By contrast, high concentrations of oxaliplatin induce TRPA1 activation probably through the platinum-containing metabolite of oxaliplatin. This response is dependent on ROS-mediated oxidation of some cysteine residues but is independent of PHD activity (Miyake et al. 2017).

In our series of studies, we show that mutant hTRPA1 lacking a hydroxylation-susceptible Pro³⁹⁴ residue mediates sensitivity to cold stimulation, although wild-type hTRPA1 is insensitive to cold stimulation, which is consistent with previous reports (Chen et al. 2013). Similar results are obtained with wild-type hTRPA1 following pretreatment with the PHD inhibitor, an oxalate analog, or oxaliplatin (Miyake et al. 2016, 2017). Furthermore, our data show that cold exposure

causes an increase in ROS levels in mitochondria that is responsible for cold-induced TRPA1 activation. These findings demonstrate that relieving hTRPA1 from prolyl hydroxylation by PHD inhibition or oxaliplatin treatment enables the channel to respond to cold through sensing even small amounts of ROS produced from the mitochondria (Miyake et al. 2016, 2017; Nakagawa and Kaneko 2017). Therefore, we suggest that TRPA1 does not directly sense noxious cold but instead acts as a noxious cold sensor in specific environments by transducing cold-induced ROS signaling into neuronal activity (Fig. 17.1).

17.5 TRPA1 in Cold Hypersensitivity During Peripheral Vascular Impairment

A relationship between peripheral blood flow and cold sensation has been observed (Davey et al. 2013). Peripheral vascular impairment in

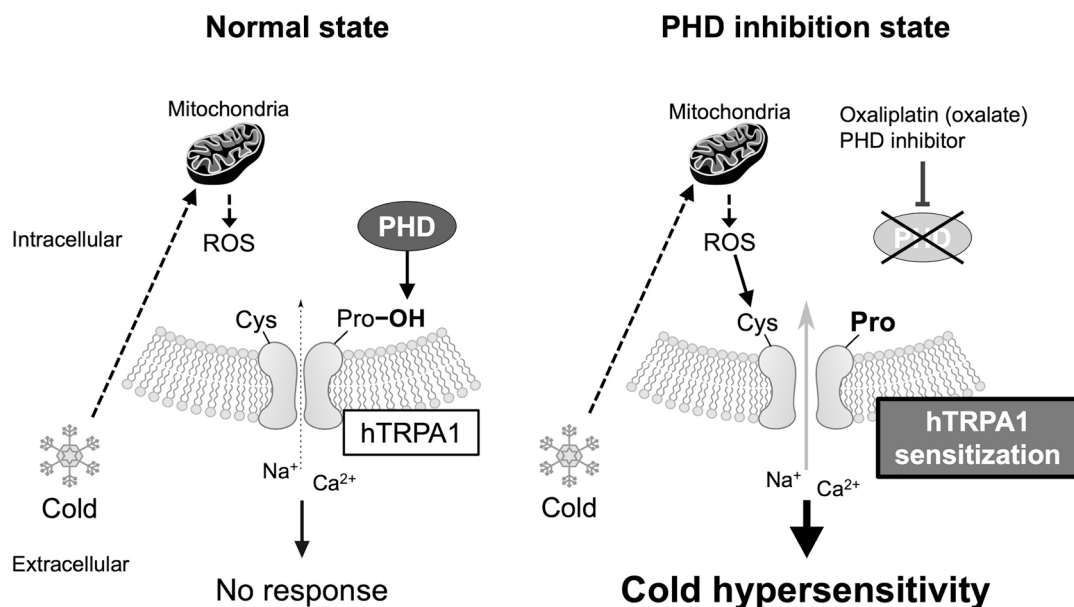


Fig. 17.1 Illustration of cold hypersensitivity through PHD inhibition-uncovers human TRPA1 (hTRPA1) sensitization. In the normal state, cold exposure induces ROS generation from mitochondria, but hTRPA1 is not activated due to conserved PHD-mediated hydroxylation of a proline residue (Pro³⁹⁴ in human TRPA1). By contrast,

oxaliplatin, or its metabolite oxalate, inhibits PHD activity, which enhances hTRPA1 sensitivity to ROS. This endows TRPA1 with indirect cold sensitivity through cold-evoked ROS (Miyake et al. 2016, 2017; Nakagawa and Kaneko 2017)

peripheral arterial diseases induces cold sensation, dysesthesia, and numbness (Rüger et al. 2008; Devulder et al. 2011). We previously reported that transient hindlimb ischemia/reperfusion evokes spontaneous licking behavior in mice. In this peripheral post-ischemic painful dysesthesia mouse model and in cultured DRG neurons, we showed that hypoxia-induced PHD inhibition enhances the sensitivity of TRPA1 through inhibition of Pro³⁹⁴ hydroxylation. Indeed, the ischemia/reperfusion evoked painful behavior is reduced by TRPA1 antagonists or TRPA1 deficiency and ROS scavengers, suggesting a mechanism wherein ROS-evoked activation of TRPA1 sensitized by hypoxia-induced PHD inhibition (So et al. 2016). Similarly, we reported that chronic hindlimb ischemia leads to TRPA1 sensitization (Hiyama et al. 2018), although spontaneous painful behavior is not observed.

Accumulating evidence suggests that diabetic neuropathy is closely related to peripheral vascular impairment and the resultant nerve ischemia (Yorek 2015). Endoneurial hypoxia induces nerve dysfunction and degeneration, especially in diabetic nerves with increased vulnerability (Cameron et al. 1991; Zochodne et al. 1996), which might cause abnormal sensations such as loss of sensitivity and diabetic neuropathic pain (Wang et al. 2015, 2016). We reported that cold hypersensitivity induced in streptozotocin-induced diabetic model mice is observed in parallel with a decrease in skin blood flow levels and is dependent on TRPA1 sensitization. A vasodilator, tadalafil, ameliorates this diabetic cold hypersensitivity by restoring the decreased skin blood flow and reducing TRPA1 sensitization. These findings suggest that diabetic vascular impairment induces TRPA1 sensitization, which is responsible for cold hypersensitivity. However, TRPA1 deficiency fails to affect mechanical hypersensitivity, as well as electrical current or mechanical hyposensitivity, or the retraction of intraepidermal nerve fibers, suggesting that TRPA1 plays little or no role in the

progression of diabetic peripheral neuropathy (Hiyama et al. 2018).

