The Sensory Coding of Warm Perception

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RICARDO PARICIO MONTESINOS

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Neural Circuits and Behavior, Group Leader Prof. Dr. James Poulet

Max Delbruck Center for Molecular Medicine (MDC)

Neuroscience Research Center (NWFZ)

Charite Universitatsmedizin Berlin

Campus Charite Mitte

Chariteplatz 1, 10117 Berlin

Supervisor: Prof. Dr. James Poulet

Second examiner: Prof. Constance Scharff, PhD.

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The Sensory Coding

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SUMMARY

Animals continuously sense the temperature in their environment, which is crucial for survival and for maintaining an optimal energy expenditure. Thermal perception is enabled by sensory afferent neurons that innervate the skin and express molecules that transduce thermal stimuli into electrical signals, which are later processed by the nervous system. In the recent years, studies in genetically modified mice have found sensory afferents and ion channels that transduce cooling in mammals, but the perceptual ability of mice to sense warmth and the underlying encoding mechanisms remain unknown.

In this work, I have investigated the neurobiological mechanisms that underlie the perception of warmth. To do so, I have employed the mouse (*Mus musculus*) as a model system due to both the phylogenetic proximity to humans and the availability of genetic tools for mechanistic studies.

Using a sensory detection behavior, I first show that mice perceive tiny (0.5^{o}C) changes in temperature of the forepaw. Like humans, mice are able to discriminate warming from cooling, they are less sensitive to warmth and the baseline temperature strongly impacts the perceptual saliency. Together, these data indicate that mice and humans share many features of thermal perception, suggesting common sensory coding mechanisms.

Cooling perception in mice is known to require cool-activated sensory afferents that express the channel TRPM8, but the neurons encoding warmth are unknown. Here, warming recruited two polymodal afferent populations: one fired upon warming (warm-activated) and the other was both warm-silenced and cool-activated. To investigate their role in perception, I used gene knockouts and optogenetic afferent stimulation and found that mice sense and encode warming without the warm-activated ion channels TRPV1, TRPM3, TRPA1 and TRPM2. However, surprisingly, despite TRPM8+ afferent stimulation evoked a cooling percept, TRPM8-null mice cannot detect warming. *Trpm8*$^{-/-}$ mice possess warm-activated afferents but lack warm-silenced neurons, suggesting that cooling input from warm-inhibited fibers is required for warmth perception.

In preliminary work I have also investigated the role of primary somatosensory cortex in warm perception. Using intrinsic optical imaging I observed that cooling and touch, but not warming, elicits robust responses; but silencing of this brain region impaired the perception of warming stimuli.

Altogether, the data from my thesis suggest that warming perception is an integrative process and requires input from both warm- and cool-activated sensory pathways.

ZUSAMMENFASSUNG

Lebewesen sichern ihr Überleben und optimieren ihren Energieaufwand, indem sie fortwährend die Temperatur ihrer Umgebung wahrnehmen. Die Wahrnehmung von Temperatur wird über sensorische afferente Nervenzellen gewährleistet, welche die Haut innervieren und Moleküle exprimieren, die thermale Reize in elektrische Signale umwandeln. Diese werden darauffolgend vom Nervensystem verarbeitet. Aktuelle Studien in genetisch veränderten Mäusen konnten zeigen, dass sensorische Afferenzen und Ionenkanäle die Kältewahrnehmung in Säugetieren bedingen. Im Gegensatz dazu ist bislang nicht bekannt, wie Mäuse Wärme wahrnehmen und welche Kodierungsmechanismen dem zugrundeliegen.

In dieser Arbeit habe ich die neurobiologischen Mechanismen, welche die Wahrnehmung von Wärme bedingen untersucht. Um dies zu adressieren, verwendete ich hier den Modellorganismus Maus (mus musculus) aufgrund der phylogenetischen Nähe zum Menschen und der Verfügbarkeit von genetischen Manipulationen für mechanistische Studien. Zunächst konnte ich mit Hilfe eines sensorischen Verhaltenstest zeigen, dass Mäuse kleinste Temperaturunterschiede (0.5^{o}C) an der Vorderpfote wahrnehmen. Meine Daten zeigen, dass Mäuse, ähnlich den Menschen, zwischen Erwärmung und Abkühlung unterscheiden können, dass sie weniger empfindlich gegenüber Wärme sind und dass die anfängliche Ausgangstemperatur stark die Salienz ihrer Wahrnehmung beeinflusst. Zusammenfassend zeigen diese Daten, dass Mäuse und Menschen ähnliche Charakteristika bezüglich thermaler Wahrnehmung aufweisen, was wiederum nahelegt, dass gemeinsame sensorische Kodierungsmechanismen existieren. Während bekannt ist, dass für die Wahrnehmung von Kälte in Mäusen Kälte-aktivierbare sensorische Afferenzen nötig sind, die den Ionenkanal TRPM8 exprimieren, ist bislang nicht bekannt, wie Nervenzellen Wärme enkodieren. Hier beschreibe ich, dass Wärme zwei polymodale afferente Faser-populationen rekrutiert: Eine der Populationen erzeugte Aktionspotentiale bei Erwärmung (Wärme-aktiviert) wohingegen die andere Population durch Wärme inhibiert und durch Kälte aktiviert wurde.

Um die Rolle dieser beiden Populationen bei der Wahrnehmung zu untersuchen, verwendete ich Knockout-Mäuse und afferente optogenetische Stimulation. Meine Daten zeigen, dass Mäuse auch ohne die Wärme-aktivierten Ionenkanäle TRPV1, TRPM3, TRPA1 und TRPM2 in der Lage sind, Erwärmung wahrnehmen und zu kodieren. Überraschenderweise zeigte sich, dass TRPM-8-Null-Mäuse nicht in der Lage waren, Erwärmung wahrzunehmen, obwohl die Stimulation von TRPM8+ Afferenzen

normalerweise die Wahrnehmung von Kälte hervorruft. Ich konnte zeigen, dass Trpm8 -/- Mäuse zwar Wärme-aktivierte Afferenzen besitzen, dass ihnen aber Wärme-inhibierte Nervenzellen fehlen. Dies lässt darauf schließen, dass durch Kälte induzierter Input von Wärme-inhibierten Fasern benötigt wird, um Wärme wahrnehmen zu können.
Desweiteren habe ich die Rolle des primären somatosensorischen Kortex in der Wärmewahrnehmung untersucht. Mit Hilfe intrinsicher optischer Bildgebung konnte ich beobachten, dass Kälte und Berührung, aber nicht Wärme, robuste Reaktionen hervorrufen. Dennoch beeinträchtigte ein Ausschalten dieser Gehirnregion die Wahrnehmung wärmebezogener Stimuli.

Zusammenfassend legen die Daten meiner Arbeit nahe, dass die Wahrnehmung von Wärme ein integrativer Prozess ist und Input von Wärme- und Kälte aktivierten sensorischen Nervenbahnen benötigt.

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1. INTRODUCTION

Animals sense the temperature in their environment to avoid tissue damage and navigate in locations (and interact with objects) that are most energy-favorable. Also, during haptic exploration, thermal and mechanical information is integrated to build up an accurate somatosensory percept. This is possible because primary sensory afferents are constantly capturing thermal information at the skin, and transmitting it to higher processing areas of the nervous system. In this introduction, I will summarize: i) the known features of human and rodent thermal perception, ii) the identified sensory peripheral neurons that encode thermal information, iii) the protein sensors expressed at these afferents that render them thermosensitive, and iv) the neural circuits of thermal perception within the central nervous system.

1.1 THERMAL PERCEPTION

Temperature is crucial for the survival and development of life. To date, some prokaryotic organisms have been found to survive and grow at temperatures between -20 and +122 degrees Celsius (^{o}C) (Clarke, 2014; Clarke et al., 2013; Hickey & Singer, 2004; McKay, 2014; Mykytczuk et al., 2013; Takai et al., 2008). Mammalian species, however, have relatively strict thermal requirements, since they need to keep their body temperatures around 30-40^{o}C (Clarke & Rothery, 2007).

One way to achieve this thermal homeostasis is to activate internal physiological processes that generate heat. As endothermic animals, mammals are able to regulate their own core temperature internally. To allow for this tight control, it is necessary not only to be equipped with mechanisms to sense internal temperature, but also to trigger physiological processes such as sweating, panting, shivering, cutaneous vasoconstriction or heat generation by the brown adipose tissue (Morrison & Nakamura, 2019). These are unconscious processes, and the major center for internal temperature control that will initiate them is located in the preoptic area of the hypothalamus (Song et al., 2016; Teague & Ranson, 1936).

Internal, physiological thermoregulation is, however, an energy-costly process (Armitage et al., 1984; Clapham, 2012). Thus, temperatures that differ greatly from this range are not only challenging energy-wise but can also be harmful and a threat to survival. Internally adapting to temperatures that differ greatly from the optimal thermal range has limits even in endotherms (Hanna & Tait, 2015).

Thus, another method to keep the body cells at a favorable temperature is to physically navigate the environment in search of optimal thermal conditions. For this purpose, and also to avoid potential thermal threats, animals (both ectotherm and endotherm) have evolved capable of sensing the temperature of their environment, a process called thermosensation or thermal perception. By constantly acquiring thermal information of their surroundings, animals can avoid unfavorable cold or heat and increase their chances of survival (Nagashima et al., 2018; Pline et al., 1988; Touska et al., 2016). Moreover, extreme temperatures can induce quick freezing and burning of tissues, which can be damaging or even lethal (Handford et al., 2017; Jeschke et al., 2020). Therefore, thermal perception of the environment is a crucial sense in animals.

The biological mechanisms underlying thermal perception in mammals are not yet completely understood. However, it is known that thermal information is detected in afferent, sensory neurons at the peripheral nervous system. These innervate the skin where they detect temperature thanks to the expression of thermally-gated ionic channels that can trigger action potentials in the neuron (Caterina et al., 1997). Sensory afferent neurons send the thermal signal to the dorsal laminae of the spinal cord, and it finally reaches encephalic structures such as the thalamus, hypothalamus and brain cortex (Bokiniec et al., 2018).

1.1.1 Human thermal perception

Humans have a high sensitivity to temperature changes in the skin. Generally, we can detect warming and cooling of less than 1^{o}C, but thermal sensitivity depends on body (skin) regions. Moreover, sensitivity to warming and cooling correlate across skin areas (Stevens & Choo, 1998). For instance, the lips, cheeks and forehead are particularly sensitive (warming and cooling threshold both of <0.5^{o}C for 20-year old subjects). On the other hand, thermal sensitivity is lower at the calves and, especially, the toes (warming threshold of >2^{o}C, and cooling >0.5^{o}C, for the same age group) (Stevens & Choo, 1998).

Cooling is therefore perceived more accurately than warming in humans. Consistent with this are reaction times to perceive and report thermal stimuli. It has been shown that humans can report cooling in the hand as fast as ~0.5 seconds while warming takes slightly longer, ~0.7 seconds (Yarnitsky & Ochoa, 1991b).

Moreover, the area of stimulation is an important factor that can determine whether a small-amplitude thermal stimulus will be detected or not. A greater stimulation area allows the detection of smaller thermal stimuli (Filingeri, 2016; Stevens et al., 1974). This

suggests that spatial summation of thermosensitive neurons is important for the perception of subtle warming and cooling. Interestingly, the perception of painful heat has been shown to depend much less on the area of stimulation (Stevens et al., 1974; Yarnitsky & Ochoa, 1991a). This might mean that thermal stimuli are encoded differently depending on whether they are painful or not.

The rate of change of the thermal stimulus also influences thermal perception. For instance, smaller cooling and warming stimuli can be detected when delivered at faster rates of change (Kenshalo et al., 1968).

Interindividual differences in thermal perception are also noteworthy. Interestingly, the thermal perceptual thresholds increase progressively with age (Stevens & Choo, 1998). Moreover, physiological responses to temperature changes have been shown to differ between human populations living in different geographical areas (Daanen & Van Marken Lichtenbelt, 2016). Although climate adaptation may account for these observed differences, genetic selection could also play an important role. For instance, genetic variations of the main cooling protein receptor have been found between populations living in distant geographical locations (Key et al., 2018). Finally, sex-dependent biological differences may also exist in human thermal perception. Females have been reported to be slightly more sensitive to temperature than males, as shown by smaller thermal thresholds (Averbeck et al., 2017). Consistent with this, it has also been shown that males require slightly (~1^{o}C) cooler ambient temperatures than females, in order to initiate shivering. At the same ambient temperature, males report feeling warmer than females (Kaikaew et al., 2018).

Another important aspect of thermal perception is valence. On one hand, humans (and mammals in general) perceive temperatures as painful or non-painful. Pain is an evolutionary signal for danger, and generally alerts of stimuli that are noxious (damaging for the tissue) (Walters & Williams, 2019). Although some variability exists among healthy individuals and different stimulation methods, heat pain perception occurs at temperatures beyond 43^{o}C (Pertovaara et al., 1996; David Yarnitsky & Ochoa, 1990). For cold, temperatures around 10^{o}C can already be classified as painful (Croze & Duclaux, 1978). Therefore, temperatures between ~10-43^{o}C are generally perceived as non-painful by humans. In addition, pain thresholds can be altered by pathological conditions such as inflammation (J. Huang et al., 2006).

On the other hand, humans classify thermal sensations on whether they are evoked by temperatures above or below our own skin temperature. At rest, the human skin perceives temperatures between ~30-34^{o}C as thermoneutral (Filingeri, 2016). Above

those values, non-painful thermal sensations are described as “warm”, whereas temperatures below the thermoneutral range are described as “cool”. However, this spectrum can vary, since long exposure of the skin to slightly lower and higher temperatures results in adaptation and a slight shift of the thermoneutral range (Filingeri et al., 2017).

Therefore, in summary, humans generally perceive temperatures below ~10ºC as cold (which is unpleasant or painful) and temperatures between ~10-32ºC as cool. Values between ~32-43ºC are described as warm, and hot temperatures (which are painful) correspond to values of ~43ºC and beyond (**Figure 1**).

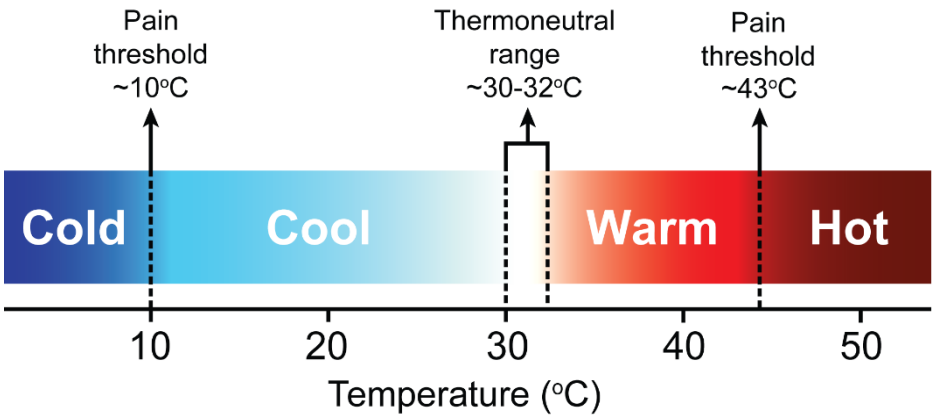

Figure 1. Human perceptual classification of temperatures.

Non-noxious temperatures between ~10-43ºC are generally perceived as non-painful by humans. Below 10ºC, humans sense cold pain (Croze and Duclaux, 1978) and, above 43ºC, heat pain (Pertovaara et al., 1996; Yarnitsky and Ochoa, 1990). Humans perceive temperatures of 30-32ºC as thermoneutral (although this depends on the baseline temperature of the skin, and can be extended to 27-34ºC, Filingeri et al., 2017). Temperatures between thermoneutrality and 43ºC are perceived as warm, whereas temperatures between thermoneutrality and 10ºC are perceived as cool. Note that pain thresholds show interindividual differences in healthy individuals (Croze and Duclaux, 1978), and can also be modulated by pathological conditions such as local inflammation (Huang et al., 2006).

1.1.2 Rodent thermal perception

In order to investigate mammal and human physiology and disease, rodents are widely used as animal models. That is the case as well for sensory perception. Mice in particular have been proven extremely useful to understand somatosensation and many aspects of thermosensation. This is primarily due to the development of transgenic mice, which have enabled researchers to test the role of single proteins to switch on and off potentially

thermosensitive neurons. In this section, I will discuss the methods employed in rodent thermosensation, as well as the main findings observed in these experiments.

The ability of rodents to sense temperature has been investigated relatively unevenly. On one hand, rodent sensitivity to noxious heat and cold stimuli has been extensively investigated. However, non-noxious thermal perception of mice and rats remains relatively unknown, with most behavioral assays being focused on thermal preference rather than thermal detection abilities.

To test for thermal pain thresholds in mice, existing assays consist in exposing one or more body parts of the animal to a hot or cold temperature and measure the time required by the mouse to withdraw from such a stimulus (Deuis et al., 2017). For instance, in the tail immersion test, the mice are under restraint (wrapped in cloth or inside a tube) and have their tails submerged into a water bath of a given temperature, typically between 45-55^{o}C for heat pain, or 0-5^{o}C for cold pain. If the water is simply mildly cool or warm, the animals do not retract their tails from the bath, or at least take a significant amount of time to do so. On the other hand, if the water is cold or hot and the animals sense it as painful, they quickly withdraw the tail (Caterina et al., 2000; Moqrich et al., 2005; Vandewauw et al., 2018). An alternative method to measure noxious heat sensation in the tail is the so-called tail flick test (D'Amour & Smith, 1941). In this assay, radiant heat (via a beam of intense light) is used instead to stimulate the tail, and the response latency of the animal is similarly quantified (Gregory et al., 2013; Vandewauw et al., 2018). Additionally, heat pain sensation is investigated in mice with the hot plate test (Woolfe & MacDonald, 1944). In this assay, the animal is placed within a chamber, standing on top of a plate that heats up. Upon heating of the surface, the animal will either lick its paw as a response to the stimulus, or even jump (Caterina et al., 2000; Vandewauw et al., 2018; Yalcin et al., 2009). If the experimenter wants to deliver a localized heat stimulus, the Hargreaves test is a widely known choice. In this case, a source of radiant heat is used to deliver the stimulus through a glass floor, pointing at a target paw or body region of the animal. However, the main limitation of using radiant heat as the stimulus is the challenge in assessing the exact temperature at which the paw is withdrawn (Hargreaves et al., 1988).

