Identification of key signaling molecules involved in hypothalamic thermoregulation

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Summary

Thermoregulation is a crucial homeostatic function orchestrated by the central nervous system for all homothermic animals to obtain an optimal thermal environment. The thermoregulatory center of homothermic animals is located in the preoptic area of the hypothalamus, which not only receives the temperature input from the peripheral thermosensors, but also detects the local brain temperature as a central thermosensor. By integrating the peripheral and central temperature information, the preoptic area orchestrates thermoregulatory responses through different pathways. The sparse warm-sensitive neurons in the preoptic area are hypothesized to play a pivotal role in controlling thermoregulation. However, the molecular basis of the hypothetical thermoregulatory mechanism has remained mysterious ever since it has been first proposed.

This doctoral thesis aims to identify the key signaling molecules underlying the thermosensitivity of warm-sensitive preoptic neurons and the corresponding thermoregulatory mechanisms. In this work, temperature-sensitive preoptic neurons were identified by calcium (Ca^{2+}) imaging *in vitro*. Thereafter, by carrying out two pharmacological and transcriptomic screening strategies on primary preoptic neurons and cell lines, respectively, we identified that Transient receptor potential cation channel, subfamily M, member 2 (Trpm2) as an important receptor in the heat response of warm-sensitive preoptic neurons in the mouse hypothalamus. Trpm2 expression was detected in the preoptic area by *in situ* hybridization. Heat responses of warm-sensitive neurons were abolished in the neurons from Trpm2 knockout mice characterized by Ca^{2+} imaging, indicating that Trpm2 channel is involved in conducting Ca^{2+} influx during heat stimuli. Corresponding behavioral studies showed that Trpm2 knockout mice developed a less pronounced increase of core body temperature during fever induction by

lipopolysaccharide (LPS) when compared to wild-type littermates. In summary, our work sheds light on the molecular basis of the temperature sensation and thermoregulatory mechanisms in the mammalian central nervous system.

Zusammenfassung

Thermoregulation ist eine lebenswichtige homöostatische Funktion aller homothermischen Organismen. Sie wird vom zentralen Nervensystem reguliert und stellt den Mechanismus dar, der eine stabile Körpertemperatur garantiert. Das thermoregulatorische Zentrum im Gehirn befindet sich in der preoptischen Region des Hypothalamus. Es empfängt nicht nur Informationen über die Körpertemperatur von peripheren thermosensorischen Neuronen, sondern misst zusätzlich die lokale Hirntemperatur um nach Integration dieser beiden Signale verschiedene thermoregulatorische Mechanismen zu koordinieren. Obwohl eine Gruppe weniger, wärmesensitiver Neuronen in der preoptischen Region nach wissenschaftlicher Erkenntnis in diesem Prozess eine wichtige Rolle spielt, sind die molekularbiologischen Grundlagen der Hirntemperatursensorik noch nicht bekannt.

Das Ziel dieser Dissertation war die Identifikation wichtiger Bestandteile von Signalkaskaden, die der Wärmesensitivität in preoptischen Neuronen zugrunde liegen. Nach der erfolgreichen Identifikation der Neuronen mit Hilfe eines kalziumbasierten (Ca^{2+}) Bildgebungsverfahrens *in vitro*, führten pharmakologische und Analysen des Transkriptoms in primären preoptischen Neuronen und einer wärmesensitiven Zelllinie letztendlich zur Identifikation von Trpm2 (Transient receptor potential cation channel, subfamily M, member 2) als ein essentieller Bestandteil der Temperaturwahrnehmung durch preoptische Neuronen des Hypthalamus.

Die Expression von Trpm2 in der preoptischen Region in Mäusen wurde durch *in situ* Hybridisierungsexperimente bestätigt. Die Kalziumaufnahme –als Reaktion auf einen Wärmestimulus– war in Trpm2 Knockout-Mäusen stark reduziert und

deutet auf eine essentielle funktionale Rolle Trpm2s in der wärmestimulusabhängigen Kalziumaufnahme hin.

In darauffolgengenden Verhaltensstudien konnte gezeigt werden, dass eine durch LPS-injektion (lipopolysaccharide) induzierte Fieberentwicklung in Trpm2-Knockout-Mäusen im Vergleich zum Wilddtyp verringert ist.

Zusammenfassend beschreibt diese Dissertation die Entdeckung von *in vitro* identifizierten, und *in vivo* verifizierten molekularen Mechanismen, die der Wärmedetektion in der preoptischen Region des Hypothalamus in Säugetieren zugrunde liegen.

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1. Introduction

1.1 Thermoregulation

Thermoregulation is the capacity of an organism to maintain its core body temperature within a certain range independent of the ambient temperature (Hardy, 1961; Hensel, 1973; 1981; Romanovsky, 2006; Benarroch, 2007; Szekely et al., 2009). It is a crucial homeostatic function orchestrated by the central nervous system in all homeothermic animals. As a result of its evolution, the ability of thermoregulation benefits homeothermic organisms by providing an optimal internal thermal environment for maintaining metabolism, molecular activities, and biochemical reactions (Rolfe and Brown, 1997; Krajewski and Narberhaus, 2014). Meanwhile, it also serves as a fundamental function for the immune system against invading pathogens by allowing the elevation of core body temperature, resulting in fever (Saper and Breder, 1994; Saper, 1998; Romanovsky et al., 2005; Steiner et al., 2006). Thermoregulation of homeotherms, including humans, is involuntarily regulated by numerous physiological heat-gain and heat-loss responses, such as shivering and non-shivering thermogenesis (Fuller et al., 1975; Janský, 2008; van Marken Lichtenbelt, 2012), cutaneous vasodilation and vasoconstriction (Kellogg, 2006; Johnson et al., 2014), panting (Robertshaw, 2006), sweating (Gleeson, 2007), and other less dominant thermoregulatory processes. Thermoregulation is one of the main components of energy homeostasis impacting health (Kluger, 1979), longevity (Kluger, 1979; Tabarean et al., 2010), and aging (Florez-Duquet and McDonald, 1998).

The thermoregulatory center of homothermic animals is located in a deep brain region – the preoptic area (POA) of the hypothalamus (Boulant, 2000; McAllen, 2004; Yoshida et al., 2009; Boulant, 2010). As a region of homeostatic integration

in the brain, the hypothalamus orchestrates several regulatory systems including the control of body temperature (Rodbard, 1948; Morrison and Nakamura, 2011), blood pressure (Guyenet, 2006), body osmolarity (Bourque, 2008), feeding behaviour (Horvath and Diano, 2004), reproductive activity (Handa and Weiser, 2014), and circadian rhythm (Saper et al., 2005a). The POA is located in the very anterior part of the hypothalamus and is involved in regulating core body temperature, reproduction and sleep (Paxinos and Franklin, 2012). A wealth of experimental evidence indicates that the POA is the thermoregulatory center. Firstly, lesions of this area in various species of mammals, including rodents, dogs, cats and rabbits, lead to dysfunction of thermoregulation when compared with lesions performed on other brain regions (Carlisle, 1969; Christensen et al., 1977). Second, with the aid of c-Fos immunostaining, researchers are able to mark the neurons activated in response to temperature stimuli in rodents. The results show that much more neurons are activated in the POA in temperature-stimulated animals than in the control group kept at room temperature, indicating that ambient temperature changes sensed by peripheral somatosensory neurons reach the preoptic area of the hypothalamus and increases the neuronal activity in this region (Scammell et al., 1993; Bachtell et al., 2003). Moreover, a direct role for temperature detection of POA neurons relevant to homeostatic temperature control is best demonstrated by the studies of mammals with implanted thermodes with which it is possible to induce local warming and cooling (Nakayama et al., 1961; 1963). A tiny change of local POA temperature by a thermode causes striking thermoregulatory responses in the peripheral organs of animals (Andersson and Larsson, 1961; Fuller et al., 1975; Martelli et al., 2014). Recently, another innovative genetic experiment performed by the Bartfai group shows that the elevated hypothalamic temperature of Hcrt-Ucp2 transgenic mice (which overexpress the Ucp2 protein in hypocretin-positive hypothalamic neurons, thereby causing increases in hypothalamic temperature) resulted in a 0.3-0.5°C reduction of abdominal temperature (Conti et al., 2006). Finally, a small population of temperature sensitive neurons in the POA of the hypothalamus has been identified and characterized by electrophysiological

approaches (Nelson and Prosser, 1981; Vasilenko, 1994; Boulant, 2000). It's believed that the set-point of core body temperature is determined by the thermosensitive neurons in this area (Boulant, 2000; Belmonte and Viana, 2008). In summary, the preoptic area does not only receive the temperature information sent from the peripheral somatosensory neurons (e.g. dorsal root ganglion (DRG) neurons), but also detects the local brain temperature. The available evidence demonstrates that the POA of the hypothalamus acts as the primary thermoregulatory center in controlling core body temperature homeostasis of homeothermic animals (Nakamura, 2011; Morrison et al., 2014).

1.1.1 Circadian rhythm of core body temperature

The circadian rhythm of daily core body temperature fluctuations is one of the best examples of thermoregulation (Refinetti and Menaker, 1992; Weinert and Waterhouse, 2007). The normal core body temperature of a human adult varies between 36.5°C–37.5°C. Thermoregulation enables humans to fine-tune their core body temperature within this narrow range no matter whether the ambient temperature is colder or warmer. The fluctuation pattern within this range is due to the circadian rhythm of core body temperature during day and night cycles (Saper et al., 2005b; Kräuchi, 2007). All diurnal, nocturnal and crepuscular homeothermic animals have their self-sustaining circadian rhythm of core body temperature due to the activity-correlated metabolic rate (Brown et al., 2002; Buhr et al., 2010).

1.1.2 Hypothermia and hyperthermia

Hyperthermia is defined as a state of elevated core body temperature due to a failure of thermoregulation resulting in a body that produces or absorbs more heat than it dissipates (Desforges and Simon, 1993; Burke and Hanani, 2012). Hyperthermia is defined as a temperature greater than 37.5°C. It occurs upon physiological conditions such as intense exercise or pathological conditions, e.g. malignant hyperthermia and heat stroke (Flouris, 2010). On the contrary,

hypothermia is a condition describing the drop of core body temperature below the threshold mandatory for normal metabolism and body functions (Hochachka, 1986; Romanovsky et al., 2005; Xanthos and Chalkias, 2014). Any core body temperature lower than 35°C is regarded as hypothermia, which usually happens when a body is unable to cope with a cold environment or has suffered from trauma.

1.1.3 Fever

Fever is a process initiated by the body as a response to bacterial or viral infection (Kluger, 1979; Mackowiak, 1998; Romanovsky et al., 2005). It's generally characterized as the 1–4°C elevation of core body temperature above the normal range of 36.5–37.5°C caused by an increased internal temperature set-point (Flier et al., 1994). In fever responses, autonomic and behavioral thermoregulatory mechanisms are initiated to either increase heat-gain or reduce heat-loss (Mitchell et al., 1970). For example, blood flow is adjusted from cutaneous to deep vascular beds in order to minimize the heat dissipation from the skin (Wanner et al., 2013). This explains why human individuals usually turn pale under fever conditions.

Several pyrogens have been shown to induce fever in animals (Conti et al., 2004; Romanovsky et al., 2005), including interleukin-1β (IL-1β) (Long et al., 1990; Nakamori et al., 1994), interleukin-6 (IL-6) (Rummel et al., 2006; Nilsberth et al., 2009), tumor necrosis factor-α (TNFα) (Cooper et al., 1994), prostaglandin E_2 (PGE_2) (Blatteis et al., 2005), and others (Conti et al., 2004; Blatteis et al., 2005; Romanovsky et al., 2005). Even though body temperature is increased during both fever and hyperthermia, they are distinct conditions caused by different mechanisms (Mackowiak, 1998). The difference between them is whether the set-point of core body temperature is elevated or not (Flier et al., 1994). Hyperthermia is a situation in which core body temperature exceeds the set-point because of improper thermoregulation or excessive heat production, while the

fever response is due to the fact that the temperature set-point is increased by pyrogens. During the fever response, the body feels cold even though core body temperature is rising since it is still under the newly elevated temperature set-point initially. Heat gain responses are normally triggered at this stage, e.g. muscular shivering and vasoconstriction. Once body temperature reaches the fever temperature set-point, heat-gain responses cease.

Several cytokines have been identified to induce fever in animals, and most of them are produced in response to the LPS-initiated fever response. As an endotoxin involved in fever induction, LPS was found in the outer membrane of Gram-negative bacteria causing strong immune responses in animals (Rietschel et al., 1994). The administration of LPS via intraperitoneal (ip.) or intraveinous (iv.) injection into rodents has been shown to induce systemic fever responses by activating Toll-like receptor 4 (TLR-4) and its intracellular downstream signaling (Poltorak et al., 1998; Nagai et al., 2002), such as NF-κB mediated pathway (Han et al., 2002). Subsequently, it induces the production of several major pro-inflammatory molecules, including IL-1β, IL-6, and TNFα (Dinarello, 2004). These cytokines upregulate the expression of the inducible isoform of cyclooxygenase (COX) – COX-2 (Conti et al., 2004), which are involved in the production of the potent inflammatory mediator PGE_2 in both peripheral macrophages and brain endothelial cells (Steiner et al., 2006). PGE_2 is the proximally-acting pyrogen that mediates fever responses by activating prostaglandin E2 receptor 3 (EP3)-dependent signaling pathways in preoptic neurons (Lazarus et al., 2007). Activating this PGE_2 signalling in POA neurons is able to drive thermogenesis in the brown adipose tissue and skin vasoconstriction to evoke the increase of core body temperature (Romanovsky et al., 2005).

1.2 Temperature-sensitive preoptic neurons in the hypothalamus

Within the preoptic area, a small population of warm-sensitive neurons has been identified and regarded as the central thermostat that integrates temperature information from peripheral and central nervous systems as well as orchestrats thermoregulatory responses (Boulant, 2010; Morrison and Nakamura, 2011). Warm-sensitive neurons were described for the first time in the early 1960s (Nakayama et al., 1963; Boulant, 1974; Boulant and Hardy, 1974). The specific property of these neurons is detecting subtle temperature increase (Griffin and Boulant, 1995). Boulant et al have identified the warm-sensitive neurons by recording the firing rate changes of preoptic neurons in brain slices during heat stimulation using electrophysiological recoding techniques (Boulant and Dean, 1986). According to their studies, about 30% of preoptic neurons are sensitive to heat stimulation judging by the firing rate criterion of warm-sensitive neurons, which -somewhat arbitrarily- has been defined to be +0.8 impulses/s/°C (Zhao and Boulant, 2005). This means, any neuron that increases its ongoing firing frequency by 0.8 Hz (or greater) when the temperature is elevated by 1°C, is considered to be a warm-sensitive neuron. Single-cell transcriptomic analysis showed that warnm-sensitive neurons are GABAergic due to the expression of the marker gene GAD1 (glutamic acid decarboxylase 1) (Eberwine and Bartfai, 2010).

Correspondingly, another small subset of preoptic neurons is identified to be cold sensitive, because their firing rate increases during a decrease of temperature (-0.6 impulses/s/°C) (Hori, 1981). However, whether these neurons are intrinsically cold-sensitive, or, alternatively, inhibited by warm-sensitive neurons remains controversial because they appear to lose cold sensitivity upon synaptic blockade on brain slices (Boulant and Gonzalez, 1977; Kelso and Boulant, 1982). Furthermore, their spontaneous firing is tonically inhibited by GABAergic warm-sensitive neurons. They respond to cold stimuli once the inhibitory signal from

warm-sensitive neurons are alleviated due to their cooling-induced firing rate decrease.

However, no matter whether they are primarily sensitive to cold, the warm- and cold- sensitive neurons enable the temperature-sensitive preoptic neurons to detect subtle temperature changes on both directions. Consequently, the thermosensitive feature endows the preoptic area with thermostat-like properties, which are believed to control the core temperature of homeothermic animals.

Therefore, characterization of the temperature-sensitive preoptic neurons is important for uncovering the molecular basis of thermoregulation. However, because of the lack of marker genes and because the temp-sensitive population is small and sparsely distributed in the POA with a salt-and-pepper-like pattern, the temperature-sensitive neurons are very difficult to characterize and isolate (e.g. for gene expression analysis) to identify the gene(s) involved in temperature sensitivity.

1.2.1 Temperature sensitive channels

Over the last 20 years, a number temperature-sensitive ion channels have been identified in different contexts (Montell and Caterina, 2007), which hold some promise to be candidates for conveying temperature-sensitivity to preoptic neurons.

Recently, various transient receptor potential (TRP) channels have been discovered to play an important role in somatosensation including temperature detection. Among the TRP channel family, there is an array of temperature sensitive TRP channels sensing heat and cold stimuli, respectively, so called “thermoTRP” channels (Patapoutian et al., 2003). Trpv1-4, Trpm2, Trpm4 and Trpm5 are heat sensitive channels (Caterina et al., 1997; Kanzaki et al., 1999; Güler et al., 2002; Peier, 2002; Smith et al., 2002; Talavera et al., 2005; Togashi

et al., 2006). TrpA1 and Trpm8 can be activated by cold stimulus (Story et al., 2003; Kwan et al., 2006; Bautista et al., 2007). Most of these thermoTRP channels are nonselective cation channels except Trpm4 and Trpm5 that are sodium selective (Moran et al., 2011). Each of them has a relatively narrow temperature range of activation spanning from noxious cold (<0ºC) to noxious heat (>43ºC) (Belmonte and Viana, 2008). More importantly, the accumulation of different temperature activation ranges for each thermoTRP channel covers a wide temperature spectrum, making them to be ideal thermosensors for organisms to detect environmental and internal temperature signals (Caterina, 2006).

Aside from TRP channels, there are other ion channels with high sensitivity for temperature changes. The Ca^{2+}-activated Cl^- channel Anoctamin-1 (ANO1), also known as Transmembrane member 16A (TMEM16A), has been shown to sense noxious heat in nociceptive DRG neurons of mice (Cho and Oh, 2013). Conditional deletion of Ano1 in DRG neurons causes the defect of noxious heat detection in mice (Cho et al., 2012). Additionally, one of the L-type voltage-gated Ca^{2+} channels (VGCC) $Ca_v1.2$ is also recognized to mediate intrinsic firing of pyramidal neurons during temperature increase (Hardy, 1961; Hensel, 1973; 1981; Szekely et al., 2009; Radzicki et al., 2013). At the temperature ≥37ºC, $Ca_v1.2$ channel becomes active at relatively lower hyperpolarized membrane potential to induce an increase in firing rate. Moreover, the ER Ca^{2+} sensor STIM1 (Stromal interaction molecule-1) has, somewhat unexpectedly, been proposed to be temperature-sensitive, it promotes activation of its counterpart, the Ca^{2+}-selective channel - Calcium release-activated calcium channel protein 1 (ORAI1), in a temperature-dependent manner (Xiao et al., 2011; Krajewski and Narberhaus, 2014).

Additionally, some two-pore K^+ channels, for instance TREK-1, TREK-2 and TRAAK, are also sensitive to temperature changes. However, these findings remain controversial due the loss of temperature sensitivity of some two-pore

channels in heterologous expression systems (Boulant, 2000; Maingret et al., 2000; Kang et al., 2005). Thus, it has been proposed that their heat sensitivity is not an inherent property and might rely on an auxiliary protein which acts as the thermosensor (Enyedi and Czirják, 2010).

1.3 Thermoregulatory circuitries

The thermoregulatory pathways are orchestrated by central nervous system to maintain core body temperature homeostasis (Janský, 2008; Morrison et al., 2008; Morrison and Nakamura, 2011; Nakamura, 2011; van Marken Lichtenbelt, 2012; Morrison et al., 2014). The preoptic area of the hypothalamus receives signals of ambient temperature information transmitted through the thermosensory afferent pathways and integrates them with the deep brain temperature detected locally by the temperature-sensitive preoptic neurons. By matching the peripheral and central body temperature information, heat-gain or heat-loss responses generated by the thermal effecter organs are initiated to sustain core body temperature within a narrow physiological range. A putative thermoregulatory pathway is shown in Fig.1.

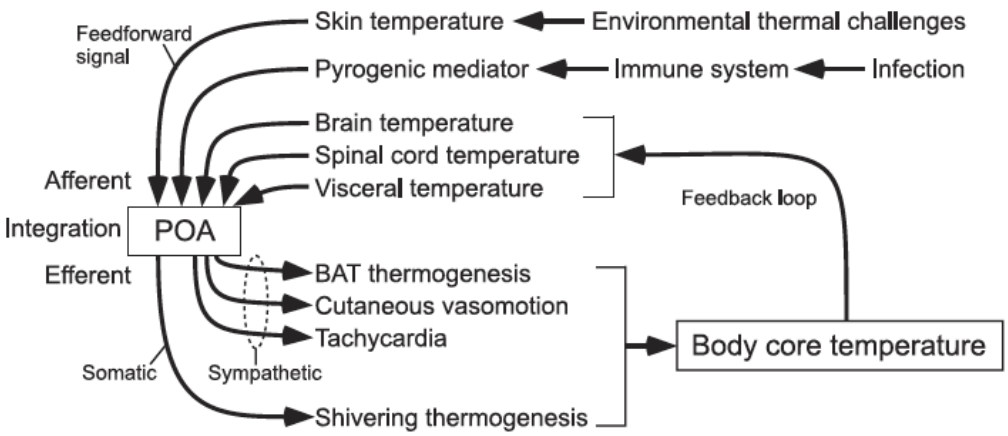


Fig.1 Schematic representation of thermoregulation system.
Temperature information detected by peripheral thermal sensitive organs is sent to the POA region of hypothalamus via the afferent circuits of temperature transduction. Temperature-sensitive neurons in the POA integrate the peripheral and central temperature signals, and initiate heat-gain

or heat-loss responses in different thermal effector organs through the corresponding efferent circuits in order to maintain core body temperature. (modified from *Am J Physiol Regul Integr Comp Physiol (2011) 301: R1207–28.*)

1.3.1 Thermosensory afferent pathways

To maintain core body temperature at a relatively constant level, homeothermic animals need to generate thermoregulatory responses before their core body temperature is affected by ambient temperature changes. For this purpose, the thermosensors in primary sensory nerve endings innervated into the skin should detect the changes of environmental temperature and instantly send the signals to the thermoregulatory center – POA in central nervous system.

The molecular mechanisms underlying the cutaneous thermosensation have been studied extensively, and the thermoTRP channels are illustrated to play an important role in periphery temperature detection (Patapoutian et al., 2003) as introduced in section 1.2.1. The principal cold sensors in the peripheral nervous system are regarded as TRPM8 (Story et al., 2003; Kellogg, 2006), although TRPA1 was used to be considered as a cold sensor at the beginning. Both TRPM8 and TRPA1 are proved to be sensitive to cooling stimulus *in vitro* and expressed in primary somatosensory neurons. The temperature threshold of TRPM8 activation is about 25ºC (moderate cold) when the initial temperature is kept at 30ºC (Robertshaw, 2006; Bautista et al., 2007), while the threshold is 17ºC for TRPA1. It is generally accepted that TRPM8 is required for the detection of innocuous cold *in vitro* and *in vivo* (Almeida et al., 2006; Tabarean et al., 2010). More importantly, Almeida et al demonstrate that Trpm8 can also affect central thermoregulation since pharmacologically blocking Trpm8 by it's selective antagonist M8-B causes hypothermia in rats (Almeida et al., 2012). On the other hand, a recent study done by de Oliveira et al excludes the involvement of TRPA1 in autonomous thermoregulation in rodents since neither genetic deletion nor pharmacological blockade of Trpa1 causes any obvious change on core body temperature and thermal effective responses in rodents during deep cold

exposure (de Oliveira et al., 2014). Therefore, Trpm8 is dominantly in charge of somatosensory cold sensation and also affects central thermoregulation.

Additionally, there is also evidence for the involvement of other thermosensitive TRP channels and the Ca^{2+}-activated Cl^- channel ANO1 in warm or heat sensation: TRPV1 (>43ºC) (Caterina et al., 1997; McAllen, 2004), TRPV2 (>52ºC) (Caterina et al., 1999), TRPV3 (33-39ºC) (Peier, 2002), TRPV4 (25-34ºC) (Güler et al., 2002; Guyenet, 2006), TRPM2 (>37ºC) (Togashi et al., 2006), TRPM4 and TRPM5 (15-35ºC) (Horvath and Diano, 2004; Talavera et al., 2005), and ANO1 (>44ºC) (Cho et al., 2012). So far, there have been no studies suggesting that any of these channels robustly affect circadian rhythm of core body temperature or thermoregulatory responses *in vivo* when analyzing the corresponding genetic knockout mice.

The capsaicin receptor, TRPV1 is expressed in small diameter DRG neurons and is involved in pain sensation. The Trpv1 knockout mice have slightly higher magnitude of circadian fluctuation of core body temperature because of their hypermetabolic living state (Garami et al., 2011). TRPV3 and TRPV4 are prominently expressed in keratinocytes in skin epidermis as well as in subset of DRG neurons. According to their temperature thresholds of activation, these two channels are sensitive to innocuous warmth. Mice lacking Trpv3 have strong deficits in response to innocuous and noxious heat (Carlisle, 1969; Christensen et al., 1977; Moqrich et al., 2005). Moreover, Trpv4 null mice have altered thermal preference comparing to wildtype littermates (Scammell et al., 1993; Bachtell et al., 2003; Lee et al., 2005). Hence, TRPV3 and TRPV4 have a specific role in warmth perception. With respect to noxious heat sensation, ANO1, also expressed in some small-diameter DRG neurons, is identified to be sensitive to heat simulus. Ano1 deficient mice show longer latency in response to noxious heat (Cho et al., 2012).

Other thermoTRP channels, TRPM2, TRPM4, and TRPM5 are not expressed in primary sensory neurons of the peripheral nervous system, which suggests no role for them in thermosensory afferent pathways. However, as a heat-sensitive channel, TRPM2 plays a role in mediating immune responses (Wehrhahn et al., 2010; Knowles et al., 2011; Sumoza-Toledo and Penner, 2011), which may potentially affect core body temperature regulation indirectly. TRPM2 is widely expressed in different immune cells, including monocytes (Yamamoto et al., 2008; Wehrhahn et al., 2010), macrophages (Kashio et al., 2012; Zou et al., 2013), neutrophils, megakaryocytes (Carter et al., 2006; Nazıroğlu, 2011), lymphocytes (Feske et al., 2012; Magnone et al., 2012) and other immunocytes, which has correlated functions in immune responses (Sumoza-Toledo and Penner, 2011). Moreover, Togashi et al report that warm temperature (>35°C) directly evokes the activation of TRPM2 heterologously expressed in HEK293 cells (Togashi et al., 2006). More interestingly, the temperature threshold of TRPM2 gating has been shown to be lowered by H_2O_2, termed “sensitization” (Kashio et al., 2012), suggesting redox signals enable TRPM2 activation at physiological body temperatures by reducing its temperature threshold. Since it is found in numerous cell types of immune system, the oxidant-dependent mechanism of TRPM2 activation indicates its significant role in pathogenic processes that need the recruitment of an increased oxidative microenvironment (Sumoza-Toledo and Penner, 2011).

Primary sensory neurons detect the temperature information by the above thermoreceptors and send it to lamina I neurons in the dorsal horn of the spinal cord. Then, the ascending thermosensory pathway bifurcates into two: one directly connects the interneurons in the thalamus that project to the primary somatosensory cortex evoking temperature perception in the brain (Craig et al., 1994); Aside from the thermo-perception pathway, the other afferent pathway is more important for autonomous thermoregulation. Temperature signals are sent to neurons in lateral parabrachial nucleus (LPB) through the second-order neurons in the spinal cord (Hylden et al., 1989; Bratincsák and Palkovits, 2004).