Unlike the rapid-onset cold hypersensitivity induced by a single administration of oxaliplatin, the cold hypersensitivity observed in oxaliplatin-induced cumulative peripheral neuropathy may be dependent on peripheral vascular impairment. Oxaliplatin-induced acute peripheral neuropathy usually improves within a week, whereas multiple cycles of chemotherapy induce cumulative peripheral neuropathy (Colvin 2019). Similar to what is observed in streptozotocin-induced diabetic model mice, acute administration of the vasodilator tadalafil reduces cold, but not mechanical, hypersensitivity by restoring the decreased skin blood flow. However, prophylactic long-term treatment with tadalafil can ameliorate cold, mechanical, and electrical current hypersensitivities, as well as thermal hypoesthesia and axon degeneration in the sciatic nerves (Ogihara et al. 2019).

17.6 Conclusion

In this review, we summarize the evidence for the role and molecular mechanism of action of TRPA1 in painful cold hypersensitivity (Fig. 17.2). Both hypoxia and oxaliplatin inhibit PHD-mediated hydroxylation of an N-terminal proline residue in TRPA1, which induces TRPA1 sensitization to ROS. Hence, chemical modification endows hTRPA1 with cold sensitivity via signal transduction of cold-induced ROS production. The experimental evidence summarized in this review suggests that TRPA1 is a promising therapeutic target for the treatment of painful cold hypersensitivity, as has been suggested in other reviews (Souza Monteiro de Araujo et al. 2020). Furthermore, the improvement in peripheral vascular impairment by vasodilators shows suitable therapeutic treatments for cold hypersensitivity induced by PHD inhibition-mediated TRPA1 sensitization, and these vasodilators may also prevent peripheral neuropathy.

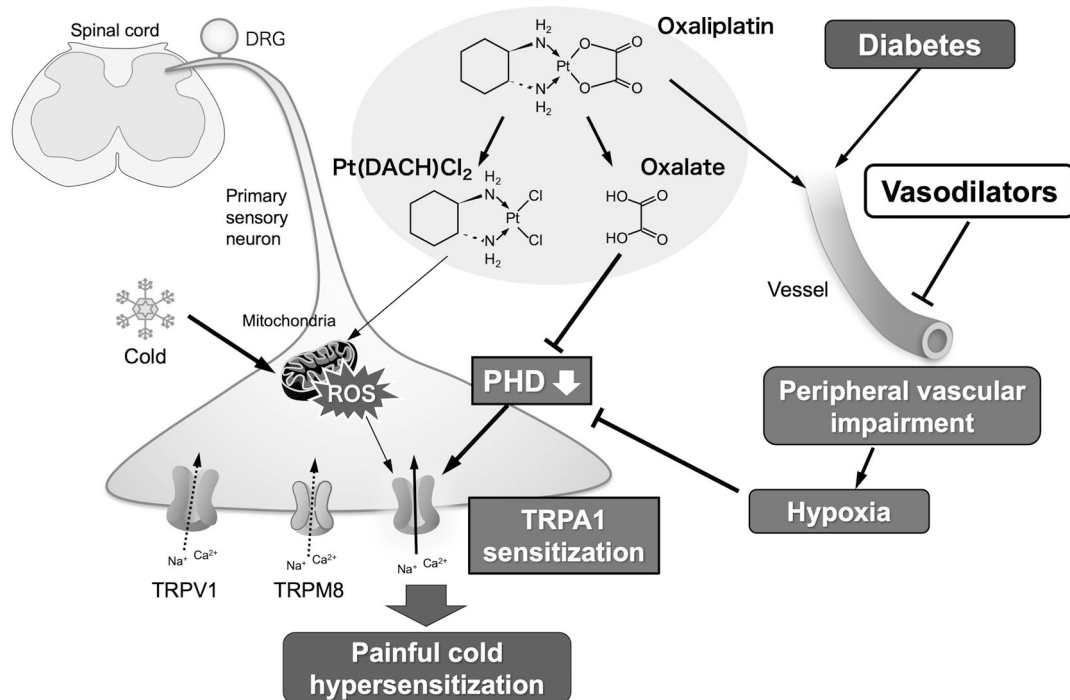


Fig. 17.2 Summary of painful cold hypersensitization through TRPA1 sensitization

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Thermal Adaptations in Animals: Genes, Development, and Evolution

18

Ako Agata and Tadashi Nomura

Abstract

Thermal adaptation to environmental temperature is a driving force in animal evolution. This chapter presents thermal adaptation in ectotherms and endotherms from the perspective of developmental biology. In ectotherms, there are known examples of temperature influencing morphological characteristics, such as seasonal color change, melanization, and sex determination. Furthermore, the timing of embryonic development also varies with environmental temperature. This review will introduce the cellular and molecular mechanisms underlying temperature-dependent embryogenesis. The evolution of thermal adaptation in endotherms is also important for survival in cold climates. Recent genome-wide studies have revealed adaptive mutations in the genomes of extant humans as well as extinct species such as woolly mammoths and Neanderthals. These studies have shown that single-nucleotide polymorphisms

in physiologically related genes (e.g., CPT1A, LRP5, THATA, PRKG1, and FADS1-3) allow humans to live in cold climates. At the end of this chapter, we present the remaining questions in terms of genetic assimilation, heat shock protein Hsp90, and embryonic development.