In order to measure pain sensitivity to cold in rodents, one of the most commonly used techniques is the cold plate test. Mirroring the heat plate method, the animals are placed onto a surface that has been previously cooled down. Then, the latency to jump, lift the paw or the number of paw licks are measured (Jasmin et al., 1998; Karashima et al., 2009; Knowlton et al., 2010; Vandewauw et al., 2018). In a similar approach, the cold

plantar assay consists in delivering cold stimuli through a glass floor where the mouse is standing. The stimulus is in this case localized: the experimenter places a pellet of dry ice below the floor area where one paw of the mouse is standing. In this case, a thermocouple filament needs to be used to monitor the temperature of the paw at all times (Brenner et al., 2012). Moreover, tail immersion at cold temperatures has also been used in rodents (Lolignier et al., 2015).

In summary, noxious thermal perception has been extensively investigated in rodents using a variety of behavioral models. This is likely due to the clinical relevance of thermal pain sensations. Thermal allodynia (or thermal pain in absence of painful stimuli) can occur in patients under inflammatory disorders and patients with neuropathy (Viana, 2018). Thus, sensitivity to thermal pain in rodents is commonly used when investigating the efficacy of drugs targeting pain at pre-clinical stages.

Regarding noxious thermosensation, it has been shown that rats and mice have similar thresholds to thermal pain as humans. For heat pain, mice have been shown to exhibit robust nociceptive responses in the hot plate test at temperatures above ~42-44ºC, as well as rats (Yalcin et al., 2009). In the tail immersion test, mice start withdrawing the tail when the bath is at ~45-48ºC (Caterina et al., 2000; H. Lee et al., 2005; Lolignier et al., 2015; Vandewauw et al., 2018). Interestingly, the tested animals take more or less time before reacting to the noxious heat, depending on the absolute temperature. Overall, both in the hot plate and tail immersion test, mice react faster to hotter temperatures (Caterina et al., 2000; Marics et al., 2014; Moqrich et al., 2005; Yalcin et al., 2009). For cold pain perception, clear nociceptive responses in mice occur at temperatures below ~3ºC, and below ~8ºC in the case of rats, as shown by limb withdrawal in cold plate tests (Yalcin et al., 2009). In tail immersion tests, mice withdraw the tail at temperatures below 10ºC (Lolignier et al., 2015). In rats, some did not observe strong behavioral responses to the cold plate test at 10, 5 and even 0ºC (Jasmin et al., 1998) but others showed that rats lift their paw to a cold plate at 5ºC (Yoon et al., 1994). The slight discrepancies between studies suggest that cold pain perception is particularly sensitive to the parameters of stimulation. Overall, rodents appear to have similar hot and cold pain thresholds as humans (~10ºC for cold and ~43ºC for heat pain).

Unlike thermal pain perception, however, rodent thermosensation within the non-noxious range is not very well characterized. This is mainly due to the technical challenge of monitoring whether the animals can or cannot perceive subtle stimuli (which do not elicit a clear aversive response, such as quick avoidance). So far, most of what is known about non-noxious thermal perception in rodents comes from thermal preference assays,

which exploit the innate preference of the animals for some specific range of temperatures. In the two-plate preference test, the animal is placed in a cage with two chambers with different floor temperatures. The animal can roam freely within the cage and the two chambers for a few minutes. The experimenter then monitors the amount of time that the animal spends in each chamber, which is normally a few (5-10) minutes. This type of assay has been useful to determine that rodents have a preference for ~30^{o}C, and tend to spend less time at temperatures above ~38-40^{o}C and below ~20-15^{o}C, despite not being noxious or painful. This has been shown for both mice (Moqrich et al., 2005) and rats (Balayssac et al., 2014; Morgan et al., 2012). Moreover, important mechanistic studies to understand thermosensation have been carried out in genetically modified mice that do not show such preference (Mishra et al., 2011; Pogorzala et al., 2013; Tan & McNaughton, 2016). A slightly more sophisticated approach is the thermal circular gradient assay (Touska et al., 2016). In this test, the mice are placed on a circular floor that has a spectrum of temperatures, so that there is a smooth transition between the coolest and warmest spots. There are in total more than 10 small regions with distinct temperatures, and the test animals are allowed to explore the area for longer time periods, up to 60 minutes. This allows for a more accurate characterization of thermal preference. Using this method, wild type mice show a strong predilection for temperatures at ~32^{o}C, and deviations from this can be observed in mice with sensory impairments in thermal perception (Touska et al., 2016). The temperature at which rodents are most comfortable appears to fit the so-called thermoneutral zone in humans (Filingeri et al., 2017).

Therefore, thermal preference assays are very useful to identify gross abnormalities in thermosensation in rodents. However, this experimental approach has a number of limitations. First, spatial control of the stimulus is not possible, and mice get thermal stimulation in the paws, face, body and tail. It is thus not clear which body parts may be sensing temperature. Second, it is a slow task and there is no temporal control of the stimulus, therefore perceptual latencies cannot be investigated. The long, full body stimulation may elicit changes in the internal temperature of the animals, which makes the data difficult to interpret. Third, these tasks measure stimulus valence rather than detection. For these reasons, it is unclear whether the observed thermal preference is a result of perception or a more innate behavior. For these reasons, sensory-driven, goal-directed tasks are needed to investigate in detail mouse thermal perception.

Recently, a goal-directed task was developed for cooling perception. In this assay, mice under head restraint are given rewards when they lick from a sensor, upon cooling stimulation of the forepaw. The stimulation was achieved via a 3x3 mm Peltier element.

Not only did the mice learn to report most of the stimuli (>80% accuracy) in a few days, but also the latencies to report cooling were of a few hundred milliseconds. Moreover, the animals perceived cooling of as little as 2^{o}C (Milenkovic et al., 2014). This indicates that mice can be aware of thermal stimuli and consciously respond to them.

Finally, another important behavioral development was the use of a two-alternative forced choice test for thermal perception. In this test, freely moving mice had to orally sample a water drop and make a choice depending on its temperature. If the water was warm (40^{o}C), the animals had to report it by licking from a sensor on one side of the cage, within a few seconds. If the water was neutral (32^{o}C), they had to lick from a different sensor. This way, mice achieved a very robust accuracy (~90%) (Yarmolinsky et al., 2016). These results indicate that mice can detect oral warm stimuli and distinguish them from a neutral temperature.

In summary, research so far suggests that mice have a good ability to sense cooling, and the ability to discriminate between different temperatures. They sense cooling very quickly (<1 second) and the threshold was found to be quite small (2^{o}C), although not as subtle as in humans (<1^{o}C).

However, the ability of rodents to perceive non-noxious warming is much less clear. Mice are able to distinguish warm from neutral temperatures, but information about warming threshold or detection times are missing. Also, whether mice cognitively classify warming and cooling as two separate percepts or, instead, ambiguously perceive deviations from the preferred “thermoneutral” temperature is still unknown. Moreover, humans are slightly more capable of sensing cooling than warming, but this has not been tested in rodents yet. Finally, more detailed psychophysical aspects of thermosensation still remain to be determined, such as the effects on thermal perception of the stimulation area, the rate of temperature change or the baseline temperature of the skin.

1.2 PERIPHERAL ENCODING OF TEMPERATURE

When the skin contacts an object, the very first step that will enable the perception of its temperature is the encoding of such a stimulus at the peripheral sensory afferent neurons. Mammalian sensory neurons are pseudounipolar: their cell bodies are located at the dorsal root ganglia (DRG), which are found parallel to the spinal cord, and their single axon splits in two branches. One of them elongates up until the skin, where it detects temperature. The other branch travels and sends the information to the dorsal

spinal cord (Vriens et al., 2014). In the head, sensory afferent neurons have their cell somata at the trigeminal ganglia (Yarmolinsky et al., 2016).

The first thermosensitive afferent neurons were discovered in mammals nearly 100 years ago (Zotterman, 1936). While it is clear by now that some of these sensory cells are required for peripheral thermosensation (Mackenzie et al., 1975; Mishra et al., 2011), their concrete identity is still unknown. So far, many studies have identified peripheral sensory neurons that respond to various thermal stimuli both in primates and rodents. Interestingly, some thermosensitive afferents have been reported to be thermal-specific (Darian-Smith, Johnson, LaMotte, Kenins, et al., 1979; Emery et al., 2016; Yarmolinsky et al., 2016), while some have been found to respond to several modalities (polymodal) (Campero et al., 2001; Wang et al., 2018). However, the causal contribution to perception of both specific and polymodal afferent types remains to be investigated.

For this reason, somatosensory scientists have long speculated on the nature of the encoding of sensations at the periphery (Craig, 2003). On one hand, a well-known postulate is the labeled line hypothesis, or specificity theory (Perl, 2007). According to this view, distinct sensations like warming, cooling, touch, pain or itch would all be encoded by specific neuron subtypes that do not carry significant information from the other sensory modalities (Chen et al., 1996; Hensel & Schafer, 1984; Yarmolinsky et al., 2016). This type of neural segregation may continue until the brain. The labeled line hypothesis was first introduced in the 19th century as the “law of specific nerve energies”, when it was found that applying electrical impulses over some specific regions of the skin elicited either warming or cooling sensations (Blix, 1882).

Opposed to the labeled line conception is the so-called population coding, or pattern theory. According to this view, sensations would instead arise from the activation of multiple types of sensory afferents that have different sensitivities to various modalities of stimuli. The integration of such varied inputs would give rise to perception (Craig, 2003; Green, 2004; Ma, 2010; Prescott et al., 2014).

Because the identity of sensory afferent neurons that encode thermal stimuli and drive perception is unclear, it has not been demonstrated whether thermosensation relies on a few distinct “labeled” neural pathways for cooling, cold, warming and heat or a mixed input from many afferent neuron types. In the following sections, the different thermosensitive afferent neurons identified so far, both for primates and rodents, will be discussed.

1.2.1 Primate sensory afferent encoding of temperature

Because primary sensory afferent neurons located in the DRGs are not easily accessible experimentally, most studies investigating their physiological responses employ recordings at the nerve neurite or "fibers" in the limbs. This has been done in *ex vivo* preparations (Koltzenburg & Lewin, 1997; Vandewauw et al., 2018; Zotterman, 1936), but and also *in vivo* (Campero et al., 1996; Darian-Smith et al., 1973; Dykes, 1975; Kumazawa & Perl, 1977). Sensory afferents that have been recorded in the sensory nerve site are commonly referred to as fibers.

Using this approach, afferent neurons that are activated (and, therefore, respond with action potentials) during thermal stimulation have been found in primates. Thermosensitive afferents have been studied for decades, and some heterogeneity can be observed in the sensory features of different afferents identified across studies.

On one hand, sensory afferents that respond to cool and cold stimuli have been found innervating the hands of rhesus monkeys (Dykes, 1975). "Cool" fibers show a firing rate that is dependent on the baseline temperature, with higher steady temperatures (>30^{o}C) eliciting low spiking and cool and cold temperatures (<25^{o}C) eliciting robust firing rates (**Figure 2A**). Some cool afferents respond more vigorously to cool than to cold temperatures, and some respond similarly to both (Campero et al., 2001) (**Figure 2A**). These neurons also increase their firing rate during cooling transitions, having transient bursts (Darian-Smith et al., 1973). Interestingly, the conduction velocity of these cooling afferents is relatively quick (3-30 m/s), as they have thinly myelinated, Aδ axons (Darian-Smith et al., 1973; Dykes, 1975). However, some cool- and cold-sensitive afferents have unmyelinated, C-fiber axons. These have been found in monkeys (Iggo, 1969) and in cats (Bessou et al., 1971; Hensel et al., 1960). In humans, microneurography studies have found both cool/cold-sensitive Aδ afferents (Susser et al., 1999) and C-fibers (Campero et al., 2001; Campero & Bostock, 2010). C-fiber afferents have slower conduction velocities, between 0.8 and 3 m/s. Their thermal response profile is similar to that of cool Aδ fibers (i.e. a low firing rate at around thermoneutral baseline, which is increased upon cutaneous cooling). Interestingly, some cooling C-fibers have been found to be polymodal in humans. They are activated by innocuous cooling, noxious heat and noxious mechanical stimulation (**Figure 2C**). Moreover, their firing rate is increased during cooling transitions and decreased during warming steps (Campero & Bostock, 2010). Additionally, some C-fibers encode noxious cold and heat, as well as noxious mechanical stimuli, but not innocuous cooling (Campero et al., 1996). C-fibers that only respond to noxious stimuli are normally termed nociceptors.

Therefore, in primates, cooling and cold activate sensory fibers that have either thinly myelinated (Aδ-type) or unmyelinated (C-type) axons. Some C-fibers are polymodal, and are also excited by noxious heat. Neurons exclusively encoding cold pain appear to be C-fibers.

For warmth and heat, two neuron populations with different response profiles have been described in rhesus monkeys (Hensel & Iggo, 1971). One population of fibers shows a monotonic dependence on absolute temperature, displaying higher firing rates at higher steady temperatures, even beyond the pain threshold (>43ºC). This suggests a role in thermal nociception. The remaining population of warming-sensitive fibers are specific for non-noxious warming. They fire robustly within the warm range (~30-40ºC) but are much less active at greater temperatures (LaMotte & Campbell, 1978) (**Figure 2B**). From these two sensory afferent populations, only the monotonic afferents have been described to be polymodal: they also discharge with mechanical stimulation (Kumazawa & Perl, 1977). Interestingly, all warm-activated afferents found in monkeys are C-fibers (Darian-Smith et al., 1979). In humans, both warming-tuned C-fibers (Konietzny & Hensel, 1977), and heat-sensitive C-fibers (Hallin et al., 1982) have been described.

Therefore, in primates, warmth and heat are both encoded via unmyelinated (C-fiber) sensory neurons that are warming-tuned or monotonic, respectively. Whereas warming-tuned appear to be specific, monotonic warming C-fibers can be polymodal.

To date, the role of each of these fiber subclasses in thermal perception is not yet understood. On one hand, some fiber types may be required for conscious thermal perception, while others may play a role in homeostasis or thermal comfort. In addition, it remains to be shown whether cooling and warming sensations arrive to the brain in separate channels (labeled lines) or, alternatively, whether the two pathways are intermixed with cool and warm information integrated in the central nervous system to trigger a perception of warming stimuli, and vice-versa. Despite the general assumption that fibers activated by one stimulus are mediating its evoked sensation, it is unknown if that is the case for thermal afferents. In fact, the absence of neuronal firing can also be interpreted by the nervous system as a signal, and contribute to generate percepts. That is the case, for example, of the so-called OFF-center retinal ganglion cells, which can alone generate visual percepts in primates (Schiller et al., 1986).

1.2.2 Rodent sensory afferent encoding of temperature

Similar to primates, rodents have also been shown to possess thermosensitive afferent neurons that fire action potentials upon temperature changes. One of the main

advantages of using mice as a laboratory model, however, is that many genetic tools are available to manipulate neural circuits. Thanks to those tools, mechanistic experiments have been carried out in recent years that describe specific features of the encoding of thermal stimuli.

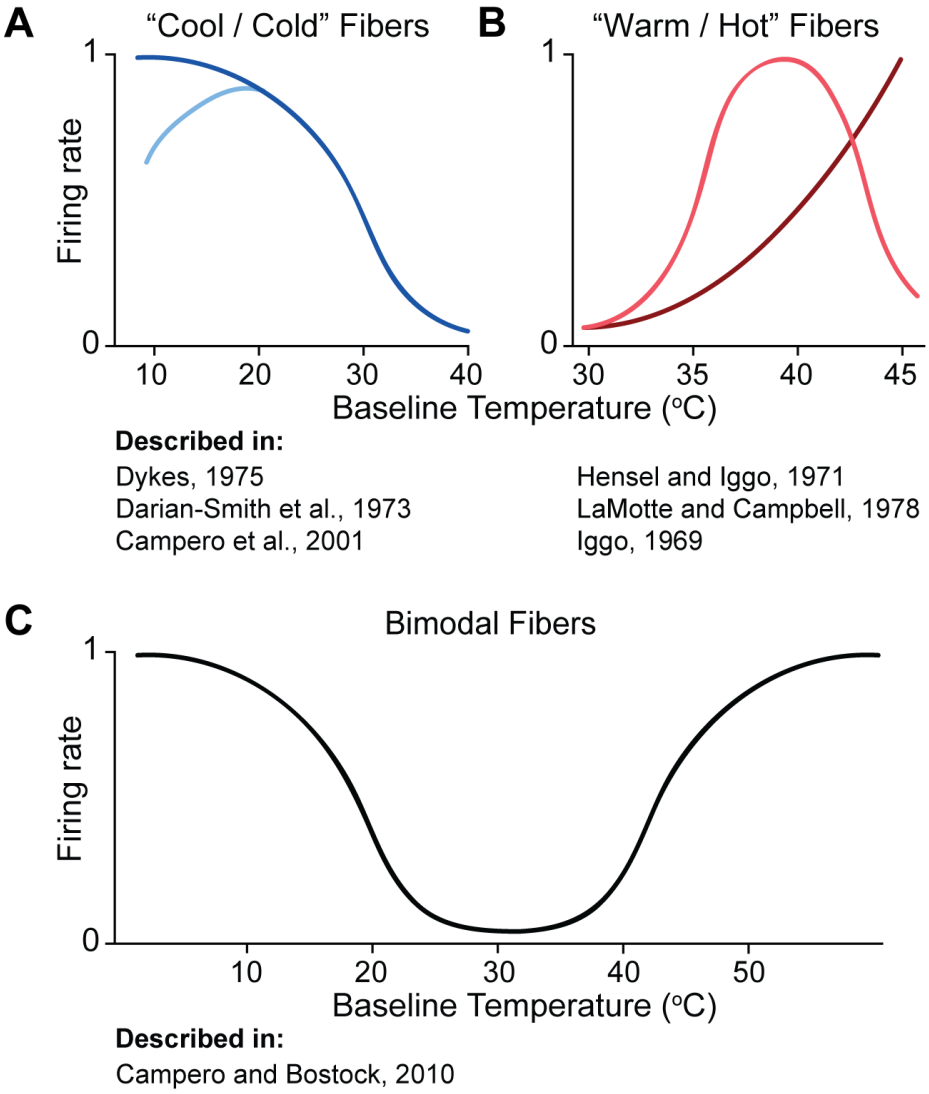

Figure 2. Schematic summary of primate sensory afferent responses to temperature.