Distinct populations of LPB neurons are activated in response to warm and cold stimuli separately. Neurons in external lateral region of LPB (LPBel) are activated by skin cooling and relay the temperature signal to the median preoptic nucleus (MnPO), a small region located in the POA, to induce cold-defensive responses in thermal effecter organs, e.g. brown adipose tissue (BAT) (Nakamura and Morrison, 2007). In contrast, skin warming stimulates the neurons in dorsal part of LPB (LPBd) and further activate MnPO neurons to cause heat-defensive responses (Nakamura and Morrison, 2010). Moreover, thermoregulatory activities evoked by nanoinjecting of N-methyl-D-aspartate (NMDA) into MnPO mimics cold-induced thermogenesis (Nakamura and Morrison, 2008), indicating a glutamatergic property of the related LPBel neurons. Temperature information transmitted by these distinct populations of LPB neurons is essential for distinguishing different temperature signals and generating rapid thermoregulatory responses to sustain core body temperature upon various ambient temperatures (Morrison and Nakamura, 2011).

1.3.2 The preoptic area as the thermoregulatory integrator

As introduced above, the preoptic area of the hypothalamus is the thermoregulatory center. GABAergic neurons and gamma-aminobutyric acid (GABA) receptor-expressing neurons within the POA are regarded to modulate thermoregulation based on the following evidence: (1) There are GABAergic neurons as well as neurons expressing GABA receptors in the POA (Flügge et al., 1986; Jarry et al., 1988; Gong et al., 2004; Saito et al., 2013). Also, GABA is endogenously released in the POA (Herbison et al., 1990). (2) Preoptic injection of GABA or GABAA receptor agonist muscimol causes hyperthermia in rats by simulating cold-induced thermal responses (Osborne et al., 1994; Osaka, 2004; Ishiwata et al., 2005). On the other hand, cold/PGE_2-evoked thermogenesis is blocked by local administration of a GABAA receptor antagonist bicuculline (Osaka, 2004; 2008). (3) The firing rate of warm-sensitive preoptic neurons can be also be modulated by agonists or antagonists of GABA receptors *in vitro*

(Yakimova et al., 1996). Furthermore, given the detection of the GABA biosynthetic enzyme Gad1 transcripts in warm-sensitive neurons (Eberwine and Bartfai, 2010), it strongly suggests that the warm-sensitive neurons are likely to express both GABA and its receptors.

In vivo studies show that skin cooling, resulting in heat-gain responses, decreases the tonic firing rate of warm-sensitive neurons in the preoptic area (Boulant and Hardy, 1974). In contrast, their firing rate is increased when the skin is warmed up, which evokes heat-loss effects. Instead of inducing the temperature changes in the periphery, directly cooling down the local temperature of the POA decreases the firing rate of warm-sensitive neurons, which induces thermogenesis *in vivo* (Hammel et al., 1960; Imai-Matsumura et al., 1984). Taken together, an emerging model suggests that warm-sensitive neurons integrate cutaneous and deep brain temperature signals and function as inhibitory preoptic neurons that are tonically active at thermoneutral temperatures to suppress thermogenesis.

Secondary neurons, residing in the dorsomedial hypothalamus (DMH) and raphe pallidus area (RPa) in the brain stem, relay the thermoregulatory signals generated in the POA to thermal effecter organs via the outflow pathways (Morrison, 1999; Cao et al., 2004; DiMicco and Zaretsky, 2006; Rusyniak et al., 2008). Neurons in these areas are mainly glutamatergic and tonically inhibited by GABAergic warm-sensitive preoptic neurons in direct or indirect ways (Nakamura et al., 2004). Activation of the neurons in the DMH or RPa results in the upregulation of thermogenesis and subsequently the increase of core body temperature (Morrison, 2001; Yoshida et al., 2003; Zhang et al., 2011; Bi, 2013). Given the anatomical locations of the POA, DMH and RPa in the brain, the thermoregulatory signals generated in the POA and processed in the DMH and RPa follow a consecutive rostal-to-caudal order, reflecting an array of specific signal transduction circuits.

1.3.3 Thermoregulatory effectors

The major thermoregulatory effectors are brown adipose tissues (BAT), skeletal muscles and heart activity for thermogenesis; cutaneous blood vessels controlling heat loss, and species-dependent mechanisms (e.g. panting, sweating and saliva spreading) for evaporative heat loss (Morrison and Nakamura, 2011).

A. BAT

BAT is an organ mainly in charge of non-shivering thermogenesis (or adaptive thermogenesis). It generates heat via proton leakiness across the mitochondrial membranes facilitated by uncoupling protein 1 (UCP1) expressed in the inner membranes of mitochondria of the brown adipocytes (Shabalina et al., 2013). Although there is basal activity of BAT for heat generation, the mobilization of BAT to defend the body temperature against a cold challenge or during fever responses is controlled by the central nervous system (Morrison et al., 2014). The neuronal signals transmitted through the POA-DMH-RPa axis control heat production by modulating the sympathetic nerve activity (SNA) (Tupone et al., 2011). The extent and rate of thermogenesis in BAT is determined mainly by the expression level of UCP1 as well as norepinephrine (NE)-induced activation of β3-adrenergic receptors (Mund and Frishman, 2013). BAT is recognized as a critical thermogenesis organ in rodents and human newborns as well as hibernating mammals. BAT has recently also gained some recognition as an important thermoregulatory organ in adult humans (Cypess et al., 2009; Virtanen et al., 2009).

B. Cutaneous vasomotion

Cutaneous blood flow transfers metabolic heat to the body surface where heat is dissipated into the environment (Johnson et al., 2014). Cutaneous blood flow can be modulated bidirectionally by vascular smooth muscle that effect vasoconstriction and vasodilation: vasoconstriction results in reducing the blood

flow, while vasodilation increases it. Hence, an increased body temperature causes cutaneous vasodilation so that the heat can dissipate more efficiently through the skin (Flouris et al., 2008; Daanen, 2009). Instead, cold exposure or fever usually induces vasoconstriction in order to preserve heat so that core body temperature is prevented from dropping, or is increased by a resetting of the set point temperature in the course of a fever response(Wilson et al., 2007; Taniguchi et al., 2011). Similar to BAT, vasoconstriction and vasodilation are also mediated by the POA-DMH-RPa axis or the POA-RPa cascade, and downstream sympathetic activities.

C. Tachycardia

During cold defensive responses or fever, cardiac thermogenesis can also be triggered via the increase of heart rate. This occurs in parallel to adaptive thermogenesis recruiting BAT or shivering thermogenesis utilyzing skeleton muscles (Nakamura and Morrison, 2011). The autonomous tachycardia accounts for the increased demand of cardiac output to supply other thermoregulatory effector organs with sufficient oxygen as well as energy substrates in response to cold exposure or fever. Neurons modulating heart rate as part of thermoregulatory control mechanisms are also found in the RPa (Cao and Morrison, 2003; Luong and Carrive, 2012). The thermoregulatory signals are conveyed by the sympathetic nervous system.

D. Shivering

Moreover, neurons in the RPa can also modulate the level of shivering thermogenesis in skeleton muscles (Tanaka et al., 2006; Brown et al., 2008). Shivering thermogenesis is recruited as the last cold-defense mechanism since its thermal threshold of activation is lower than that of either cutaneous vasoconstriction or BAT thermogenesis. Although it is regarded as an essential mechanism in cold defense and fever induction in mammals (Saper and Breder, 1994), the central neural mechanisms underlying shivering thermogensis and the related neuronal circuits are almost entirely unknown. Rhythmic bursts of

activities in the α-motor neurons innervating skeletal muscle fibers contribute to the muscle contractions of shivering (Schäfer and Schäfer, 1973).

Aside from autonomous thermoregulation utilizing effector organ activity, cold/warm-seeking behavior is also a manner of thermoregulatory response to sustain core body temperature against ambient temperature challenges or fever responses (Konishi et al., 2003; Almeida et al., 2006).

2. Aims of the thesis

Unraveling the molecular basis of intrinsic temperature-sensitivity in preoptic neurons in the hypothalamus is crucial for understanding the mechanisms of thermoregulation. However, due to the lack of marker genes for the temperature-sensitive preoptic neurons, it has been difficult to characterize these neurons. To solve the problem, we established a Ca^{2+} imaging-based assay that allowed us to examine temperature responses in cohorts of preoptic neurons.

Using a pharmacologic screening approach, siRNA interference and transcritpome analysis, this thesis work describes the identification of candidate molecules required for temperature sensitivity in POA neurons. Furthermore, using a genetic mouse model, combined with temperature telemetry, the most relevant candidate molecule has been characterized for its role in POA neuron temperature sensitivity and thermoregulation.

As a side project, given the important role of Trpm8 in peripheral cold sensation, we also evaluated the potential function of Trpm8 in mediating core body thermregulation by using c-Fos immunostaining.

3. Materials and Methods

3.1 Animals

C57Bl/6N mouse pups were obtained from Charles River Labs and Janvier Labs. The Trpm2 genetic knockout mouse line was originally generated by Prof. Yasuo Mori's lab at Kyoto University. With his permission, we obtained this mouse line from Prof. Marc Freichel's lab at Heidelberg University. The care of all mice was within Institutional Animal Care and Use Committee guidelines. Mice were housed with ad libitum access to food and water in a room air-conditioned at about 22°C with a standard 12h light/dark cycle (light on at 6:00 and off at 18:00 every day).

3.2 Cell culture

Different cell lines were maintained in DMEM (31966-021, Invitrogen, USA) supplemented with 10% heat inactivated FBS in a humidified incubator (CB-150I, BINDER GmbH, Germany) with 5% CO_2 at 37°C. Cells were split when their growth reached 90% confluence.

3.3 Warm and cold exposure

Mice used in the experiments were transferred to individual cages and kept under standard laboratory conditions specified above. After 2-3 days of habituation, acute warm or cold stimuli were applied by transferring mouse cages to a cold (7°C) or warm (37°C) climate incubator (KB720, BINDER GmbH, Germany) for 2 hours. Concurrently, the control mice were kept at basal environmental temperature (The basal temperature was RT (≈22°C) if not indicated otherwise). This rapid temperature stimulus was used for the investigation of c-Fos

expression by immunohistochemistry. For monitoring core body temperature changes during the process of ambient temperature heating or cooling, mice were housed in the climate incubator equipped with the DSI telemetry system at RT at least 2 days before any temperature stimulus. Heat or cold stimulus was applied to mice by adjusting the temperature of the climate incubator to either 37°C or 7°C. It normally takes 4-5 hours to reach the desired temperature from 22°C.

3.4 Preparation of frozen brain sections

Mice were anesthetized with Ketaset-Rompun anaesthetic (Bayer, Germay) and sacrificed by transcardiac perfusions with 20ml phosphate buffer saline (PBS, pH=7.4) followed by 30ml fixative solution (4% wt/vol paraformaldehyde (PFA) in PBS, pH=7.4). Brains were dissected and post-fixed in 4% PFA for 2h at 4°C. Brains were embedded after overnight incubation in 30% sucrose at 4°C, and free-floating coronal sections at 50μm thickness were cut on a sliding microtome (SM2010R, Leica Biosystems, Germany). Sections containing POA were collected. For *in situ* hybridization experiments, thinner brain sections (20μm) were prepared with a freezing microtome (CM3050S, Leica Biosystems, Germany).

3.5 Immunohistochemistry

c-Fos immunostaining was performed on mouse free-floating brain sections using the avidin-biotin-peroxidase method. Briefly, the sections were washed in 0.2% Triton X-100 in PBS three times and incubated in 2% normal donkey serum in PBS for 2h at RT. Then sections were incubated in rabbit anti-c-Fos antibody (1:5000; Calbiochem, USA) overnight at 4°C. After washing sections with PBS three times for 10min each, the secondary antibody, biotinylated anti-rabbit IgG (1:200; Vector Lab., USA), was applied to the sections for 2h at RT. Subsequently, sections were treated with avidin-biotinylated peroxidase complex

(PK-6101, Vector Lab, USA) for 1h. The c-Fos-immunoreactivity was revealed by DAB solution (0.2mg/ml DAB, 0.03% H_2O_2 in PBS, pH=7.4) applied for about 5 min. After washing in PBS, brain sections were mounted on slides with ImmuMount (99-904-12, Thermo Scientific, USA).

3.6 *In situ* hybridization

Trpm2 expression in mouse brain sections was detected by using cyanine-2 (Cy2)-labeled antisense riboprobes against mouse Trpm2 (NM_138301.2). Trpm2 cDNA was cloned into PCI-neo plasmids provided kindly by Prof. Yasuo Mori at Kyoto University. Plasmids were linearized with EcoRI and transcribed with T3 RNA polymerase to produce Trpm2 antisense RNA probes for *in situ* hybridization. The rhodamine-labelled RNA probes for detecting GAD1 were obtained from Prof. Carmen Birchmeier-Kohler at Max-Delbruck-Center in Berlin. Adult mouse brains were mounted and freshly frozen at -80°C. *In situ* hybridization was performed on 20μm thick cryosections as described previously (Watakabe et al., 2007; Abe et al., 2009).

3.7 Primary preoptic neuronal culture

Preoptic neurons were isolated from the brains of 1-3 day-old postnatal C57Bl/6N mouse pups. In brief, brains were isolated quickly, and brain slices containing the preoptic area of the hypothalamus were cut by a vibratome (HM650V, Thermo Scientific, USA) at the thickness of 200μm in artificial cerebral spinal fluid (ACSF: 135.5mM NaCl, 1mM KCl, 2mM $CaCl_2$, 1.3mM $MgCl_2$ 1.26mM K_2HPO_4, 26mM $NaHCO_3$ and 9mM glucose oxygenized in carbogen (95% O_2 and 5% CO_2)). The preoptic area was dissected from each brain slice in the HBSS solution (14175-053, Invitrogen, USA) under a dissection microscope (SZ61, Olympus, Japan). Small POA tissue pieces were collected and incubated in the HBSS solution containing 0.5mg/ml papain at 37°C for 20 min. After the digestion procedure, tissue was mechanically dissociated into single-cell suspensions by triturating it

in growth medium with a Pasteur glass pipette. After centrifugation of the cell suspension (1000rpm), the cell pellet was resuspended in growth medium and plated on poly-D-lysine (P4832, Sigma, USA) and laminin-coated (L2020, Sigma, USA) coverslips (5mm diameter). The isolated POA neurons were cultured in a humidified incubator at 37°C, 5% CO_2.

3.8 Gene knockdown with siRNA

To knockdown the expression of target genes in cell lines, 3 individual siRNAs targeting different sequences of the genes were designed by the algorithm on the website of Thermo Scientific (http://www.thermoscientificbio.com/design-center). The custom-designed siRNAs were ordered from Sigma. Sequences of each siRNA used in this study are listed below.

siRNA sequences:

Gene	Accession No.	siRNA sequences (5'→3')
Orai1	NM_001013982	CUCAAAGCCUCCAGCCGAA
		GCCAUAAGACGGACCGACA
		CGAACAACAGCAAUCCGGA
Orai2	NM_001170403	GCCACAACCGUGAGAUCGA
		GCCACAAGGGCAUGGAUUA
		GCAUGCACCCGUACAUCGA
Orai3	NM_001014024	CCAACGACUCCACCGAUAU
		GGGUCAAGUUUGUGCCCAU
		CUGUGGGACUAGUGUUUAU
Trpm2	NM_001011559	CAGAAGAGCAUGAACGCUU
		CGUCAUCACCAUCGGCAUA
		AACCAGAUUGUGGAAUGGA
Trpc1	NM_053558	GAACAUAAAUUGCGUAGAU
		CGCUGAAGGAUGUGCGAGA
		CCAUAACUAUUGAAAACGA
Trpc4	NM_001083115	CCUUGAAGAUUGUCGCAUU
		GCCAUUAAGUACCGUCAAA
		TACCAAGAGGTGATGAGGA

For knocking down the expression of a certain gene, siRNA was transiently transfected into cells cultured in 6-well plates with Lipofectamine 2000 (13778, Invitrogen, USA) and serum-free opti-MEM (11058021, Invitrogen, USA) according to the manufacturer's instructions. GFP plasmid was also transfected into cells together with siRNAs in order to label the cells that likely had taken up the transfected siRNAs. The gene knockdown effect was evaluated by Ca^{2+} imaging 3 days after the transfection. Only GFP-positive cells were selected for data analysis.

3.9 Measurement of $[Ca^{2+}]_i$

Cells in culture were first washed once with Ringer's solution (140mM NaCl, 5mM KCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 10mM glucose, and 10mM HEPES at pH=7.4) and then incubated with 10μM Ca^{2+}-sensitive ratiometric fluorescent probe Fura-2 AM (F1201, Molecular Probes, USA) for 90min at RT in Ringer's solution. After the loading procedure, coverslips were mounted in a perfusion chamber (RC-22, Warner Instruments, USA) and perfused by an 8-channel modular perfusion system (ValveBank-8, Science-Products, Germany). Ca^{2+} imaging was performed by an inverted fluorescent microscope (Axio Vert.A1, Carl Zeiss, Germany) equipped with a 10X objective (Fluar 10x/0.5 M27, Carl Zeiss, Germany) and a cooling CCD (CoolSNAP-HQ2, Photometrics, USA). Fura-2 AM-loaded cells were excited at the wavelengths of 340nm and 380nm (Chroma Fura-2 filter set), and the fluorescence was collected at 510nm. The obtained images were analyzed for changes in the fluorescence intensity within the ROI using MetaFluor 7.1 (Molecular Devices, USA). The $[Ca^{2+}]_i$ change was represented by the normalized fluorescence ratio F340/F380. The calcium-free Ringer's solution was prepared by substituting $MgCl_2$ for $CaCl_2$ at the same concentration and adding 2mM EGTA. The N-methyl-d-glucamine chloride (NMDG-Cl) Ringer's solution was prepared by replacing NaCl with the strong base NMDG (140mM) and HCl was added to adjust the pH to 7.4.

To change the temperature of the perfusion solution for the application of heating or cooling stimuli, Ringer's solution flowed through a glass coil with a small hermetic glass tank surrounding it (a custom solution built at the MDC). Water was pumped into the tank by a water-bath with heating and cooling functions (MultiTemp III, Pharmacia Biotech, Sweden). The temperature of the Ringer's solution was adjusted by changing the temperature of water circulating between the glass tank and the water-bath. The temperature in the perfusion chamber was monitored by a thermocouple probe (IT-18, Physitemp Instruments, USA) connected with an electric digital thermometer (BAT-12, Physitemp Instruments, USA).

3.10 Transcriptomic analysis by deep sequencing

Total RNA was extracted from the samples of interest by Trizol reagent (15596-026, Invitrogen, USA) according to the manufacturer's protocol. 1µg total RNA from each sample was supplied to the deep sequencing core facility of the Max-Delbrück-Center in Berlin for transcriptomic analysis by deep sequencing. The degradation of the total RNA samples was minimized during the whole extraction procedure with ribonuclease-free buffers. Their quality was proved to be sufficient for sequencing by determination of the RNA integrity number (RIN) (>9.0) of the bioanalyzer (2100, Agilent Technologies Genomics, USA) results. The estimation of gene expression level is presented as reads per kilobase per million reads (RPKM).

3.11 Temperature transmitter implantation

The mouse surgical procedure was performed aseptically under anesthesia. Mice were kept in anesthesia by inhaling a gas mixture (30ml/min O_2 and 60ml/min N_2O) containing 1.5% (v/v) isoflurane through a mask. The fur of the mouse abdomen was shaved and completely removed by depilatory cream (Pilca,

Sodalco, Italy). After opening the abdominal cavity, the sterile DSI transmitter (TA11TA-F10, Data Sciences International, USA) was inserted, which can detect the core body temperature of the mouse and its activity. Then suture was carefully done for each mouse with sterile absorbable-needled sutures (Marlin® 17241041, Catgut, Germany). During the whole operation procedure, mice were kept on a heating pad at 37°C. After the surgery, mice were transferred to their original cages exposed to warming infrared lamps (SIL-16, Sanitas, Germany) overnight to help them recover from the surgery. They were allowed to recover from surgery for at least seven days before the start of any experiments.

3.12 Core body temperature measurement by telemetry system

Mice implanted with transmitters were housed individually in cages visually separated from each other in the climate chamber. They were allowed to move freely with ad libitum access to food and water. Receivers (RSC-1, Data Sciences International, USA) under each mouse cage can detect the radio signals sent by the implanted transmitter and convey this information to a computer where the core body temperature and the locomotion activity were acquired and stored by the acquisition software Dataquest A.R.T. version 4.3.1 (Data Sciences International, USA). The information about the core body temperature and locomotion activity of mice was collected once every 5 minutes. The basal environmental temperature was set at 22°C in the climate chamber.

3.13 Fever induction

Fever was induced in mice by the ip injection of LPS (E. coli 0111:B4, Sigma L3024 lot: 043M4107V). Mice were housed in a soundproof environment for at least a week after the surgery of transmitter implantation. Core body temperature was monitored by the DSI telemetry system starting at least 1 day before the injection to record the baseline of the core body temperature. Mice were injected with LPS at the does of 100µg/kg dissolved in sterile saline. The injection for

each mouse was done in the morning to minimize the effect of the circadian rhythm on the core body temperature, and the telemetry recording was continued for the following 8h.

3.14 Data analysis

Analysis was performed with either Excel (Microsoft Office 2008 for Mac) or SPSS (Ver.13.0) software using the 2-tailed Student's *t*-test for unpaired comparisons unless indicated otherwise. All results are presented as mean ± standard error of the mean (SEM). P values < 0.05 were considered statistically significant.

In the statistic analysis of Ca^{2+} imaging data, normalization was done by Clampfit (Ver.10.3) software if the normalized peak values are shown in the figures containing statistics. In brief, the first heat-induced $[Ca^{2+}]_i$ peaks of each cell were treated as 1. Peak values during other heat stimuli were normalized to the corresponding value of the first peak to obtain their normalized peak values of the heat-induced $[Ca^{2+}]_i$ response.

4. Results

4.1 Identification of temperature-sensitive preoptic neurons *in vitro*

To identify temperature-sensitive neurons in the POA, we stimulated cultured primary preoptic neurons with temperature stimuli to identify different subtypes of temperature-sensitive neurons by Ca^{2+} imaging *in vitro*. Neurons isolated from newborn mouse pups (P1-P3) were cultured on glass coverslips (Fig.1-1A). The attached POA neurons spontaneously developed neuronal-like morphology *in vitro*. Their round soma and long processes can be clearly recognized in the bright-field image (Fig.1-1A: BF). The chemical Ca^{2+} indicator Fura-2 can be homogeneously loaded into these cells (Fig.1-1A: Fura-2).

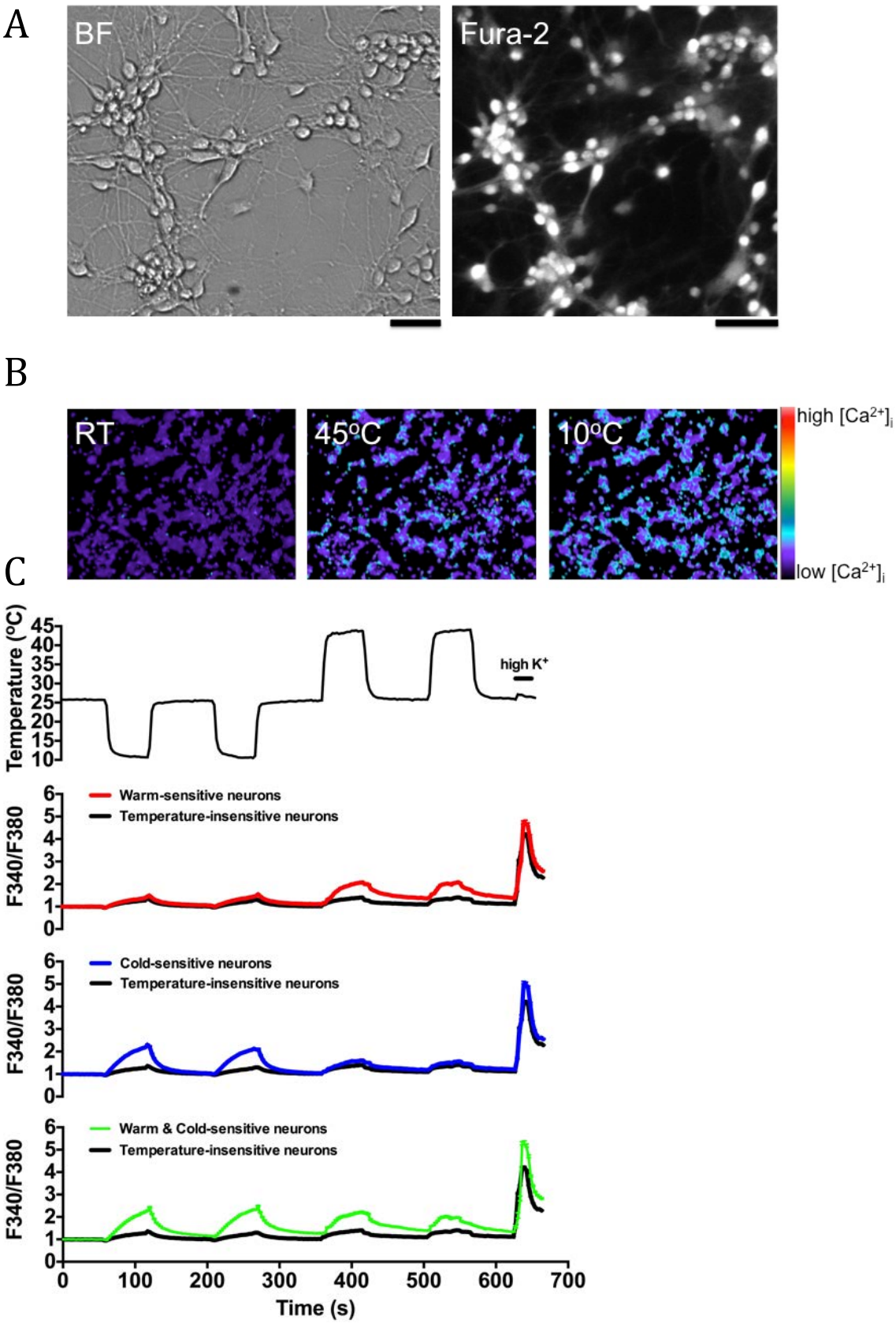
To identify the temperature-sensitive neurons, the primary cultured POA cells were stimulated by 10°C and 45°C Ringer's solution twice at each temperature sequentially, and high potassium (K^+) Ringer's solution was applied to the cells at the end of the experiment to detect the excitable neuronal population. The bath temperature was recorded throughout the experimental procedure (Fig.1-1C, upper panel). Images of pseudo-color $[Ca^{2+}]_i$ responses of all cells within the imaging region are shown at different stimulation conditions (Fig.1-1B). Almost no cells were activated at RT. During the stimuli, some cells were activated by 45°C heat stimulus, while some were more sensitive to 10°C cold treatment. The result of high K^+ stimulation suggested that the majority of the cells in culture were neurons because of their excitability.