Keywords

Thermal adaptation · Ectothermy · Endothermy · Cold adaptation · SNPs · Development · Evolution

18.1 Introduction

Since the publication of *On the Origin of Species* (Darwin 1859), it has been widely accepted that diversified traits of organisms have evolved through the selection of phenotypes adapted to specific environments. Sporadic, periodical, and persistent changes in climate are thought to have great impacts on organisms, shaping the variation of species as a strong selective pressure. Particularly, an increase or decrease in environmental temperatures significantly affects the size of animal populations. For example, Darwin noted that “*periodic extremes of cold or drought are the most effective checks of all ... the winter 1854-1855 destroyed four-fifths of the birds on my*

A. Agata
Developmental Neurobiology, Kyoto Prefectural
University of Medicine, Kyoto, Japan

T. Nomura (✉)
Developmental Neurobiology, Kyoto Prefectural
University of Medicine, Kyoto, Japan

Applied Biology, Kyoto Institute of Technology,
Kyoto, Japan
e-mail: tadnom@kit.ac.jp

own grounds” (Darwin 1859). In contrast, animals are not always sensitive to dramatic changes in climates; a number of species display amazing flexibility and robustness to altered ambient temperatures and survive in challenging environments (Gilbert 2015). A wide range of plasticity to temperature changes in animals is an issue of great interest in biology, although the underlying mechanisms remain to be elucidated.

The embryogenesis of ectothermic animals is significantly affected by environmental temperature. A lower temperature prolongs developmental processes and delays hatching in a variety of egg-laying species (Romanoff 1936). The temperature sensitivity of embryogenesis has been thought to be a passive physiological event because many enzymatic reactions essential for cellular metabolism exhibit decreased rates at lower temperatures. However, recent studies have shown active regulation of temperature-dependent embryogenesis because of adaptive evolution. Furthermore, genome-wide association studies have identified unique genetic signatures in various endothermic species, including humans, that are thought to be associated with adaptive traits related to cold climates. Here, we review the plasticity and robustness of animals against a wide range of environmental temperatures from the perspective of developmental and evolutionary biology. First, we describe several examples of phenotypic changes by altered environmental temperatures in a variety of ectothermic species. Next, we describe the cellular and molecular mechanisms that actively regulate the plasticity and rigidity of temperature-dependent embryogenesis. Finally, we discuss recent progress from genome-wide studies that have revealed genetic changes contributing to thermal adaptations in mammalian species as well as ancestral and modern humans. Understanding thermal adaptations in embryogenesis will shed light on the tremendous potential of animals to cope with extreme environments, providing significant insight into the impact of global warming on the ecosystem.

18.2 Temperature-Dependent Plasticity of Phenotypes in Ectothermic Animals

18.2.1 Seasonal Changes in Butterfly Wings

Some Lepidoptera species exhibit seasonal differences in the colors of their wings (Nijhout 1991). Distinct patterns regarding wing color or the size of pigmented spots between spring and summer forms have evolved in the same genetic backgrounds (i.e., the same species). Several researchers have clarified that environmental temperature and the length of daytime during larval and early pupal stages are key factors controlling seasonal dimorphism (Nijhout 1999; Watt 1968). For example, August Weismann, who denied Lamarck’s concept of the inheritance of acquired traits, focused on the European common map butterfly *Araschnia levana* and revealed that experimental changes in the rearing temperature during larval stages reproduced seasonal dimorphism in the wing colors of the adult butterfly (Churchill 2015). In this species, higher temperatures and longer daytime periods increased wing pigmentation. Conversely, in *Copaodes*, a kind of skipper, a higher rearing temperature and a long-day photoperiod reduced the size of dark scales at the hind wing margin (Lewis 1985). Thus, the magnitude of pigmentation in response to environmental changes varies among species, which reflects adaptive evolution depending on specific ecological niches. Some portion of this seasonal phenotypic dimorphism is governed by 20-hydroxyecdysone (20E), a kind of steroid hormone that triggers numerous morphological changes associated with insect growth and metamorphosis (Nijhout 1999, 2003). In *Araschnia*, a lower temperature and a shorter day period during late larval and early pupal stages inhibit the pulse of 20E, which induces a spring form with reduced pigmentation. Intriguingly, the administration of 20E in the pupa results in various levels of pigmentation, including intermediate phenotypes between spring and summer forms, although these phenotypes are never observed in the wild population (Nijhout 2003).

20E binds to a specific nuclear receptor and regulates the expression of various target genes. *Distal-less (Dll)* is a gene encoding a homeodomain transcription factor, whose expression is activated by 20E-dependent transcriptional regulation. The expression of *Dll* is responsible for the development of eyespots in certain butterflies: the continuous expression of *Dll* delineates prospective eyespots on their wings (Brakefield et al. 1996; Koch et al. 2003). In *Bicyclus anynana*, living in southeast Africa, seasonal dimorphic wings with different sizes of eyespots are observed. The wet-season form develops larger eyespots on the wings, which might be beneficial to escape from predators, while the dry-season form shows smaller eyespots and lack of obvious wing coloration, which are presumably adaptations for hiding in abundant fallen leaves during dry seasons. Several studies have shown that the seasonal phenotypes of *Bicyclus* depend on environmental temperature, as a higher temperature during late larval and early pupal stages increases the level of 20E, which maintains *Dll* expression in the anlage of wing discs and consequently promotes eyespot formation in the adult wings (Brakefield et al. 1996).

18.2.2 Temperature-Dependent Melanization

Most seasonal phenotypes of Lepidoptera are consequences of differential levels of pigmen-

tion (i.e., melanization). For example, in a species of tiger moth (*Utetheisa ornatrix*), cold treatment during larval and pupal stages increased melanin marks on the fore- and hindwings (Fig. 18.1). Melanin synthesis is considered to be beneficial for animals for several reasons, including the prevention of UV-dependent DNA damage, particularly in lower-latitude areas with higher UV irradiation. Melanization is also advantageous to efficiently absorb sunlight, through which animals can maintain their body temperature in cold climates. Conversely, melanin synthesis is metabolically costly and confers the risk of being found by predators. Thus, seasonal changes in melanin synthesis are adaptive traits resulting from different selective pressures.