(A) Sensory afferent neurons responding to cool and cold temperatures show little spiking activity at steady temperatures above the thermoneutral range (~30°C) but strong activity at lower temperatures. Some cool afferents partially reduce their firing during cold (<15°C, light blue) whereas some do not (dark blue). These fibers are either thinly myelinated (Aδ-type) or non-myelinated (C-type).

(B) Two types of warm-excited sensory afferents have been described in primates. On one hand, monotonic fibers (dark red) fire action potentials during both warmth and heat, even beyond the pain threshold (>43°C). On the other hand, warm-tuned afferents respond more strongly to non-

noxious warmth (light red). Both are C-type fibers, but only the monotonic neurons are polymodal (they respond to noxious mechanical stimuli).

(C) C-fibers that respond to the cool/cold and also the hot range have been described. These fibers are also polymodal.

Electrophysiological recordings have been employed to identify sensory afferent fibers that activate upon warming and cooling in rats (Heinz et al., 1990; Hellon et al., 1975) and mice (Milenkovic et al., 2014; Schäfer et al., 1988).

For cooling, both myelinated and unmyelinated cool-activated afferents have been found in mice, as in humans. Namely, Aβ-, Aδ-, monomodal and polymodal C-fibers have been described (Li et al., 2011; Milenkovic et al., 2014; Winter et al., 2017). Cold afferents of the rat fire action potentials in the 15-30^{o}C range, but are much less active at warmer temperatures (Heinz et al., 1990), and only some fire vigorously during intense cold (Heinz et al., 1990; Milenkovic et al., 2014), similar to the primate cool afferents (**Figure 2A**). A fraction (~20%) of cooling fibers of the mouse also respond to heat ramps (Milenkovic et al., 2014), resembling primate polymodal C-fibers (**Figure 2C**). However, unlike in primates, a large proportion of murine cooling afferents are unmyelinated: polymodal C-fibers account for ~85% of all cold-sensitive afferents (Milenkovic et al., 2014). Moreover, genetically-targeted manipulations that completely disable cooling sensitivity in mice are accompanied by a loss in responsiveness to cooling, primarily, in polymodal C-fibers (Bautista et al., 2007; Dhaka et al., 2007; Milenkovic et al., 2014). This indicates that cooling perception in mice mainly depends on the encoding carried out by polymodal C-fibers. Similar to cooling, noxious cold activates polymodal C-fibers that respond to noxious mechanical and, sometimes, heat stimuli (Winter et al., 2017). This, together with the fact that some cool afferents also respond to noxious cold (Heinz et al., 1990; Milenkovic et al., 2014), suggests that cool and cold sensations in mice may be mediated by overlapping populations of polymodal C-fiber afferents.

For heat sensation in mice, polymodal C-fibers are also the main mediators (Caterina et al., 2000; Vandewauw et al., 2018; Woodbury et al., 2004). These afferents are silent at rest and fire action potentials when temperature reaches a given value. At higher temperatures, they spike more vigorously, as observed in primates (Woodbury et al., 2004). Consistent with this, spiking deficits in C-fibers have been shown to impair heat perception in rodents (Vandewauw et al., 2018). Non-noxious warming encoding, however, still lacks a detailed characterization in rodents, although some warm-sensitive responses have been found. Warm-tuned afferents shown in rats respond weakly during noxious heat (Hellon et al., 1975), and some heat C-fiber nociceptors in mice show

spiking activity below 40ºC (Vandewauw et al., 2018). Recent calcium imaging studies have investigated polymodality in warm-sensitive neurons. Neurons from the mouse trigeminal ganglia were reported to be mostly (>90%) unimodal, so few heat-responsive cells were activated by cold (Yarmolinsky et al., 2016). In neurons from the dorsal root ganglia of the hindpaw, however, polymodality was reported to be very common, and neurons responding to warmth, heat, cool, cold and touch partially overlapped (Wang et al., 2018).

Therefore, studies in rodents suggest that unmyelinated C-fibers are the main mediators of cool, cold and hot sensations, but the afferent neurons that mediate warm perception are unknown. Additionally, cooling-sensitive A-fibers do exist in the mouse, but they are relatively rare.

1.3 MOLECULAR TRANSDUCERS OF TEMPERATURE

Thermosensitive afferent neurons are equipped with a battery of biomolecules that enable them to alter the firing rate as a response to thermal stimuli. Of particular importance are the protein ion channels that are thermally gated. Since the discovery of the capsaicin (chili) receptor, which opens at hot temperatures (Caterina et al., 1997), many protein sensors have been identified and causally linked to different features of thermal perception.

The most investigated thermosensitive proteins belong to the so-called Transient Receptor Potential (TRP) ionic channel family. This group of ion channels includes several subfamilies, with some having physiological roles unrelated to thermal perception. For thermal sensing, the most relevant TRP subgroups are TRPV (vanilloid), TRPM (melastatin) and TRPA (ankyrin). These three subfamilies have at least one protein with a major role in thermal perception (Palkar et al., 2015; Venkatachalam & Montell, 2007). Moreover, besides TRP channels, other proteins have also been shown to respond to temperature. The identified proteins responding to cooling, cold, warming and heat will be discussed.

1.3.1 Cool and Cold TRP channels

Several protein channels have been found to activate upon cooling in mice. Here, I will discuss *Transient Receptor Potential Melastatin 8* (TRPM8), *TRP Ankyrin 1* (TRPA1), *TRP Canonical 5* (TRPC5), *Glutamate receptor, Kainate type 2* (GluK2) and *receptor Guanlylyl Cyclase G* (GC-G).

TRPM8

The most investigated thermosensitive protein is the main sensor for cooling perception, TRPM8. When expressed in heterologous systems *in vitro*, cells expressing TRPM8 exhibit Ca^{2+} (calcium ion) influx upon cooling stimuli or application of its agonist menthol (De La Peña et al., 2005; McKemy et al., 2002; Peier et al., 2002). TRPM8 is mainly expressed in sensory afferents that are activated by non-noxious cooling (Jankowski et al., 2017) and afterwards carry the information to the external laminae of the dorsal spinal cord (Dhaka et al., 2008). The cooling activation threshold of TRPM8 is around ~25^{o}C (Bautista et al., 2007; Brauchi et al., 2004). When TRPM8 is genetically ablated in mice, the animals have a much smaller proportion of cooling-sensitive C-fibers than wild types (Milenkovic et al., 2014). Moreover, mice lacking TRPM8 have severe deficits in the perception of cooling, as shown in thermal preference tests and a detection task (Bautista et al., 2007; Dhaka et al., 2007; Knowlton et al., 2013; Milenkovic et al., 2014).

TRPM8 is also activated by chemical compounds, which is a common feature in thermosensitive TRP channels. Menthol, as well as synthetic agonists such as icilin, activate TRPM8 (Andersson et al., 2004; Peier et al., 2002). Chemical activation of TRPM8 elicits cooling-like behavioral responses *in vivo*, as shown by the so-called wet-dog shakes in mice, upon TRPM8 agonist injection (Dhaka et al., 2007; Knowlton et al., 2013). On the other hand, pharmacological blockade of TRPM8 has also been shown to suppress its natural response to temperature (Knowlton et al., 2011).

Interestingly, loss of the TRPM8 channel alone causes a similar cooling-sensing phenotype (in the range of ~20-30^{o}C) to complete loss of TRPM8-positive neurons. This suggests that the mechanisms of mild cooling sensing depend on the TRPM8 channel almost entirely (Pogorzala et al., 2013). However, TRPM8-deficient mice are still sensitive to intense cold, as shown by cold avoidance behaviors (Pogorzala et al., 2013). Interestingly, for cold sensing (<20^{o}C), the phenotype of TRPM8 knockout mice is no longer similar to that of animals devoid of TRPM8+ neurons. In this case, deletion of TRPM8 channel has a milder effect on cold sensing, and disabling TRPM8+ neurons almost entirely suppresses intense cold perception (Pogorzala et al., 2013). This shows that, first, TRPM8 channel plays a partial role in cold sensing. Secondly, these findings also demonstrate that, in some cold-sensitive afferents, TRPM8 is co-expressed with one (or multiple) cold thermosensitive protein(s). Finally, it appears that cold sensation is mediated mostly by neurons expressing TRPM8, despite the fact that this channel only activates the neurons during mild or innocuous cooling.

TRPA1

The protein channel that has been most extensively investigated in cold processing, aside from TRPM8, is TRPA1. However, the role of this channel in cold sensation is controversial.

TRPA1 is expressed mainly in somatosensory neurons, where, when activated by intense cold (<17ºC, *in vitro*) or cooling drug agents, it allows the passage of calcium ions across the cell membrane (Story et al., 2003). TRPA1 is also expressed in visceral afferents, where it could play a homeostatic function (Fajardo et al., 2008). Because of its cold temperature threshold, TRPA1 is thought to mediate painful or unpleasant cold sensations, but this is debated.

Consistent with this view, genetically ablating TRPA1 in mice causes a partial loss of cold afferents, which in turn has been shown to decrease the sensitivity of the animals to noxious cold temperatures (Karashima et al., 2009; Vandewauw et al., 2018). In humans, a gain-of-function mutation of TRPA1 causes episodic pain (Kremeyer et al., 2010). Altogether, these findings indicate that TRPA1, unlike TRPM8, serves mainly as a nociceptive cold transducer.

Interestingly, TRPA1 is not co-expressed with the innocuous cooling sensor TRPM8. This is unexpected from a cold sensor because, as stated above, ablation of TRPM8+ afferents almost entirely inactivates cold perception. Instead, this cold channel is found in heat-activated sensory afferents expressing the heat-sensitive channel TRPV1. For this reason, it has been suggested that TRPA1 is located in polymodal C-fibers which fire action potentials during cold and heat (Campero et al., 1996; Story et al., 2003). Moreover, pungent natural compounds found in mustard, cinnamon and ginger oils activate TRPA1 and elicit nociceptive behaviors in mice (Bandell et al., 2004; Jordt et al., 2004). Bradykinin, an inflammatory compound, also activates TRPA1. This suggests that TRPA1-mediated sensations are noxious. Moreover, these findings might explain the phenomenon of paradoxical heat sensation, which is a percept that resembles burning pain but occurs when the skin is in contact with intense cold objects (Susser et al., 1999).

Despite the reports linking TRPA1 and cold, some studies have failed to observe a strong cold-sensing phenotype in mice lacking TRPA1 (Bautista et al., 2006; Knowlton et al., 2010). Also, it has been shown that TRPA1 is activated by high concentrations of intracellular calcium (Zurborg et al., 2007), suggesting that its activation may only enhance already existing cold signals (Caspani & Heppenstall, 2009). Similarly, the cold sensitivity of TRPA1 has been shown to depend on the concentration of reactive oxygen species (Miyake et al., 2016). Finally, recent reports indicate that TRPA1 is also activated

by heat. Thus, this channel could act as a bidirectional thermal transducer in sensory afferents (Moparthi et al., 2016; Sinica et al., 2019; Vandewauw et al., 2018). TRPA1 activation has been shown at around ~15-18^{o}C for cold, and ~45^{o}C for heat (Karashima et al., 2009; Vandewauw et al., 2018). Therefore, whether TRPA1 plays a significant role in sensing cold is still debated.

Importantly, genetically ablating both TRPM8 and TRPA1 in mice does not completely disable cold sensation (Knowlton et al., 2010). This indicates that there are additional thermosensitive proteins expressed in cold-activated C-fibers.

TRPC5

Transient receptor potential canonical channel 5 (TRPC5) has been identified as an additional cool/cold sensor in the DRGs of mice and humans. This channel has been shown to mediate responses to cool or cold in sensory neurons, in a relatively wide range of temperatures (~30-10^{o}C). However, genetically deleting TRPC5 alone in mice does not cause observable cool- or cold-sensing phenotypes (Zimmermann et al., 2011). This suggests that the implication of TRPC5 in thermal perception is much more subtle than that of TRPA1 and TRPM8.

GluK2

Recently, an additional cold responsive protein candidate has been found in *C. elegans* (*Glutamate receptor-like protein 3*, GLR-3). Importantly, its mammalian homolog, GluK2 (*Glutamate Kainate receptor type 2*), is expressed in mouse DRGs. The *in vitro* cooling threshold of GluK2 is around ~18^{o}C. Moreover, its suppression reduces the responses of cold-sensitive afferents to cold but not cooling *in vitro* (Gong et al., 2019). This suggests that GluK2 could be a cold channel that co-expresses with TRPM8 and drives cold perception. *In vivo* physiological and behavioral studies will be required to confirm whether GluK2 plays a major role in mediating cold sensation.

GC-G

Finally, it is worth noting that there is another murine cool-sensitive protein that may not be involved in haptic somatosensation but is able to trigger neuronal activation. *Receptor Guanylyl Cyclase type G* (GC-G) is specifically expressed in neurons from the Grueneberg ganglion, an olfactory structure located at the nasal cavity in rodents. Unlike ionic channels, GC-G activates Grueneberg ganglion neurons upon cooling (<30^{o}C, but maximal response is at ~15^{o}C) by generating second messengers (Chao et al., 2015; Schmid et al., 2010). Cooling-driven activation of Grueneberg ganglion neurons triggers ultrasound vocalization in mouse pups, which is thought to elicit maternal behaviors.

Altogether, physiological and behavioral evidence suggests that TRPM8 is the main sensor for cooling perception. For cold, TRPA1 may play a larger role than TRPC5, and GluK2 might be a promising candidate to be investigated. Finally, GC-G may be a homeostatic sensor in mice for unpleasant cooling.

1.3.2 Hot and Warm TRP channels

As in cold transduction, many ionic channels have been identified that become activated by heat in mice. However, complete loss of acute heat sensation has only been achieved recently in mice (Vandewauw et al., 2018). For warmth sensation, some TRP channels have been suggested to play a partial role, but there is no known protein – or set of proteins – that, when eliminated, suppress non-noxious warming completely.

Here, I will summarize the most important thermosensitive proteins that have been shown to play a role in the perception of heat or warmth: *Transient Receptor Potential Vanilloid 1, 2, 3 and 4* (TRPV1, TRPV2, TRPV3 and TRPV4), *TRP Melastatin 2 and 3* (TRPM2 and TRPM3), *TRP Ankyrin 1* (TRPA1), *Anoctamin 1* (ANO1), *Stromal Interaction Molecule 1* (STIM1) and *TWIK-Related Potassium channels* (TREK-1, TREK-2 and TRAAK).

TRPV1

The first identified member of the Transient Receptor Potential family was TRPV1, a heat sensor. TRPV1 is expressed in the sensory neurons of the dorsal root (and trigeminal) ganglia and is activated by heat in the noxious range (with activation threshold around 43ºC), as well as by the “hot” component of chili peppers: capsaicin (Caterina et al., 1997). Moreover, TRPV1 channel ablation in mice decreases heat-evoked afferent (Yarmolinsky et al., 2016) and spinal cord responses, and was initially reported to reduce sensitivity to heat pain (Caterina et al., 2000). However, the role of TRPV1 in heat sensation is controversial, and evidence suggests that this channel may only be a partial contributor to the perception of heat. For instance, some studies report that thermoreceptors lacking TRPV1 still respond vigorously to heat (Woodbury et al., 2004) and TRPV1-null mice have no or mild defects in heat sensation (Davis et al., 2000; Marics et al., 2014). However, TRPV1 activation does induce heat or burning pain sensation (Black et al., 2020; Siemens et al., 2006; Yilmaz et al., 2007). In inflammatory conditions such as skin burning or tissue damage, moreover, the capsaicin receptor is upregulated in sensory neurons. This triggers thermal hyperalgesia (Davis et al., 2000; Yarmolinsky et al., 2016). In addition, TRPV1 function itself has been shown to be modulated by biochemical changes such as phosphorylation (Bhave et al., 2002). Thus,

the function of the capsaicin receptor in somatosensation appears to be highly dependent on context; and TRPV1 is commonly investigated as a drug target to induce pain relief (Szallasi et al., 2007).

Additionally, TRPV1 may contribute to non-noxious warming. Recently, it has been shown that pharmacological inhibition of TRPV1 induces a decrease in warmth sensitivity in the trigeminal ganglia of mice. Specifically, WT mice were trained to sample a lick spout with their tongue and report whether it was warm (40^{o}C) or neutral (32^{o}C). While control mice reported the temperature with ~90% accuracy, systemic administration of a TRPV1 antagonist decreased their accuracy to about 70% (Yarmolinsky et al., 2016). This suggests that TRPV1 plays a role in non-noxious warm sensation. However, more studies are required to elucidate to what extent warming sensation relies on this ion channel.

Importantly, besides its thermal sensing properties in adult animals, TRPV1 has been shown to be very useful as a sensory neuron marker. TRPV1 is expressed in most thermal sensory neurons that are activated by warmth or heat (Ran et al., 2016; Yarmolinsky et al., 2016). Furthermore, adult mice require TRPV1-positive sensory afferents in order to avoid hot temperatures. For this reason, these neurons have been proposed to be a labeled line for heat sensation (Pogorzala et al., 2013). However, during development, TRPV1 appears to be widely expressed in the lineage of cells that will give rise to all thermosensitive afferent neurons (Mishra et al., 2011). Therefore, targeting of TRPV1-expressing neurons during development or in adulthood are promising strategies to manipulate thermal circuits. Thus, afferents driving heat sensation express TRPV1; but this channel itself only drives part of the heat response (Pogorzala et al., 2013).

In summary, the TRPV1 channel plays only a partial role in heat nociception and warming perception. Under inflammatory conditions, TRPV1 function is modulated to transmit pain.

TRPM3

TRPM3 is a Ca^{2+}-permeable non-selective cation channel that is activated by heat (activation threshold ~40^{o}C) and is expressed in sensory neurons of the dorsal root and trigeminal ganglia. When missing, mice exhibit reduced encoding and behavioral responses to noxious heat but remain partially sensitive. Similarly, its selective activation (by administration of pregnenolone sulphate, a TRPM3 agonist) evokes pain reactions in mice. Finally, as expected, TRPM3 shows partial co-expression with TRPV1. (Held et al., 2015; Vriens et al., 2011). Similar to TRPV1, TRPM3 has been shown to mediate

heat hypersensitivity and spontaneous pain after injury (Su et al., 2021). Together, these findings support a nociceptive role for TRPM3 in noxious heat perception.