For further analysis, all the high K^+ responding cells were selected to include all excitable cells for data analysis. Based on their responses to different temperature treatments, neurons were categorized into four different groups: (a)

temperature-insensitive neurons, (b) warm-sensitive neurons, (c) cold-sensitive neurons, and (d) the ones that are sensitive to both warm and cold stimuli (Fig.1-1C). Neurons were included in one of the three categories of temperature-sensitive neurons if their heat- or cold-induced $[Ca^{2+}]_i$ response exceeded the value of the mean value plus 3 standard deviations of all neurons.

Based on this criterion, the majority of the neurons (78%) are temperature-insensitive (Fig.1-1C,D). A small increase of the Fura-2 fluorescence ratio was detected during the temperature stimuli in the temperature-insensitive neurons (black trace, Fig.1-1C), which was due to the small known temperature sensitivity of the Ca^{2+} indicator Fura-2 fluorescence. Presumably, this sensitivity is because of the change of K_d (dissociation constant) value of Fura-2 during heating and cooling. Similar technical background can be found in all our Ca^{2+} imaging experiments during temperature stimuli no matter whether the cells are temperature-sensitive or not, indicating that the effect is not cell-type specific but it rather reflects a technical issue. Therefore, it was treated as the background signal in this Ca^{2+} imaging assay.

About 7% of all neurons (Fig.1-1D) were only warm-sensitive since they were reproducibly activated by heating twice but not by cooling (red trace, Fig.1-1C). In contrast, cold-sensitive neurons (10%, Fig.1-1D) responded to cold treatment only (blue trace, Fig.1-1C). Interestingly, there was a small population of neurons (5%, Fig.1-1D) that was sensitive to both heat and cold stimuli, implying a degree of heterogeneity of the temperature-sensitive neurons in the POA (green trace, Fig.1-1C). For either warm or cold sensitive neurons, temperature-induced $[Ca^{2+}]_i$ increase indicated that there were certain channel(s) or receptor(s) conducting Ca^{2+} flux during temperature stimuli. Thus, we decided to try to identify which genes are involved in the thermosensitivity of the neurons. Due to the putatively intrinsic warm-sensitivity of preoptic neurons (Boulant, 2010), we mainly focused on characterizing warm-sensitive neurons in the following experiments.


A
BF
Fura-2
B
RT
45°C
10°C
high [Ca2+]i
low [Ca2+]i
C
Temperature (°C)
high K+
Warm-sensitive neurons
Temperature-insensitive neurons
Cold-sensitive neurons
Temperature-insensitive neurons
Warm & Cold-sensitive neurons
Temperature-insensitive neurons
F340/F380
Time (s)

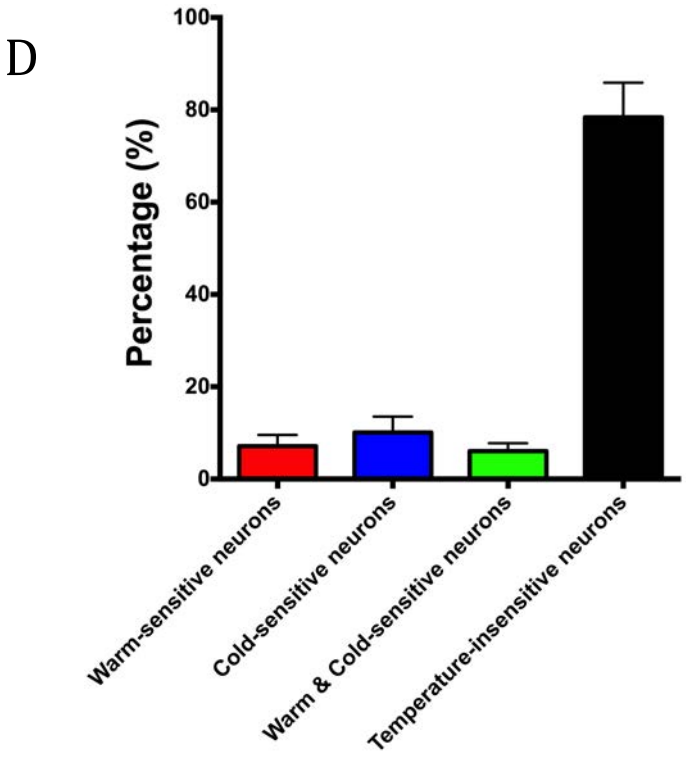


Fig.1-1 Temperature sensitivity of primary POA neurons.

Identification of temperature-sensitive primary cultured POA neurons by Ca^{2+} imaging. (A) The bright field (BF) and fluorescent (Fura-2) images of POA neurons in culture. Scale bar: 50μm. (B) Cell response images of Ca^{2+} imaging with Fura-2 during RT, 45°C and 10°C stimuli. (C) Average Ca^{2+} imaging traces of different groups of temperature-sensitive POA neurons. The trace in the top panel shows the temperature stimulus recorded during the experiments. At the end of every experiment, high K^+ Ringer's solution was applied to the cells. Ca^{2+} responses of different temperature-sensitive neurons were plotted in the following panels: warm-sensitive neurons (red, n=111); cold-sensitive neurons (blue, n=101); neurons that are sensitive to both warm and cold (green, n=45); temperature-insensitive neurons (black, n=1537). (D) Summary of the percentage of each goup of temperature-sensitive neurons among all the high K^+ responders (data were obtained from 4 independent experiments).

To unravel the mechanism underlying the warm sensitivity of the preoptic neurons, we carried out a screening strategy to identify the heat-activated channel expressed in the warm-sensitive neurons by Ca^{2+} imaging and transcriptomic analysis. The warm-sensitive preoptic neurons were characterized by pharmacological profiling and transcriptomic analysis to obtain a short list of candidate channels. To further identify the channel conducting the heat response

among these candidats, given the potential technical obstacles during the verification of candidate channels using a siRNA knockdown method in primary neurons, we decided on a screening strategy based on easily cultivated and transfected cell lines. In brief, we hoped to find a warm-sensitive cell line that could recapitulate the warm sensitivity of preoptic neurons. Candidate channels arising from the pharmacological and transcriptomic screen in the cell line could then be easily verified by siRNA knockdown apporach, and the identified channel will be tested in the preoptic neurons eventually to determine its involvement in their warm-sensitivity.

4.2 Characterization of warm-sensitive preoptic neurons

4.2.1 Temperature threshold of the warm-sensitive POA neurons

To charaterize the warm-sensitive preoptic neurons in detail, we first investigated the temperature threshold for activating warm-sensitive neurons by Ca^{2+} imaging. Preoptic neurons were stimulated by ascending temperature steps (each was increased by 1-2°C) starting from the basal physiological temperature of ≈37°C (Fig.2-1). The average Ca^{2+} response of the warm-sensitive neurons is shown in Fig.2-1. Neurons were activated once the temperature stimulus reached 45.6°C. However, there was no obvious response at the temperatures lower than 44.5°C (Fig.2-1). It suggested that the threshold of the warm-sensitive neurons was between 44.5°C and 45.6°C in the experimental condition.

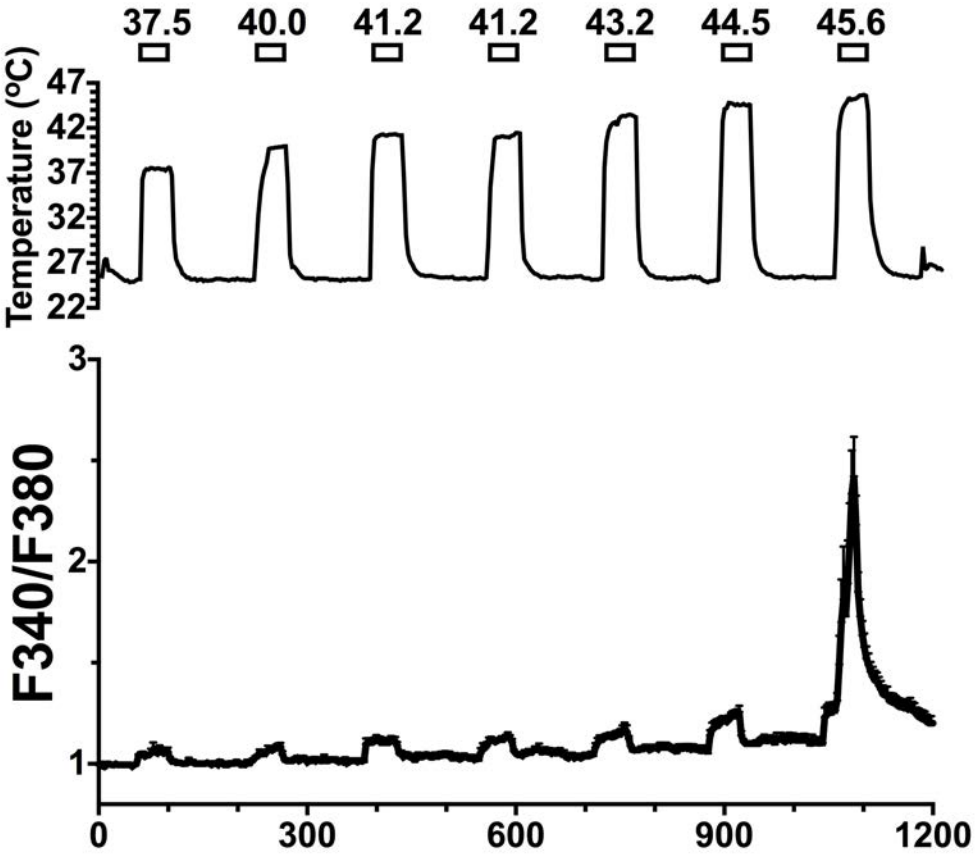


Fig.2-1 Temperature threshold of warm-sensitive neurons
The temperature threshold of warm-sensitive neurons was studied by Ca^{2+} imaging. The upper panel shows the temperature stimuli applied to the POA neurons. The corresponding intracellular Ca^{2+} response is plotted in the lower panel.

4.2.2 Effects of pyrogens on warm-sensitive POA neurons

As a class of fever inducers, pyrogens have been shown to play an important role in modulating core body temperature. Mechanistically, it has been proposed that they cause fever by decreasing the firing rate of the warm-sensitive neurons, which in turn relieve their inhibitory GABAergic input on the downstream heat-generating neuronal circuits. Subsequently, the heat-gain response is initiated to increase the core body temperature. In agreement with this hypothesis, some pyrogens, for instance PGE_2, LPS, IL-1β, were studied and shown by others to have mild inhibitory effects on the firing rate of warm-sensitive neurons during heat stimuli as determined by patch clamp recordings (Shibata and Blatteis,

1991; Xin and Blatteis, 1992; Ranels and Griffin, 2003; Tabarean et al., 2004). To determine whether pyrogens would affect the thermosensitivity of the warm-sensitive preoptic neurons identified by our Ca^{2+} imaging assay, we compiled a list of different pyrogens widely used for inducing fever in rodents and evaluated their effects on the heat response of preoptic neurons by Ca^{2+} imaging. Cells were treated by heat stimuli and then incubated with a certain pyrogen for about 10min and subsequently exposed to another heat stimulus at the same temperature. The magnitude of the heat-induced $[Ca^{2+}]_i$ responses right before and after the application of the corresponding pyrogen was compared in order to detect the potential inhibitory effect on the warm sensitivity of the preoptic neurons.

First, we studied the effect of LPS on the warm-sensitive preoptic neurons. The temperature of the heat stimuli was decreased until it reached the one evoking a small heat response, as indicated by the first arrow in Fig.2-2. Neurons were then treated with 1μg/ml LPS for 10min. The LPS incubation did not induce any $[Ca^{2+}]_i$ elevation, indicating that preoptic neurons cannot be activated by 1μg/ml LPS alone (Fig.2-2 lower panel). Furthermore, the heat-induced $[Ca^{2+}]_i$ response was similar before and after LPS treatment (Fig.2-2 lower panel), suggesting that LPS had no effect on the warm sensitivity of the neurons.

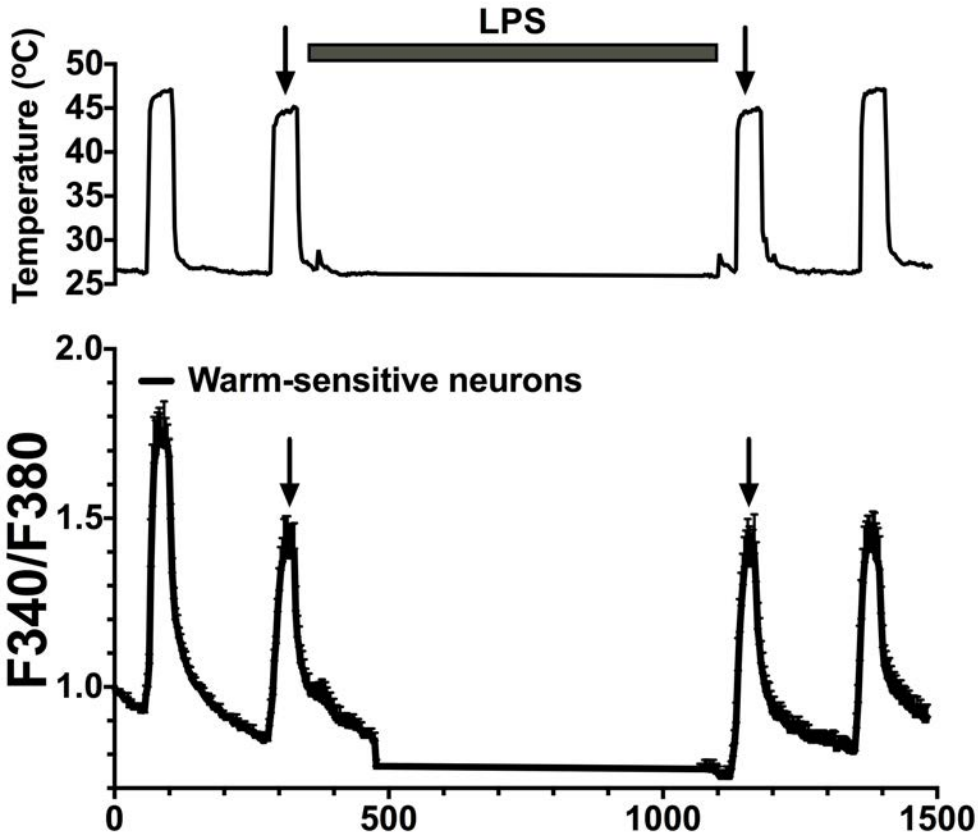


Fig.2-2 LPS sensitization effect on warm-sensitive neurons
A potential LPS sensitization effect on warm-sensitive neurons was studied by Ca^{2+} imaging. The upper panel shows the temperature stimuli applied to the preoptic neurons. Neurons were incubated with LPS (1μg/ml) for 10min. The corresponding intracellular Ca^{2+} response is plotted in the lower panel. There was no statistical difference between the $[Ca^{2+}]_i$ responses right before and after the application of LPS indicated by the arrows. (n=4)

The effect of another pyrogen, the interleukin IL-1β, on preoptic neurons was studied by Ca^{2+} imaging. Similar to the LPS experiment described before, 50ng/ml IL-1β was applied to neurons for 10 minutes. There was no obvious difference of heat stimulus-induced Ca^{2+} responses before and after IL-1β treatment (Fig.2-3). Therefore, as LPS, IL-1β likely does not play an important role in mediating the thermosensitivity of the warm-sensitive neurons.

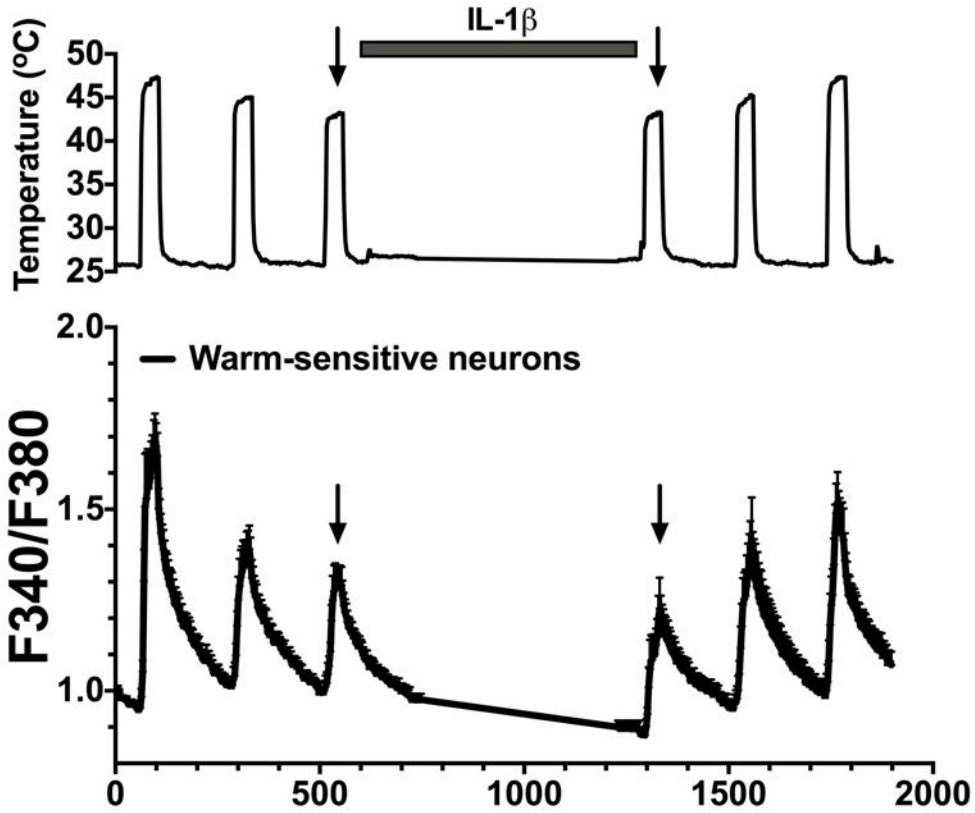


Fig.2-3 IL-1β sensitization effect on warm-sensitive neurons
IL-1β sensitization effect on warm-sensitive neurons was studied by Ca^{2+} imaging. The upper panel showed the temperature stimuli applied to the preoptic neurons. IL-1β (50ng/ml) was incubated with the neurons for 10min. The corresponding intracellular Ca^{2+} response was plotted in the lower panel. There was no statistical difference between the $[Ca^{2+}]_i$ responses right before and after the application of IL-1β indicated by the arrows. (n=6)

Using the identical experimental protocol, a slightly inhibitory effect of TNFα was observed by comparing the amplitudes of heat-induced Ca^{2+} increases before and after treatment with TNFα (30ng/ml, 10 minutes; Fig.2-4). It indicates that TNFα appears to be potentially involved in modulating the Trpm2-conducted Ca^{2+} response induced by the heat stimulus.

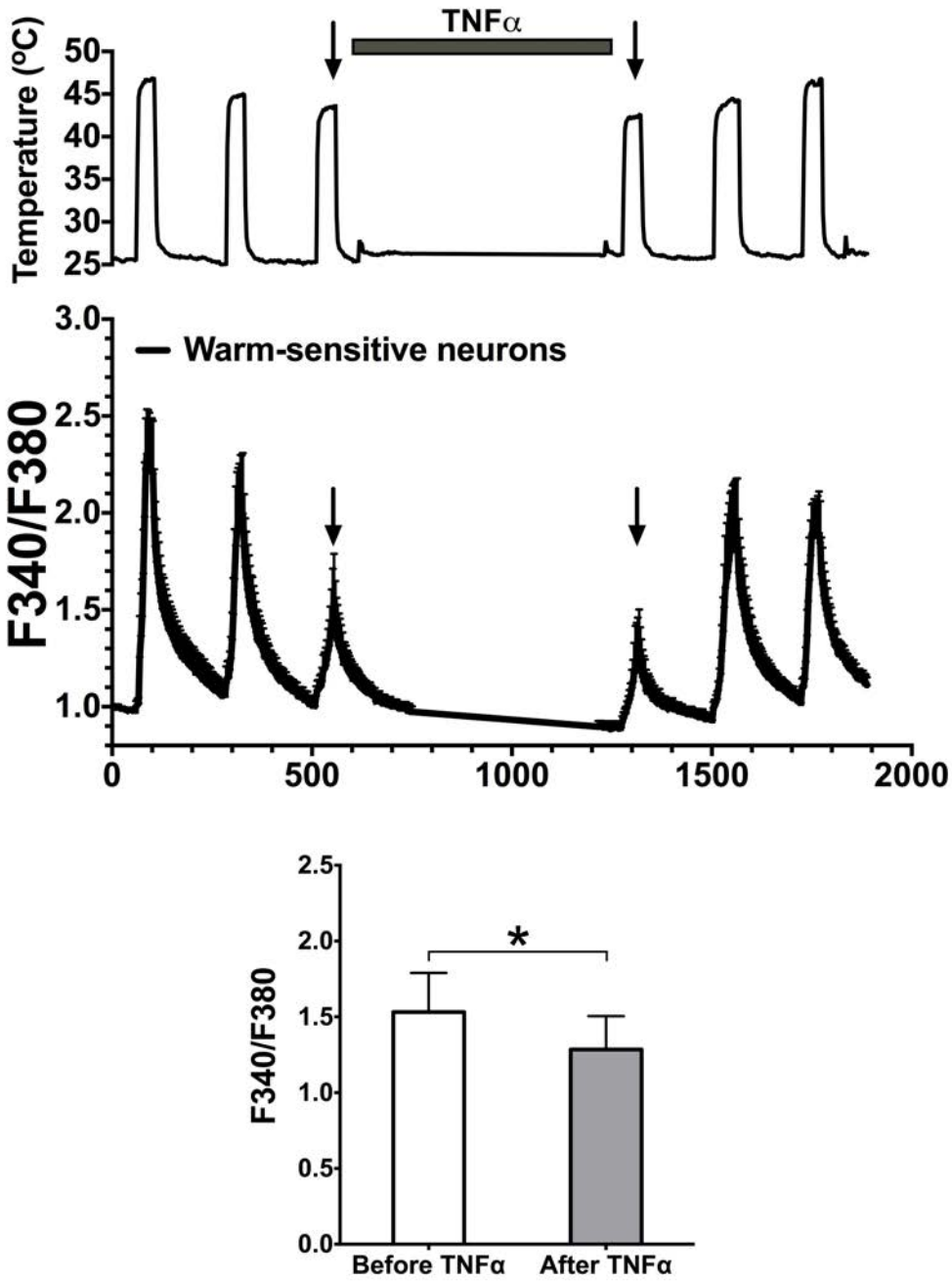


Fig.2-4 TNFα sensitization effect on warm-sensitive neurons
TNFα sensitization effect on warm-sensitive neurons was studied by Ca^{2+} imaging. The upper panel showed the temperature stimuli applied to the preoptic neurons. TNFα (30ng/ml) was incubated with the neurons for 10min. The corresponding intracellular Ca^{2+} response was plotted in the middle panel. There was significant difference between the $[Ca^{2+}]_i$ responses right before and after the application of TNFα indicated by the statistical analysis shown in the lower panel. (Student's *t*-test (paired), *p<0.05, n=6)

In contrast to the pyrogens discussed above, PGE_2 induces a fever response by activating its receptors expressed in the brain. Saper and his colleagues have shown that one of the four PGE_2 receptors called EP3R is expressed in the median preoptic nucleus, and plays a critical role in inducing fever responses (Lazarus et al., 2007). Mice lacking EP3R in this region developed a much abated fever response induced by either LPS or PGE_2 injection. This conclusion leads us to investigate if the Trpm2-mediated warm sensitivity of preoptic neurons can be modulated by PGE_2. Following the same experimental procedure as described for the previous pyrogens, preoptic neurons were incubated with 500nM PGE_2 for 10 minutes between two identical heat stimuli. Unfortunately, no difference in heat responses before and after PGE_2 treatment was observable by Ca^{2+} imaging (Fig.2-5), indicating that the heat response is not modulated by PGE_2 under these conditions.

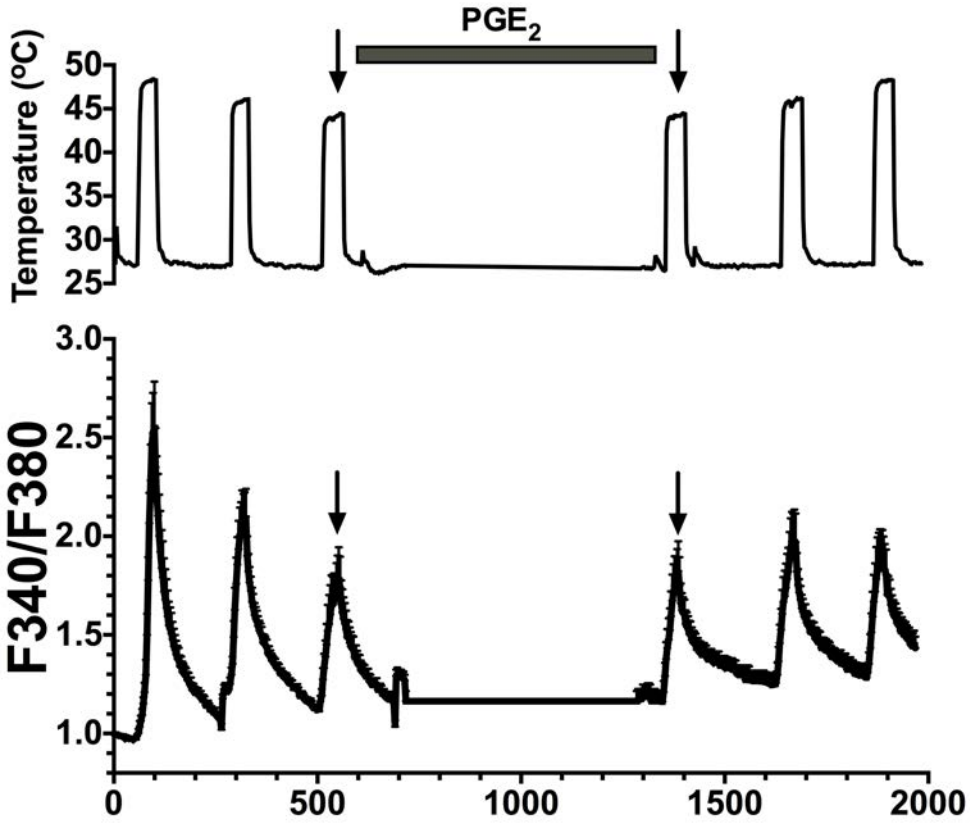


Fig.2-5 PGE_2 sensitization effect on warm-sensitive neurons.
PGE_2 sensitization effect on warm-sensitive neurons was studied by Ca^{2+} imaging. The upper panel showed the temperature stimuli applied to the preoptic neurons. PGE_2 (500nM) was incubated with the neurons for 10min.