Temperature-dependent melanization is also reported in vertebrates. Tyrosinase is a rate-limiting enzyme that regulates the synthesis of melanin. Intriguingly, temperature-dependent albinism has been reported in cats (Siamese), Himalayan rabbits, mice, and humans (Iljin and Iljin 1930; Searle 1968). In these cases, pigmentation is largely restricted to distal areas of body, such as the tips of tails, legs, or faces. These conspicuous coloring phenotypes are due to a temperature-sensitive mutation of tyrosinase, resulting in a protein that retains enzymatic activity at lower temperatures, while it loses its function at higher temperatures (around body temperature in endothermic animals), due to the

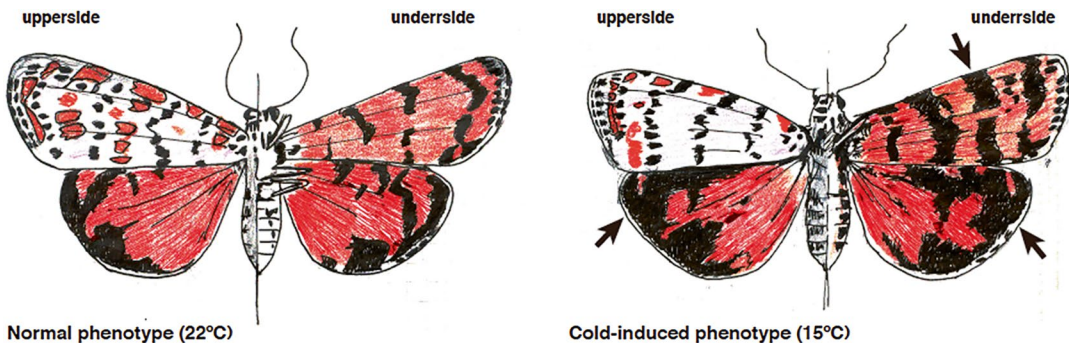


Fig. 18.1 Temperature-dependent phenotypic plasticity of the tiger moth (*Utetheisa ornatrix bella*). Compared with control moths raised at 22 °C (left), cold treatment (15 °C, right) during the last instar of larvae and the pupal stage decreased the number of orange/red spots and

increased melanization in adult forewings and hindwings (indicated by arrows). *U. ornatrix bella* occurs from the Caribbean and the southern United States to Nova Scotia. (The illustration is based on Sourakov 1995)

loss of functional protein folding. Relatively lower temperatures in the peripheral parts of bodies allow melanization to occur, mediated by mutant tyrosinase, while higher temperatures in the center of the body interfere with the production of functional enzymes, resulting in specific patterns of albinism. In humans, the missense mutation of tyrosinase also results in peripheral pigmentation (Giebel et al. 1991; King et al. 1991). In this case, amino acid substitution (arginine to glycine) induces thermosensitivity of tyrosinase, through which the mutant protein loses enzymatic activity at higher temperatures, as in the case of temperature-dependent albinism in other vertebrates.

18.2.3 Temperature-Dependent Sex Determination

Temperature-dependent sex determination (TSD) is a unique developmental program in which environmental temperature during embryogenesis influences the sex determination of offspring (Gilbert 2015). TSD is reported in a number of egg-laying animals, such as alligators (Ferguson and Joanen 1982), turtles (Bull and Vogt 1979; Ewert et al. 1994), lizards (Harlow and Shine 1999), salamanders (Sakata et al. 2005), and teleosts (Ospina-Alvarez and Piferrer 2008). TSD is classified into two patterns: (1) lower temperatures produce females, while higher temperatures produce males (or vice versa), or (2) both lower and higher temperatures produce females, while intermediate temperatures produce males. Thus, the former pattern displays a single transition zone of sex determination in response to temperatures, while the latter exhibits multiple transition zones with female offspring dominating at extreme temperatures (Merchant-Larios 2001).

TDS is mediated by estrogen, a well-studied steroid hormone that regulates feminization in many vertebrates. The administration of estrogen in turtle embryos dramatically increases the proportion of females regardless of temperature (Rhen and Lang 1994). In the European pond turtle *Emys obicularis*, the expression of aromatase, an enzyme that is essential for the biosyn-

thesis of estrogen, is significantly increased by higher temperatures, at which females are dominant in the offspring (Desvages et al. 1993). Blocking aromatase activity with specific inhibitors induces masculinization in turtle offspring independent of temperature (Dorizzi et al. 1994; Rhen and Lang 1994). Although the evolutionary advantages of TSD remain to be elucidated, biased sex ratios might be relevant to the survival of offspring if males and females exhibit differential fitness under distinct environmental conditions and nest temperatures are associated with such variable conditions. In agamid lizards, differential nest temperatures result in biased sex ratios in offspring; intriguingly, embryogenesis proceeds more rapidly in the warmer nests than in the cooler nests, and female offspring from the warmer nests hatch much earlier than males (Warner and Shine 2005). This might be beneficial for short-lived, early-maturing species, wherein the number of females has a large impact on the size of the population (Warner and Shine 2005).