TRPA1

Because TRPV1 and TRPM3 both play a partial role in noxious heat encoding and sensation, a recent study investigated the effects of combined ablation of these two channels in mice (Vandewauw et al., 2018). Double knockout mice for TRPV1 and TRPM3 still had heat-activated afferent responses and showed heat avoidance. However, additional deletion of the TRPA1 channel (discussed above and considered to be, despite controversy, a cold sensor) significantly reduced heat-evoked responses in sensory neurons of mice. Triple knockout mice for TRPV1, TRPM3 and TRPA1 did not avoid harmful temperatures of >48^{o}C that resulted in tissue damage (Vandewauw et al., 2018). Double knockout combinations of these TRP channels were also investigated, but all resulting mice retained acute heat sensitivity.

Consistent with these findings, other studies have also reported both heat and cold sensitivity of TRPA1, supporting the view that this channel is a bidirectional thermal sensor (Moparthi et al., 2016; Sinica et al., 2019). Altogether, these data indicate that TRPV1, TRPM3 and TRPA1 are redundant heat sensors that act in concert to mediate acute noxious heat perception. Interestingly, however, mice lacking this channel trio had a normal thermal preference behavior within the non-noxious range. This argues against a role for TRPV1, TRPM3 and TRPA1 in innocuous thermal sensing.

ANO1 (or TMEM16A)

Besides these channels, other molecular sensors have been implicated in heat sensation. *Anoctamin 1* (ANO1, also referred to as TMEM16A) is a Ca^{2+} activated chloride channel that is expressed in small afferent sensory neurons and has been shown to activate during noxious heat (>44^{o}C), inducing depolarization. Importantly, deletion of ANO1 decreases nociceptive responses in mice (Cho et al., 2012). ANO1 also contributes to the exacerbated excitability in DRG neurons upon inflammation, leading to pain (Lee et al., 2014).

TRPV2

TRPV2 is a non-selective cation channel that is also expressed in sensory neurons and activates during heating. Strikingly, the heat threshold of TRPV2 is very high, at ~53^{o}C as shown *in vitro* (Yao et al., 2011). This channel could therefore appear to be a candidate for heat pain sensation. However, TRPV2 knockout studies carried out in mice have shown no observable defects in heat perception (Katanosaka et al., 2018; U. Park

et al., 2011). Therefore, it is unlikely that TRPV2 plays a major role in mammalian thermosensation. The role of TRPV2 in mechanical nociception has also been discussed: some have reported that TRPV2-null mice have normal mechanical pain (U. Park et al., 2011), but others found pain deficits in these animals (Katanosaka et al., 2018).

TRPV3

TRPV3 is a warm- and heat-activated ion channel that is expressed in keratinocytes (instead of sensory afferents) and has a relatively low activation threshold (~30-40^{o}C) (Peier et al., 2002; Smith et al., 2002; Xu et al., 2002). TRPV3 induces warm-evoked currents in cultured mouse keratinocytes (Chung et al., 2004). Deleting TRPV3 in mice was initially reported to impair the warming thermal preference and sensitivity to heat (Moqrich et al., 2005). However, others tried replicating these findings in different mouse strains and did not observe major thermosensation deficiencies in TRPV3-null mice (S. M. Huang et al., 2011). Therefore, the role of TRPV3 in warming and heat perception is unclear.

TRPV4

TRPV4 is another nonselective cation channel that has been implicated in the detection of warm and hot stimuli, since it has an *in vitro* activation threshold of ~34^{o}C when expressed in mammalian cells (Güler et al., 2002). TRPV4 is found both in afferent neurons (Alessandri-Haber et al., 2003) and keratinocytes, where it evokes currents upon warming (Chung et al., 2004). Similar to TRPV3, TRPV4 was initially thought to mediate thermal preference behaviors in mice (H. Lee et al., 2005), but later it was suggested that TRPV4 does not play a major role in thermosensation (S. M. Huang et al., 2011).

TRPM2

TRPM2 is another TRP channel that has been recently suggested to convey warming percepts in mice. TRPM2 is activated *in vitro* upon warming above 35^{o}C (Togashi et al., 2006). This channel is expressed in DRG sensory neurons and was reported to mediate responses to heat (>40^{o}C) (Tan & McNaughton, 2016). Importantly, genetic ablation of TRPM2 suppressed the thermal preference behavior in mice, in the 33-38^{o}C range. However, TRPM2-null mice still showed avoidance of temperatures near the heat pain threshold (43^{o}C), as well as cooling temperatures (23 and 28^{o}C), suggesting a specific role for this channel in non-noxious warming sensitivity (Tan & McNaughton, 2016). Moreover, TRPM2 has also been postulated as a thermal sensor (activated at >38^{o}C) in

the preoptic area of the hypothalamus, being required for normal thermoregulatory control (Song et al., 2016). Together, these findings suggest that TRPM2 could be a major transducer of warming sensation.

STIM1 (and Orai)

Stromal Interaction Molecule 1 (STIM1) is a calcium sensor found in the endoplasmic reticulum of keratinocytes. In response to warmth and moderate heat (activation threshold ~35-37ºC) (Xiao et al., 2011), it physically couples to the plasma membrane channels *Orai1 and Orai3* (in Greek mythology, Orai are the keepers of the gates of heaven), which leads to an increase of intracellular calcium in these cells (Mancarella et al., 2011). This is thought to modulate the activity of thermosensitive afferent neurons (Sadler et al., 2020). Behaviorally, deletion of STIM1 in keratinocytes leads to a slight shift (~2ºC) in thermal preference of mice, making them prefer subtly warmer temperatures within the non-noxious range (Liu et al., 2019). However, since the behavioral effects of deleting this channel are subtle, it seems likely that STIM1 (and Orai) only play a supporting role in non-noxious thermosensation.

TREK-1 (and TREK-2 and TRAAK)

A particular case is that of *TWIK-Related Potassium channel 1* (TREK-1), a member of the protein family of 2-pore domain potassium channels (TWIK stands for *Tandem of P-domains in a weak inwardly rectifying potassium channel*) (Honoré, 2007). Its function is to regulate neuronal excitability, and maintaining the voltage of the cell membrane below the action potential threshold (Djillani et al., 2019). Contrary to the "classical" thermosensitive channels described above, TREK-1 activation causes hyperpolarization (R. Xiao & Xu, 2021). TREK-1 is opened by mechanical stimuli and by temperatures around ~40ºC. TREK-1 is expressed in DRG afferent neurons (where it co-expresses with TRPV1) as well as in hypothalamic thermoregulatory center (preoptic area), suggesting a role for this channel in temperature sensing (Maingret et al., 2000). Deletion of TREK-1 increases C-fiber afferent excitability and increases pain perception of both tactile and thermal stimuli in mice (Alloui et al., 2006). These findings suggest that TREK-1 is a regulatory protein in sensory afferents that encode noxious heat and mechanical pain.

Moreover, 2 other channels also belonging to the TWIK family are *TWIK-Related Potassium channel 2* (TREK-2) and *TWIK-Related Arachidonic acid Activated Potassium channel* (TRAAK). Both have been shown to activate upon heat in a similar manner to TREK-1, and they may therefore play a similar function (Kang et al., 2005). Importantly, TREK-1, TREK-2 and TRAAK have been shown to co-localize with TRPV1 and, to a

lesser extent, TRPM8 (Y. Yamamoto et al., 2009). A model where TREK-1 and TRAAK keep sensory afferents silent (hyperpolarized) while temperatures are non-noxious has been suggested (Noël et al., 2009).

Altogether, there are many proteins proposed to be involved in warm and heat sensation (**Table 1**). For noxious heat sensation, the main transducers seem to play a redundant role and are TRPV1, TRPM3 and TRPA1. ANO1 may also contribute, whereas a role for TRPV2 is more unlikely. For warm sensing, TRPM2 may be the most promising candidate, and the keratinocyte-expressed STIM1 as well as TRPV1 could play a minor role. Moreover, TREK-1 (and TREK-2 and TRAAK) may play an indirect role in both warm and heat sensation, by silencing afferent neurons while temperatures are non-noxious. Finally, TRPV3 and TRPV4 have shown conflicting results and the latest reports suggest that they are dispensable for both warm and heat perception. To date, however, no combination of protein sensors has been found to disable warming perception in mice.

Protein	Threshold	Role	Behavior?	Selected References
TRPM8	25ºC	C	Yes	Bautista et al., 2007; Dhaka et al., 2008., Pogorzala et al., 2013
TRPA1	17ºC	C,H	Yes	Story et al., 2003; Vandewauw et al., 2018
TRPC5	30-10ºC	C,C	No	Zimmermann et al., 2011
GluK2	18ºC	C	No	Gong et al., 2019
GC-G	30ºC	C	No	Chao et al., 2015
TRPV1	43ºC	W,H	Yes	Caterina et al., 2000; Yarmolinsky et al., 2016; Vandewauw et al., 2018
TRPM3	40ºC	H	Yes	Vriens et al., 2011; Vandewauw et al., 2018
ANO1	44ºC	H	Yes*	Cho et al., 2012
TRPV2	52ºC	H?	No	Yao et al., 2011; Park et al., 2011; Katanosaka et al., 2018
TRPV3	30-40ºC	W	Conflicting	Peier et al., 2002; Moqrich et al., 2005; Huang et al., 2011
TRPV4	34ºC	W	Conflicting	Güler et al., 2002; Lee et al., 2005; Huang et al., 2011
TRPM2	35-40ºC	W	Yes	Tan and McNaughton., 2016
STIM1-Orai	35-37ºC	W	Yes*	Xiao et al., 2011; Liu et al., 2019
TREK-1	40ºC	H	Yes	Maingret et al., 2000; Alloui et al., 2006

* small effect

Table 1. Summary of molecular sensors of temperature.

Protein candidate sensors for temperature are listed on the left column, followed by their reported activation threshold (approximated, *in vitro* or *in vivo*) and their putative role on specific thermal ranges (light blue C: cooling; dark blue C: cold; light red W: warmth; dark red H: heat). The fourth column indicates whether the ablation of the protein candidates has been found to impact thermal perception at the behavioral level in mice. Finally, selected references implicating each protein in thermal perception are cited.

1.4 CENTRAL ENCODING OF TEMPERATURE

Following encoding of temperature by the peripheral, primary sensory neurons innervating the skin, the information is sent to the central nervous system (CNS). First, afferents send their information to the dorsal (sensory) spinal cord. Later, thermal signals reach the thalamus, hypothalamus and brain cortex (Bokiniec et al., 2018). While neural responses have been found at those stages of the central nervous system, the exact pathways and connections between them are unclear. Here, I will summarize the main findings of the neural encoding of thermal information in the CNS.

1.4.1 Thermal encoding at the spinal cord

Anatomical studies have shown that thermosensitive afferent projections target the most external layers of the dorsal spinal cord. For instance, projections coming from TRPM8-positive afferents can be seen in lamina I/II (Dhaka et al., 2008; Jankowski et al., 2017; Wrigley et al., 2009), and TRPV1-expressing neurons target these laminae as well (Park et al., 2008; Takashima et al., 2010). Physiological studies, in turn, have shown that neurons from the superficial laminae are activated by cooling and warming (Bester et al., 2000; Burton, 1975; Dostrovsky & Craig, 1996; Hachisuka et al., 2016), although responses to noxious temperatures have also been reported in deeper spinal laminae (Burton, 1975). In line with this, mechanistic experiments have demonstrated that cool/cold- and warm/hot-evoked firing in the dorsal spinal cord depend greatly on TRPM8- and TRPV1-expressing sensory afferents (Ran et al., 2016).

Within the spinal cord, the temperature processing is not completely understood. As in sensory afferents, stronger deviations from thermoneutral temperature (~30-32^{o}C) drive higher spike rates (Craig & Dostrovsky, 2001). Stronger thermal stimuli also activate a greater number of spinal neurons (Ran et al., 2016). Also, despite some thermosensitive spinal neurons are specific to heat or cold, a significant amount of thermal bimodality has been observed (Han et al., 1998). Measured by calcium imaging, 7% of lamina I spinal neurons fired action potentials during both subtle cooling (29^{o}C) and warming (37^{o}C). Meanwhile, 44% of cells were activated during cold (5^{o}C) and heat (50^{o}C) (Ran et al., 2016). Therefore, the bidirectional activation of spinal neurons encoding skin temperature is enhanced for larger amplitudes. However, the local circuitry and the processing of thermal information by specific neuron subpopulations remains poorly understood. Generally, sensory information in the dorsal laminae of the spinal cord travels upwards (Kato et al., 2009), with projection neurons (the output of the spinal cord)

being found in lamina I. Interestingly, a study investigated projection neurons and found that there is a proportion of cells that fire action potentials upon cold and heat (Hachisuka et al., 2016). Regarding encoding mechanisms, the dynamics of the responses during heat and cold seem to differ at the spinal cord. Warm and hot stimuli trigger activation that is proportional to the absolute skin temperature value whereas cold responses are mainly evoked by the magnitude of temperature change, and later show a greater degree of adaptation (*i.e.* firing is high when the temperature is dropping, then the response is reduced if the temperature remains cold) (Ran et al., 2016).

To date, three information pathways have been reported at the spinal cord, with only two of them reaching central structures beyond the spinal cord: i) First, sensory afferents can signal the presence of noxious heat or cold to spinal sensory neurons, leading to the activation of spinal motor neurons. The activation of this pathway results in the thermal withdrawal reflex, which is crucial to quickly avoid harmful temperatures (Vriens et al., 2014). ii) Second, thermal information travels through spinothalamic neurons, whose cell bodies are found mainly at lamina I. In this pathway, the signal reaches the contralateral thalamus and eventually the neocortex, leading to the perception or sensation of temperature (Craig & Dostrovsky, 2001). iii) Finally, and parallel to the spinothalamic circuit, cutaneous (and visceral) thermal information follows the spinoparabrachial pathway. In this case, projection neurons from lamina I of the spinal cord (Allard, 2019) send the signals to cells of the lateral parabrachial nucleus (found in the midbrain-pons junction) (Yahiro et al., 2017; Yang et al., 2020), which later target the preoptic area (POA) of the hypothalamus. There, the sensed temperatures may impact thermoregulatory processes that aim to maintain the body temperature at an optimal range (Clapham, 2012; Morrison & Nakamura, 2011; Nakamura & Morrison, 2008).

1.4.2 The thermal thalamus

Within the thalamus, the nuclei that are thought to mediate thermal perception in rodents are the ventral posterolateral (VPL) (Hellon & Misra, 1973; Zhang et al., 2006), posterior medial (POm) (Hellon & Misra, 1973; Schingnitz & Werner, 1980) and the posterior triangular (PoT) (Gauriau & Bernard, 2004b) nuclei. VPL and VPM are sometimes investigated together, and referred to as the ventro-basal complex (VB) (Hellon & Misra, 1973; Koyama et al., 1998; Schingnitz & Werner, 1980). Consistent with rodent data, in primates, thermal responses have been found in VB (Burton et al., 1970; Poggio & Mountcastle, 1963; Poulos & Benjamin, 1968) and the ventromedial posterior nucleus (VMpo) (Craig et al., 1994), which is missing in rodents but has been proposed to be the

primate homolog of PoT, due to proximity to other structures and similar output projections (Gauriau & Bernard, 2004a).

The targets of these thalamic nuclei will be neocortical neurons. VPL and POm project to primary somatosensory cortex (S1) (Ohno et al., 2012). POm also projects to the secondary somatosensory cortex (S2) (Viaene et al., 2011). The rodent PoT projects to both S2 and insular cortex (IC) (Gauriau & Bernard, 2004b). In turn, primate VMpo has been reported to project to IC (Craig, 2014; Craig & Blomqvist, 2002).

1.4.3 Cortical representation of temperature

There is substantial evidence that cool thermal information is represented in the mammalian primary somatosensory cortex. In rodents and cats, cooling temperatures have been shown to activate contralateral S1 neurons (Hellon et al., 1973; Milenkovic et al., 2014; Tsuboi et al., 1993). In humans, S1 has also been shown to respond to innocuous cooling and warming, both via functional imaging (Egan et al., 2005; Guest et al., 2007) and electroencephalography (Chatt & Kenshalo, 1977). Lesions in the human S1 (postcentral gyrus) can ablate the perception of thermal stimuli (Ploner et al., 1999). Importantly, pharmacological inactivation of S1 disrupts cooling perception in mice and, similarly, S1 responses to cooling are strongly reduced when switching off the afferent mechanism to encode it (Milenkovic et al., 2014). Altogether, S1 cortex appears to play an important role in thermal perception in mammals. However, while mechanistic studies have been conducted to demonstrate a causal impact of S1 in cooling perception, the role of S1 in warmth sensation is still unclear.

Besides S1, temperature has been shown to elicit activation in contralateral S2 and IC. In humans, noxious heat has been correlated with activity in S2 and IC (Casey et al., 1996; Davis et al., 1998). Heat intensity has also been linked to S2 in humans (Moulton et al., 2012). Finally, IC and the cingulate cortex have been shown to activate upon noxious and non-noxious thermal stimulation in human studies (Brooks et al., 2002; Craig et al., 2000; Egan et al., 2005; Guest et al., 2007). Interestingly, abnormal thermal and pain sensitivities have been observed in human patients with parietal and insular lesions (Starr et al., 2009; Veldhuijzen et al., 2010). Consistent with these findings, noxious heat stimulation elicits S1, S2 and IC activation in rodents (Reimann et al., 2016), and ablation of S1 and S2 impairs thermal discrimination in rats (Porter et al., 1993).

In summary, human and rodent studies have shown that thermal stimulation of the skin elicits responses in S1, S2 and IC. However, mechanistic studies are scarce and whether

these regions are redundant in the processing of temperature or they each encode different aspects of thermal sensation is unknown. In mice, it has been demonstrated that S1 is indispensable for the detection of innocuous cooling (Milenkovic et al., 2014), but whether this is also the case for warming sensation is unknown. Similarly, the contribution of S2 and IC in sensing cooling or warming remains to be investigated.