The corresponding intracellular Ca^{2+} response was plotted in the lower panel. There was no statistical difference between the $[Ca^{2+}]_i$ responses right before and after the application of PGE_2 indicated by the arrows. (n=6)

4.2.3 Pharmacological study on the temperature-sensitive POA neurons

In order to unravel the mechanism of warm sensitivity of preoptic neurons, we investigated their heat responses during application of pharmacological agents. First, ethylene glycol tetraacetic acid (EGTA)-containing Ringer's solution and cyclopiazonic acid (CPA) were applied to the primary preoptic neurons to determine the source of the heat-induced $[Ca^{2+}]_i$ increase. As a Ca^{2+} chelating agent, EGTA is widely used to deplete extracellular free Ca^{2+} ions so that Ca^{2+} cannot enter through any channels or ionotropic receptors. The heat-induced $[Ca^{2+}]_i$ increase was abolished by the removal of extracellular Ca^{2+} ions comparing to the heat response before the application of EGTA (Fig.2-6B) and the control sample (Fig.2-6A). On the contrary, the inhibitor of the intracellular Ca^{2+} pump CPA did not attenuate the heat-induced $[Ca^{2+}]_i$ elevation at all (Fig.2-6C). The statistical summary of the blocking effects of EGTA and CPA on the heat response of preoptic neurons is shown in Fig.2-6D. Furthermore, the combination of EGTA and CPA treatments could abolish any $[Ca^{2+}]_i$ increase due to the Ca^{2+} depletion of both intracellular Ca^{2+} store and extracellular solution (Fig.2-6D). The small remaining increase of F340/F380 ratio in EGTA & CPA-treated sample was the background of the Fura-2 dye in response to the heat stimulus as mentioned before. Taken together, these data clearly suggested that the heat-induced $[Ca^{2+}]_i$ increase mainly resulted from extracellular Ca^{2+} influx and not intracellular Ca^{2+} store release.

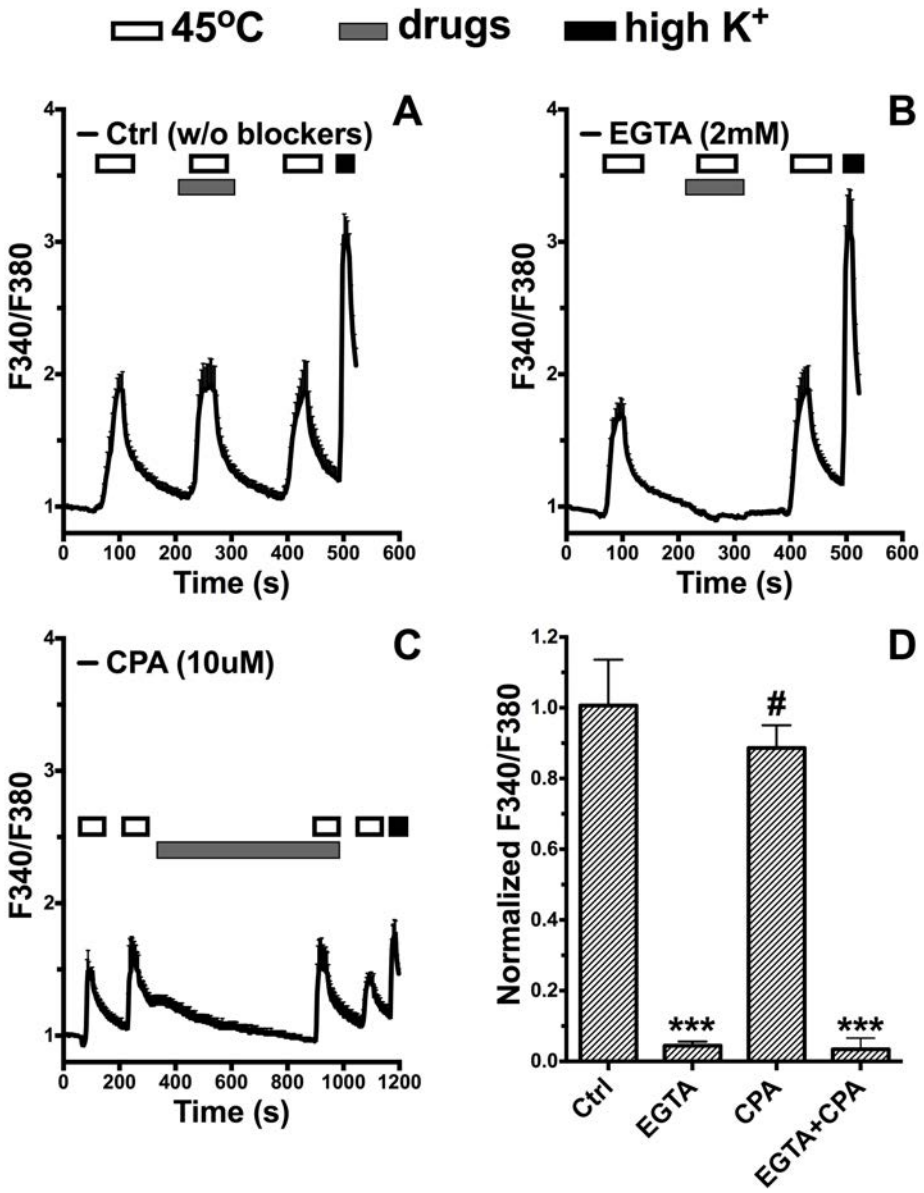


Fig.2-6 Heat-induced Ca^{2+} influx in warm-sensitive neurons.
The source of heat-induced $[Ca^{2+}]i$ increase was evaluated by Ca^{2+} imaging. (A) Control: without applying any blockers to the preoptic neurons. (B) Ca^{2+}-free Ringer's solution containing 2mM EGTA. (C) 10μM intracellular Ca^{2+} pump inhibitor CPA treatment. (D) Statistical summary of the blocking effects on the warm sensitivity of preoptic neurons. Student's *t*-test, ***p<0.001, #p>0.05, n≥3.

Furthermore, we wanted to know which channel or receptor conducted the heat-induced Ca^{2+} current in the warm-sensitive preoptic neurons. Candidates were studied by application of their corresponding antagonists in Ca^{2+} imaging. Voltage-gated sodium channels, which play an essential role in generating action

potentials in neurons, were considered to be the potential temperature-sensitive candidate channels since their gating can be affected by temperature (Thomas et al., 2009; Saura et al., 2010). To test the contribution of voltage-gated sodium channels to the heat response, preoptic neurons were stimulated by heat in the presence of 1μM voltage-gated sodium channel blocker, tetrodotoxin (TTX). Interestingly, two subpopulations of warm-sensitive neurons were identified according to the distinct effects of TTX on them (Fig.2-7B,C). About 50% warm-sensitive neurons were resistant to TTX since the amplitude of their heat response was the same with or without TTX treatment (Fig.2-7B,J), while others were sensitive to TTX because of the partial blocking effect on their heat response (Fig.2-7C,J). The divergent TTX-blocking effects on the heat response indicated the there are at least two distinct populations the warm-sensitive preoptic neurons.

Voltage-gated Ca^{2+} channels (VGCC) were also possible candidates as the activation of L-type VGCCs has been shown to be affected by temperature (Allen and Mikala, 1998). Nifedipine, an antagonist of L-type VGCCs, was found to block the heat response by 37% in warm sensitive preoptic neurons (Fig.2-7D,J). Since the majority of temperature-dependent $[Ca^{2+}]_i$ increase was maintained in the presence of nifedipine, L-type VGCCs are likely not the primary channels activated by the heat stimulus. Nevertheless, given the partial blocking effect of nifedipine, it is possible that L-type VGCCs function as channels may amplify the initial heat-induced $[Ca^{2+}]_i$ influx.

Additionally, the involvement of voltage-gated K^+ channels was evaluated by applying the general blocker tetraethylammonium (TEA) to the preoptic neurons. Since potassium channels are normally in charge of maintaining the resting membrane potential of neurons, we predicted that the heat response would be affected by the TEA treatment. Not surprisingly, more heat-induced $[Ca^{2+}]_i$ increase was found in the warm-sensitive neurons by comparing the peak values with and without TEA treatment (2nd peak vs. 1st & 3rd peak, Fig.2-7E,J).

Additionally, TEA treatment also turned some warm-insensitive neurons (1st peak, Fig.2-7F) into warm-sensitive ones (2nd peak, Fig.2-7F) probably by depolarizing those neurons to some extent. Thus, the TEA modulated the warm sensitivity of the preoptic neurons by changing their resting potential.

Moreover, Calcium Release-Activated Channels (CRAC) were recently reported to regulate heat responses in immune cells (Xiao et al., 2011). The authors showed that clustering of Stim1 was induced during a heating stimulus above 35°C without depleting intracellular Ca^{2+} stores, and the Ca^{2+} selective channel Orai1 mediated Ca^{2+} influx during the re-cooling procedure as a “heat off” response. 2-APB, a nonspecific blocker of CRACs, was shown to modulate (either activate or inhibit) several temperature-sensitive channels, serving as a useful tool to evaluate the involvement of these channels in certain heat responses. In order to investigate whether CRACs and other 2-APB sensitive channels could contribute to the heat sensitivity of preoptic neurons, we included 2-APB in the Ca^{2+} imaging experiments. Surprisingly, 2-APB (30µM) abolished the heat response completely (Fig.2-7G,J). The result indicated that a 2-APB sensitive channel was involved in regulating the heat-mediated Ca^{2+} influx in warm-sensitive preoptic neurons.

Other candidate channels were the thermoTRP channels, a subset of Trp channels, of which the members Trpv1-4 can be blocked by ruthenium red (RR) efficiently. However, RR only caused a slight inhibitiory effect on the heat response, which indicated a limited involvement of Trpv channels in the heat response (Fig.2-7H,J). Furthermore, considering that the temperature threshold of Trpv1 activation (43°C) is very close to our temperature stimulus applied to warm-sensitive preoptic neurons, capsaicin (CAP), the specific agonist of Trpv1, was applied to the preoptic neurons to evaluate the role of the Trpv1 channel on warm sensitivity. The result showed that CAP neither activated any preoptic neuron by itself nor modulated the heat-induced Ca^{2+} influx (Fig.2-7I).

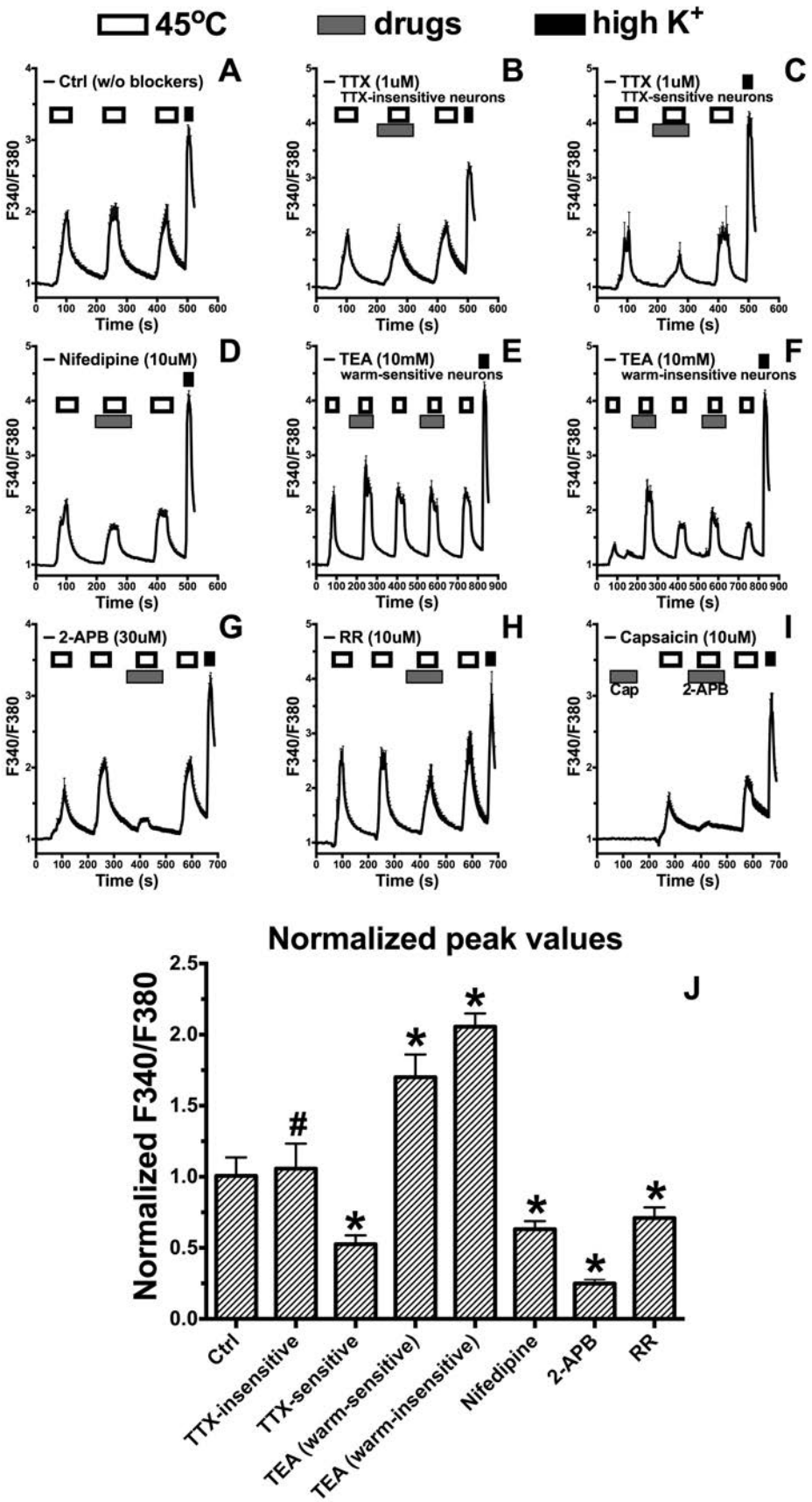

45°C
drugs
high K+
A
– Ctrl (w/o blockers)
B
– TTX (1uM)
TTX-insensitive neurons
C
– TTX (1uM)
TTX-sensitive neurons
D
– Nifedipine (10uM)
E
– TEA (10mM)
warm-sensitive neurons
F
– TEA (10mM)
warm-insensitive neurons
G
– 2-APB (30uM)
H
– RR (10uM)
I
– Capsaicin (10uM)
Cap
2-APB
F340/F380
Time (s)
Normalized peak values
J
Normalized F340/F380
Ctrl
TTX-insensitive
TTX-sensitive
TEA (warm-sensitive)
TEA (warm-insensitive)
Nifedipine
2-APB
RR

Fig.2-7 Pharmacological study on the heat response of primary preoptic neurons.
The heat response of primary cultured preoptic neurons was characterized by Ca^{2+} imaging. Traces of average $[Ca^{2+}]_i$ response was shown in the following panels: (A) Control: without applying any blocker to the neurons; (B) 1μM TTX (TTX-insensitive preoptic neurons); (C) 1μM TTX (TTX-sensitive preoptic neurons); (D) 10μM nifedipine; (E) 10mM TEA (warm-sensitive neurons); (F) 10mM TEA (warm-insensitive neurons); (G) 30μM 2-APB; (H) 10μM RR; (I) 10μM CAP; (J) Statistical summary of normalized heat response. Student's *t*-test, *p<0.05, #p>0.05, n≥10.

In summary, the results of the pharmacological characterization of preoptic neurons led us to conclude that different cation channels, such as voltage-gated sodium, potassium and calcium channels, mediated the heat-induced Ca^{2+} response by different means. Two populations (TTX-sensitive and insensitive) of warm-sensitive neurons were found by Ca^{2+} imaging. Morevoer, Trpv channels other than Trpv1 might also contribute to the heat response but only slightly. The strongest phenotype was found after 2-APB treatment. The dramatic blocking effect of 2-APB indicated that a 2-APB sensitive channel was most likely the main mediator conducting the heat-induced Ca^{2+} influx in the warm-sensitive preoptic neurons. Therefore, we aimed to identify and investigate the channel(s) in more detail in the following experiments.

4.2.4 Screening of warm-sensitive candidate channels in the POA by RNA-sequencing

As a membrane permeable nonselective pore blocker, 2-APB blocks a series of channels and receptors including the inositol 1,4,5-trisphosphate (IP_3) receptor (Splettstoesser et al., 2009), CRAC channels (Peinelt et al., 2008) and different Trp channels including Trpv6, Trpm2, Trpm3, Trpm7, Trpm8, Trpp2, and Trpc channels (Hu et al., 2009; Togashi et al., 2009). Additionally, it can also activate Trpv1-3 channels at very high concentrations (>100μM) (Colton and Zhu, 2007; Zimmermann et al., 2005). Since 2-APB only caused an inhibiting effect in this study, we did not consider the involvement of Trpv1, Trpv2 and Trpv3 channels to be likely. In addition, IP_3 receptor was also excluded from the list of candidates since the heat response was not due to intracellular Ca^{2+} release (Fig.2-6).

Due to the unspecific nature of 2-APB, further investigations were necessary to identify the heat-sensitive channel(s) underlying the warm sensitivity of the preoptic neurons. First, we performed a transcriptomic analysis of mouse tissue containing the POA by deep sequencing to obtain the gene expression information of channels in this brain area. Thereafter, by comparing the information of channels expressed in the POA and known 2-APB sensitive channels, we obtained a candidate list (Table.1). Among the list representing all 2-APB targets that are expressed in the POA, only one of them, called Trpm2, has been demonstrated to be responsive to heat stimuli (Togashi et al., 2006). Therefore, Trpm2 was the most promising candidate channel involved in the heat-induced Ca^{2+} response of warm-sensitive neurons. Nevertheless, other candidate channels can also participate in this procedure potentially.

Gene name	RPKM
Trpc1	6.98
Trpc2	3.61
Trpc3	4.45
Trpc4	6.01
Trpc5	4.34
Trpc6	1.40
Trpc7	3.87
Trpm2	4.29
Trpm3	4.17
Trpm7	7.87
Trpv2	4.85
Orai1	2.29
Orai2	11.33
Orai3	5.79

Table.1 A list of 2-APB sensitive channels expressed in the POA
Ion channels expressed in the POA were detected by transcriptomic analysis with deep sequencing. The expression level was represented by RPKM (reads per kilo base per million). Its value >1 was regarded as significant expression.

4.3 Screening and characterization of warm-sensitive cell lines

Given the technical obstacle of identifying molecules responsible for temperature detection among the short candidate list by gene knockdown approach in primary neuronal cultures, we further carried out the screening project by investigating warm-sensitive cell lines that recapitulate the warm sensitivity of preoptic neurons.

4.3.1 Screening of warm-sensitive cell lines

By applying heating and cooling stimuli to different cell lines, it is possible to evaluate their temperature sensitivity by Ca^{2+} imaging. It was the aim to find a temperature-sensitive cell line with the expectation that it could recapitulate the temperature sensitivity of preoptic neurons. Several hypothalamic cell lines (GT1-1, CLU101, CLU116, CLU172) and neuroblastoma cell lines (SH-SY5Y, PC12, IMR-32, N2A) were included together with a rat insulinoma β-cell line in our screening experiment. The pseudocolor fluorescent images of Ca^{2+} imaging represent the Ca^{2+} responses before and during temperature stimuli (Fig.3-1). At the end of each experiment, high K^+ Ringer's solution was applied to test if these cell lines were excitable. Among all the lines in our study, only three of them were responsive to the stimulus of high K^+ solution, namely PC12, SH-SY5Y and the insulinoma β-cell line. None of the other cell lines did showed robust responses to the high K^+ stimulus. In addition, none of them responded to temperature stimuli. In contrast, among the three excitable cell lines, the insulinoma β-cell line showed nice heat responses upon a 42°C stimulus indicating this cell line is heat sensitive.

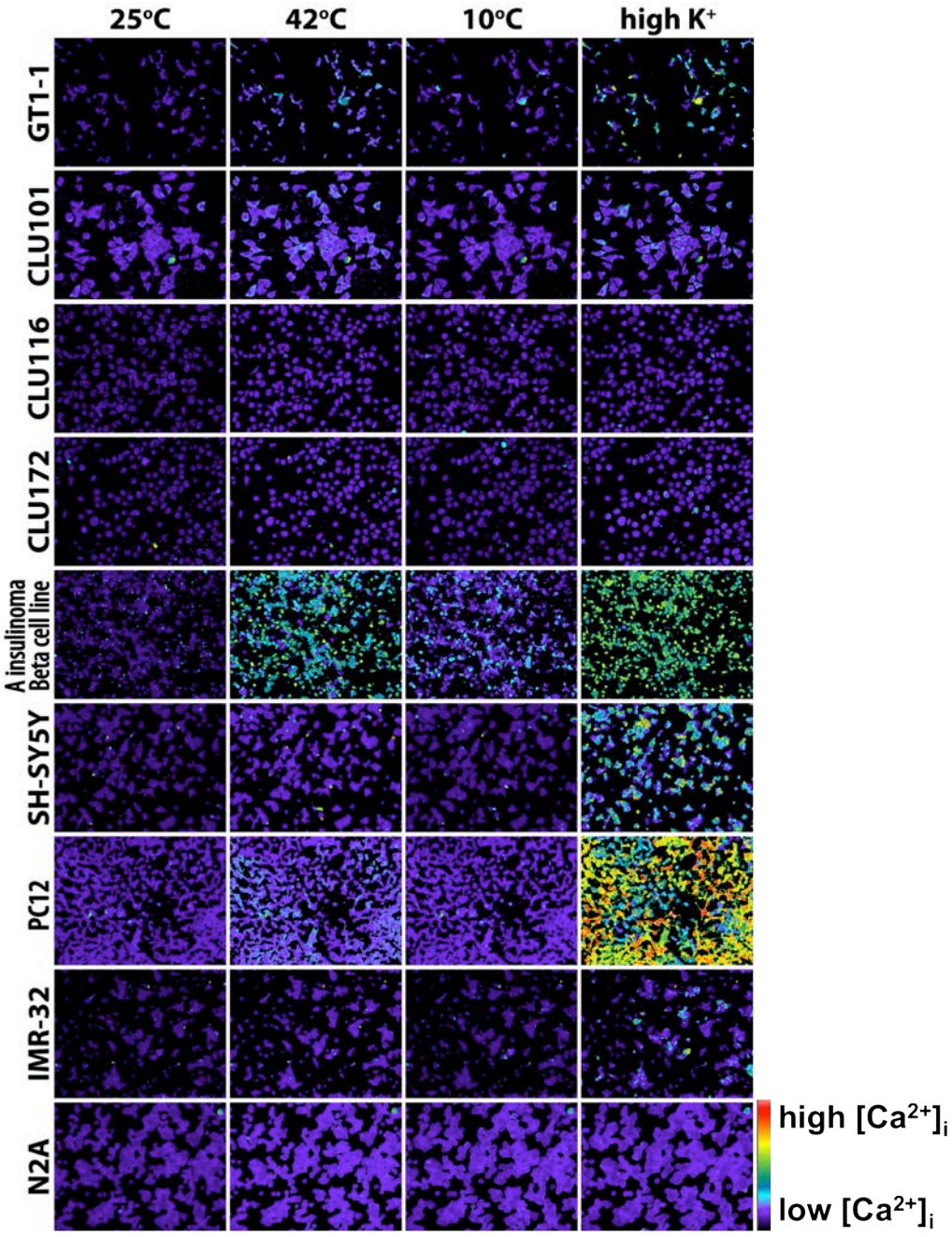


Fig.3-1 Screening for temperature-sensitive cell lines by Ca^{2+} imaging
Ca^{2+} imaging pictures of each cell line is shown in rows corresponding to the responses to RT, 45°C, 10°C and high K^+ stimuli, respectively.

4.3.2 Pharmacological study of a warm-sensitive cell line

The heat sensitivity of the insulinoma β-cell line represented a powerful tool to investigate a potentially similar mechanism underlying the heat response of

preoptic neurons. Hence, we pharmacologically investigated the heat response of the insulinoma β-cells by Ca^{2+} imaging. Different antagonists were applied to cells in order to evaluate the involvement of the targeted ion channels/pumps in response to the heat-stimuli as well as the similarity between the cell line and warm-sensitive preoptic neurons.

In the control condition in which no antagonists were present, the heat response was reproducible (Fig.3-2A). To identify the source of heat-induced $[Ca^{2+}]_i$ increase, Ca^{2+}-free Ringer's solution containing 2mM EGTA or the intracellular Ca^{2+} pump inhibitor thapsigargin were applied to the cells, testing for extracellular calcium influx and internal calcium storage release, respectively (Fig.3-2B,C). The heat response was fully abolished by Ca^{2+}-free Ringer's solution (Fig.3-2B). The remaining response is due to the temperature sensitivity of the Ca^{2+} indicator Fura-2 and can be considered the background signal. In contrast, the heat response was not altered by blocking the Ca^{2+} pumps in intracellular Ca^{2+} stores with thapsigargin (Fig.3-2C). This suggested that the heat-induced $[Ca^{2+}]_i$ increase resulted exclusively from extracellular Ca^{2+} influx but not intracellular Ca^{2+} release, which is the same as primary warm-sensitive preoptic neurons.

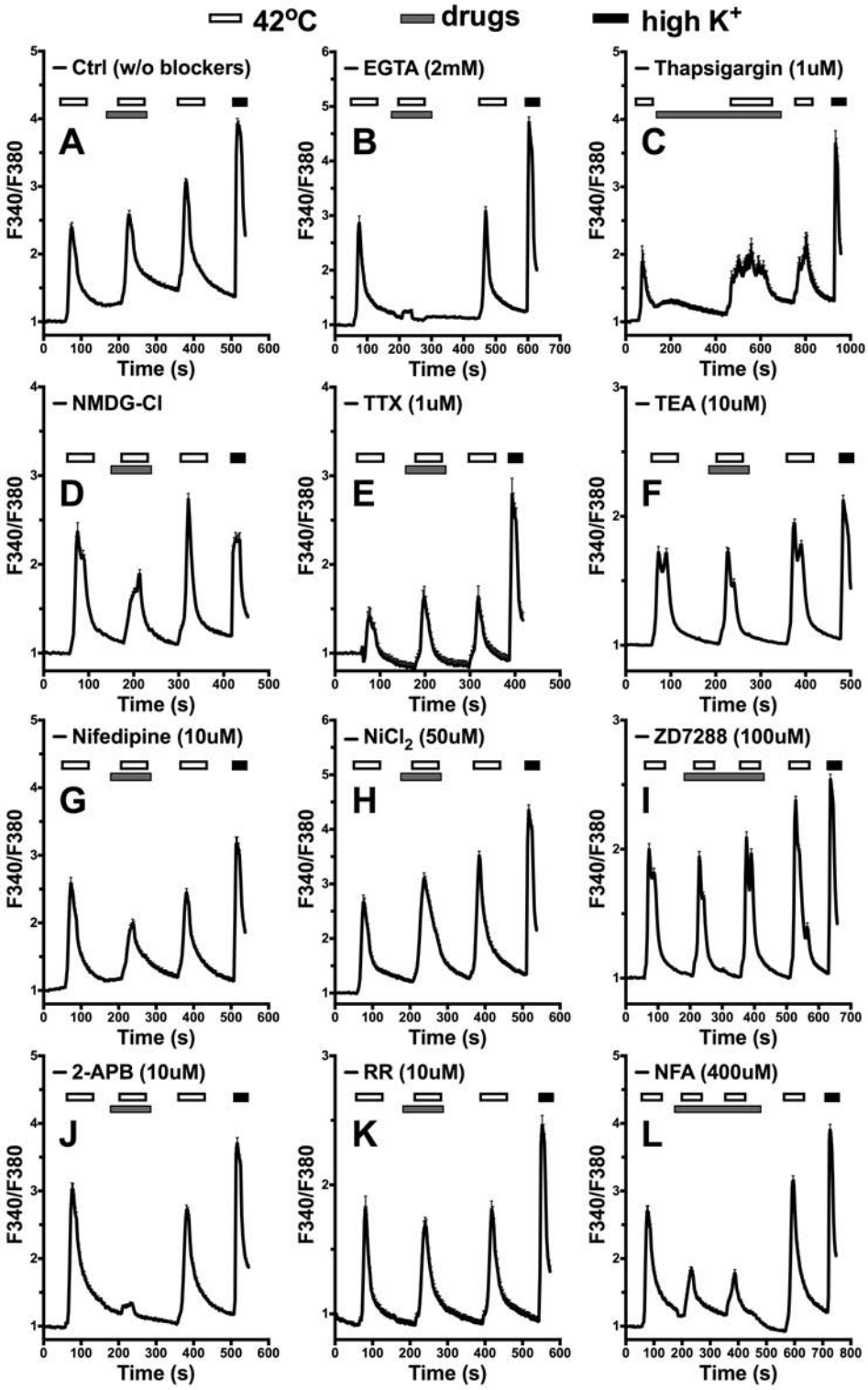


Fig.3-2 Pharmacological study on the insulinoma β-cells
The heat response of the rat insulinoma β-cell line was characterized by Ca^{2+} imaging. Traces of the average $[Ca^{2+}]_i$ response is shown in the following panels: (A) Control: without applying any blocker to the cells; (B) Ca^{2+}-free Ringer's solution with 2mM EGTA; (C) 1μM thapsigargin; (D) NMDG-Cl

Ringer's solution; (E) 1μM TTX; (F) 10μM TEA; (G) 10μM nifedipine; (H) 50μM $NiCl_2$; (I) 100μM ZD7288; (J) 10μM 2-APB; (K) 10μM ruthenium red (RR); (L) 400μM niflumic acid (NFA).