18.2.4 Temperature-Dependent Growth and Differentiation: Plasticity and Rigidity

It is widely known that extraembryonic temperature affects the timing of embryogenesis. An increase in temperature accelerates the speed of embryogenesis in a variety of ectothermic animals. In *Drosophila melanogaster*, the total period of embryogenesis is approximately 33 h at 17.5 °C, while it is shortened to 16 h at 27.5 °C (Kuntz and Eisen 2014). Intriguingly, the relative timing of developmental events is constant at any temperature, suggesting that scaling mechanisms regulate the progress of embryogenesis. Comparative studies of temperature-dependent embryogenesis in various *Drosophila* species derived from different climates revealed species-specific responses during the progression of embryogenesis (Kuntz and Eisen 2014); for example, *Drosophila persimilis* and *Drosophila pseudoobscura* isolated from Alpine areas show reduced acceleration of embryogenesis at 22.5 °C

compared with tropical species, such as *D. melanogaster*. Despite variable responses to the absolute length of embryogenesis, the relative timing of early developmental events is uniformly scaled among species regardless of temperature. Thus, the temperature-dependent regulation of *Drosophila* embryogenesis consists of two distinct developmental mechanisms including (1) conserved scaling mechanisms of developmental timing, especially at early stages of embryogenesis, and (2) species-specific regulation of the total length of the developmental period as a result of evolutionary adaptation in variable climates. In *Caenorhabditis elegans*, cell division intervals decrease exponentially with increasing temperature, indicating temperature-dependent cell division timing according to the Arrhenius equation, the function describing the temperature dependence of chemical reactions (Begasse et al. 2015). Similar kinetics of temperature-dependent cell divisions are evident in *Caenorhabditis briggsae*, a sister species isolated from a warmer climate, suggesting that conserved chemical reactions underlie the temperature-dependent scaling of early embryogenesis (i.e., cell cycle control). In contrast, these nematode species show a distinct range of physiological tolerance to temperatures. Although both species exhibit a slowed rate of cell division at extremely high temperatures, the Arrhenius range of *C. briggsae* is higher by 2 °C, indicating that *C. briggsae* tolerates higher temperatures than *C. elegans* (Begasse et al. 2015).

The molecular mechanisms underlying temperature-dependent embryogenesis are poorly understood. In insects showing seasonal changes in melanization, a pulse of ecdysone also stimulates the growth of larvae and accelerates the timing of embryogenesis (Hartfelder and Emlen 2012). In addition, it has been suggested that the rate of mitochondrial activity acts as a limiting factor for cell division timing, through changes in the available amount of adenosine triphosphate (ATP). A recent study showed that *C. briggsae* exhibits a lower mitochondrial membrane potential (Hicks et al. 2012), although extensive analyses in different clades of animals will be required to verify the causality.

The temperature-dependent scaling of developmental timing implies the robustness of developmental programs in response to variable rates of different developmental periods. Lucchetta et al. indicated that unusual temperature conditions in *Drosophila* embryos did not alter segment patterns at later developmental stages (Lucchetta et al. 2005). In their experiments, the anterior and posterior halves of the embryos were incubated at different temperatures by using microfluidic devices. As a result, the gradient of Bicoid protein and the frequency of mitosis at early stages were significantly altered within a single embryo depending on the temperature; however, subsequent gene expression, such as the position of stripes of Even-skipped and Hunchback proteins, was highly stable and was not disturbed by temperature, suggesting that correction mechanisms compensate for the initial perturbation of developmental programs (Lucchetta et al. 2005).

In *Drosophila*, the level of Notch receptor signaling is highly stable within a wide range of temperatures. Upon the binding of membrane-bound ligands such as Delta, Serrate, and Lag2, the Notch receptor is activated and cleaved into several fragments by Kuzbanian and Presenilin, which are specific metalloproteinases. The intracellular domain (ICD) of the receptor is translocated to the nucleus and incorporated into a transcriptional regulatory complex (Kopan and Ilagan 2009). Alternatively, Notch receptor activation occurs through a cytoplasmic protein Deltex independent of DLS ligands, which promotes the endocytic trafficking of the receptor into lysosomes (Wilkin et al. 2008). DSL-dependent and -independent pathways exhibit different temperature sensitivities; a higher temperature (~29 °C) promotes the ligand-dependent activation of the receptor, while a lower temperature (~18 °C) facilitates the ligand-independent signaling pathway (Shimizu et al. 2014). The balance of these signaling routes is controlled by Suppressor of Deltex [Su(dx)], an intracellular protein that blocks the function of Deltex-induced activation of the Notch receptor in a temperature-dependent manner. Thus, opposing pathways positively or negatively regulate Notch signaling

with differences in their temperature dependence, establishing the thermal robustness of Notch signaling under the wide range of environmental temperatures (Shimizu et al. 2014).

18.3 Evolution of Thermal Adaptation in Endothermic Animals

Endothermy is a unique physiological trait in which a constant body temperature is maintained by producing heat within the body. During the evolution of vertebrates, endothermy was acquired independently in mammals and birds. These animal groups are thought to be derived from the common ancestor of amniotes living approximately 300 million years ago (MYA), and ancestral amniotes may have exhibited ectothermic characteristics similar to those of modern reptiles. However, endothermy and ectothermy are not clade-specific traits, as several extant species of reptiles and fishes present the potential for endogenous heat production during egg brooding (Brashears and DeNardo 2015; Farmer 2000; Tattersall et al. 2016). Nevertheless, endothermy is tightly linked with several anatomical and physiological characteristics that are specific to extant mammals and birds, suggesting that the evolution of endothermy might have been triggered by multiple changes in genetic, developmental, and environmental backgrounds (Farmer 2000). Based on paleontological and physiological perspectives, Lovegrove hypothesized that the evolution of endothermy was promoted through three major steps: (1) increased metabolism and body temperature in ancestral animals living in the Permian and Triassic, (2) miniaturization of body size and the associated evolution of fur or feathers for the insulation of the body temperature, and (3) further specialization of anatomical and behavioral traits, such as powered flight in birds and terrestrial cursoriality in mammals, during the Cretaceous and Cenozoic (Lovegrove 2017). Although this tri-phasic model provides a fascinating scenario in which sequential changes in multiple traits contribute to the evolution of endothermy, it is difficult to verify

the validity of the hypothesis due to the lack of fossil evidence preserving physiological characteristics. In contrast, recent technological advancements in the isolation and sequencing of archaic DNA materials have provided significant information about the genetic backgrounds of extinct animals, allowing us to ascertain the thermogenic properties of ancestral species.