1.4.4 Thermal parabrachial and hypothalamic neurons

Parallel to the spinothalamic pathway that sends thermal information to the neocortex, some axons from spinal thermosensitive neurons reach the lateral parabrachial nucleus (LPN, at the junction of midbrain and pons), which in turn projects to the hypothalamus. While thalamic and neocortical neurons are thought to mediate perception of thermal stimuli, the parabrachial and hypothalamic neurons are believed to play an important role in homeostasis (*i.e.* keeping the body within an optimal thermal range).

Studies have shown that populations of parabrachial neurons are activated by cool and warm temperatures (Xue et al., 2016), and project to the preoptic area (POA) of the hypothalamus (Nakamura & Morrison, 2008). As a response to high temperatures, warm-activated LPN neurons drive the inhibition of thermogenesis and increase peripheral vasodilation for enhanced cutaneous body heat loss (Yang et al., 2020). Importantly, it has been shown that LPN neurons drive behavioral thermal preference in absence of the thermal thalamus (Yahiro et al., 2017). This supports the view that thermal perception and preference follow separate neural pathways (spinothalamic and spinoparabrachial, respectively).

Within the hypothalamus, there are also thermosensitive neurons that detect internal warming and act as a brain thermostat. Warm-activated POA hypothalamic neurons control body temperature (Song et al., 2016). Furthermore, cool-sensitive neurons have been found at the dorsomedial hypothalamus (DMH) that additionally modulate core temperature. Interestingly, neurons from the POA modulate the activity of those from the DMH (Zhao et al., 2017).

In summary, thermal information travels through the afferents, spinal cord, thalamus and neocortex (S1, S2 and IC). Also, in parallel, some signals travel from the spinal cord to the LPN and reach the POA of the hypothalamus to mediate thermoregulatory responses (both physiological and behavioral) (**Figure 3**). However, it is currently unknown how each of these stages of thermal processing contribute to the different aspects of thermal sensation (*e.g.* intensity, localization, valence). Recently, S1 cortex has been shown to be required for the thermal detection of cooling stimuli (Milenkovic et al., 2014),

suggesting that S1 is involved in the encoding of stimulus intensity or localization. However, the role or activity of S1 in warming perception is still unknown.

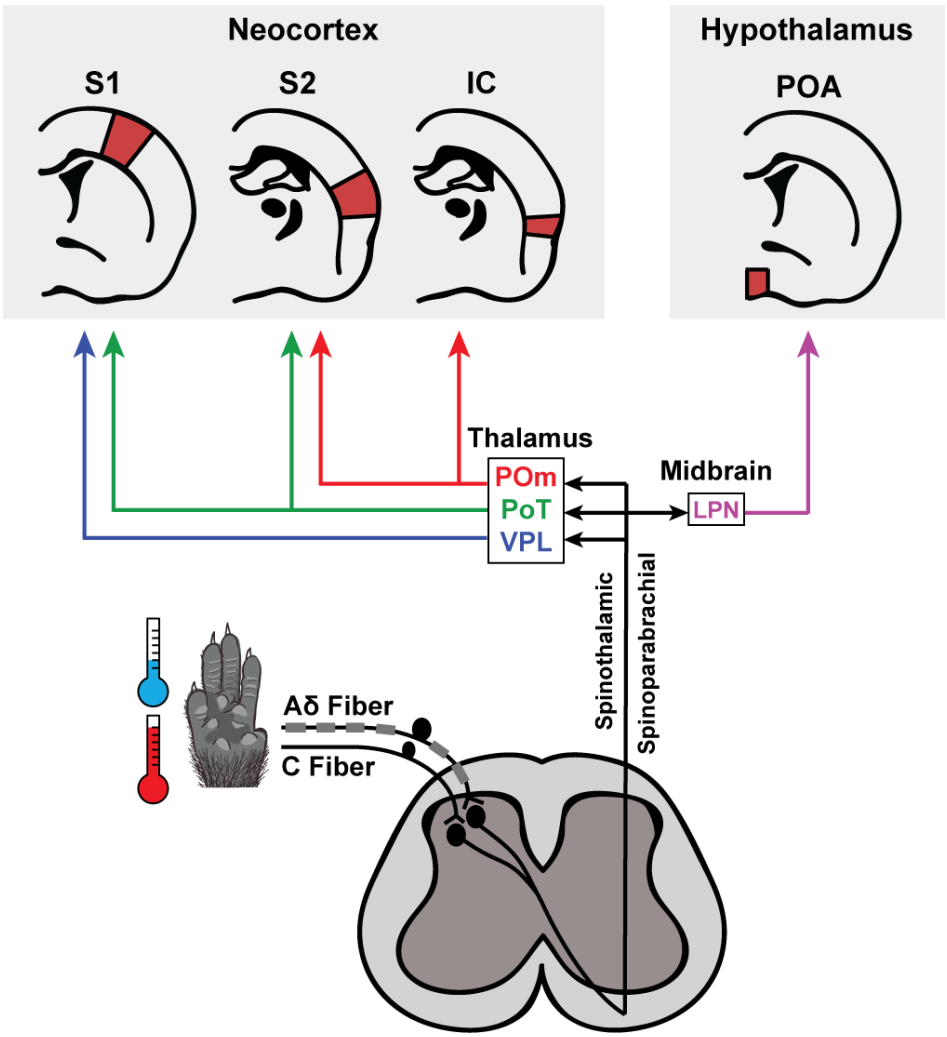

Figure 3. Putative neural circuits of thermal perception.

First, sensory afferents innervating the skin target the external laminae of the spinal cord. Spinal projection neurons decussate at the spinal cord and send axons to thalamic nuclei (POm, PoT and VPL) and the lateral parabrachial nucleus (LPN). Thalamic nuclei will send the information to the neocortex (S1, S2 and IC), which is thought to mediate conscious perception of thermal stimuli. LPN, in turn, will send the information to the preoptic area (POA) of the hypothalamus, which is thought to main center for homeostatic control of temperature.

2. METHODS

2.1 ANIMALS

All experiments presented here were approved by the Berlin animal ethics committee and carried out under the European animal welfare law. Both male and female wild-type mice (*Mus musculus*) from the C57Bl/6J strain were used, and there were no obvious differences observed between sexes. Besides WT mice, the following strains of transgenic mice were used: 1) *trpv1*$^{-/-}$ mice on a mixed background, from Jackson Laboratories (B6.129X1-Trpv1^{tm1Jul}) (Caterina et al., 2000). 2) *trpm2*$^{-/-}$ mice on a mixed background (129/SvJ and C57Bl/6N), kindly provided by Yasuo Mori, Kyoto University (S. Yamamoto et al., 2008). 3) *trpm8*$^{-/-}$ mice on a mixed background, from Jackson Laboratories (B6.129P2-Trpm8^{tm1Jul}) (Bautista et al., 2007). 4) *trpv1:trpa1:trpm3*$^{-/-}$ triple knockout mice on a C57Bl/6J background, kindly provided by Thomas Voets and Joris Vriens (Vandewauw et al., 2018). 5) Trpm8-ChR2 mice were generated by crossing *trpm8*^{Cre} (Yarmolinsky et al., 2016) and Ai34^{fl} mice (ref. 012569, Jackson Laboratories), both from the C57Bl/6J background. 6) VGAT-ChR2 mice (ref. 014548, Jackson Laboratories). All mice were maintained on a daily 12h light / 12h dark cycle and under *ad libitum* food and water conditions unless during behavioral training, where a water restriction protocol was applied. Mice under water restriction had part of their daily water intake during the behavioral training (in the form of rewards) and the rest was given after the session, up to ~1.5 mL/day. Animal body weight was daily monitored to ensure that mice did not lose more than 20% of their weight.

2.2 IMPLANTING OF MICE FOR BEHAVIORAL TRAINING

During surgery, mice were kept at 37^{o}C using a heat pad. Mice were anesthetized with isoflurane (3-4% in O_2 for induction and 1.5% maintenance), and injected subcutaneously with Metamizol (200 mg per kg of body weight). A light metal support was implanted onto the dorsal side of the skull with glue (UHU dent) and dental cement (Paladur). After the procedure, mice were placed back in the home cage and had Metamizol (200 mg/mL) in the drinking supply for 1-3 days.

2.3 BEHAVIORAL TRAINING

Implanted mice that had recovered from surgery were first habituated to head-restraint in the behavioral setup for three days with increasing duration (15, 30 and 60 minutes). The right forepaw was also tethered to the ground, with medical tape, except in the first habituation session. Next, mice underwent a water restriction protocol and two consecutive behavioral sessions ("pairing') where the sensory stimulus (warming, cooling, touch or sound) to be detected was presented together with free rewards that the mice would obtain from a lick spout. The goal of these sessions was to initiate a stimulus-reward association in the mice.

Following pairing sessions, the behavioral protocol required the mice to lick from the water spout during the time window of stimulus presentation (3.5 s). Upon the first lick, a water droplet (4-7 µL) was given as a reward, in every stimulus correctly reported (hits). Catch trials, where no stimulus was presented and therefore no reward was given if the mice licked from the sensor, were interleaved with stimulus trials as 50% of the total session trials. Licks during catch trials were also quantified (false alarms) and their proportion was compared to that of hits to measure performance. Trials were presented at randomized trial intervals between 3 and 30 s. A training session typically consisted of about 100 trials (50 stimulus + 50 catch), but in some experiments mice continued to lick for a few more trials.

Thermal stimuli were delivered using a 3 x 3 or 8 x 8 mm Peltier element. The structure of thermal stimuli was that of a ramp-hold-ramp (0.5, 3 and 0.5 s, respectively). Tactile stimuli were used in controls and consisted on a single contact delivered by a piezo bending actuator on the glabrous skin of the forepaw. Sound controls were performed in which a light sound (~40 dB SPL) was presented using a magnetic buzzer. The sound and tactile stimuli lasted for as long as the thermal stimulus (3.5 s).

For light stimulation in afferent optogenetics experiments, the right forepaw of mice was tethered to a glass surface (similar to thermal detection experiments), with a blue LED (ref. M470L3, Thorlabs) underneath for light delivery (470 nm, 100 Hz, 5 ms ON – 5 ms OFF pulses of light, average power ~5 mW; calibrated with a power meter photodiode at the forepaw spot). A nylon "blanket" made of optical blackout fabric (Thorlabs) was used to cover the paw and the stimulator completely, to ensure that mice could not see the light.

For the behavioral unrewarded detection sessions, cooling (32-22^{o}C) and warming (32-42^{o}C), or cooling, vibrotactile (60 Hz sine wave) and multisensory (simultaneous cooling

and vibrotactile) stimuli were presented in an interleaved manner and no water reward was given to the animals if they licked during stimulus presentation. In some mice, a water reward was given only between stimulus trials to increase motivation, if licking behavior ceased. For thermal discrimination, stimuli were 2.5 s long; for discrimination between cooling and mechanical stimuli, length of stimuli was 1 s. For vibrotactile stimulation, a force-feedback mechanical stimulator was used (Aurora Scientific, Dual-Mode Lever Arm systems 300-C). To ensure that the force amplitude was sufficiently salient, a group of wild type mice was trained to report this stimulus configuration (d' > 2, n = 5 mice, data not shown).

2.4 SKIN-NERVE PRIMARY AFFERENT RECORDINGS

Skin-nerve recordings were carried out by Dr. Fred Schwaller. Mice were euthanized by CO_2 inhalation (2-4 min) followed by cervical dislocation. Then, dissection of the saphenous nerve and shaved hairy skin of the hind limb was carried out for hindpaw experiments in Trp knockout mice and their corresponding C57Bl/6J controls. For forepaw experiments in a separate group of WT mice, the forepaw glabrous skin and the innervating medial and ulnar nerves were dissected. The tissue was then maintained in a buffer bath perfused with a synthetic interstitial fluid (SIF buffer): 123 mM NaCl, 3.5 mM KCl, 0.7 mM $MgSO_4$, 1.7 mM NaH_2PO_4, 2.0 mM $CaCl_2$, 9.5 mM sodium gluconate, 5.5 mM glucose, 7.5 mM sucrose and 10 mM HEPES (pH 7.4). The nerves were kept in an adjacent chamber in mineral oil, where fine filaments were teased from the nerve and placed on the recording electrode. The temperature of the organ bath was kept at 32^{o}C or 27^{o}C, depending on the experiment (details in results section).

The receptive fields of thermosensory units were identified by pipetting hot (48^{o}C) and cold (5^{o}C) SIF buffer onto the surface of the skin. To classify the fibers according to conduction velocity, electrical stimuli (1 Hz, square pulses of 50-500 ms) were delivered to their receptive fields (C fibers: < 1.2, A-delta: 1.2-10 and A-beta: >10 m/s). To test for mechanosensitivity in fibers, mechanical stimuli (3 s ramp and hold, duration 4 s) of increasing amplitude (20-400 mN) were delivered by a computer controlled nanomotor (Kleindieck, Germany).

To investigate the afferent encoding of temperature, thermal stimuli were delivered with a 3x3 mm Peltier element, placed at the center of the receptive field. In hindpaw experiments, an increasing (1^{o}C/s) heat ramp from 32 to 48^{o}C, and a decreasing (1^{o}C/s) cold ramp from 32 to 12^{o}C was used. Heat and cold stimulation ramps were repeated three times each, with 2 minute intervals between stimuli. In forepaw experiments,

thermal stimuli that matched the behavioral experiments were delivered: ramp-hold-ramp (0.5, 3 and 0.5 s) warming and cooling stimuli, of different amplitudes, were used. Thermal ramps were repeated 3-7 times to create average cell responses. Sensory fiber receptive fields were also stimulated using 1ºC/s 32-48ºC heat and 32-12ºC cold ramps. Cells that exhibited signs of wind up or spontaneous activity after multiple stimulations were discarded from analysis.

2.5 FOREPAW TRANSDERMAL INJECTIONS

Mice that had undergone thermal detection training behavior (6 sessions) were briefly anesthetized with isoflurane (3-4% in O_2 for induction, 1.5-2% for maintenance). After assessing that no pain reflexes were present due to anesthesia, a syringe of gauge 30 G (0.3 mm) was used to inject transdermally 10 µL of solution containing a TRPM8 antagonist drug (4 µL DMSO with 0.1 mg of PBMC, diluted in 6 µL of saline; PBMC catalog number: 10-1413, Focus Biomolecules) in the right forepaw of the animals. Next, mice recovered from anesthesia and, 15 minutes post injection, all mice were active and were tested in the thermal detection task as described previously. To control for possible effects of the injection procedure on animal performance, mice were also injected, in a different day, with DMSO control solution (4 µL of DMSO in 6 µL of saline).

2.6 INACTIVATION OF S1

The ability of mice to detect thermal (and sound, in controls) stimuli was tested under S1 inactivation in WT and VGAT-ChR2 mice.

For pharmacological injections, S1 was first located in WT mice via intrinsic signal optical imaging (see below). Afterwards, a small (<1 mm) craniotomy was performed under anesthesia in forepaw S1. The dura mater was left intact. Afterwards, the area was covered with Kwik-cast (World Precision Instruments) and the mouse recovered in the home cage. On the day of the injection, awake mice were head fixed and muscimol (or vehicle control solution) was microinjected across the whole S1 cortical column (30 mM of muscimol in Ringer's solution, 200 nL in 4 injections of 50 µL at depths 200, 400, 600 and 800 µm. Total muscimol injected: 684 ng). Microinjection was carried out with an oil hydraulic injection system (MO-10, Narishige). After injection, mice were tested for sensory detection behavior after 10-40 minutes. Injections were performed in V1 for a group of controls, following stereotaxic coordinates (Paxinos & Frankin, 2008). Prior to injection, mice were placed in the injection setup on 1-2 days for habituation.

For optogenetic inactivation of S1, primary somatosensory cortex was first located via intrinsic signal optical imaging in VGAT-ChR2 mice. Then, a clear-skull preparation was employed (Guo et al., 2014). The intact skull was covered by a thin layer of transparent dental cement (ref. 9002679, Henry Schein) and the forepaw S1 region was painted with clear nail polish. Black ink was used to cover the surrounding areas beyond forepaw S1 to avoid the light reaching other cortical areas. In the day of the experiment, trained VGAT-ChR2 mice underwent behavioral training and half the stimulus and catch trials were accompanied by blue (465 nm, 5 mW, 100 Hz pulses) LED light stimulation over S1 cortex (LED stimulation system, Campden Instruments). Light stimulation over S1 lasted for as long as the thermal (or sound) stimulus. Masking, blinking light of the same wavelength was used throughout all training sessions to prevent mice from seeing when light was shone over S1. Masking lights were placed bilaterally near the head of the animals.

2.7 INTRINSIC SIGNAL OPTICAL IMAGING

Intrinsic signal optical imaging (ISOI) was carried out to locate S1 cortex of wild-type and VGAT-ChR2 mice (for S1 inactivation experiments) and to investigate sensory responses to tactile and thermal stimuli in wild type mice. Mice under light isoflurane anesthesia (3-4% in O_2 for induction, 1-1.5% for maintenance) had their skull exposed and covered with pre-warmed Ringer's solution (135 mM NaCl, 5 mM KCl, 5 mM HEPES, 1.8 mM $CaCl_2$ and 1 mM $MgCl_2$). To locate S1 responses, the skull was illuminated with red light (630 nm) and thermal (3 second, 32-42 or 32-22^{o}C steps) or tactile (10 Hz contacts) stimuli were applied to the plantar side of the contralateral (right) forepaw. The thermal and mechanical stimulators were the same as used in behavior. Imaging was captured via a monochrome QIcam CCD camera (QImaging). Green light (530 nm) was used to view the blood vessel pattern.

ISOI data was processed using custom-written scripts in Igor (IGOR Pro) and Matlab (Mathworks). Reflection of red light was calculated over time and averaged across trials. The largest response for every S1 field of view was plotted across time and averaged across mice to generate a population mean for touch, cool and warm.