Then we evaluated the potential involvement of different ion channels in the heat responses of this cell line by application of their antagonists. To test if the participation of voltage-gated Na^+ channels is required, we first replaced the extracellular Na^+ ions with N-methyl-d-glucamine (NMDG). As a large organic monovalent cation, NMDG is impermeable to voltage-gated Na^+ channels. The heat response was partially blocked by NMDG-Cl Ringer's solution (Fig.3-2D). However, TTX, a potent inhibitor of voltage-gated Na^+ channels, did not attenuate the heat response at all (Fig.3-2E). There are two possibilities to explain the results of samples treated with NMDG-Cl and TTX. One is that voltage-gated Na^+ channels are not involved in the heat-induced $[Ca^{2+}]_i$ increase, and the lowered heat response during the application of NMDG-Cl resulted from hyperpolarization. This interpretation is based on the hypothesis that the heat sensitivity is dependent on the membrane potential of the insulinoma β-cells. Since replacement of extracellular Na^+ with NMDG normally leads to a hyperpolarized membrane potential and hyperpolarized cells need a greater driving force (heat–stimulus, in this case) to be activated, the NMDG effect could be independent of voltage-gated Na^+ channels. In contrast to the Na^+ replacement experiment, the application of TTX does not alter resting membrane potential so the heat response was unaltered.

As the other possibility, we cannot exclude the involvement of TTX-insensitive Na^+ channels, even though there are only a few Na^+ channels insensitive to TTX. Independent of which possibility holds true, voltage-gated Na^+ channels did not seem to play a pivotal role in heat-induced $[Ca^{2+}]_i$ increases since neither NMDG nor TTX completely blocked the heat response. A similar conclusion could be drawn for voltage-gated K^+ channels because their antagonist TEA elicited no blocking effect on the heat response at all (Fig.3-2F).

Furthermore, we treated the cells with different antagonists of Ca^{2+} channels to test if some of them might participate in the heat-induced response. Nifedipine only partially blocked the heat response (Fig.3-2G), suggesting that it might play a role in amplifying the heat response instead of acting as the primary thermosensor in the insulinoma β-cells. Moreover, we tested inhibitors of T-type Ca^{2+} channels and HCN channels in the next experiments since they were reported to be sensitive to heat (Iftinca et al., 2006; Biel et al., 2009). $NiCl_2$, a blocker of T-type Ca^{2+} channels, did not alter the heat response at all (Fig.3-2H). Similar results were obtained when the cells were treated with the HCN channel blocker ZD7288 (Fig.3-2I). These results clearly exclude the involvement of T-type Ca^{2+} channels and HCN channels in the heat response.

Moreover, 2-APB was included in our experiments since it blocked the most promising candidate channels identified by the screening on primary preoptic neurons. Interestingly, it almost completely abolished the heat response (Fig.3-2J), indicating that a certain 2-APB sensitive channel plays an important role in the heat sensitivity of the insulinoma β-cells.

In addition, we tested the antagonists of some known heat-sensitive ion channels. The non-selective blocker of TRPV channels RR did not obviously inhibit the heat response (Fig.3-2K), while niflumic acid (NFA), a blocker of Ca^{2+}-activated Cl^- channels (Osorio et al., 2013), partially blocked the heat response (Fig.3-2L). From this result, it initially seemed that Ano1, but not Trpv channels, could be involved in the heat response. However, NFA is not a specific antagonist of Ano1 only. In fact, it is also able to partially block Trpm2 channels (Nazıroğlu et al., 2007) as well as other Ca^{2+}-activated Cl^- channels.

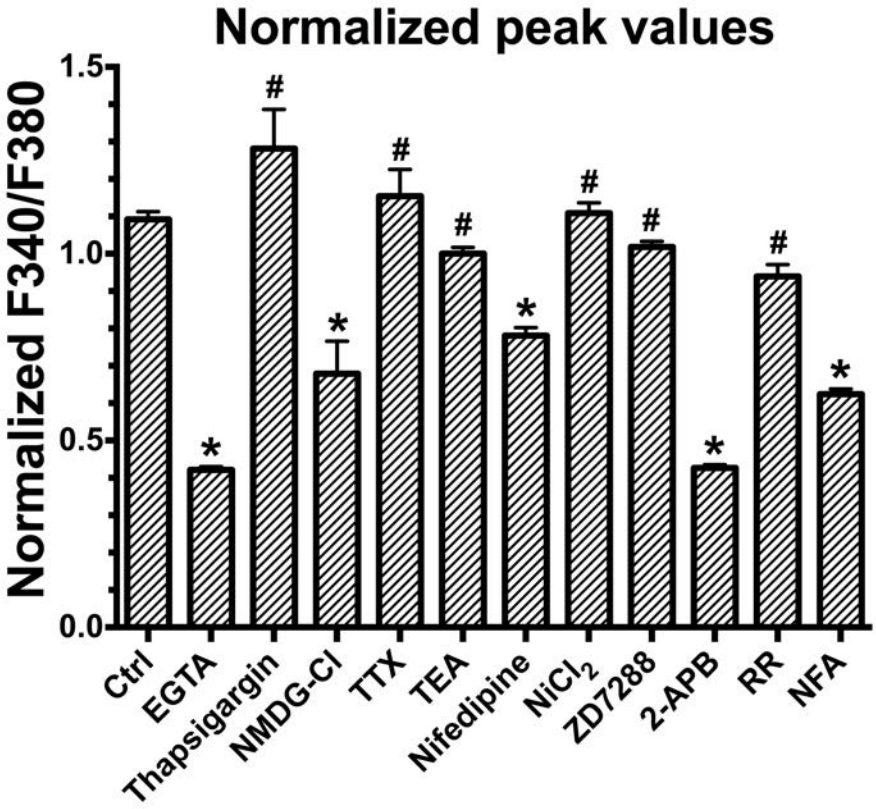


Fig.3-3 Statistical analysis of the inhibitory effects on the insulinoma β-cells
The peak values of the 1st heat responses of each experiment were normalized to 1 in order to compare the blocking effect of these experiments with each other. The bar graph showed the peak values of the 2nd heat responses of each experiment in the presence of a pharmacological agent. The control experiment did not include any agent but only Ringer's solution during the 2nd heat stimulus. Student's *t*-test, *p<0.05, #p>0.05, n≥10.

Fig.3-3 summarizes the statistical analysis of the pharmacological studies shown in Fig.3-2. Peak values during the treatment with channel blockers were normalized to the peak values of the 1st heat response. In conclusion, the specific heat response found in the insulinoma β-cells was sensitive to 2-APB, indicating that a channel sensitive to 2-APB is involved in the heat-induced $[Ca^{2+}]_i$ response. The similarity of the in-depth pharmacological profiling between the primary warm-sensitive neurons and the insulinoma β-cell line, especially the blocking effect of 2-APB on their heat responses, suggests that the rat insulinoma β-cell line is very likely to share the same mechanism underlying the temperature-sensitivity of the warm-sensitive preoptic neurons. Since 2-APB can

inhibit the activation of several different channels, further investigation is required to identify the specific channel(s) responsible for the heat response.

4.3.3 Identification of the heat-sensitive channel in the warm-sensitive insulinoma β-cell line by siRNA knockdown experiments

As a nonselective antagonist, 2-APB has been reported to affect the activation of different cation channels and receptors including several Trp channels, CRACs, and IP_3 receptors as mentioned in the section 4.2.4 before (Bootman et al., 2002; Hu et al., 2009; Splettstoesser et al., 2009; Togashi et al., 2009; Li et al., 2014). Since 2-APB caused an inhibitory effect similar to what it did on the warm-sensitive preoptic neurons in this study (Fig.3-3), we excluded the involvement of Trpv channels as well as IP_3 receptor. To identify the most promising candidate channels, we compiled a list of channels, which are known to be blocked by 2-APB as well as one based on the expression profile of all ion channels in this cell line obtained by transcriptomic analysis. After comparing the 2-APB sensitive channels with those being members in both lists, we carried out gene knockdown experiments of the following channels – Orai1, Orai2, Orai3, Trpm2, Trpc1, and Trpc4 in the insulinoma β-cells. As the only known heat-sensitive channel among them, Trpm2 was regarded to be the most promising candidate as it was in the primary preoptic neurons.

To guarantee the knockdown efficiency, three siRNA oligonucleotides targeting different sequence regions of the same gene were included in the experiment. In addition, various gene delivery reagents were tested to optimize the number of the insulinoma β-cells transfected (data not shown), leading to the selection of lipofectame 2000 (Invitrogen) as the final transfection agent. In the gene knockdown experiment, siRNA was transfected together with GFP plasmids, and only the data of GFP-positive cells was analyzed to evaluate the knockdown effect on the heat response by Ca^{2+} imaging. As a control, scrambled siRNA was included to rule out any artificial effect in the experimental set-up.

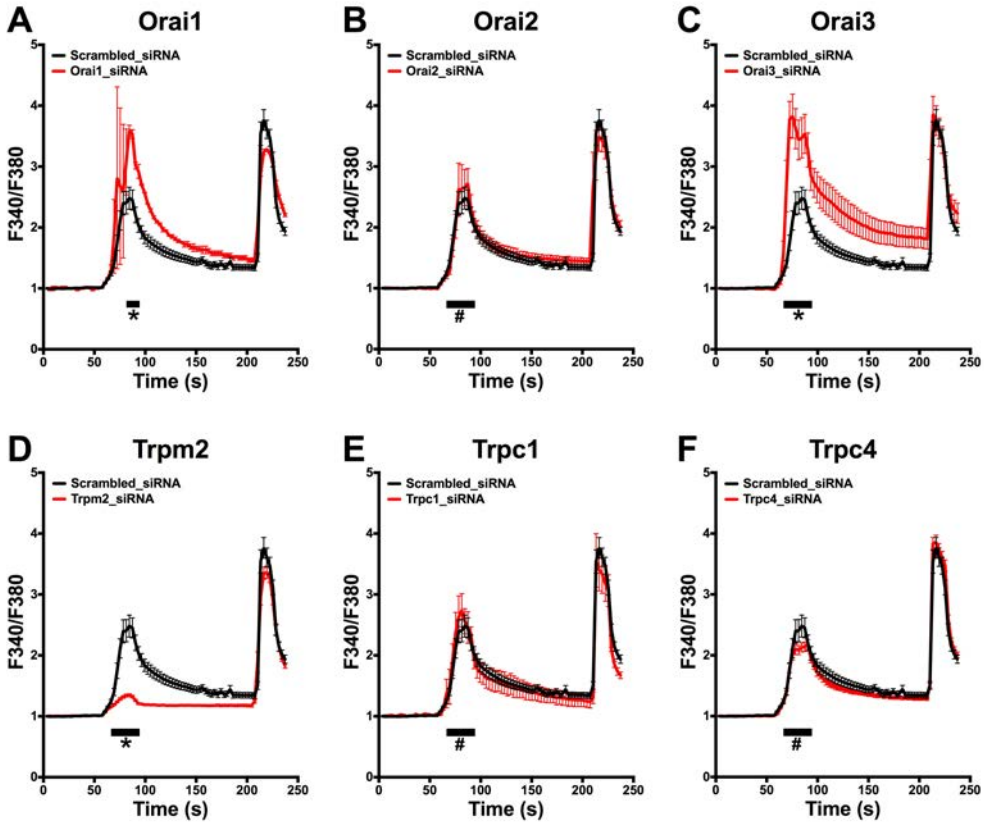


Fig.3-4 siRNA knockdown experiments in the insulinoma β-cells
The expression of target channels in the insulinoma β-cells was knocked down by siRNA transfection. The heat response of siRNA transfected insulinoma β-cells was characterized by Ca^{2+} imaging. Average $[Ca^{2+}]_i$ responses of heat sensitive cells are shown as the red traces in the following panels: (A) siRNA against Orai1; (B) siRNA against Orai2; (C) siRNA against Orai3; (D) siRNA against Trpm2; Due to the lack of heat responders identified after the knockdown of Trpm2, the Ca^{2+} response of heat insensitive cells was plotted here. (E) siRNA against Trpc1; (F) siRNA against Trpc4. In comparison, cells were transfected with the scrambled siRNA as the control (shown as black traces in each panel). Student's *t*-test, *p<0.05, #p>0.05, n≥4.

As shown in Fig.3-4, cells responded more or less the same to high K^+ stimuli (2nd peak of Ca^{2+} responses), indicating that knocking down the candidate genes did not alter the excitability of the insulinoma β-cells. Knocking down Orai1 and Orai3 enhanced the heat response (Fig.3-4A,C). No obvious effect was discovered in the Orai2, Trpc1 and Trpc4 siRNA transfected samples in comparison to the scrambled siRNA control, suggesting that these channels were not involved in the response to the heat stimulus (Fig.3-4B,E,F). Importantly,

knocking down the expression of Trpm2 almost completely blocked the heat-induced $[Ca^{2+}]_i$ increase in the insulinoma β-cells (Fig.3-4D). This suggested that Trpm2 is crucially involved in the heat response. Since it has been reported before that Trpm2 is a heat-sensitive channel (Togashi et al., 2006), we concluded that the extracellular Ca^{2+} influx during the heat stimulus was due to activation of Trpm2 in the insulinoma β-cells.

4.4 Trpm2 was identified to conduct the heat response of primary warm-sensitive preoptic neurons

Based on the aforementioned screening strategy, Trpm2 identified as the channel conducting the heat-induced Ca^{2+} response in the insulinoma β-cell line has a very high possibility to also contribute to the heat response of the primary warm-sensitive preoptic neurons because of the similarity of the pharmacological profiles of their heat responses. Therefore, we hypothesized that Trpm2 contributes to the heat response of primary warm-sensitive preoptic neurons. To test the hypothesis, we treated the preoptic neurons with H_2O_2, a oxidative molecule has been shown to be able to sensitize the activation of Trpm2 by reducing its temperature threshold of activation (Kashio et al., 2012), and check if the same sensitization effect would happen in the warm-sensitive preoptic neurons.

4.4.1 H_2O_2 sensitization effect on warm-sensitive POA neurons

To study the sensitization effect on Trpm2 in preoptic neurons, warm-sensitive neurons were identified by the criteria that the heat responses could be abolished by 2-APB (30μM). The temperature of the heat stimulus was afterwards adjusted to be lower (40°C) than the threshold of activation determined in the previous experiment (44.5°C<T<45.6°C, Fig.2-1). Neurons were then incubated with 1mM H_2O_2 for 8min and subsequently exposed to another heat stimulus at the same

temperature. The magnitude of the heat-induced $[Ca^{2+}]_i$ responses before and after the application of H_2O_2 was compared in order to detect the sensitization effect on the warm sensitivity of the preoptic neurons. Fig.4-1 shows the sensitization effect induced by the H_2O_2 treatment. Before being incubated with H_2O_2, cells were unresponsive to the 40°C heat stimulus. However, the heat response of 3 out of 14 warm-sensitive neurons was increased after the incubation of H_2O_2 (1mM), indicating that the temperature threshold was lowered by H_2O_2 treatment. This phenomenon was not observed in the other 11 neurons, showing that individual preoptic neurons responded to H_2O_2 differently. The $[Ca^{2+}]_i$ level was not affected by H_2O_2 during the 8min incubation and thereafter, indicating that 1mM H_2O_2 and its downstream signaling molecules did not directly activate Trpm2 channels. On the contrary, they potentiated the heat-induced Trpm2 activation by reducing their temperature threshold.

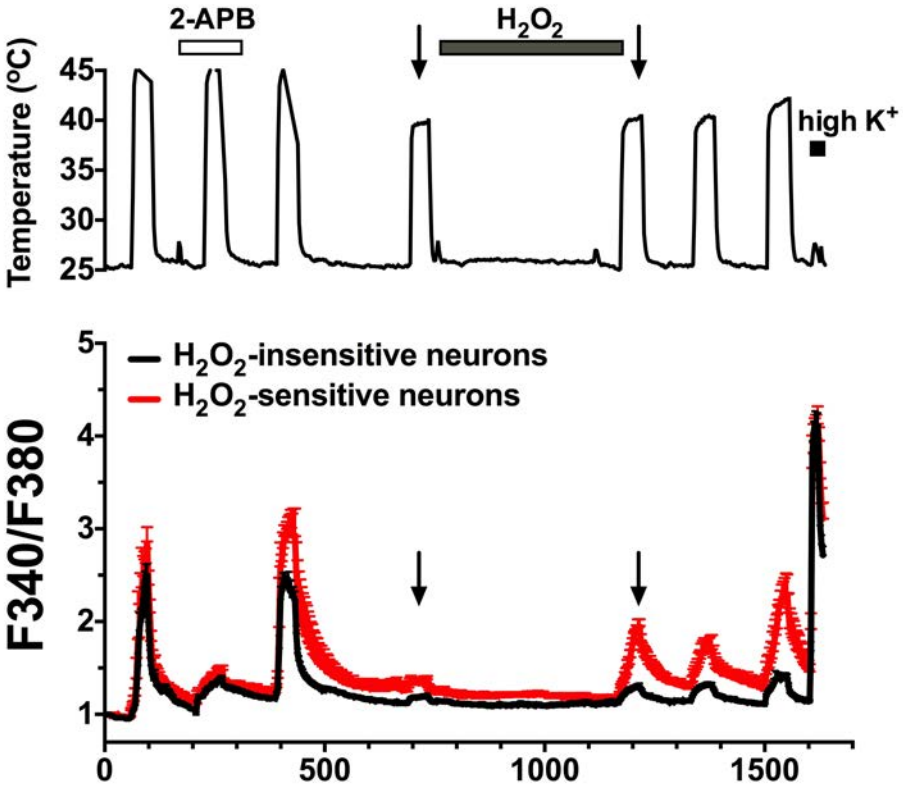


Fig.4-1 H_2O_2 sensitization effect on warm-sensitive neurons
The temperature threshold of warm-sensitive neurons was studied by Ca^{2+} imaging. The upper panel shows the temperature stimuli applied to the POA neurons. The neurons were incubated with the H_2O_2 (1mM) for 8 minutes. The corresponding intracellular Ca^{2+} response was plotted in the lower panel. 2-

APB (30μM) was applied after the detection of warm-sensitive neurons. The sensitization effect of H_2O_2 was evaluated by comparing the heat responses immediately before and after the application of H_2O_2 indicated by the arrows. Among 14 warm-sensitive preoptic neurons, the heat response of 3 cells were sensitized by the incubation of H_2O_2 (red trace), while the other 11 neurons were resistant to H_2O_2 since their Ca^{2+} responses during the second heat stimulus were unchanged (black trace). (n=14)

Obvious sensitization effect of H_2O_2 was observed in some warm-sensitive preoptic neurons, although not all of them were sensitized by the treatment of H_2O_2. It strongly suggested that Trpm2 probably conducted the heat response in these neurons, which prompted us to include the Trpm2 knockout mouse line in the following experiment in order to characterize the functional role of Trpm2 in primary preoptic neurons.

4.4.2 Charaterization of the heat response in the primary preoptic neurons by using Trpm2 knockout mice

The heat response was evaluated on primary cultured preoptic neurons isolated from Trpm2 wildtype and knockout littermate mouse pups by Ca^{2+} imaging. In order to compare $Trpm2^{+/+}$ and $Trpm2^{-/-}$ cells in an unbiased way, all the cells activated by the high K^+ stimulus were selected for data analysis. Warm-sensitive neurons were defined by the same criterion as before. Importantly, warm-sensitive neurons were identified only in $Trpm2^{+/+}$ but not in $Trpm2^{-/-}$ preoptic samples (Fig.4-2). The averaged $[Ca^{2+}]_i$ traces of warm sensitive and insensitive neurons from $Trpm2^{+/+}$ and $Trpm2^{-/-}$ samples are plotted in Fig.4-2A, and the peak values during heat stimulus are summarized in Fig.4-2B. In $Trpm2^{+/+}$ preoptic neurons, 11.7±1.9% high K^+ responders were sensitive to the heat stimulus. This percentage was consistent with our previous result (≈12%, Fig.1-1D). The 46°C heat stimulus caused a $[Ca^{2+}]_i$ increase in the subpopulation of high K^+ responders, which was blocked by 30μM 2-APB (Fig.4-2A). In contrast, no warm-sensitive responders were identified in the $Trpm2^{-/-}$ preoptic neurons (Fig.4-2A,B), indicating that the activation of Trpm2 is required for the heat-induced $[Ca^{2+}]_i$ elevation in warm-sensitive preoptic neurons. Since Trpm2 is a

heat-sensitive channel, it is reasonable to suggest that Trpm2 acts as the thermo-transducer in the process of heat-induced Ca^{2+} entry.

Since there were no heat responders among $Trpm2^{-/-}$ neurons and all the heat responders in $Trpm2^{+/+}$ neurons were sensitive to 2-APB, we further used 2-APB as a powerful pharmacological tool to identify and study warm-sensitive neurons, as it is specifically blocked the heat responses in these neurons.

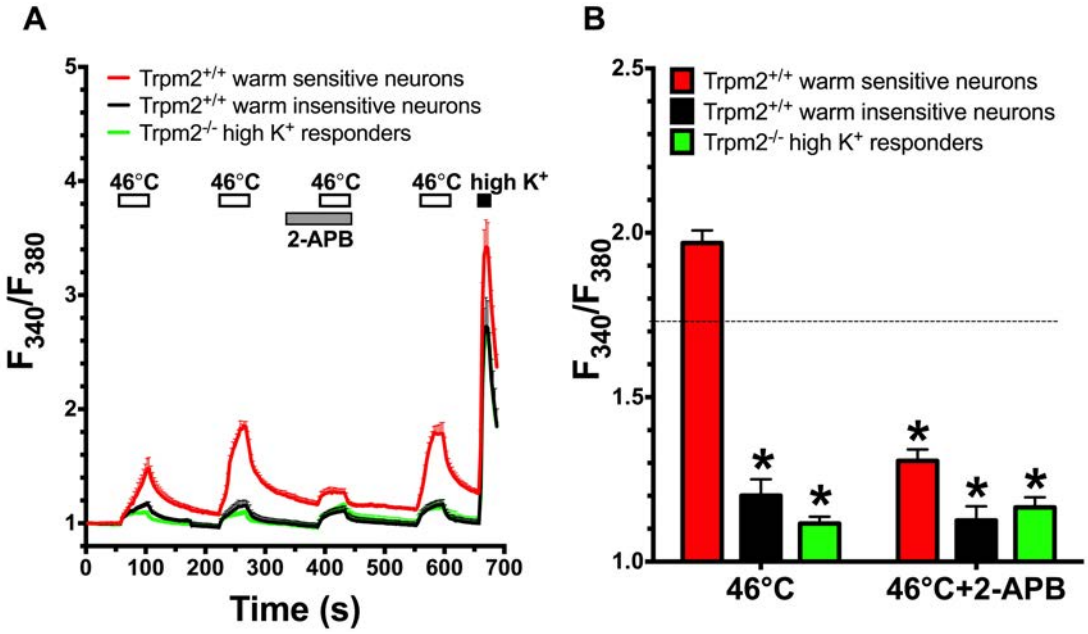


Fig.4-2 Heat-induced Ca^{2+} responses in $Trpm2^{+/+}$ and $Trpm2^{-/-}$ preoptic neurons
The heat-induced Ca^{2+} responses were evaluated in $Trpm2^{+/+}$ and $Trpm2^{-/-}$ preoptic neurons by Ca^{2+} imaging *in vitro*. (A) Average traces of warm-sensitive and insensitive neurons from $Trpm2^{+/+}$ and $Trpm2^{-/-}$ mice. (B) Statistical analysis of the peak values during heat stimuli with and without the application of 2-APB. Student's *t*-test, *p<0.05, n=4. (The desh line indicates the threshold value for identification of warm-sensitive preoptic neurons according to their heat-induced Ca^{2+} increases.)

4.5 Trpm2 expression in mouse preoptic area of hypothalamus

Based on the Ca^{2+} imaging results (Fig.4-2), Trpm2 appears to be expressed in the preoptic area of the mouse hypothalamus. Due to the lack of a useful Trpm2 antibody, we applied fluorescent *in situ* hybridization on mouse frozen brain

sections with anti-sense RNA probes against mouse Trpm2 (performed by Dr. Hagen Wende) in order to verify the expression of Trpm2 in the POA. The expression pattern of Trpm2 in the POA is shown in Fig.5-1. There were some Trpm2 expressing cells (green) scattering in the POA, the distribution of which was more concentrated in the medial septal nucleus (MS) and ventrolateral preoptic area (vlPOA) (indicated by the white dash-lined square, Fig.5-1A). DAPI staining (blue) marked nuclei of all cells in the POA section. The magnified view of the MS and vlPOA regions were shown in Fig.5-1C,D. In addition, Trpm2 expression was colocalized with the marker gene of GABAergic neurons - GAD1 (red) in some preoptic neurons (Fig.5-1B), especially within the MS and vlPOA regions (Fig.5-1E,F). It suggested that some of the Trpm2-expressing neurons were GABAergic.