18.3.1 Genomic Signatures of Thermal Adaptation During Mammalian Evolution

Cold tolerance is a major thermal adaptation in several mammalian species, which are assumed to have evolved during an ice age. Woolly mammoths (*Mammuthus primigenius*) are extinct elephants adapted to an extremely cold environment, having evolved small ears and tails, thick subcutaneous fat tissue, and long thick fur, to minimize heat loss (Callaway 2015) (Fig. 18.2). These specialized traits are thought to have evolved relatively recently at the geological time scale because woolly mammoths and their close relatives, including modern Asian elephants, diverged around 5 MYA, and colonization of the steppe-tundra has occurred at approximately 1–2 MYA

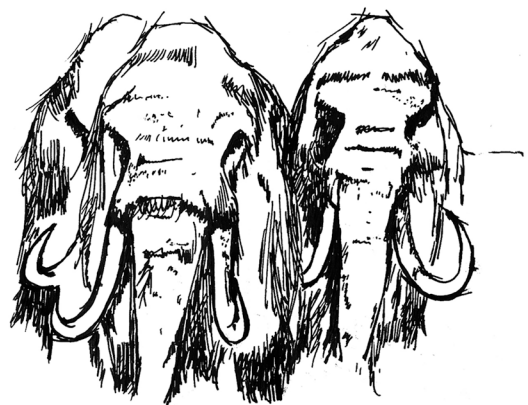


Fig. 18.2 Walking woolly mammoths. Woolly mammoths (*M. primigenius*) are an extinct elephant species characterized by long hair coats, thick fat layers, and small ears, as adaptive traits to arctic climates. The majority of the population in Siberia disappeared approximately 14,000–11,500 years ago, but the last colony is thought to have remained on Wrangel Island until 2000 B.C.

(Debruyne et al. 2008). Identification of whole genomic sequences of woolly mammoths provided genetic changes that are relevant to cold adaptation in this extinct species (Campbell et al. 2010; Lynch et al. 2015; Rompler et al. 2006). For example, mammoth-specific amino acid substitutions have been found in genes regulating circadian clocks, which are tightly associated with temperature homeostasis and thermogenesis (Lynch et al. 2015). The most intriguing signature found in mammoth genomic sequences consists of amino acid changes in genes encoding temperature-sensitive transient receptor potential channels (TRP channels). In particular, a TRPV3 channel with a woolly mammoth-specific N647D substitution (asparagine is changed to aspartate) is less responsive to a range of temperatures than the ancestral version of the channel (Lynch et al. 2015). Given the pleiotropic roles of TRPV3 in temperature sensation, hair growth, and adipose cell generation, it is possible that the woolly mammoth-specific changes in TRPV3 contributed to the evolution of a variety of traits associated with cold adaptation (Lynch et al. 2015).

Mitochondrial uncoupling protein 1 (UCP1) plays a crucial role in thermogenesis. UCP1 is localized in the mitochondrial inner membrane of brown adipose tissue and facilitates substrate oxidation and heat generation (Cannon and Nedergaard 2004; Nicholls and Locke 1984; Oelkrug et al. 2015). This process is referred to as nonshivering thermogenesis (NST) and is a characteristic of placental mammals (Oelkrug et al. 2015). However, functional UCP1 is not produced in several mammalian lineages due to inactivating mutations in coding sequences (Gaudry et al. 2017). Intriguingly, the loss of UCP1 function occurred during the period corresponding to global cooling during the Paleogene, and UCP1 inactivation was associated with the subsequent acquisition of unique traits adapted to cold environments in several mammalian species, such as woolly mammoths that had a large cervical mass of fat. An increase in body mass is another adaptive trait observed in response to global cooling; however, it is also linked to low species diversity in mammalian clades lacking functional UCP1 (Gaudry et al. 2017).

18.4 Thermal Adaptation During Human Evolution

18.4.1 Phenotypic and Genetic Characteristics of Neanderthals

Anatomically modern humans (AMHs), including the present human population and our direct ancestors, originated in Africa approximately 330,000 years ago and spread around the globe over the past 100,000 years (Reich 2018). During migration and colonization, AMHs adapted to a variety of climates and ecological niches, shaping their (and our) genetic backgrounds and phenotypic variations. Some currently observed genetic signatures are derived from extinct human populations, such as Neanderthals and Denisovans, who lived during the Pleistocene (Meyer et al. 2012; Prufer et al. 2017).

Neanderthals are hominins that lived in Europe and the Middle East from approximately 400,000 to 28,000 years ago (Finlayson et al. 2006). Over the time period, repetitive cycles of glacial and interglacial periods occurred, and Neanderthals are considered to have been physically adapted to cold environments. However, it is not clear which of their biological traits were relevant to cold acclimation. The bone features of Neanderthals suggest that they exhibited robust physical characteristics. Compared with AMHs, Neanderthals had shorter limbs in relation to their trunks (Ben-Dor et al. 2016), as well as a wider pelvis and larger thorax (Stegmann et al. 2002). The proportion of muscle mass per unit height in Neanderthals was higher than that in modern humans, which might be beneficial for physical activity in cold conditions (Ruff et al. 1997). In addition, the larger sinus of Neanderthals suggests that they had protruding noses, which has been assumed to be an adaptive trait to cold temperatures (Churchill 1998). However, various hominin species inhabiting northern areas with cold climates exhibit relatively smaller maxillary sinuses (Rae and Koppe 2000). Because the nasal index, which is calculated from nose width against nose height, is negatively correlated with vapor pressure (Weiner 1954), protracted and

narrower noses might be rather beneficial to protect the respiratory tract in cold air. Through advanced three-dimensional analysis, de Azevedo et al. reconstructed the nasal respiratory cycles of Neanderthals and compared them with those of two modern human populations: admixed Argentinians of southwestern European (SWE) origin and northeastern Asians (NEA) (de Azevedo et al. 2017). The data indicated that the most anterior part of the nose affects temperature uniformity of airflows, which was achieved more rapidly in NEA than in Neanderthals and SWEs. Based on these results, the authors concluded that the nasal morphology of Neanderthals cannot be regarded as an adaptive characteristic to cold conditions (de Azevedo et al. 2017). Similarly, comparative analysis of maxillary and frontal sinuses among various hominins and human populations revealed that these structures of Neanderthal were not different from those of recent Europeans (Rae et al. 2011). These lines of evidence rebutted the hypothesis that a larger paranasal sinus in Neanderthals provided increased pneumatization and was advantageous for survival in a cold climate (Rae et al. 2011).