2.8 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Following painless killing and perfusion, the spinal cord and DRG tissue from adult Trpm8-ChR2 mice was fixed in 4% PFA for 90 min, incubated with 30% sucrose

overnight at 4ºC, frozen in Tissue-Tek and cryo-sectioned (30 µm slices, Leica cryostat). Sections were collected in superfrost glass slides. Afterwards, immunohistochemical stainings were carried out to visualize Trpm8-expressing neurons or projections. Antibodies used were sheep anti-GFP 1:2000 (Bio-Rad) (followed by donkey anti-sheep 1:1000, 488 nm, FITC) and NeuroTrace Nissl staining 1:200 (Thermo Fisher). Images were obtained via a confocal microscope (Zeiss LSM 800).

Images were analyzed using ImageJ / Fiji.

2.9 ANALYSIS OF BEHAVIORAL DATA

Mouse licks were recorded throughout the experiment by a capacitance sensor placed at the tip of the water spout that delivered rewards. Temperature of the thermal stimulator was controlled by a thermocouple wire located at the top of the Peltier element (at the interface between Peltier and glabrous skin of the forepaw). Licks that happened within a window of opportunity (3.5 s) after stimulus onset were categorized as “hits”, and their proportion was later compared to that of “false alarms”, *i.e.* licks during catch trials of the same time length where no stimulus was presented, in the same training session. The latency to the first lick reporting each stimulus (first lick latency, or reaction time) was also quantified, as well as the first lick during each catch trial.

To compare performance across sessions and between groups, d’ (sensitivity index) was used instead of percentage of correct trials. This was to take into account bias in the licking criterion (Carandini & Churchland, 2013). To calculate d’, the formula used was: $d' = z(h) - z(fa)$, where z(h) and z(fa) are the normal inverse of the cumulative distribution function of the hit and false alarm rates, respectively. To avoid infinity d’ values, when all (rate = 1) or none (rate = 0) of the trials were reported, the rates were replaced by 1-(1/2N) or (1/2N), respectively, where N is the number of total trials where the stimulus was presented (Macmillan & Kaplan, 1985). The z scores were calculated with OpenOffice Calc (Apache Software Foundation) using the function NORMINV.

Behavioral data was collected using custom-written routines in Lab View (National Instruments) at sampling rate 1 kHz, and custom-written Python (Python Software Foundation) scripts were used for analysis.

2.10 ANALYSIS OF SKIN-NERVE RECORDINGS

Cutaneous forepaw and hindpaw sensory afferent units were categorized based on their conduction velocity and their sensitivity to thermal and mechanical stimuli. Single unit responses to temperature represent a mean response of ≥3 stimulus repetitions. Thermal and mechanical thresholds of units were calculated as the temperature or mechanical amplitude required to elicit the first action potential. Skin-nerve electrophysiology data was collected and analyzed with Spike2 (CED Software). Spike latency data was analyzed using custom-written scripts in Python. Spike histogram graphs represent pooled data from multiple responses within and between C-fiber recordings in different animals.

2.11 STATISTICAL TESTS

Statistical analyses were carried out with GraphPad Prism 5.0/6.0 (GraphPad Software) and Python. Statistical tests for significance are stated in the text, and include two-way repeated-measures ANOVA with Bonferroni's post hoc test, Student t test, Mann Whitney test and Wilcoxon matched pairs test. Kolmogorov-Smirnov test was used to assess normality of the data. Asterisks in figures indicate statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3. RESULTS

3.1 MOUSE THERMAL PERCEPTION

3.1.1 Mice detect warming stimuli in a Go/No Go detection task.

In order to investigate the neurobiological mechanisms underlying warming perception, the first step was to characterize to what extent mice are capable of sensing warming. To do this, I used a Go/No Go detection task in mice, where warming (32-42ºC) stimuli were delivered to the plantar side of the right forepaw. This task was employed in Milenkovic et al., 2014., where cooling stimuli (32-22ºC) were delivered via a 3 x 3 mm thermal stimulator. The reason for choosing 32ºC as a baseline temperature was that human psychophysical studies have reported that temperatures around 30ºC are perceived as thermoneutral (Filingeri et al., 2017) and 32ºC is commonly chosen as a baseline temperature in afferent recording studies (Koltzenburg et al., 1997; Milenkovic et al., 2008; Vandewauw et al., 2018; Walcher et al., 2018; Zimmermann et al., 2009). On the other hand, temperatures above 43ºC are considered noxious and trigger pain responses (Deuis et al., 2017). Therefore, 42ºC was chosen as the highest temperature delivered in my experiments.

Prior to training mice to report forepaw warming, head-restrained wild type mice were first habituated to the behavioral setup and to tethering of the forepaw. Afterwards, mice underwent two sessions of pairing, where forepaw warming stimuli were delivered together with a reward, which was a water drop coming from a lick spout. This was done to help mice form a stimulus-reward association. Because water was used as a reward in the task, mice underwent a water scheduling protocol from the first pairing session. Once mice had shown interest to lick from the water spout during the pairing, they started the warming detection training.

In the behavioral training, head restrained mice had their right forepaw tethered to a thermal stimulator, with the glabrous skin contacting a Peltier element at a baseline of 32ºC (**Figure 4A**). At random intervals between 3 - 30 s, warming stimuli (32-42ºC) of short duration (4 s) were delivered. More specifically, stimuli consisted on a 0.5 s increasing ramp, followed by a 3 s hold phase, and finally 0.5 seconds decreasing ramp. During stimulus trials, mice had a time window of 3.5 seconds to lick from the water spout to obtain a reward. To account for spontaneous licking of the mice, catch trials of the same length were introduced, where no thermal stimulus or reward were delivered. The warming detection performance of mice was then assessed at the end of the session,

computing the percentage of stimulus trials that were correctly reported (hits), and comparing it to the percentage of catch trials when the mice licked from the sensor (false alarms) (Figure 4B). In most cases, the behavioral task lasted 100 trials (50 stimulus and 50 catch trials). However, the session was interrupted if mice missed 5 stimulus trials in a row, which indicated that mice were no longer interested in the water reward and motivation for the task was low.

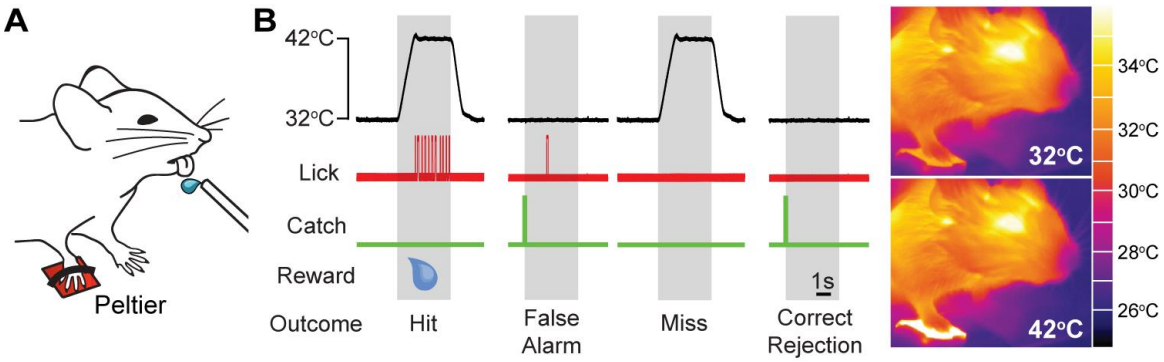

Figure 4. A behavioral task for warming detection.

(A) Schematic representation of the warming detection task. Head-restrained mice were trained to lick from a water spout when warming stimuli are delivered to the right forepaw, which was tethered to a thermal stimulator containing a Peltier element.

(B) (left) Structure of the goal-directed warming detection task. Warming stimuli of 32-42ºC were delivered in stimulus trials, randomized with catch trials. All trials were 3.5 seconds long (grey area). If mice licked during this time window (hit), they got a water reward. Hits were then compared to licks during catch trials (false alarms), to assess performance. (right) Thermal imaging capture of a trained mouse during a warming stimulus, without tethering of the paw.

Surprisingly, wild type mice did not reliably learn to report warming stimuli when delivered to the center of their forepaw in a 3 x 3 mm stimulation area (n = 7, 14 days of training, Figure 5A). This contrasted with cooling training, where delivery of stimuli with this Peltier size was shown to trigger robust perception in mice (Milenkovic et al., 2014). Seeing the inability of mice to report warming under these conditions, I increased the stimulation area and used a larger (8 x 8 mm) Peltier element, which covered most of the glabrous skin of the forepaw. Now, when warming stimuli were applied to the whole forepaw area during training, wild type mice successfully learnt to report 32-42ºC warming steps in 3-4 sessions, as indicated by statistically significant differences between hit and false alarm rates (n = 12, 10 days of training, Figure 5B-C). By the end of the training, hit rates were near 100%, whereas false alarms were roughly 30%

(Figure 5C). These results indicate that mice are able to reliably sense warming stimuli, but that warming detection depends greatly on spatial summation.

To further characterize warming perception in mice, I tested the perceptual threshold of warming-trained mice. To do this, the amplitude of the warming stimuli was progressively reduced in consecutive detection sessions in trained mice. Amplitudes of 6, 4, 2, 1 and 0.5ºC were tested, with the baseline always at 32ºC. Statistically significant differences between hit and false alarm rates were observed when the warming stimulus amplitude was larger or equal to 1ºC, but not when the stimulus was of 0.5ºC (n = 11, Figure 5D).

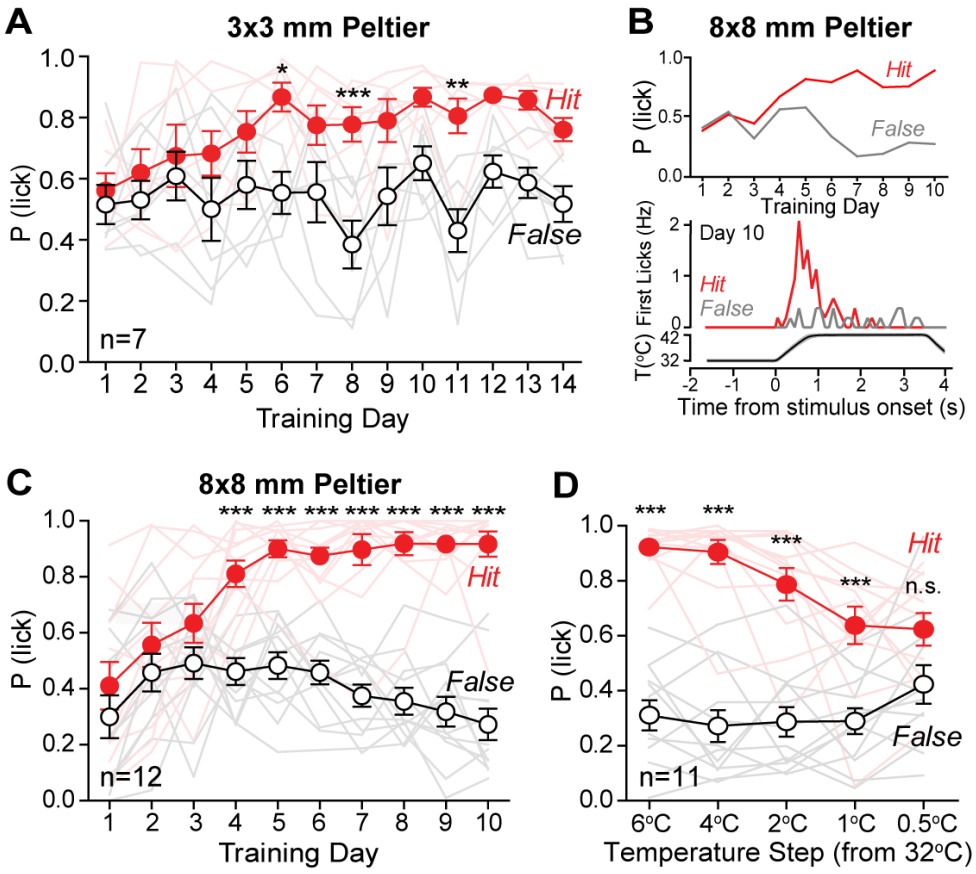

Figure 5. Mice report forepaw warming.

(A) WT mice did not reliably learn to report forepaw warming when delivered with a 3x3 mm thermal stimulator (n=7, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(B) (top) Hit and false alarm data from a representative mouse at session 10, trained to report warming with a larger 8x8 mm thermal stimulator. (bottom) Lick latencies during stimulus trials (red) and catch trials (grey) are time-locked to the onset of the warming stimulus.

(C) WT mice learnt to report warming when delivered with a 8x8 mm thermal stimulator, after 3-4 days of training (n=12, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(D) The warming stimulus amplitude was reduced over consecutive sessions and mice could still report 1-2ºC but not 0.5ºC as shown by differences between hit and false alarms (n=11, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

3.1.2 Mice detect warming with lower fidelity than cooling.

Because the stimulation area required by mice to perceive warming is larger than the surface required for cooling (**Figure 5A-C**) (Milenkovic et al., 2014), I hypothesized that mouse thermal perception is more sensitive for cooling than for warming. To test this, wild type mice were trained to report cooling steps of 32-22ºC (10ºC amplitude, as in warming), delivered via the same 8 x 8 mm Peltier element used for warming training (**Figure 5A**). Surprisingly, unlike warming-trained animals, mice trained to report cooling had a very high performance in the task from the very first session (n = 7 mice, **Figure 6B-C**). By the end of the training, mice could report cooling stimuli with near 100% of accuracy (hit rate), while keeping spontaneous licking (false alarms) below 10%. The behavioral training in this section (3.1.2) was carried out experimentally by Dr. Annapoorani Udhayachandran, and I performed all the analyses of the data.

Next, I compared the warming- and the cooling-detection performance of mice. Because I observed that warming-trained mice had higher false alarm rates, and differences in spontaneous licking may indicate a difference in criterion (willingness to participate in the task), I used d' (pronounced "d prime", an measure of sensitivity) instead of computing the percentage of correct trials (Macmillan & Creelman, 1991, 2005; Macmillan & Kaplan, 1985). D prime is commonly used in detection tasks to control for criterion (Carandini & Churchland, 2013). The formula to calculate it is d' = z(h) – z(fa), where z(h) and z(fa) are the normal inverse of the cumulative distribution function of the hit and false alarm rates, respectively (Macmillan & Kaplan, 1985). In this task (50 trials), the d' limit values are 0 and (roughly) 4. A sensitivity (d') value of 0 indicates that the hit and false alarm rates are the same (i.e. chance level). On the other hand, an almost perfectly-performing mouse that reports 49 / 50 stimuli (98 % hit rate) and licks to 1 / 50 catch trials (2 % false alarm rate) has a d' of 4.11 for that session (percentage correct = 98 %). In the literature, values of d' above 1.5 are considered good performance (Peron et al., 2015). For example, a mouse reporting 45 / 50 stimulus trials (90 % hit rate) and licking to 20 / 50 catch trials (40 % false alarm rate) would have d' = 1.53 (percentage correct = 75 %). Finally, negative d' values are also possible, and indicate that the false alarm rate is

higher than the hit rate. This can occur if mice actively avoid licking during the stimulus trials.

Warming-trained mice had lower d' values throughout the training sessions, compared to cooling-trained mice, indicating that mice find cooling easier to detect than warming (n = 12 warming-trained and n = 7 cooling-trained mice, **Figure 6D**).

Previously, the cooling perceptual threshold in mice was found to be 2ºC (starting from a 32ºC baseline) when delivering the cooling stimulus in a very small forepaw area with a 3 x 3 mm stimulator (Milenkovic et al., 2014). However, here I show that mice are capable of warming detection of stimuli as small as 1ºC, when delivered to the whole forepaw area with an 8 x 8 mm Peltier (**Figure 5E**). This, together with the fact that cooling-trained mice performed better throughout the training sessions than warming-trained mice (**Figure 6D**), led me to hypothesize that the cooling perception threshold is smaller than the warming-detection threshold. Therefore, cooling-trained mice were tested for smaller amplitudes in consecutive sessions (6, 4, 2, 1 and 0.5ºC, starting from a baseline of 32ºC). Mice could easily report cooling of 0.5ºC (n = 7 mice, $p < 0.0001$, **Figure 6E**). Therefore, as expected, mice can report cooling of smaller amplitude than warming.

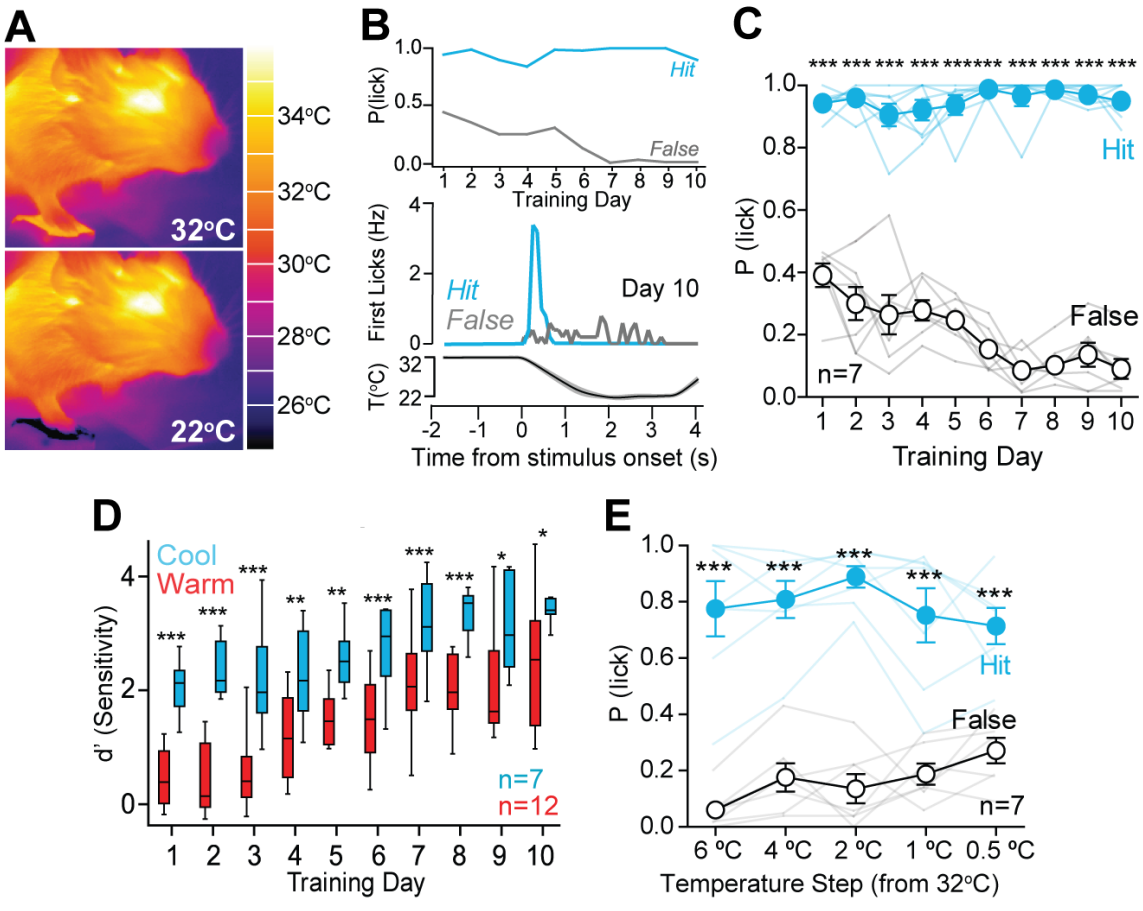

Figure 6. Mice report forepaw warming with less fidelity than cooling.