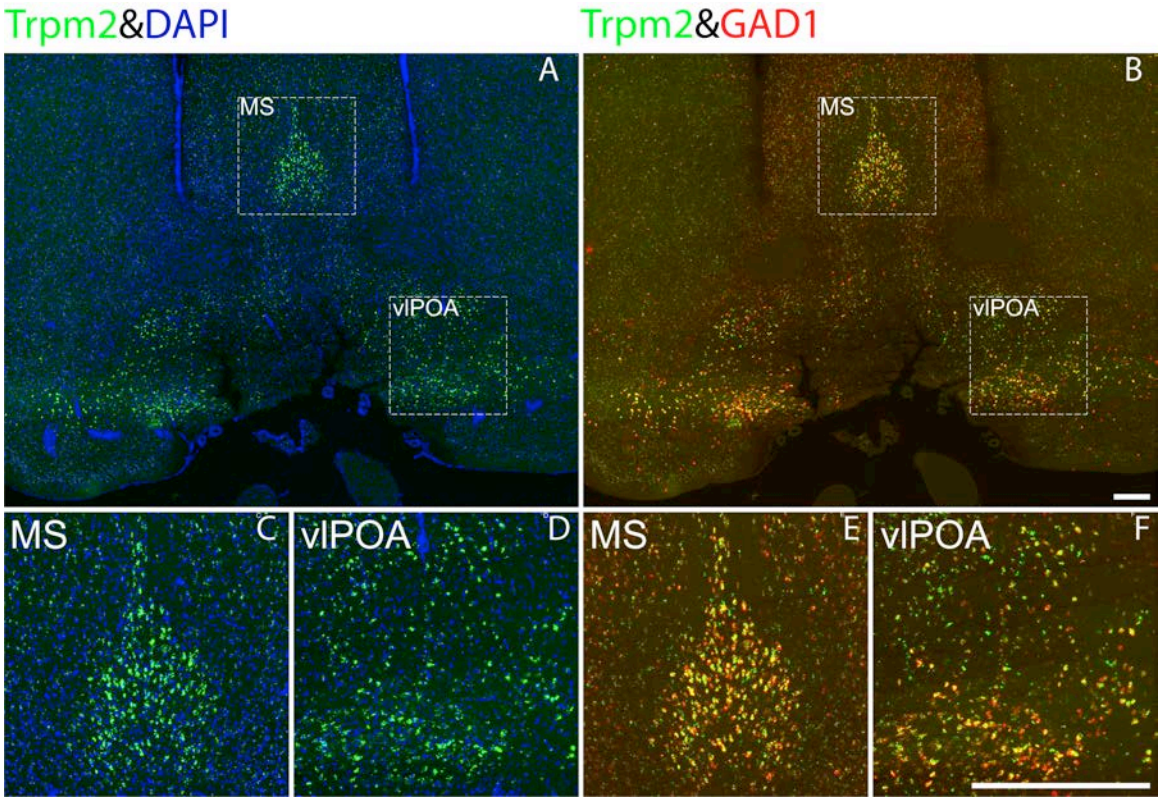


Fig.5-1 Expression of Trpm2 in the POA characterized by fluorescent *in situ* hybridization
Expression of Trpm2 transcripts in the POA was analyzed by fluorescent *in situ* hybridization with Cy2-labeled probes against mouse Trpm2 (green). GABAergic neurons were analyzed by a GAD1 probe (red). DAPI staining marks nuclei of all cells (blue). (A) Overview of the Trpm2 expression pattern in the POA. (B) Colocalization of Trpm2 and GAD1 in the same POA section

as (A). (C) and (D) Magnified view of the MS and vlPOA indicated in (A). (E) and (F) Magnified view of the MS and vlPOA in (B). MS: medial septal nucleus, vlPOA: ventrolateral preoptic area. Scale bar: 300μm.

4.6 Characterization of Trpm2 functions *in vivo*

As Trpm2 channels are functionally expressed in warm-sensitive neurons of the POA, we further addressed the question of whether there is a thermoregulatory fever phenotype of Trpm2 knockout mice. There are several reasons for characterization of the Trpm2 knockout mice in the context of fever.

(1) As mentioned in section 1.3.1, Trpm2 is expressed in different immunocytes involved in systemic inflammation, a process that is usually accompanied by changes in body temperature. It has been shown that genomic deletion of Trpm2 in different immune cells functionally affects inflammatory responses owing to a defect in febrile cytokine secretion *in vitro* and *in vivo* (Yamamoto et al., 2008; Di et al., 2011; Knowles et al., 2012).

(2) There is a mechanistic basis for the hypothesized Trpm2 activation in febrile conditions: Trpm2 is sensitive to temperature and oxidative stress. These two Trpm2-modulating factors are usually altered during a fever response. Furthermore, the sensitization effect of the redox molecule H_2O_2 has been shown in Trpm2-expressing HEK293 cells and macrophages by reducing its activation temperature threshold (Kashio et al., 2012). Given the expression of Trpm2 in many immunocytes and the upregulation of ROS production during immune responses, ROS-sensitized Trpm2 could serve as a thermosensor to mediate immune activities during a febrile state.

(3) Beyond modulating the function of the immune system, heat-sensitive Trpm2 channels are also expressed in the POA of the hypothalamus – the thermoregulatory center controlling core body temperature. Its expression pattern in the brain provides the potentially functional association of Trpm2 with thermoregulation during the fever response.

(4) It has been shown that Trpm2 knockout mice developed more high-dose LPS-induced lung inflammation resulting in a higher lethality rate than the wildtype mice, indicating that Trpm2 is involved in LPS-induced inflammatory responses (Di et al., 2011).

Given the considerations above, we decided to characterize the functional role of Trpm2 in thermoregulation in the context of the fever response by means of a Trpm2 knockout mouse model.

4.6.1 Core body temperature of $Trpm2^{+/+}$ and $Trpm2^{-/-}$ mice at different ambient temperature

At first, we evaluated whether the basal core body temperature might be different between $Trpm2^{+/+}$ and $Trpm2^{-/-}$ mice by recording their temperature circadian rhythm at 22°C. Core body temperature and activities of $Trpm2^{+/+}$ and $Trpm2^{-/-}$ mice (6 mice for each genotype) were detected by a DSI telemetry system over 4 days using 5 minute recording intervals. There was no obvious difference in core body temperature and basal activities during either light or dark phases between them (Fig.6-1A,B). Only subtle differences in core body temperature between them were found (indicated by blue bars marking individual sampling time points in Fig.6-1A). This effect was negligible since the maximal continuous time span showing statistical differences was very short (at most 10 minutes, the equivalent of two consecutive data points). As a nocturnal animal, mice are more active in the dark phase than the light phase, which was reflected by their activity pattern (Fig.6-1B), indicating that the telemetry system was successful in recording this difference. Further analysis was performed considering mouse genders. No obvious difference was detected in terms of circadian core body temperature or activity between the $Trpm2^{+/+}$ and $Trpm2^{-/-}$ male mice (Fig.6-1C,D). Similar results were obtained for the female mice (Fig.6-1E,F). Thus, the $Trpm2^{-/-}$ mice behaved the same as $Trpm2^{+/+}$ mice in terms of their circadian rhythm of core body temperature and daily activities.

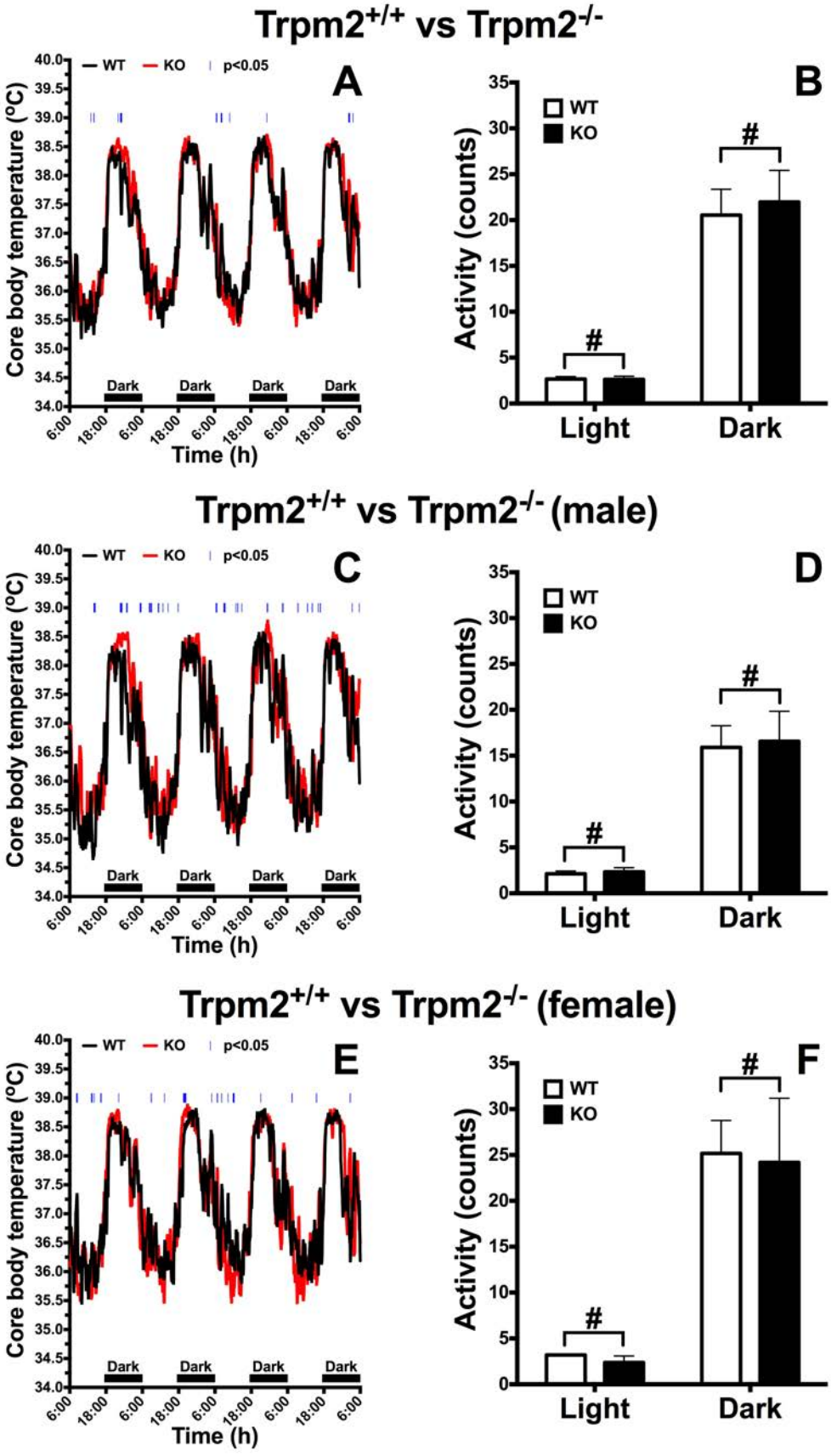

Trpm2+/+ vs Trpm2-/-
A
WT
KO
p<0.05
Core body temperature (°C)
Dark
Time (h)
B
Activity (counts)
Light
Dark
Trpm2+/+ vs Trpm2-/- (male)
C
D
Trpm2+/+ vs Trpm2-/- (female)
E
F

Fig.6-1 Circadian rhythm of core body temperature in Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice at 22°C
Basal core body temperature and activities of 12-week-old Trpm2 wildtype and knockout mice were recorded by a DSI telemetry setup over 4d. (A) Core body temperature of Trpm2$^{+/+}$ (black trace) and Trpm2$^{-/-}$ (red trace) mice showed the pattern of circadian rhythm over light and dark phases. (n=6, Student's *t*-test, blue bars correspond to the time point showing statistical difference of core body temperature between Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice. $p<0.05$). (B) Average activities of Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice during light and dark phases. (n=6, Student's *t*-test, #$p>0.05$). To study if there was gender-dependent effect on circadian rhythm, the data of male and female mice were separated and shown in (C) core body temperature of male mice, (D) average activities of male mice, (E) core body temperature of female mice, and (F) average activities of female mice. (n=3 for each gender and genotype, Student's *t*-test, #$p>0.05$).

Sucessively, the core body temperature of Trpm2 knockout mice was evaluated during temperature stimuli. Twelve Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice (6 for each genotype) were challenged by heat (37°C) and cold (7°C) for about 6h on different dates. The core body temperature traces of the two groups are shown in Fig.6-2A,C. Heat treatment caused a tiny increase in core body temperature for both Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice (Fig.6-2A). In contrast, the cold stimulus induced transient hypothermia (T_b<35°C) (Fig.6-2C). However, no statistical difference of core body temperatures was revealed during either heat or cold stimuli, indicating that there is no robust thermoregulatory phenotype in Trpm2 knockout mice during mild ambient temperature challenges. In addition, their activity was also similar during the temperature stimuli (Fig.6-2B,D).

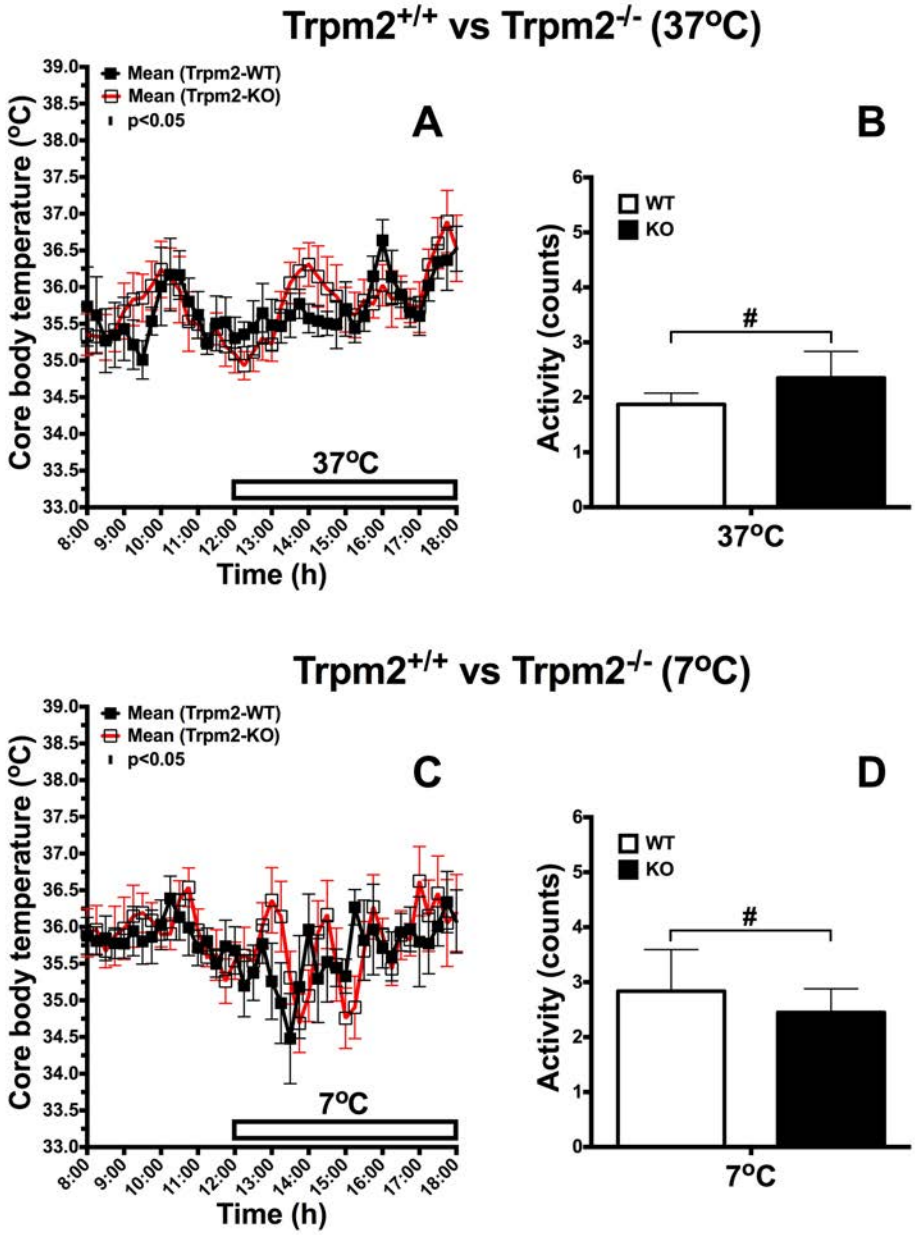


Fig.6-2 Core body temperature and activities of Trpm2$^{+/+}$ and Trpm2$^{-/-}$ littermates during heat and cold stimuli

Trpm2 wildtype and knockout mice were treated with heat (37°C) and cold (7°C) stimuli by changing the ambient temperature. (A) Core body temperature of Trpm2$^{+/+}$ (black trace) and Trpm2$^{-/-}$ (red trace) mice was recorded during a 37°C heat stimulus without showing any significant difference. (B) Average activities of Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice during the heat stimulus (12:00pm-18:00pm). (C) Core body temperature of Trpm2$^{+/+}$ (black trace) and Trpm2$^{-/-}$ (red trace) mice was recorded during a 7°C cold stimulus without showing any significant difference. (B) Average activities of Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice during the cold stimulus (12:00pm-18:00pm). (n=6, Student's *t*-test, #p>0.05).

4.6.2 Core body temperature of Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice during a fever response

To induce a fever response, Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice were injected (ip.) with 100µg/kg LPS dissolved in sterile saline. Normally, the LPS-induced fever response lasts for 4-6 hours. To avoid interference by the circadian fluctuation of body temperature, injection was done between 9:00am and 10:00am so that there was enough time for the development of the fever response. After injection of LPS, the core body temperature of all mice peaked immediately short after the handling of the animals because of the injection-induced stress (Fig.6-3A). Their core body temperature returned to the basal level within 1h after injection. Such observation was also reported in other studies (Romanovsky et al., 2005; Lazarus et al., 2007). Subsequently, the fever response was developed in both Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice according to their slowly increased core body temperature from 36°C to 37.5°C (Fig.6-3A). There was a tendency that the Trpm2$^{-/-}$ mice developed a less pronounced fever response than the Trpm2$^{+/+}$ mice, especially from 15:00pm to 17:00pm. The core body temperature showed a statistically significant difference between the Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice within this specific time frame. Activity results showed that there was no significant difference between the Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice, indicating that the phenotype on core body temperature during fever was not a result of altered mouse activity (Fig.6-3B). The phenotype of the fever response was found in both male and female Trpm2$^{-/-}$ mice (Fig.6-3C-F). In summary, Trpm2$^{-/-}$ mice developed a less pronounced core body temperature increase during LPS-induced fever when compared to Trpm2$^{+/+}$ mice. Since the activity of Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice was not different, the mechanism underlying the phenotype remains, at this point of time, elusive.

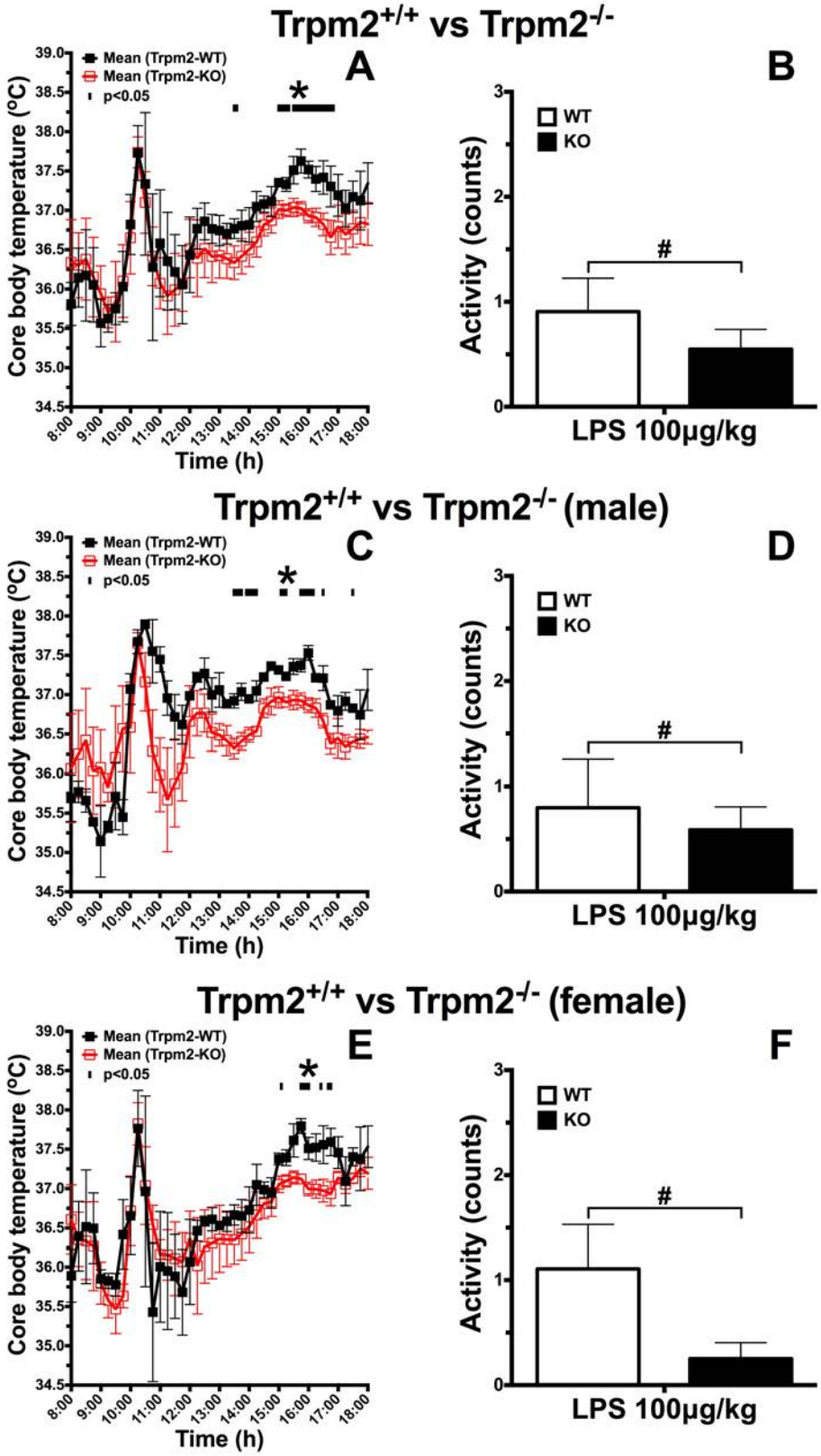

Trpm2+/+ vs Trpm2-/-
A
B
Mean (Trpm2-WT)
Mean (Trpm2-KO)
p<0.05
Core body temperature (ºC)
Time (h)
Activity (counts)
WT
KO
LPS 100µg/kg
Trpm2+/+ vs Trpm2-/- (male)
C
D
Trpm2+/+ vs Trpm2-/- (female)
E
F

Fig.6-3 Core body temperature and activities of Trpm2$^{+/+}$ and Trpm2$^{-/-}$ littermates during the LPS-induced fever response
100μg/kg LPS was injected i.p. into Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice housed at 22°C between 9:00am and 10:00am. (A) Core body temperature of Trpm2$^{+/+}$ (black trace, n=5) and Trpm2$^{-/-}$ (red trace, n=6) mice was recorded until 18:00pm. Core body temperature differences were detected which lasted about 2 hours (Black bars corresponded to the time point showing statistical difference of core body temperature between Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice. Student's *t*-test, *p<0.05). (B) Average activities of Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice during the time frame showing NO statistical difference between Trpm2$^{+/+}$ and Trpm2$^{-/-}$ mice induced by LPS injection (#p>0.05, Student's *t*-test). To study if there was a gender-dependent effect on LPS-induced fever between the two genotypes, the data of male and female mice were separated and shown in (C) core body temperature of male mice, (D) average activities of male mice, (E) core body temperature of female mice, and (F) average activities of female mice. (n=3 for each gender and genotyped mice except for Trpm2$^{+/+}$ male mice: n=2, Student's *t*-test, *p<0.05, #p>0.05).

4.7 Characterization of the functional role of Trpm8 in thermoregulation by c-Fos immunostaining

As a cold-sensitive cation channel, Trpm8 is regarded to be the principal cold transducer in the peripheral nervous system. Mice lacking Trpm8 show deficiency in detecting mild cold (Bautista et al., 2007). It suggests the importance of Trpm8 for cold sensation in the primary somatosensory neurons, which provides peripheral cold temperature input to the POA for thermoregulatory responses (Almeida et al., 2012). Consequently, it leads us to evaluate the functional role of Trpm8 in thermoregulation.

For this purpose, we first made use of c-Fos immunostaining to verify the POA and LPB as the brain regions containing neurons activated by ambient temperature stimuli, which suggests these two regions are involved in thermoregulation. Second, we challenged Trpm8$^{+/+}$ and Trpm8$^{-/-}$ mice with a cold stimulus (7°C) and performed c-Fos immunostaining on brain sections containing the POA and LPB areas. By quantifying the c-Fos positive cells in these two regions, we aimed to evaluate the involvement of Trpm8 in the central thermoregulatory response.

4.7.1 Neurons in the POA and LPB regions were activated by temperature stimuli.

To verify that the POA and LPB are the brain regions involved in thermoregulation, we carried out c-Fos immunostaining on brain slices to mark the activated cells during temperature challenges *in vivo*.

As a marker gene of cell activation, c-Fos immunostaining is widely applied to detect activated neurons upon a certain stimulus (Morgan and Curran, 1986; Sagar et al., 1988). Here, we stimulated two C57Bl/6N wildtype mice with temperature stimuli by exposing them to either 37°C (heat) or 7°C (cold) ambient temperatures for 2h. The third mouse was kept at its original basal ambient temperature 22°C as a control. The c-Fos immunostaining procedure was performed on PFA-fixed brain sections containing POA and LPB areas. The results of the c-Fos immunostaining showed that c-Fos was expressed in the POA sections to different extents in all the mice in this experiment (Fig.7-1). As c-Fos is a transcription factor, its antibody labeled the nuclei of the cells in the brain sections shown as black dots (Fig.7-1,7-2). The number of c-Fos positive cells from the mouse kept at 22°C (Fig.7-1A) was very low. However, many more c-Fos positive cells were detected in the POA sections of the brains from both heat- and cold-treated mice (Fig.7-1B,C). The c-Fos positive cells mainly distributed in the dorsomedial and ventrolateral POA with a scattered pattern.

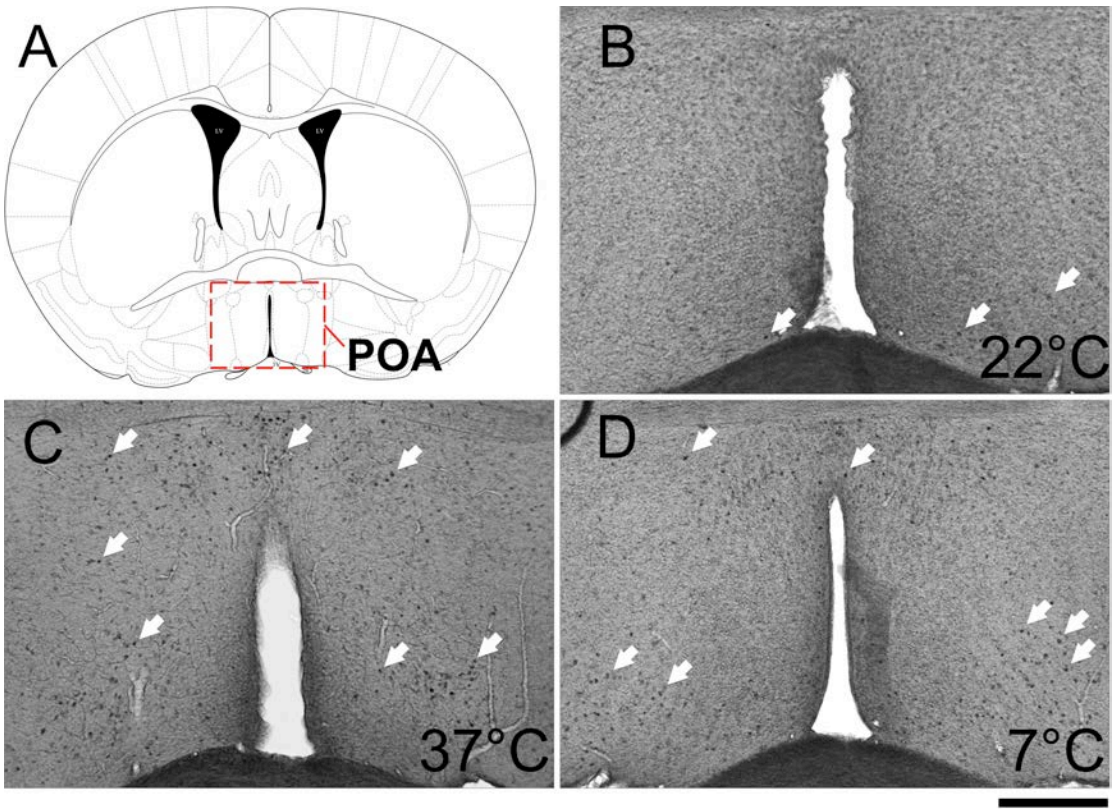


Fig.7-1 Identification of cells in the POA activated during ambient temperature stimuli
Cells activated by ambient temperature changes were identified by c-Fos immunostaining in the POA. (A) Diagram showing the location of the POA of the hypothalamus in a coronal section of the mouse brain (bregma: +0.14mm, modified from Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates, 3rd ed). Mice were stimulated by different ambient temperatures: (B) 22°C; (C) 37°C; and (D) 7°C. c-Fos positive cells are indicated by white arrows. Scale bar: 1mm.