Recent comprehensive studies of genomic sequences derived from fossil specimens suggested that Neanderthals coped with cold climates through changes in various physiological characteristics. Single-nucleotide polymorphisms (SNPs) unique to the Neanderthal genome were identified at the loci of the *LEPR*, *PPARG*, *PPARGC1B*, and *NRF1* genes, which are associated with BAT metabolism (Sazzini et al. 2014). The SNPs in *PLIN1* and *PPARGC1B* are shared by modern humans, including European, East Asian, and Latin American populations, but not African populations (Sazzini et al. 2014), suggesting that these genetic signatures in modern human populations were derived from Neanderthals by introgression. Furthermore, Neanderthal infants could have potentially presented a reduction of the energy costs required for growth, which might be due to metabolic adaptation to cold ambient temperatures (Mateos et al. 2014). Thus, BAT-mediated NST in Neanderthals might have contributed to their unique manner of infant growth. In addition, it

has been reported that Neanderthal-derived variants of genes associated with the lipid catabolism pathway (LCP) have been positively selected in the European population. These genetic variants shared with Neanderthal might be linked with diversified lipid concentrations and the expression levels of LCP-related genes in modern Europeans (Khrameeva et al. 2014).

Some Neanderthals might have pale skin and red hair, similar to some modern Europeans. Genomic sequences derived from two Neanderthal specimens contained a nonsynonymous variant in the gene encoding Melanocortin 1 receptor (MC1R), a heterotrimeric G protein-coupled receptor (Lalueza-Fox et al. 2007). MC1R is involved in hair and skin pigmentation by regulating eumelanin and pheomelanin synthesis, and various SNPs in MC1R are associated with pale skin color and light hair of the modern European population (Harding et al. 2000). Compared with wild-type MC1R, a unique variant in Neanderthals (R307G) decreased agonist-induced intracellular cAMP levels due to reduced cell surface expression of MC1R (Lalueza-Fox et al. 2007). The authors speculated that homozygosity for the variant contributed to less eumelanin production in Neanderthals, which increased their sensitivity to solar radiation and supported UV-mediated vitamin D synthesis (Kislev and Barkai 2018).

18.4.2 Thermal Adaptations in Modern Humans

Recent advancements in high-throughput analyses of genomic sequences have shed light on intriguing genetic differences in modern local human populations and their functional link to adaptive radiation (Fan et al. 2016). Ectodysplasin A receptor (EDAR) is a cell surface receptor for Ectodysplasin A and plays a crucial role in the development of ectodermal tissue. Recent studies have revealed an amino acid substitution in EDAR (V370A) in specific human populations, including East Asian and Native American populations (Bryk et al. 2008). Demographic analysis of haplotypes suggested that the V370A allele origi-

nated from central Asia approximately 30,000 years ago. Knock-in mice with the V370A substitution in the *EDAR* locus showed increases in hair thickness, the number of eccrine glands, and mammary gland branching, which correlated with phenotypic traits of East Asian people (Kamberov et al. 2013). It is suggested that the increased density of eccrine glands resulting from the V370A allele has been beneficial for ancestral hunter-gatherers in warm and humid seasons. In addition to being physiologically advantageous, phenotypic changes associated with the V370A allele might have been positively selected through mate preference (Kamberov et al. 2013).

Indigenous people living in Siberia (Fig. 18.3) exhibit a number of specific physiological traits, such as an increased metabolic rate and blood pressure and low levels of serum lipids which are thought to be adaptations to extreme cold climates (Leonard et al. 2005; Snodgrass et al. 2008). Association studies of the SNPs in the Siberian population revealed significant positive selections in chromosome regions containing genes including carnitine parmitoyltransferase 1a (*CPT1A*), low-density lipoprotein receptor-related protein 5 (*LRP5*), thyroid adenoma-associated protein (*THADA*), which are associated with metabolic

regulation, and cGMP-dependent protein kinase 1 (*PRKG1*), which controls smooth muscle contraction (Cardona et al. 2014). Furthermore, recent studies have identified unique genetic signatures of Greenland Inuit people at the genomic loci of fatty acid desaturases (*FADS1-3*) (Fumagalli et al. 2015). FADS plays essential roles in the metabolism of polyunsaturated fatty acids (PUFAs), and FAD1 and FADS2 are rate-limiting enzymes converting omega-3 to omega-6 fatty acids. Thus, the Inuit-specific SNPs in *FADS* loci are attributed to their traditional diets, with a high content of omega-6 PUFAs derived from sea foods. Intriguingly, some of these SNPs in *FADS* loci are associated with weight and height in Inuit people but not in Europeans, suggesting that changes in pleiotropic roles of FADS have contributed to multiple phenotypes specific to Inuit people (Fumagalli et al. 2015). In addition, a Greenlandic Inuit-specific polymorphism was identified in the genomic region containing *TBX15*, a transcription factor that regulates the differentiation of brown and beige adipocytes. These cell types have been shown to express UCP1 by exposure to a cold environment, suggesting that genomic differences in *TBX15* are associated with cold adaptation in Inuit (Fumagalli et al. 2015).