(A) Thermal imaging capture of a cooling-trained mouse during a cooling stimulus, without tethering of the paw.

(B) (top) Hit and false alarm data from a representative mouse at session 10, trained to report cooling (32-22^{o}C). (bottom) Lick latencies during stimulus trials (blue) and catch trials (grey) are time-locked to the onset of the cooling stimulus.

(C) WT mice learnt to report cooling stimuli of 32-22^{o}C, at the first day of training (n=7, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(D) Sensitivity (d') to report the stimulus was higher, throughout the 10 training sessions, in mice trained to report cooling (n = 7 cooling, n = 12 warming, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(E) The cooling stimulus amplitude was reduced over consecutive sessions and mice could still report 0.5^{o}C (n=7, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

Finally, I analyzed the latency to report the thermal stimuli of both warming- and cooling-trained mice (**Figure 7**). Throughout the training, the latencies to report warming were longer than the cooling detection latencies (**Figure 7A-D**). Warming-trained mice could report the 32-42^{o}C warming stimulus as quickly as 0.87 ± 0.07 s, whereas cooling-trained mice were able to report 32-22^{o}C with a latency of 0.31 ± 0.03 s (n = 12 warming-trained and n = 7 cooling-trained mice, data selected for each mouse from the training session with the fastest average latency, only from high performance sessions, i.e. d > 1.5). Interestingly, the latencies to report thermal stimuli were longer as the stimulus amplitude was reduced (**Figure 7E**).

Together, these results indicate that mice perceive thermal stimuli with high acuity but warming is perceived with lower fidelity than cooling. Both findings are similar to prior studies in humans and indicate that the mouse thermal system is an excellent, new model system for studies of sensory neurobiology (Claus et al., 1987; Stevens & Choo, 1998).

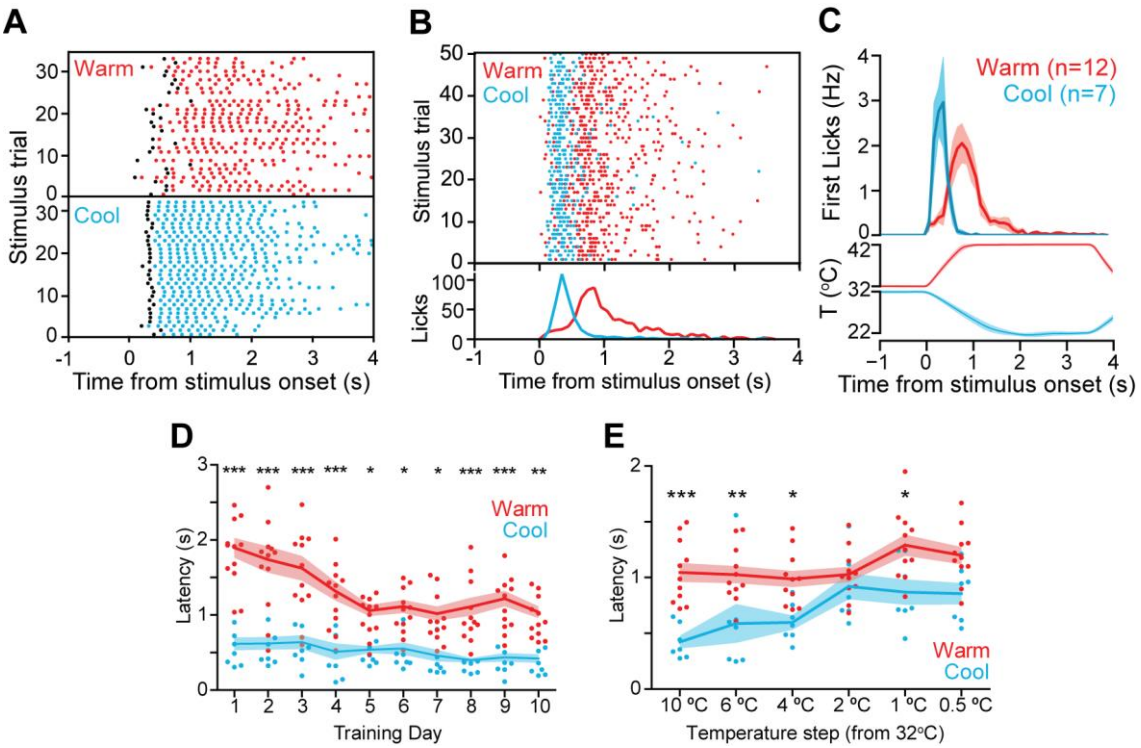

Figure 7. Mice report forepaw warming more slowly than cooling.

(A) Lick latencies of a warming-trained (top) and cooling-trained (bottom) mice on the training session 10. In each trial, the first lick is shown in black.

(B) First licks only, for all mice, throughout the last (10th) training session of cooling (blue) and warming (red) detection.

(C) (top) First lick latency PSTH of all mice trained for warming and cooling on their fastest session, chosen from sessions of good performance (d' > 1.5 or highest). (bottom) average temperature trace during cooling and warming detection sessions shows similar dynamics, with the cooling stimulus having a slightly slower ramp.

(D) Latency to report warming and cooling stimuli, across training sessions (n = 12 warming and n = 7 cooling, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(E) Latency to report warming and cooling stimuli of decreasing amplitudes (n = 11 warming and n = 7 cooling, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

3.1.3 Mice discriminate warming from cooling.

Next, I investigated whether warming and cooling stimuli elicit unique percepts in mice (i.e. if mice can discriminate warming from cooling stimuli). To do so, both warming (32-42°C) and cooling (32-22°C) stimuli were delivered to the forepaw of warming-trained

mice (**Figure 8A**). Warming-trained mice withheld licking to the 32-22°C cooling stimuli and only reported the 32-42°C warming steps, which indicated that mice discriminate warming from cooling (n = 7, **Figure 8B**). Interestingly, these mice licked during the re-warming phase (22-32°C) of the cooling stimuli (**Figure 8B**). This indicated that mice learn to report relative warming, rather than absolute temperature, in this task.

A similar experiment but in cooling-trained mice (**Figure 8C**) led to a similar result. Mice trained to report cooling (32-22°C) did not lick to the unrewarded warming stimuli (32-42°C), but licked to the re-cooling phase (42-32°C) taking place immediately after (n = 7 mice, **Figure 8D**).

Together, these data indicate that, as in humans, warming and cooling elicit unique and distinguishable percepts in mice. My work provides the ground work for future studies to train mice on two-alternative forced choice discrimination tasks (warm: lick left; cool: lick right) that will allow physiological recordings to be combined with behavior.

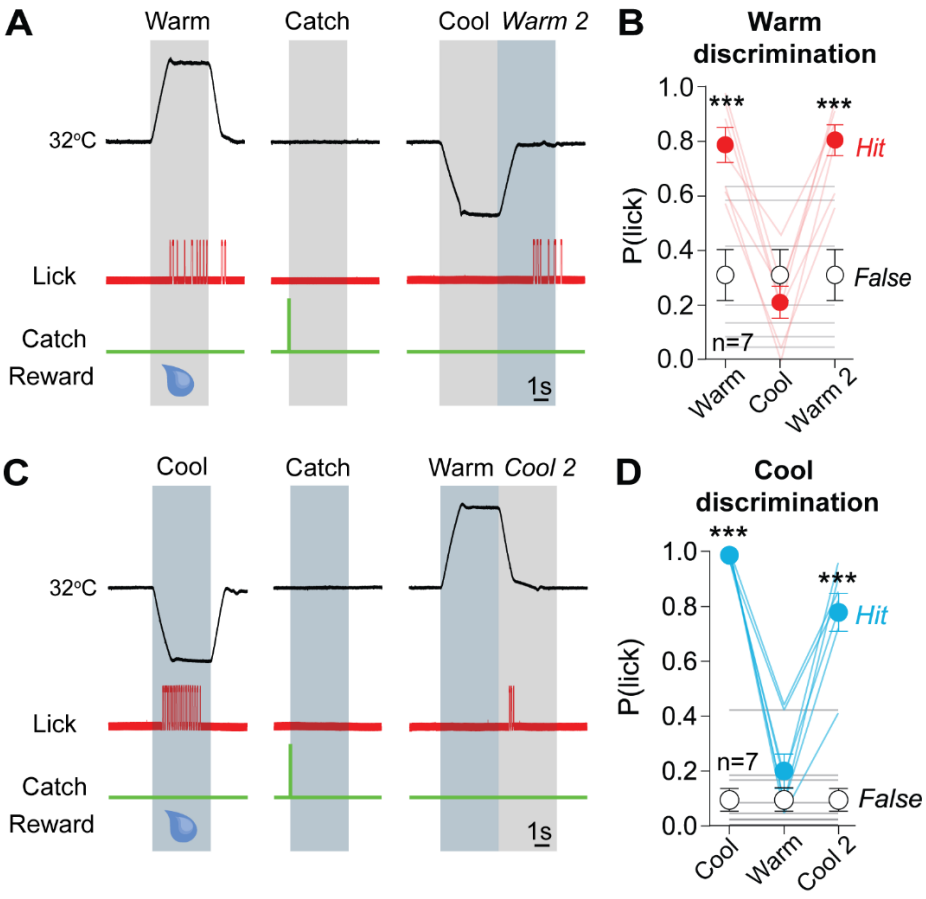

Figure 8. Mice discriminate warming from cooling.

(A) Structure of the warming discrimination task. Cooling trials (32-22°C, unrewarded) were introduced in the warming detection task.

(B) Warming-trained mice withheld licking to cooling stimuli in the warming discrimination task. Moreover, they licked to the re-warming phase (Warm 2, unrewarded) occurring when the temperature returned to baseline after a cooling stimulus.

(C) Structure of the cooling discrimination task. Warming trials (32-42ºC, unrewarded) were introduced in the cooling detection task.

(D) Cooling-trained mice did not lick the sensor during warming trials, but licked during the re-cooling phase taking place after a warming stimulus (Cool 2, unrewarded).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

3.1.4 Warming perception is enhanced at a lower baseline

In humans, thermal perception has been shown to depend on baseline temperature even within the non-noxious range (Hilz et al., 1995). To test whether this is true also for mice, I investigated warming perception at a lower baseline of 22ºC. Wild type mice were trained to report warming steps of 22-32ºC, and quickly learnt the task (n = 6 mice, p < 0.0001 training session 2, **Figure 9A**). Furthermore, warming perceptual threshold testing revealed that, with a baseline of 22ºC, mice can sense warming steps of as small as 0.5ºC (n = 6, p < 0.0001, **Figure 9B**).

Because mice trained to report warming of 32-42ºC needed longer to learn the task (**Figure 5C**) and could not reliably detect stimulus amplitudes below 1ºC (**Figure 5D**), I hypothesized that warming sensitivity is enhanced with a lower baseline. To investigate this, I first carried out an analysis of detection performance (d') between mice trained at 32-42ºC and at 22-32ºC, which revealed that mice reporting warming at the lower baseline had a better performance on several sessions (**Figure 9C**). Similarly, computing the average sensitivity across all training sessions showed that mice reported warming of 22-32ºC with higher precision than 32-42ºC (n = 12 and d' = 1.42 ± 0.12 for mice trained at 32-42ºC, n = 6 and d' = 2.48 ± 0.24 for mice trained at 22-32ºC, p = 0.0004 student t test, **Figure 9D**).

To further test this hypothesis in the same mice, I assessed the ability of warming-trained mice to detect warming stimuli at a low (22ºC) and high (32ºC) baseline, in consecutive days. Despite being able to report both types of stimuli (**Figure 9E**), mice performed better when detecting warming at a lower baseline (22-32ºC), as shown by higher sensitivity (d') indices than when reporting warming of 32-42ºC (n = 6 mice, p = 0.0014, mean d' = 3.43 ± 0.26 for 22-32ºC versus d' = 2.05 ± 0.36 for 32-42ºC, **Figure 9F**).

Next, I compared the warming detection latencies between mice trained to report warming at low (22ºC) and high (32ºC) baselines. Analysis of the population-averaged peri-stimulus time histograms (PSTHs) for both groups revealed a slightly sharper lick distribution for the mice trained at 22-32ºC on the session with the fastest latency (**Figure 9G**). Moreover, the fastest (session average) latency achieved by mice trained at 32-42ºC was 0.87 ± 0.07 s, and was significantly slower than the latency of mice trained at 22-32ºC, which was of 0.59 ± 0.04 s (n = 12 for mice at 32-42ºC, n = 6 for mice at 22-32ºC, p = 0.023, **Figure 9H**).

Together, these findings suggest that, in mice, perception of warming stimuli is enhanced when the baseline is below the human thermoneutral (~30) range (Filingeri et al., 2017).

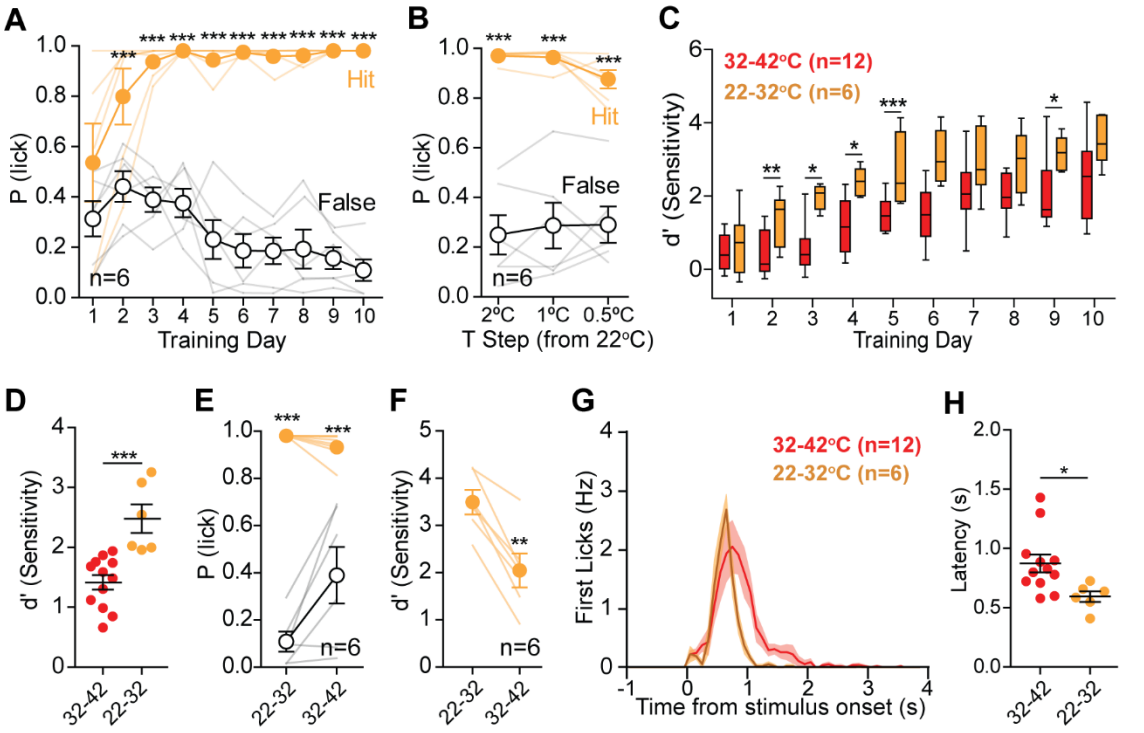

Figure 9. Mouse warming perception is enhanced with lower baseline.

(A) WT mice learn to report warming stimuli with lower baseline (22-32ºC), on average, since the 2nd training session (n = 6, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(B) WT mice detect a warming stimulus of as little as 0.5ºC when delivered from a lower baseline (22ºC) (n = 6, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(C) Sensitivity (d') to report warming stimuli of 10ºC, throughout the training, in mice trained at 32ºC (red, n = 12) and 22ºC (orange, n = 6) baseline (two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(D) Average sensitivity (d') achieved during the 10 training sessions was higher for mice trained to report warming of 10ºC from the lower baseline (n = 12 higher baseline and n = 6 lower baseline WT mice, unpaired t test).

(E) WT mice trained to report warming of 22-32ºC were tested for warming detection of 32-42ºC, and correctly reported these warming stimuli as well (n = 6, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(F) However, mice trained to report 22-32ºC reported stimuli of 32-42ºC with less fidelity (n = 6, p = 0.0014, paired t test).

(G) First lick latency PSTH of mice trained for warming of 22-32 and 32-42ºC on their fastest session, chosen from sessions of good performance (d' > 1.5 or highest).

(H) Mean latency to report warming of 22-32ºC (n = 6) was shorter than 32-42ºC (n = 12) on the fastest session, chosen from sessions of good performance (d' > 1.5 or highest) (p = 0.023, unpaired t test).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

3.1.5 Cooling perception is similar, but slower at a lower baseline

Next, I aimed to investigate whether the cooling perceptual abilities of mice are also different when using a lower (22ºC) baseline. Therefore, I trained wild type mice to report forepaw cooling stimuli of 22-12ºC. As in cooling-trained mice at 32-22ºC, mice trained at 22-12ºC had a robust stimulus detection performance since the first training session (n = 6, p < 0.0001, **Figure 10A**), and these mice could also perceive cooling steps as little as 0.5ºC very robustly (n = 6, p < 0.0001, **Figure 10B**). A comparison between the sensitivity of the two cooling-trained animal groups revealed similar performance throughout the training sessions (n = 7 for mice trained at 32-22ºC, n = 6 for mice trained at 22-12ºC, **Figure 10C**). Likewise, the average detection performances across all training sessions was not different between the two cooling-trained groups (**Figure 10D**), which greatly contrasted with warming (**Figure 9D**).