Moreover, c-Fos positive cells were also found in the LPB area of the brain stem, which has been suggested to serve as relay station for peripheral temperature information destined for the POA region, as a part of the afferent circuit of thermoregulation (Nakamura and Morrison, 2007; 2010). Almost none of the c-Fos immunoactivity was detected in the control mouse kept at 22°C (Fig.7-2B). However, a lot more c-Fos positive cells are detected in the dorsal part of LPB (LPBd) in the mouse at 37°C (Fig.7-2C). In comparison, cold responsive cells were labeled by c-Fos antibody in the external lateral part of LPB (LPBel) from the 7°C-treated mouse (Fig.7-2D).

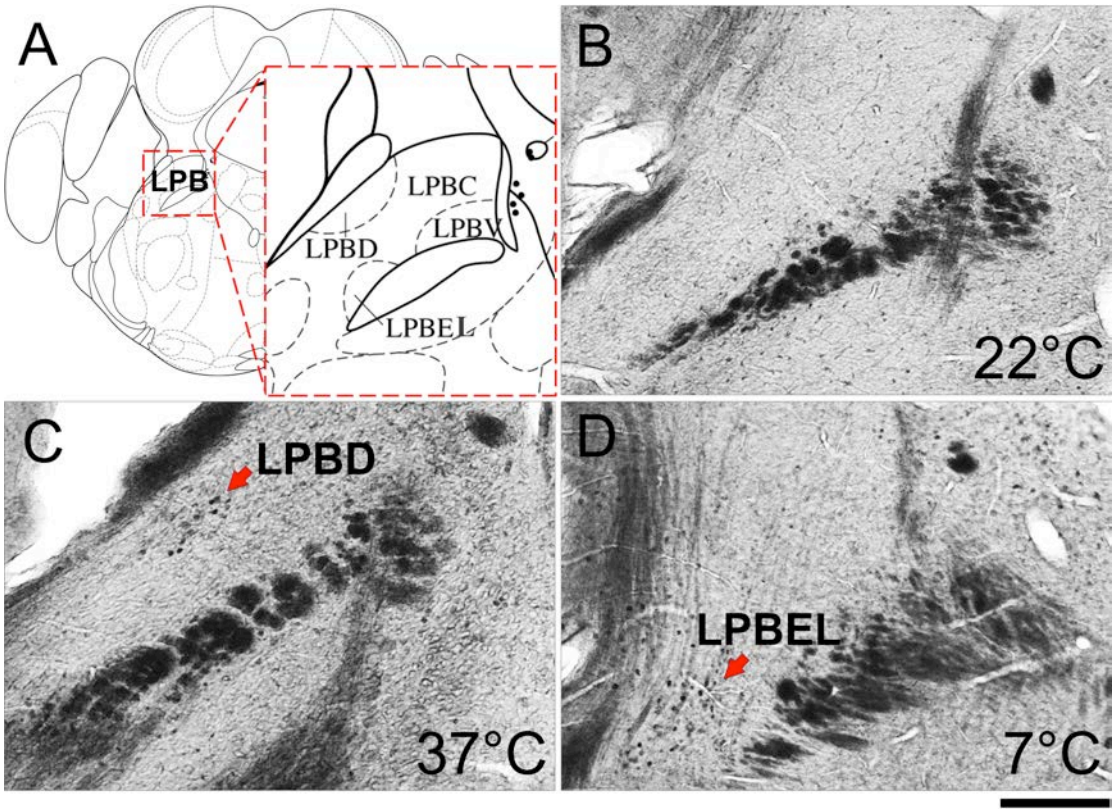


Fig.7-2 Identification of cells in the LPB activated during ambient temperature stimuli
Cells activated by ambient temperature changes were identified by c-Fos immunostaining in the LPB. (A) Diagram showing the location of the LPB in a coronal section of the mouse brain (bregma: -5.20mm, modified from Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates, 3rd ed). The enlarged image depicts the sub-regions of LPB shown in the red frame. Mice were stimulated by different ambient temperatures: (B) 22°C; (C) 37°C; and (D) 7°C. c-Fos positive cell populations are indicated by red arrows. LPBC: central LPB, LPBD: dorsal LPB, LPBEL: external lateral LPB, LPBV: ventral LPB. Scale bar: 200μm.

To sum up, the induction of c-Fos expression in the brain sections of both cold and warm stimulated mice clearly suggested that some neurons in the POA and LPB regions were involved in central thermoregulation during ambient temperature stimuli.

4.8.2 Comparison of the c-Fos expression in the POA and LPB between Trpm8$^{+/+}$ and Trpm8$^{-/-}$ mice.

To evaluate the possible functional role of Trpm8 in thermoregulation, we compared the neuronal activation in the POA and LPB areas of Trpm8$^{+/+}$ and Trpm8$^{-/-}$ mice by the quantification of c-Fos positive cells in these two regions induced by 7°C cold stimulus.

Results clearly showed that there were many more c-Fos positive cells in the POA and LPB regions after the 7°C stimulus when compared to the condition at 22°C for both Trpm8$^{+/+}$ and Trpm8$^{-/-}$ mice (Fig.7-3A,C). This indicated that the thermoregulatory pathway was activated in these mice by cold treatment and evoked neuronal activation in these areas as published previously (Nakamura and Morrison, 2007). To compare the two genotypes, the number of c-Fos positive cells counted after the cold stimulus was normalized to the corresponding 22°C basal counts (Fig. 7-3B,D). There was no statistical difference in the number of c-Fos positive cells between Trpm8$^{+/+}$ and Trpm8$^{-/-}$ mice after the 7°C stimulus, suggesting that Trpm8 does not play a crucial role in the signaling of cold ambient temperature challenges. However, there seemed to be a tendency of fewer c-Fos positive cells in the LPB for Trpm8 knockout mice.

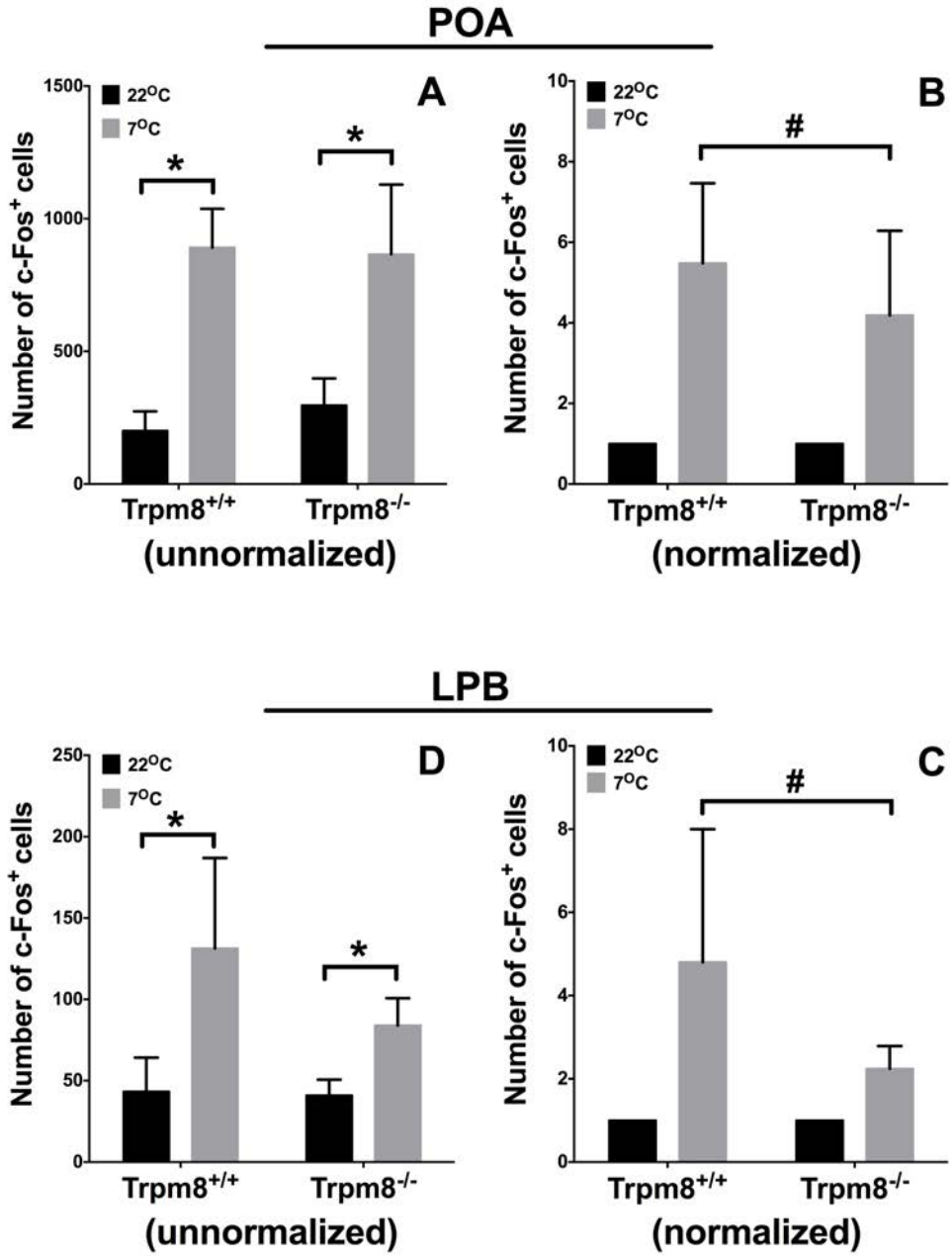


Fig.7-3 Quantification of c-Fos positive cells in the POA and LPB from Trpm8$^{+/+}$ and Trpm8$^{-/-}$ mice after a cold stimulus
c-Fos positive cell number counted in the POA (A) and LPB (C); c-Fos positive cell number in the POA (B) and LPB (D) normalized to the corresponding basal counts. Student's *t*-test, *p<0.05, #p>0.05, n=4.

5. Discussion

Persevering endeavors have been undertaken in an effort to elucidate the mechanisms underlying the temperature sensitivity of the preoptic neurons since their discovery in the POA in 1963 (Nakayama et al., 1963). Although the molecular mechanisms have been elusive until now, increasing evidence shows that temperature-sensitive preoptic neurons play a pivotal role in deep brain temperature detection and thermoregulation (Boulant, 2010; Nakamura, 2011). To contribute to these efforts, the present work employed screening strategies based on Ca^{2+} imaging assay and identified a heat-sensitive channel, Trpm2, responsible for the intrinsic thermosensitivity of preoptic neurons. Trpm2 knockout mice were studied to validate its specific role in modulating the heat response of preoptic neurons *in vitro* as well as its functional contribution to the fever response *in vivo*.

5.1 Identification of thermosensitive preoptic neurons *in vitro*

Given that only a small subpopulation of neurons in the POA is temperature-sensitive, we established a Ca^{2+} imaging-based assay to detect them in sufficient numbers to gain statistical confidence. In contrast to single-cell or single-unit recordings that have been extensively used in preoptic neurons, our Ca^{2+} imaging approach enabled us to study hundreds of neurons simultaneously.

A new criterion based on Ca^{2+} imaging data was developed to define temperature-sensitivity in preoptic neurons. According to previous studies, the majority of preoptic neurons are temperature-insensitive. Thus, we analyzed $[Ca^{2+}]_i$ responses of all isolated neurons to obtain their mean responses and standard deviations. Under the assumption that these values, to a large extent,

represented the temperature-insensitive neurons due to their much greater number (even though the inclusion of temperature-sensitive neurons leads to a slight overestimation), we made use of the “three standard deviations rule” to identify the temperature-sensitive subpopulation. Using this definition, almost all responses of the cells (99.73%) lie within three standard deviations of the mean. If the $[Ca^{2+}]_i$ response of a cell exceeded the value of the mean plus three standard deviations, it was regarded as temperature-sensitive. Using this strategy, warm- and cold-sensitive preoptic neurons were robustly identified in our experiments (Fig.1-1C,D).

In accordance with the previous reports, most preoptic neurons (78%) were determined to be temperature-insensitive. However, fewer warm-sensitive neurons (12%) were identified in our experiments when compared to previous studies (30%) (Boulant and Dean, 1986). The discrepancy could arise from the following three possibilities:

(1) The overestimation of the mean and standard deviation values made our criterion of warm-sensitivity more stringent. Therefore, more neurons were assigned to the temperature-insensitive population.

(2) Preoptic neurons in our experiments and others’ were studied in different contexts. Warm-sensitive neurons were identified by electrophysiology in anaesthetized animals or on brain slices in other studies (Boulant and Hardy, 1974; Kelso and Boulant, 1982; Dean and Boulant, 1989a; Griffin and Boulant, 1995; Griffin et al., 2001; Boulant, 2010). Heat-induced increases of firing rates in some warm-sensitive neurons could be driven by synaptic inputs derived from neighboring projecting neurons (Tabarean et al., 2005; Boulant, 2010). Since the neurons used in our experiments were dispersed and no longer part of a synaptic network, this methodological difference could be responsible for the differing observations. In fact, the pharmacological blockade of synaptic transmission resulted in the electrophysiological detection of fewer warm-sensitive neurons (Nelson and Prosser, 1981).

(3) The age of the investigated neurons could alter thermosensitivity. Although previous work has shown that the thermosensitivity of preoptic neurons even at the embryonic stage can, to a large extent, recapitulate results obtained by using adult brain slices (Tabarean et al., 2005), this has not been verified in our Ca^{2+} imaging assay. The primary cultured neurons in this studied were isolated from postnatal mouse pups (P1-P3) while the other studies often obtained brain slices from adult mice.

The percentage of cold-sensitive neurons has been reported to be about 10% (Boulant and Silva, 1989; Dean and Boulant, 1989b). Currently, the main viewpoint argues that cold-sensitive preoptic neurons are not intrinsically temperature-sensitive since their cold sensitivity is lost upon synaptic blockade (Boulant and Gonzalez, 1977; Kelso and Boulant, 1982). However, Abe et al has also found primary cold-sensitive preoptic neurons by using Ca^{2+} imaging on dispersed preoptic neurons (Abe et al., 2003). In accordance with their result, we found that about 15% of preoptic neurons were activated by a cold stimulus, providing evidence for the existence of intrinsically cold-sensitive neurons in the POA. Boulant has speculated that the sodium-potassium pump (Na^+/K^+ pump) possibly underlies the mechanism of the cold sensitivity since cooling could decrease pump activity, thereby causing a neuronal depolarization and a subsequent increase in firing rate (Boulant, 2010). However, the antagonist of Na^+/K^+ transporter ouabain failed to block the cold response (Curras and Boulant, 1989), indicating that the Na^+/K^+ pump does not account for the cold sensitivity. Therefore, the molecular basis of cold-sensitivity remains unknown. In the future, our assay and approaches described in this thesis that led to the successful identification of Trpm2 might also prove successful in elucidating the molecular basis for cold-sensitivity in preoptic neurons.

5.2 Identification of the heat-sensitive channel based on a screening strategy

To identify the heat-sensitive channel involved in the heat response of warm-sensitive preoptic neurons, we applied a screening strategy based on *in vitro* pharmacological characterization of cells with the readout assay being Ca^{2+} imaging.

To find out the candidate heat-sensitive channel(s), the screening project was started with a pharmacological profiling of primary warm-sensitive preoptic neurons in culture, which is a direct investigation of the relevant cells since the channel is expressed endogenously. The result of blocking effect of 2-APB on the heat response dramatically reduces the number of candidate channels in this study. However, it is quite difficult to further identify the specific channel contributing to the heat-induced Ca^{2+} response among the candidates since validation experiments for candidate channels in primary neuronal cultures are technically challenging because an efficient transfection of neurons with siRNA for gene knockdown experiments is often very difficult. A low efficiency of transfection is especially detrimental in this study because of the small number of warm-sensitive neurons in the preoptic neuronal cultures.

Therefore, we searched for an excitable cell line that could recapitulate the warm sensitivity of the preoptic neurons and attempted to reveal the molecules involved in the heat response of the cell line. Under the assumption that the underlying molecular mechanisms of warm sensitivity were identical in primary neurons and the cell line, it would allow for a “high throughput” siRNA-based screening not possible in the primary neuronal cultures. This is due to the fact that cell lines are homogeneous, easily cultivated, and more easily transfected. Using a very similar strategy, the Patapoutian lab successfully identified the Piezo proteins that are essential for mechanotransduction in the somatosensory system by

investigating the N2A cell line (Coste et al., 2010). The challenge of this strategy lies in its assumption to find a warm-sensitive cell line containing the same molecular repertoire responsible for heat sensitivity as employed in the preoptic neurons. Luckily, we found a rat insulinoma β-cell line and identified the same heat-sensitive channel, namely Trpm2, which was also putatively identified in the pharmacological screen of the primary neurons. The most critical step in the screening approach was the discovery that 2-APB could block the heat-induced Ca^{2+} influx in preoptic neurons, which narrowed down the list of candidate genes to be screened by siRNA or transcriptomic analaysis.

5.2.1 Identification of the heat-sensitive channel Trpm2 in the warm-sensitive preoptic neurons

Our Ca^{2+} imaging results clearly showed that Trpm2 was involved in the heat response of the warm-sensitivie preoptic neurons by studying the proptic neurons from Trpm2 wildtype and knockout mice (Fig.4-2). The expression of Trpm2 in the POA *in vivo* was further confirmed by *in situ* hybridization with Trpm2 RNA probes (Fig.5-1).

Pharmacological studies on warm-sensitive preoptic neurons suggested that the Trpm2-dependent heat response was also influenced by other channels (Fig.2-7). The Ca^{2+} signal was attenuated by at least 50% after application of TTX in some warm-sensitive neurons (Fig.2-7C,J), indicating the involvement of TTX-sensitive voltage-dependent Na^{+} channels. This result also indicated that there are at least two populations of Trpm2-dependent warm-sensitive neurons, one being TTX-sensitive and the other not. Moreover, the sustained $[Ca^{2+}]_i$ increase was also delayed as indicated by the gentler slope of the post-TTX application peak in comparison to those prior to TTX addition (Fig.2-7C). Since the activation of voltage-dependent Na^{+} channels usually results in membrane potential depolarization, it is probable that the resting membrane potential is important for shaping the kinetics and magnitude of the heat-induced $[Ca^{2+}]_i$ response. In line

with this argument, a previous study has shown that temperature sensitivity of thermoTRP channels can be mediated by transmembrane voltage (Voets et al., 2004).

Temperature changes cause graded shifts of the voltage dependence of channel activation, indicating the important role of membrane potential in thermoTRP channel activation. Although a comparable study specifically investigating the Trpm2 activation by heat has not been conducted so far, it is possible that the same mechanism could be at work here. This would mean that the higher the resting membrane potential is at basal temperatures, the more sensitive the neurons are to heat stimuli.

Consequently, the TTX-sensitive voltage-dependent Na^+ channels could serve as amplifiers to boost the Trpm2-conducted heat response by depolarizing the neurons synergistically in concert with Trpm2-dependent Ca^{2+} influx during a heat stimulus. Notably, there was also TTX-insensitive heat response in some warm-sensitive preoptic neurons with slower $[Ca^{2+}]_i$ increase upon heat exposure (Fig.2-7B) in comparison to the TTX-sensitive one (Fig.2-7C, 1st and 3rd peaks). The insensitive effect on TTX could result from the lack of TTX-sensitive voltage-dependent Na^+ channel expression.

Nevertheless, this study did not exclude the expression and involvement of the TTX-insensitive Na^+ channels, such as Nav1.5, Nav1.8 and Nav1.9. Kiyohara et al detected a similar phenomenon when characterizing warm-sensitive preoptic neurons by electrophysiological means (Kiyohara et al, 1990). Their data suggested that neurons with a high warm sensitivity (Q_{10}=4.3-7.0) could be reversibly blocked by TTX. On the contrary, neurons with a low warm sensitivity (Q_{10}=2) were resistant to TTX. In accordance with their observations, we found two subsets of warm-sensitive neurons that responsed to TTX differently (Fig.2-7B,C), indicating an active role of voltage-dependent sodium channels in modulating at least one subpopulation of warm sensitive preoptic neurons as well

as the fact that there are at least two functionally different warm-sensitive populations (TTX-sensitive and insensitive).

Furthermore, L-type VGCCs seemed to augment the heat response as indicated by the partial blocking effect of nifedipine (Fig.2-7D). Former studies show that as the main route for Ca^{2+} influx in cardiomyocytes (Bodi et al, 2005), L-type VGCCs conduct Ca^{2+} current during the action potential dependent depolarization and thereby trigger a series of events resulting in contraction (Bers, 2008). This might describe a general mechanism in which L-type VGCC activation is initiated during cellular depolarization. Since heat-evoked activation of Trpm2 depolarizes warm-sensitive preoptic neurons, we hypothesize that once the depolarized membrane potential reaches the activation threshold of L-type VGCCs, they could be activated to amplify the Ca^{2+} influx. Given that Ca^{2+} is an essential secondary messenger (Clapham, 2007), the enhancement of heat-induced Ca^{2+} entry by L-type VGCCs could potentially affect the physiological response of warm-sensitive preoptic neurons by activating the relevant downstream Ca^{2+}-dependent intracellular pathways. However, the validation of this hypothesis requires further investigation.

Interestingly, blocking voltage-gated K^+ channels with TEA increased the heat-induced Ca^{2+} entry in warm-sensitive preoptic neurons (Fig.2-7E). The increased Ca^{2+} influx could result from the following two possible mechanisms:
(1) Inactivation of potassium channels causes neuronal depolarization, which might facilitate the activation of the cation channels in response to the heat stimulus.
(2) On the other hand, blocking voltage-dependent K^+ channels with TEA could delay the hyperpolarization of neurons. Thus, the activation of cation channels could be prolonged in response to the heat stimulus.
It is also of interest to note that TEA application turned some temperature-insensitive neurons into warm-sensitive ones (Fig.2-7F), indicating that the

resting potential plays an important role in defining the heat sensitivity of preoptic neurons.

However, since TEA treatment alone could induce $[Ca^{2+}]_i$ signal of some preoptic neurons without a heat stimulus (data not shown), it is possible that the effect of TEA on temperature-insensitive neurons was not necessarily dependent on the expression of Trpm2. To test this hypothesis, we performed the experiment on $Trpm2^{-/-}$ preoptic neurons. In accordance with previous results indicating a crucial role of Trpm2 in temperature sensing we were unable to detect warm-sensitive neurons during the heat stimulus in $Trpm2^{-/-}$ cells (Fig.4-2). However, a few neurons "became" warm sensitive upon TEA treatment (data not shown), suggesting the involvement of other channel(s) and mechanisms in the TEA-dependent warm sensitivity of preoptic neurons.

The pharmacological study on preoptic neurons provided evidence for the dependence of warm sensitivity on Trpm2. The TEA experiments, however, raised the possibility that there are alternative mechanisms that could also contribute to warm sensitivity of preoptic neurons, which is independent of Trpm2 activation. This is in line with the observation that while Trpm2 is involved in the fever response, $Trpm2^{-/-}$ mice did not display any other obvious thermoregulatory phenotype.

5.2.2 Identification of the heat-sensitive channel in a warm-sensitive cell line

As one of the important steps in the screening study, Trpm2 was identified to be crucially involved in the heat-induced $[Ca^{2+}]_i$ elevation in the rat insulinoma β-cell line by means of pharmacological and siRNA knockdown experiments followed by a functional Ca^{2+} imaging assay. Nonetheless, according to the data of the gene knockdown experiments we cannot exclude the participation of other ion channels in the heat response that might modulate the Trpm2-dependent temperature sensing. For instance, Orai1 and Orai3 channels could suppress the

heat response observed in the insulinoma β-cells since their knockdown augmented $[Ca^{2+}]_i$ increases in response to the heat stimulus (Fig.3-4A,C). As Ca^{2+} selective channels, Orai proteins have been shown to play a critical role in modulating Ca^{2+} signaling in a variety of physiological and immunological conditions by tethering the intracellular Ca^{2+} sensor Stims to the endoplasmic reticulum (ER) (Feske et al., 2012). However, there is no evidence at this time suggesting that the expression of Orais can suppress Ca^{2+} entry into the insulinoma β-cells after heat stimuli. It would be interesting to elucidate their mechanism of action in temperature sensation in the future.

The partial blocking effect of nifedipine (Fig.3-2G) furthermore indicates the involvement of L-type VGCCs. The possible mechanism in the insulinoma β-cells is likely similar to the one hypothesized for preoptic neurons as described above (section 5.2.1), in that the activation of VGCCs might amplify the heat-evoked Ca^{2+} entry.

The blocking effect of NFA on the heat response (Fig.3-2L) is more likely due to the inhibition of Trpm2 and not Ano1 or some other Ca^{2+}-activated Cl^- channel; however, we cannot completely exclude the latter possibility due to the broad spectrum of channels being inhibited by NFA. Nevertheless, given the complete inhibition of the heat-stimulus response after Trpm2 knockdown (Fig.3-4D), it is reasonable to conclude that Trpm2 plays the most important role in the temperature sensitivity of the insulinoma β-cells.

5.2.3 Ionic models of warm-sensitive preoptic neurons

The mechanism of preoptic thermosensitivity remains controversial due to inconsistent results obtained under differing experimental conditions. There have been two ionic models proposed for elucidating the temperature sensitivity of warm-sensitive preoptic neurons (Kobayashi, 2005).

On the one hand, Boulant has suggested that a transient, outward hyperpolarizing K^+ current, like an A-type potassium current (I_A), underlies the heat-induced increase of firing rate in warm-sensitive neurons. Heating increases the rate of I_A inactivation (Pahapill and Schlichter, 1990), which in turn elicits the prepotential to depolarize at a faster rate. This would result in a shortening of the interval of action potentials of preoptic neurons (Griffin et al., 1996), or more simply stated, an increased firing rate. On the other hand, Kobayashi has proposed that preoptic warm-sensitivity is due to heat-activated, inward cation currents that evoke a slow depolarization of the resting membrane potential (Kobayashi 2005). Trpm2 is a heat-sensitive nonselective cation channel whose activation causes a membrane depolarization. Consequently, our finding fits into Kobayashi's model. However, it is too early to rule out any other possibilities.