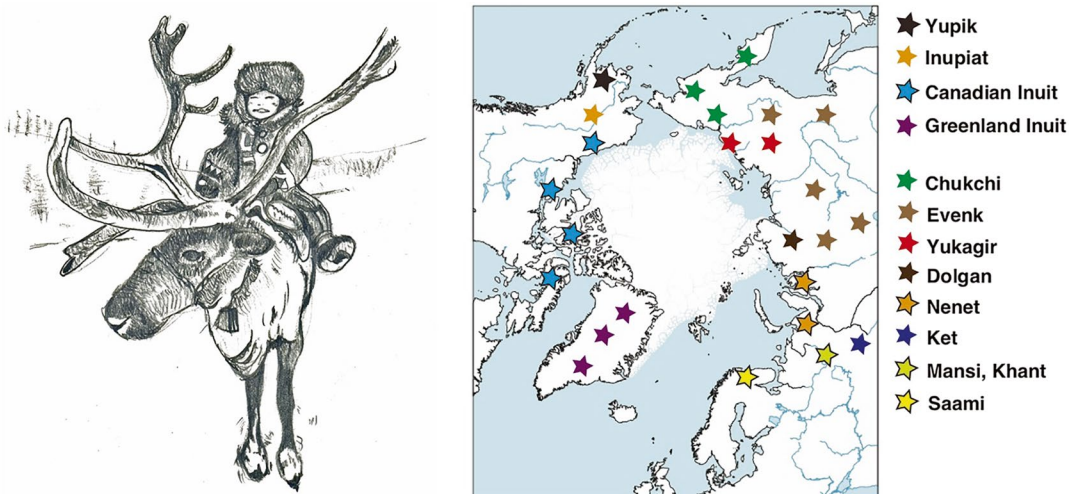


Fig. 18.3 Indigenous people living in the Arctic area. (Left) A boy of the Evenks people. The drawing is based on a picture by Alexander Khimushin. (Right) Geographical distribution of indigenous Arctic people.

The illustration is based on information from ANSIPRA (Arctic Network for the Support of the Indigenous Peoples of the Russian Arctic) and Strata360

A recent study revealed an extreme population difference in the genetic variation of TRP cation channel subfamily M member 8 (TRPM8), the only temperature receptor known to mediate moderate cold responses (Key et al. 2018). The allele frequency of the variant of *TRPM8* was significantly higher in populations living in cold climates (c.f., 88% in Finland) than in indigenous people in Africa (approximately 5% in Nigeria). The variant is a SNP localized at the upstream of *TRPM8*, suggesting the possible alteration of regulatory sequences. Intriguingly, the variant is associated with migraine and inflammatory bowel disease. Thus, it is possible that genetic adaptation to cold climates in our ancestors also conferred both variability and potential risks for specific diseases in present-day populations (Key et al. 2018).

18.5 Remaining Questions and Future Directions

As introduced in the first part of this review, the plasticity of embryogenesis in response to variable environmental temperatures plays a critical role in the development and evolution of ectothermic animals. However, how phenotypic plasticity is linked to the origin of new traits or species remains to be elucidated. Considering the evolution of novel characteristics, the phenotypic plasticity of ancestors must be suppressed in descendants, and selected phenotypes with maximum fitness in relation to the environment should be fixed in the population. This process is thought to be associated with genetic assimilation, a phenomenon in which an environment-induced phenotype loses its sensitivity in response to the environment and develops into a rigid phenotype without an environmental trigger (Waddington 1952). Although the mechanistic understanding of genetic assimilation remains extremely poor, it is inferred that cryptic genetic variations below the threshold of phenotypic manifestation become permanently exposed by lowering the threshold (Ehrenreich and Pfennig 2016). A recent study suggested that allelic differences in gene expression contribute to cryptic genetic

variations in response to environmental stressors, such as extreme temperatures (Chen et al. 2015). Furthermore, several studies have shown that the heat shock protein Hsp90, a well-known chaperone protein induced by various cellular stresses, plays a critical role in masking cryptic genetic variations that have the potential to contribute to novel traits (Rutherford and Lindquist 1998). Thus, it is possible that Hsp90 acts as an “evolutionary capacitor” that determines the threshold of phenotypic manifestation beyond neutral mutations. However, genetic insights into evolutionary capacitors and their regulatory mechanisms are largely lacking. In addition, whether small genetic variations, such as a variety of SNPs in specific human populations, could have the potential to contribute to phenotypic plasticity and genetic assimilation should be examined in the future.

Temperature sensitivity in developing endothermic animals also remains an intriguing question to be addressed. It has been believed that the embryogenesis of endothermic animals, such as mammals and birds, proceeds under a constant temperature because of intrinsic thermoregulation in parental animals. In contrast, higher or lower temperatures beyond the physiological ranges (hyper or hypothermic conditions) are deleterious for embryogenesis. For example, maternal fever during the first trimester of human embryogenesis causes serious defects in craniofacial and heart development (Dreier et al. 2014). A recent study revealed that some hyperthermia-induced congenital defects are mediated by the TRPV1 and TRPV4 ion channels expressed in neural crest cells (Hutson et al. 2017). Treatment with TRPV antagonists was shown to block hyperthermia-induced facial and heart defects, while treatment with a TRPV channel antagonist replicated defective phenotypes in the developing chick and zebrafish embryos. Thus, the TRPV-dependent thermosensitivity of neural crest cells is highly conserved among ectothermic and endothermic animals (Hutson et al. 2017), resulting in irreversible changes in offspring phenotypes. Alternatively, TRPV-dependent thermosensitivity may limit the range of reaction norms under-

lying phenotypic assimilation, although this hypothesis remains to be addressed.

Temperature-dependent plasticity and rigidity of embryogenesis are fundamental traits in both ectothermic and endothermic animals. Improving our understanding of biological responses to temperature and the underlying mechanisms of thermal adaptations will provide novel insight into the flexibility and robustness of developmental programs. Furthermore, accumulating evidence reveals that temperature-dependent phenotypic changes are tightly linked with adaptive radiation in a variety of species, including humans, in a wide range of climate conditions. Revealing the genetic backgrounds underlying thermal adaptations and their causal relationships with specific environments may significantly expand our knowledge of biodiversity and the rapid and catastrophic changes in ecosystems induced by human activity.

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