First of all, the preoptic neurons in our dispersed culture almost never spontaneously fire found by my colleague M. Moroni by means of electrophysiology, thereby from the outset excluding the possibility of studying the model suggested by Boulant. The excitability of preoptic neurons could be different in brain slices and the dissociated condition at any rate, and both conditions could be different from the true *in vivo* situation. It would be of great interest, therefore, to electrophysiologically characterize the warm sensitivity of Trpm2-expressing preoptic neurons in brain slices where spontaneous neuronal firing is retained.

Second, the two models are not mutually exclusive and might well be at play in parallel. The TTX experiments have shown that there are at least two distinct populations identifiable in Ca^{2+} imaging experiments of dispersed preoptic neuron cultures, and it is, of course, possible that there are others that work by a mechanism completely independent of Trpm2 as discussed on TEA experiments (section 5.2.1).

5.2.4 Functional correlation of subsets of warm-sensitive neurons to thermoregulatory mechanisms

Warm-sensitive preoptic neurons are not homogenous as indicated by our pharmacological results as well as numerous studies (Boulant, 2000; 2010; Eberwine and Bartfai, 2010), so that the underlying mechanisms of central thermosensation and thermoregulation are likely numerous. This heterogeneity might provide the cellular basis for the thermoregulatory diversity that allows homeothermic animals to maintain their core body temperature in a variety of environmental conditions using different pathways.

In Boulant's modified model, he categorized warm-sensitive neurons into three subsets according to their firing rates and correlated these subsets with distinct thermoregulatory functions (Boulant, 2010). In brief, the warm-sensitive neurons with the lowest firing rate (<5 impulses/s) are thermosensitive in the hyperthermic range. Therefore, their thermoregulatory role is most likely the control of heat loss responses (for example, sweating and panting) that are evoked only when the hypothalamus is warmed above a threshold. Moreover, the warm-sensitive neurons with the highest spontaneous firing rates (15-60 impulses/s) are supposed to control heat gain responses (e.g. shivering and nonshivering thermogenesis) necessary in the hypothermic range. His reasoning is that it is more likely for neurons with a high firing rate to show their greatest thermosensitivity during hypothalamic cooling when their firing rate decreases. In addition, the medium-firing warm-sensitive neurons (5-15 impulses/s) were proposed to control behavioral thermoregulation and skin blood flow (a response that is often influenced by emotion) since much of their excitatory input comes from the hippocampus (Boulant and Gonzalez, 1977), a brain region linked to emotion, behavior and memory.

Although Boulant's hypothesis requires substantiation by further investigations, he proposes an intuitive and interesting model to tie distinct thermoregulatory functions to warm-sensitive preoptic neurons with diverse thermosensitivities. Along the same line of argument, the variety of different sensitivities to the heat stimuli of distinct preoptic neurons identified in our study could be explained by the differential expression of ion channels including Trpm2 and votage-gated $Na^+/K^+/Ca^{2+}$ channels (Fig.2-7B-G). This "specific heterogeneity" could correspond to distinct thermoregulatory functions as proposed by Boulant.

5.3 Trpm2 expression in the POA

In this study, the expression of Trpm2 in mouse POA was shown in two different ways. On the one hand, Ca^{2+} imaging results (Fig.4-2) clearly showed robust heat responses in the Trpm2 wildtype preoptic neurons that were absent in those isolated from Trpm2 knockout mice, indicating the functional expression of Trpm2. On the other hand, the expression pattern of Trpm2 in the POA was revealed by *in situ* hybridization (Fig.5-1A,C,D), indicating that Trpm2 was highly expressed in the vlPOA. Moreover, the double-fluorescent *in situ* hybridization result showed that the Trpm2 was co-expressed with GAD1 in some vlPOA neurons (Fig.5-1B,E,F). This is in line with some of the Trpm2-expressing neurons being GABAergic, in agreement with the observations of warm-sensitive preoptic neurons being mainly GABAergic (Tabarean et al., 2005; Morrison et al., 2008).

The vlPOA is a small brain region known to be involved in regulating sleep and wakefulness (Sherin et al., 1996). Some neurons located in the vlPOA are more active during sleep when they secrete inhibitory neurotransmitters like galanin and GABA (Gallopin et al., 2000; Gaus et al., 2002) to inhibit neurons involved in wakefulness and arousal (Saper et al., 2005b; Saito et al., 2013). The presence of the Trpm2 channel in this area suggests the possibility of its involvement in

mediating sleep and arousal, a notion that might stimulate further investigation in the future.

5.4 Functional role of Trpm2 at the cellular and behavioral levels

So far, there have been no reports on a phenotype of Trpm2 knockout mice regarding core body temperature regulation. Uchida et al have showed that the basal core body temperature is the same in $Trpm2^{+/+}$ and $Trpm2^{-/-}$ mice, even though the Trpm2-deficient mice have an abnormal glucose metabolism (Uchida et al., 2010). The lack of relevant studies could be due to the following two reasons:

(1) The expression of Trpm2 has never been convincingly shown in somatosensory neurons, making it a less likely candidate to act as a thermosensor in the peripheral nervous system and thereby affect body temperature regulation.

(2) Its diverse expression in cells of the immune system has stimulated research interests in the direction of various immunological responses.

However, our cellular results as well as others' studies provide us promising indications to check whether Trpm2 may mediate thermoregulation during inflammatory responses, such as fever. First, the functional expression of Trpm2 in the warm-sensitive preoptic neurons suggests that Trpm2, to a large extent, contributes to the temperature-sensitivity of these neurons at least at the cellular level. Given the pivotal role of warm-sensitive preoptic neurons in thermoregulation, Trpm2 expressed in these neurons may also be functionally important for core body temperature regulation at the behavioral level. Second, the wide expression of Trpm2 in immunocytes has already been proven to account for different phenotypes during certain inflammatory responses (Sumoza-Toledo and Penner, 2011), indicating it is functionally active during inflammation. Third, the oxidative stress commonly introduced during

inflammatory responses has been shown to be able to sensitize the activation of Trpm2 by reducing its temperature threshold (Kashio et al., 2012), which provides a potential mechanism for Trpm2 activation in the processes of inflammatory responses, especially for fever. The upregulated oxidative condition and an increased body temperature during fever may synergically sensitize the activation of Trpm2 *in vivo*. The downstream signaling pathways of Trpm2-conducted cation influx may affect the regulation of core body temperature, resulting in more or less fever responses.

Based on the reasons mentioned above, we characterized the functional role of Trpm2 in the context of fever responses at both cellular and behavioral levels. Several important pyrogens were applied to the preoptic neurons individually in order to test their role in mediating the heat response by Ca^{2+} imaging. The potential function of Trpm2 in fever responses was characterized with a telemetry system by using Trpm2 knockout mice. In the cellular assay, a small inhibitory effect of TNFα on heat-induced Ca^{2+} increase was found in the warm-sensitive preoptic neurons. Importantly, we have shown in the behavioral study that in the absence of Trpm2, mice developed a less pronounced fever in response to LPS than their wildtype littermates.

5.4.1 Effects of pyrogens on warm-sensitive preoptic neurons

To investigate the possible involvement of Trpm2 in the preoptic neurons during fever, we applied H_2O_2 and pyrogens to simulate the fever condition in warm-sensitive neruons *in vitro*. Ca^{2+} imaging results showed that the warm sensitivity was unaltered upon stimulation with different pyrogens (Fig.2-2,3,5) except for TNFα (Fig.2-4). It was not a surprise that LPS did not directly alter the warm sensitivity of preoptic neurons since it is unable to efficiently cross the blood-brain-barrier and therefore never reached these neurons in the *in vivo* condition (Banks and Robinson, 2010).

TNFα was found to slightly inhibit the heat-induced Ca^{2+} increase in this study (Fig.2-4), indicating that it reduces the warm sensitivity of the warm-sensitive preoptic neurons. This is consistent with the conclusion of a former report that the application of TNFα decreased the firing rate of some warm-sensitive preoptic neurons (Shibata and Blatteis, 1991). Given that warm-sensitive neurons in the POA are mainly GABAergic, the inhibitory effect on warm sensitivity of these neurons may result in less tonic inhibition on the heat-gain thermoregulatory pathways, which in turn causes more heat generation or less heat dissipation. Consequently, the inhibitory effect of TNFα may induce an elevation of core body temperature during fever responses. To verify this speculation, a behavioral study is necessary to be preformed by comparing the core body temperature changes of Trpm2 wildtype and knockout mice during the fever response induced by locally preoptic injection of TNFα.

However, other pyrogens investigated here, such as IL-1β and PGE_2, did not show any effect on the heat response of preoptic neurons, although previous studies have indicated that these pyrogens can decrease the firing rate of warm-sensitive preoptic neurons in brain slices (Shibata and Blatteis, 1991; Xin and Blatteis, 1992; Ranels and Griffin, 2003; Tabarean et al., 2004), which is thought to cause the upregulation of heat-gain responses during fever as what TNFα does. The discrepancy between our results and those of others may be due to the following two reasons:

(1) The potentially subtle effects of the two pyrogens (IL-1β and PGE_2) on preoptic neurons may be difficult to detect by Ca^{2+} imaging because of its limited dynamic range of measuring $[Ca^{2+}]_i$ changes. Electrophysiological recordings in brain slices might simply be more sensitive. However, we were able to detect a strong sensitization effect of H_2O_2 (Fig.4-1) and a small inhibitory effect of TNFα (Fig.2-4) on some warm-sensitive neurons, indicating that the relevant biological effects can in principle be detected with our set-up.

(2) The lack of synaptic input in our dissociated cultures could render the neurons insensitive to some of the pyrogens studied. Tabarean et al suggest that PGE_2

decreases the firing rate of some warm-sensitive GABAergic neurons by affecting voltage-gated K^+ channels (Tabarean et al., 2004). This suggests that pyrogens could affect the warm-sensitivity of some preoptic neurons through altering the activities of ion channels other than the heat-sensitive channel itself. In experimental conditions in which synaptic inputs are conserved, more ion channels that are the true targets of pyrogens might contribute to the response to heat stimuli. Thus, the potential “indirect” effect of pyrogens is likely masked in our experimental conditions.

Furthermore, this could explain why the temperature activation threshold of warm-sensitive neurons in our study was determined to be between 44.5°C and 45.6°C, which is much higher than the basal and febrile temperatures *in vivo*. We propose the hypothesis that the threshold would be lower if synaptic inputs would have remained intact.

Taken together, to further study the potential role of Trpm2 in warm-sensitive preoptic neurons, the neurons should be characterized by a more sensitive approach at a more physiological condition. For example, brain slice electrophysiological recordings would be an informative approach to include in future experiments.

5.4.2 Functional role of Trpm2 in the fever response

The telemetry results showed that Trpm2 is functionally involved in the LPS-induced fever response. However, the exact mechanism remains at this point unresolved.

Trpm2 is an oxidant-sensitive Trp channel activated by the generation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) (Di et al., 2011; Kashio et al., 2012; Knowles et al., 2012; Zhong et al., 2013). It has been functionally identified to mediate ROS-induced apoptosis or tissue damage via

conducting Ca^{2+} influx followed by the activation of downstream signalling pathways (Hecquet et al., 2008; Nazıroğlu and Lückhoff, 2008; Yamamoto et al., 2008; Bari et al., 2009). Moreover, intracellular ROS has been indicated to sensitize the activation of Trpm2 by decreasing its temperature threshold to a physiological level (Kashio et al., 2012). Since Trpm2 is expressed in numerous cell types of immune system, the oxidant-dependent sensitization effect on Trpm2 activation indicates its involvement in pathogenic immune responses that need the recruitment of an increased oxidative microenvironment, such as fever. According to pathways previously shown to be involved in the LPS-induced fever response and the expression pattern of Trpm2, the phenotype could result from the lack of Trpm2 in at least two different cell populations:
(1) immune cells in the periphery;
(2) warm-sensitive preoptic neurons.

Recently, a few studies have shown that the release of different cytokines or chemokines are down-regulated in several types of immune cells isolated from Trpm2 knockout mice *in vitro*, which in turn cause decreased inflammatory responses. Since some of these cytokines are also pyrogens (e.g. TNFα and interleukins) (Wehrhahn et al., 2010; Kashio et al., 2012), a similar mechanism could also explain the fever phenotype in our study. The deficiency of Trpm2 in different immunocytes could lead to a decreased production of certain pyrogens, which might result in a milder fever response.

To investigate this hypothesis, a bone marrow transplantation experiment is currently being undertaken. In order to specifically “knockout” Trpm2 in the peripheral immune system, bone marrow ablated $Trpm2^{+/+}$ and $Trpm2^{-/-}$ mice will be transplanted with bone marrow from wildtype mice. Subsequent telemetric recording will clarify whether the phenotype of LPS-induced fever is dependent on immunocyte-specific Trpm2 expression.

Trpm2-expressing warm-sensitive preoptic neurons in the brain could also contribute to the fever phenotype by being involved in modulating core body temperature regulation. One model that has put warm-sensitive preoptic neurons in the context of thermoregulation and fever has been proposed by Shaun Morisson and his colleagues (Fig.8). In this model warm-sensitive neurons in the medial preoptic area (MPO) are GABAergic cells (shown in the square of black desh line in Fig.8) that tonically inhibit their downstream glutamatergic neurons located in the DMH and RPa in order to suppress the heat-gain responses. A warm temperature input excites the warm-sensitive preoptic neurons, which enhances the inhibition of heat generation in the peripheral thermoeffector organs. In contrast, a cold temperture input can inhibits these neurons to relieve the inhibitory effect on the downstream pathways and to induce heat production. During the fever response, the firing rate of the warm-sensitive preoptic neurons can be inhibited by the pyrogen - PGE_2, which subsequently causes the heat gain responses to increase core body temperature. This model proposes a possible mechanism of central thermoregulation in which the excitability of warm-sensitive preoptic neurons, which determines the temperature set-point, plays a fundamental role. The flexibility of the temperature set-point in warm-sensitive neurons modulated by the peripheral temperature inputs and pyrogens is critical for regulating core body temperature, especially during fever when it is upregulated so that thermogenesis can be initiated in thermal effecter organs, such as brown adipose tissues, to elevate core body temperature.

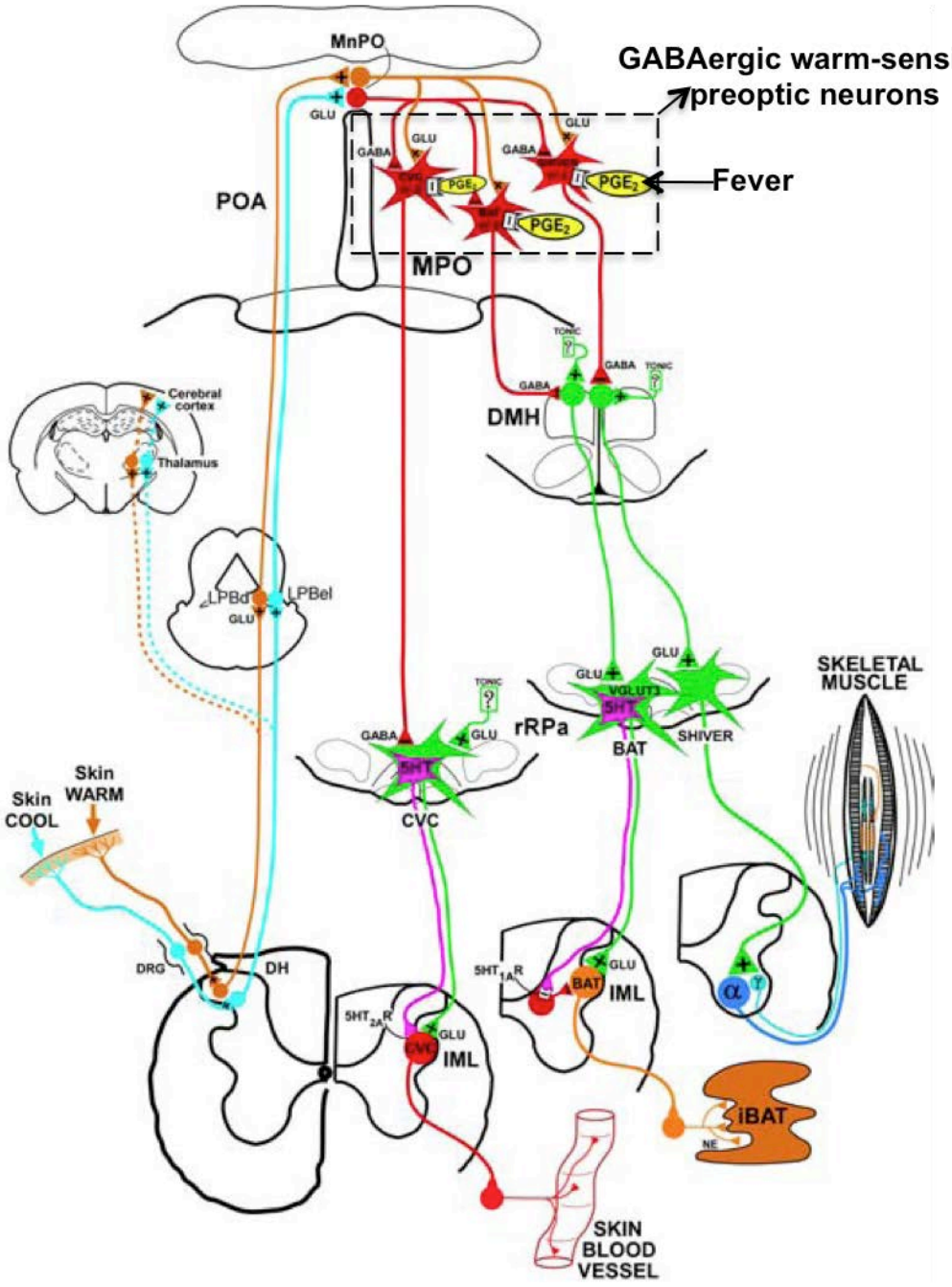


Fig.8 Functional neuroanatomical and neurotransmitter model for the fundamental pathways of thermoregulation

Cool and warm cutaneous thermal sensory receptors transmit signals to respective primary sensory neurons in the DRG that relay this information to second-order thermal sensory neurons in the dorsal horn (DH) of the spinal cord. Cool/warm sensory DH neurons glutamatergically activate third-order sensory neurons in the LPBel/LPBd. Thermosensory signals from DH neurons are also relayed to the thalamus and then to the cortex for conscious thermal perception and localization. Thermosensory signals for thermoregulatory responses are transmitted from the LPB to the POA where GABAergic interneurons in the median preoptic nucleus (MnPO) are activated by

glutamatergic inputs from cool-activated neurons in LPBel and inhibit the distinct populations of warm-sensitive neurons in the medial preoptic area (MPO). In contrast, glutamatergic interneurons in the MnPO, postulated to be excited by glutamatergic inputs from warm-activated neurons in LPBd, excite warm-sensitive neurons in the MPO. Under a fever conditioin, PGE_2 binds to EP3 receptors in warm-sensitive neurons in the MPO to inhibit their activity. Preoptic warm-sensitive neurons provide thermoregulatory control of cutaneous vasoconstriction (CVC), BAT and shivering conducted by the downstream glutamatergic neurons located in the DMH and RPa. (modified from *Front Biosci (2011) 16: 74-104*.)

In the present study, we find that Trpm2 knockout mice have a normal basal core body temperature as the wildtype littermates but a reduced fever response. Thus, according to the above model of themregulation, one could envision that genomic deletion of Trpm2 in the warm-sensitive preoptic neurons does not affect their firing activity at the steady state. However, it may result in the inability to upregulate the temperature set-point in warm-sensitive neurons during fever, eliciting a decreased activation of the heat-generating circuitry and a milder fever. In other words, the inhibitory effect of pyrogens (e.g. PGE_2) on the Trpm2 knockout preoptic neurons is less than the one on the wildtype neurons.

To test this hypothesis, it is necessary to compare the inhibitory effects of PGE_2 on the firing rate of the warm-sensitive preoptic neurons between Trpm2 knockout and wildtype mice. Moreover, in order to investigate the role of preoptic neurons in this phenotype at the behavioral level, a robust and well-establlished model of fever induction in the form of the intracerebroventricular (icv.) injection of PGE_2 will be applied to Trpm2 wildtype and knockout mice. In the future, it might also be possible to further dissect this phenotype by creating tissue-specific Trpm2 knockout mice.

With the aforementioned experiments, we aim to elucidate the mechanisms underlying the fever phenotype in more detail. Of course, it is also possible that both of the suggested possibilities contribute to the fever phenotype.

5.5 Investigation the functional involvement of Trpm8 in thermoregulation

Given the importance of Trpm8 in peripheral cold sensation, we want to investigate whether it may also affect central thermoregulation. For this purpose, we focused on the two thermoregulatory brain regions, the POA and LPB, and were able to identify the neurons there activated during temperature stimuli by c-Fos immunostaining. Then, by using Trpm8 knockout mice, we evaluated the involvement of Trpm8 in core body temperature regulation induced by the acute cold stimulus.

5.5.1 Labeling thermoregulatory neurons by c-Fos immunostaining *in vivo*

To verify previous reports of POA and LPB neurons being part of the thermoregulatory curcuit, we used c-Fos staining, a marker for neuronal activity, in the POA and LPB after cold and warm ambient temperature stimuli. A strong increase in the number of c-Fos positive cells was detected in both regions after either stimulus (Fig.7-1,7-2), suggesting that POA and LPB regions contain neurons that are part of the neuronal circuits that responds to ambient temperature changes. These results verified previous reports that subsets of POA neurons responded to ambient temperature changes *in vivo*, indicating that they are involved in core body temperature regulation (Scammell et al., 1993; Tsay et al., 1999; Bachtell et al., 2003; Plessis et al., 2006).

Additionally, two distinct sub-regions of the small LPB area – LPBd and LPBel showed c-Fos expression corresponding to cold and warm stimuli (Fig.7-2), which is consistent with the results reported by Nakamura et al (Nakamura and Morrison, 2007; 2010). The LPB has been shown to receive the ascending cutaneous temperature signals from lamina I of the spinal cord and to relay them to the POA (Craig, 1995; Al-Khater and Todd, 2009). Distinct groups of LPB

neurons were activated in response to cold and warm stimuli, indicating a temperature-dependent regional specificity of the thermoregulatory afferent circuits.

5.5.2 Evaluation of the involvement of Trpm8 in central thermoregulation by c-Fos immunostaining

In the Trpm8 study in which c-Fos expression profiles were used to investigate the involvement of Trpm8 in the thermoregulatory process of defending core body temperature against a 7°C cold stimulus, no obvious difference in the number of c-Fos positive cells could be determined between $Trpm8^{+/+}$ and $Trpm8^{-/-}$ mice (Fig.7-3) in either the POA or LPB. This indicates a minor role, if any, of Trpm8 in the core body temperature maintenance in response to a cold stimulus.

In contrast to our finding, Almeida et al recently showed telemetrically that the pharmacological blockade of Trpm8 by the application of a newly identified specific Trpm8 antagonist attenuated autonomic and behavioral cold defense responses that led to a decreased core body temperature (Almeida et al., 2012). There are two possible reasons for this difference:

(1) The c-Fos positive cell quantification assay is not an ideal way to pick up small differences due to the large variation of c-Fos positive cells between individual mice. The biological variation of core body temperature recordings by a telemetry system as used in Almeida's study is much smaller. While there was an indication of a trend of fewer c-Fos positive cells in the LPB of Trpm8 knockout mice (Fig.7-3D), the difference was not statistically significant because of the high variation in the dataset.

(2) Genomic deletion of Trpm8 does not cause mice to be completely insensitive to cold (Bautista et al., 2007), indicating that there are compensatory and/or other mechanisms that contribute to cold sensation in mice. In accordance with this observation, we found an increased number of c-Fos positive cells in the POA

and LPB of both Trpm8 wildtype and knockout mice after the cold stimulus, many of which were possibly due to Trpm8-independent cold sensation. If this were the case, the Trpm8-independent thermoregulatory mechanism might have caused a high “background” in our c-Fos assay, masking the Trpm8-specific effects. In comparison, the study of Almeida et al made use of a specific agonist of Trpm8. This enabled them to dissect the Trpm8-specific effects on thermoregulation in a wildtype background, circumventing both the issue of a knockout background in which compensatory mechanisms might have developed and the difficulty of an assay which has both high biological variation and is not entirely Trpm8-specific (Almeida et al., 2012).

6 Conclusions

In this thesis, temperature-sensitive preoptic neurons were identified in primary dispersed cultures, which provided the opportunity to unravel a novel mechanism of temperature-sensitivity in a subpopulation of preoptic neurons. The use of Trpm2 knockout mice in combination with in-depth pharmacological and transcriptomic screening experiments enabled us to identify Trpm2 as the thermosensor responsible for conducting the extracellular Ca^{2+} influx in response to heat stimuli in both primary warm-sensitive preoptic neurons and a second warm-sensitive cell line. A subsequent behavioral study showed that Trpm2 knockout mice developed a less pronounced response in a model of LPS-induced fever, indicating the potential importance of Trpm2 in the thermoregulatory process of systemic fever. The mechanism underlying the fever phenotype in Trpm2 knockout mice needs further investigation to determine the potential involvement of central thermoregulation and peripheral immune responses. Moreover, as a side project, we did not find an apparent involvement of peripheral cold-sensor Trpm8 in centrol thermoregulation by applying c-Fos immunostaining. Taken together, the present work summarizes our efforts and newly provides insights into the molecular basis of thermoregulatory mechanisms in mammals.

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Appendix I. Abbreviations

BAT	Brown adipose tissues
CAP	Capsaicin
COX	Cyclooxygenase
CPA	Cyclopiazonic acid
CRAC	Calcium release-activated channel
CVC	Cutaneous vasoconstriction
Cy2	Cyanine-2
DAPI	4',6-diamidino-2-phenylindole
DH	Dorsal horn
DMH	Dorsomedial hypothalamus
DRG	Dorsal root ganglion
EGTA	Ethylene glycol tetraacetic acid
EP3	Prostaglandin E2 receptor 3
GABA	Gamma-aminobutyric acid
GAD1	Glutamic acid decarboxylase 1
H_2O_2	Hydrogen peroxide
I_A	A-type potassium current
icv.	intracerebroventricular
IL-1β	Interleukin-1β
IL-6	Interleukin-6
ip.	intraperitoneal

iv.	intravenous
LPB	Lateral parabrachial nucleus
LPS	Lipopolysaccharide
MnPO	Median preoptic nucleus
MPO	Medial preoptic area
MS	Medial septal nucleus
NE	Norepinephrine
NMDA	N-Methyl-D-aspartate
NMDG	N-methyl-D-glucamine
PFA	Paraformaldehyde
PGE_2	Prostaglandin E_2
POA	Preoptic area
RIN	RNA integrity number
ROS	Reactive oxygen species
RPa	Raphe pallidus area
RPKM	Reads per kilobase per million reads
RR	Ruthenium red
RT	Room temperature
SEM	Standard error of the mean
SNA	Sympathetic nerve activity
Stim1	Stromal interaction molecule 1
TEA	Tetraethylammonium
TG	Trigeminal ganglion
TLR-4	Toll-like receptor 4
TNFα	Tumor necrosis factor-α

TRP	Transient receptor potential
TTX	Tetrodotoxin
UCP1	Uncoupling protein 1
VGCC	Voltage-gated Ca^{2+} channel

Appendix II. Units

°C	degrees Celsius
bp	base pairs
d	day
g	gram
h	hour
Hz	Hertz
kb	kilobase
kDa	kilodalton
l	liter
M	molar
min	minute
ml	milliliter
mM	millimolar
ng	nanogram
nm	nanometer
rpm	Revolutions per minute
s	second
µg	microgram
µM	micromolar