Next, I analyzed the mouse lick latencies to report cooling of 22-12ºC and compared them to those of mice trained at 32-22ºC. Surprisingly, the latencies to report cooling at the lower baseline (22-12ºC) were significantly slower than the latencies to report cooling at 32-22ºC, as shown by population PSTHs of the fastest session (n = 7 for 32-22ºC and n = 6 for 22-12ºC, **Figure 10E**) and fastest session-average latency achieved. High baseline cooling (32-22ºC) was reported as fast as 0.31 ± 0.03 s, whereas low baseline cooling was reported more slowly, 0.75 ± 0.06 s after stimulus onset (n = 7 for 32-22ºC and n = 6 for 22-12ºC, p < 0.0001, **Figure 10F**).

Together, these results indicate that perception of cooling is also remarkably robust in mice with a lower baseline of 22ºC, with no sensitivity differences in detecting amplitudes between 0.5 and 10ºC compared to experiments with baseline at 32ºC. However, unexpectedly, the detection of cooling stimuli at a lower baseline is slower than at a baseline around thermoneutral (~30ºC) values.

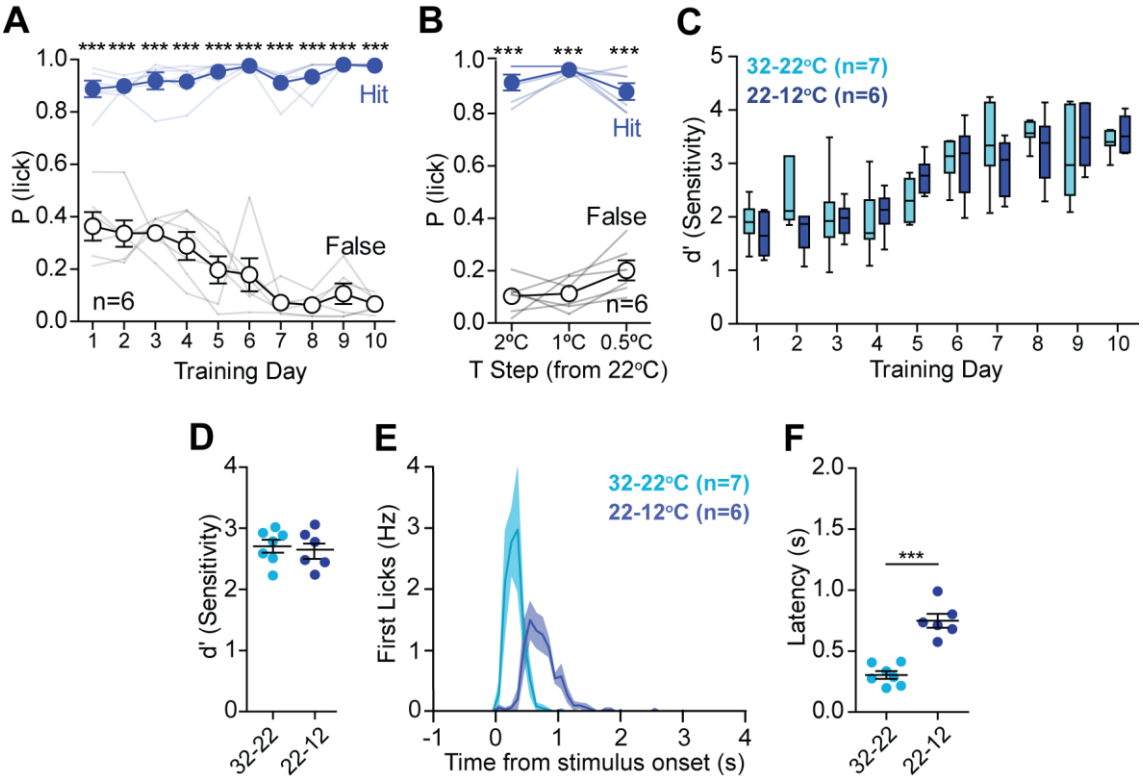

Figure 10. Mouse cooling perception is similar, but slower with lower baseline.

(A) WT mice learn to report cooling stimuli with lower baseline (22-12ºC) since the 1st training session (n = 6, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(B) WT mice detect a cooling stimulus of as little as 0.5ºC when delivered from a lower baseline (22ºC) (n = 6, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(C) Sensitivity (d') to report cooling stimuli of 10ºC, throughout the training, in mice trained at 32ºC (light blue, n = 7) and 22ºC (dark blue, n = 6) baseline (two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(D) Average sensitivity (d') achieved during the 10 training sessions was similar between mice trained to report cooling of 10ºC from 22ºC and 32ºC baseline (n = 7 higher baseline and n = 6 lower baseline WT mice, unpaired t test).

(E) First lick latency PSTH of mice trained for cooling of 22-12ºC and 32-22ºC on their fastest session, chosen from sessions of good performance (d' > 1.5 or highest).

(F) Mean latency to report cooling of 32-22ºC (n = 7) was shorter than 22-12ºC (n = 6) on the fastest session, chosen from sessions of good performance (d' > 1.5 or highest) ($p < 0.0001$, unpaired t test).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

3.1.6 Mice detect warming and cooling with slower ramps

Because there is evidence in humans that the ability to perceive small thermal stimuli depends on rate of temperature change (Kenshalo et al., 1968; Pertovaara & Kojo, 1985; Swerup & Nilsson, 1987), I went on to test whether mice were still able to reliably report warming and cooling stimuli when delivered to the forepaw in a slower, constant ramp. For that purpose, animals that were trained to report quickly ramping (10ºC/s) warming (22-32ºC and 32-42ºC) and cooling (of 22-12ºC and 32-22ºC) were tested for the perception of slowly-ramping warming or cooling stimuli, respectively. In order to keep the same stimulus amplitude (10ºC) and length (3.5 s), the slow stimuli were presented as ramps with a constant rate of change of 2.86ºC/s.

Despite the stimulus rate of change was reduced, warming-trained mice could report slow warming of 22-32ºC and of 32-42ºC, and cooling-trained mice could report slow cooling of 32-22ºC and of 22-12ºC, very reliably (n = 6 for all groups, $p < 0.0001$ hit vs false alarm rates, **Figure 11**, **Figure 12**). However, when comparing the performance in the same mice reporting fast and slow thermal stimuli, a false alarm increase while reporting slow stimuli was observed in most cases (all except warming at 32-42ºC, **Figure 13A**). As a consequence, sensitivity (d') was significantly reduced for slow warming at 22-32ºC and for slow cooling of 32-22ºC and 22-12ºC, when comparing it to the performance of the same mice reporting these stimuli delivered with a fast ramp (n = 6 for all groups, **Figure 13B**). Interestingly, the false alarm rates and d' when detecting fast or slow warming at 32-42ºC remained similar (**Figure 13A-B**). This may be due to the fact that false alarm rates while detecting fast ramping 32-42ºC were already higher than with the rest of the fast stimuli.

Together, these results indicate that mice can sense warming or cooling stimuli when delivered with fast and slow ramps. However, as in humans, the rate of temperature change seems to positively correlate with the detection performance (Green & Akirav, 2010), suggesting conserved mechanisms of temperature encoding in both species.

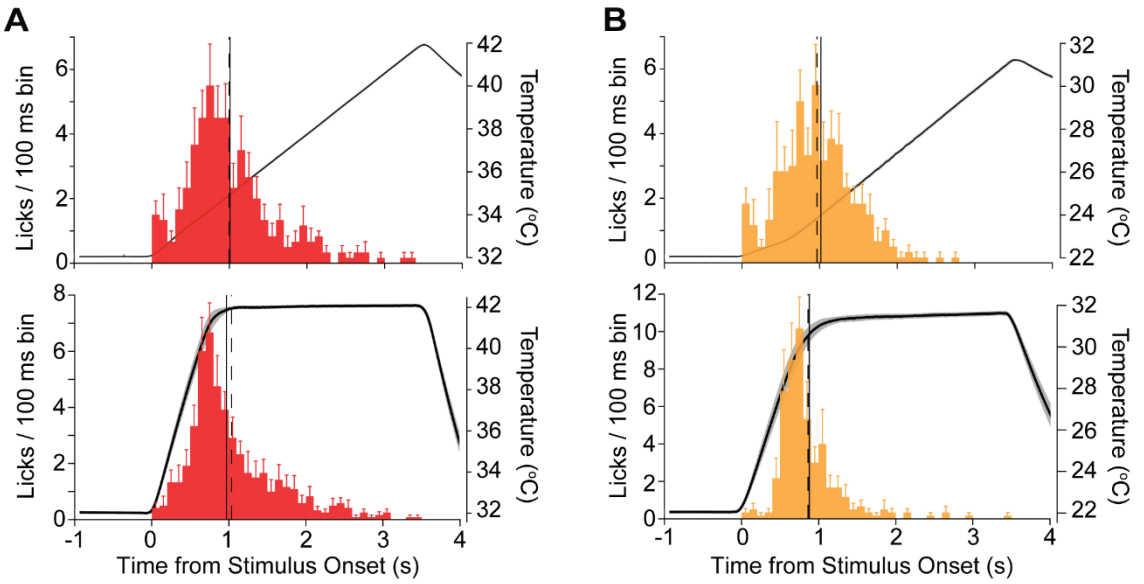

Figure 11. Mice detect warming delivered in slower ramps.

(A) First lick PSTH of WT mice reporting slow (top, n = 6) and quick (bottom, n = 6) warming of 32-42°C, overlaid with the average temperature trace during stimulus trials. Median and mean are shown as a line and a dotted line, respectively, in all panels.

(B) First lick PSTH of WT mice reporting slow (top, n = 6) and quick (bottom, n = 6) warming of 22-32°C, overlaid with the average temperature trace during stimulus trials.

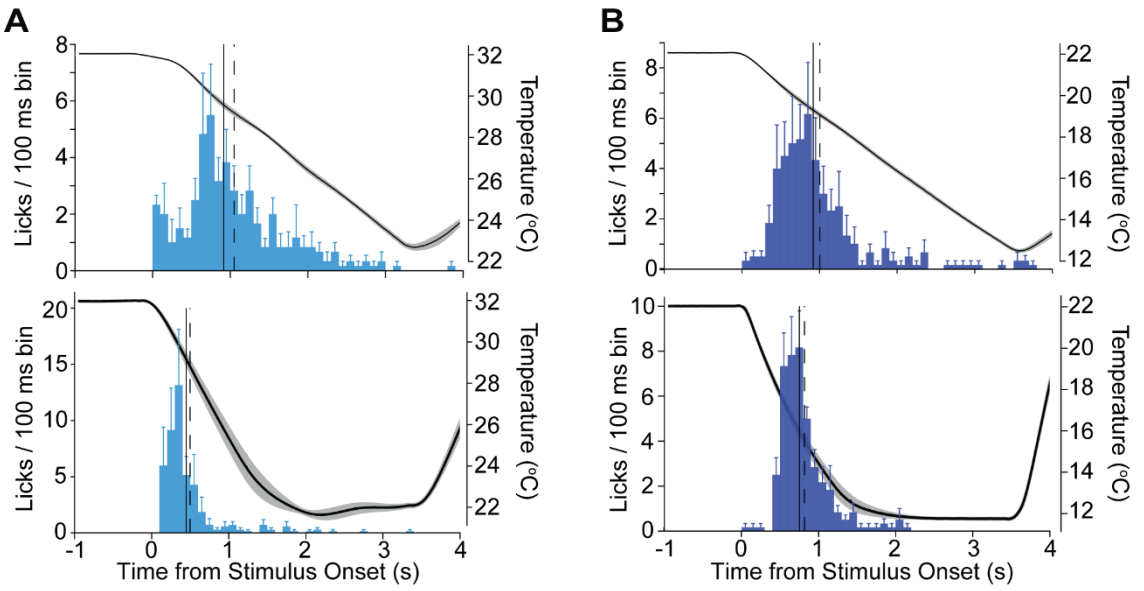

Figure 12. Mice detect cooling delivered in slower ramps.

(A) First lick PSTH of WT mice reporting slow (top, n = 6) and quick (bottom, n = 6) cooling of 32-22°C, overlaid with the average temperature trace during stimulus trials. Median and mean are shown as a line and a dotted line, respectively, in all panels.

(B) First lick PSTH of WT mice reporting slow (top, n = 6) and quick (bottom, n = 6) cooling of 22-12ºC, overlaid with the average temperature trace during stimulus trials.

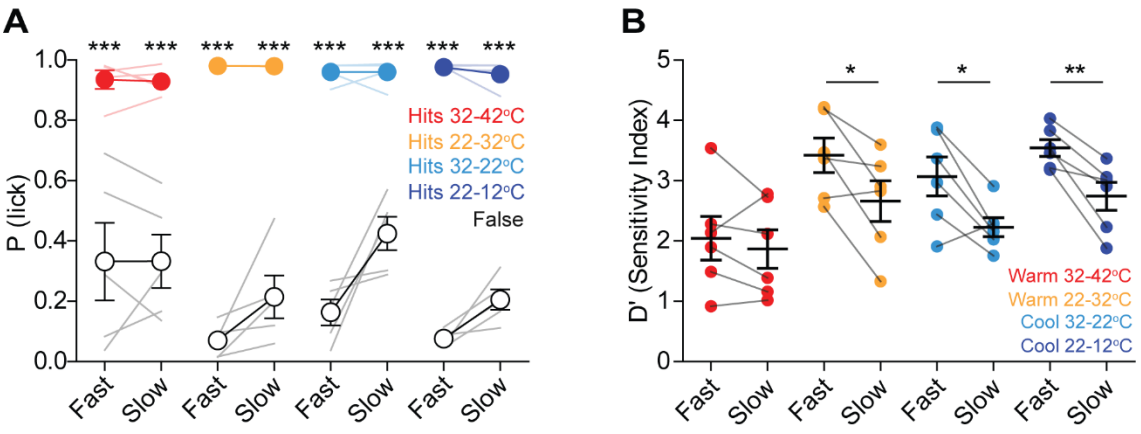

Figure 13. Thermal detection at slow and fast ramp stimulation.

(A) WT mice are able to report fast and slow thermal ramps of warming (32-42 and 22-32ºC) and cooling (32-22 and 22-12ºC), as shown by differences between hits and false alarms (n = 6 for all groups, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(B) Mice had a higher sensitivity (d') when reporting fast stimuli, for warming of 22-32ºC (n = 6, p = 0.0364, paired t test) and cooling of 32-22ºC (n = 6, p = 0.0285, paired t test) and of 22-12ºC (n = 6, p = 0.0065, paired t test). This was likely due to an observed increase of false alarms in (A), as the hits remained always near 100%.

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

3.2 SENSORY AFFERENT ENCODING OF WARMING

To comprehend the peripheral mechanisms that lead to distinct and robust percepts of warming and cooling in mice, I collaborated with Dr. Frederick Schwaller, a scientist from the laboratory of Professor Dr. Gary R. Lewin. Dr. Schwaller performed *ex vivo* recordings of dissected sensory afferents that innervate the mouse forepaw (medial and ulnar nerves), using a well-established skin-nerve protocol (Koltzenburg et al., 1997; Koltzenburg & Lewin, 1997; Walcher et al., 2018). He obtained single unit recordings of afferent fibers in response to thermal stimuli, which we jointly analysed and interpreted in combination with my behavioral data (Paricio-Montesinos et al., 2020). Data from behavior motivated some afferent recording experiments, and vice-versa. The results presented here are therefore generated from experiments carried out by Dr. Frederick Schwaller. My contribution in this section consisted on planning, analysis and interpretation of these experiments.

3.2.1 Warming activates a subset of C-fiber afferents

Skin-nerve recordings of single afferent fibers from medial and ulnar nerves of wild type mice were carried out to identify the sensory neurons that underlie forepaw thermosensation. The forepaw skin together with the ulnar and medial nerves were dissected and the tissue was maintained in a buffer at 32°C, as it is a standard in the field (Koltzenburg & Lewin, 1997; Milenkovic et al., 2014; Walcher et al., 2018). Afterwards, in order to screen for thermosensitive neurons, slow (1°C/s) ramps of thermal stimuli (32-48°C to find warm- and heat- sensitive fibers and 32-12°C to find cool-sensitive units) were applied to the skin via a Peltier element (3 x 3 mm surface), and action potential responses were recorded in afferent fibers.

From all thermosensory afferent fibers found, a large subset (28 / 37, n = 9 animals) showed spiking responses to non-noxious warming and noxious heat (**Figure 14A-B**). All fibers sensitive to temperature increases (either warm- or heat-sensitive) had little or no activity at rest (32°C), and monotonically raised the firing rate as temperature increased (**Figure 14B**). Fibers activated by warming within the noxious range (below 42°C) were termed warm-activated. Interestingly, all warm-activated fibers had conduction velocities below 1.2 ms^{-1}, indicating they have unmyelinated (C) axons (**Figure 14C**).

C-fibers can be classified according to the stimulus modalities that they respond to (Fleischer et al., 1983; Lewin & Mendell, 1994). C-fibers activated by heat and tactile stimuli are termed C-Mechanoheat (C-MH) and, if they also respond to cold, C-Mechanoheatcold (C-MHC). C-fibers activated by touch and cold are called C-Mechanocold (C-MC). Finally, C-Cold (C-C) and C-Heat (C-H) are fibers insensitive to touch but activated by cold and heat, respectively. In these experiments, all warm-activated fibers were found to be polymodal, as they were sensitive to mechanical stimuli (20 / 37 C-MH and 6 / 37 C-MHC, **Figure 14D**). Moreover, 23% (6 C-MHC out of 26 warm-activated) of all warm-activated fibers showed spiking activity also in response to cooling stimuli (**Figure 14C-D**).

Next, to investigate the afferent responses evoked by the forepaw warming with the same stimuli used in behavioral experiments, short (3.5 s) warming steps of 32-42°C were used as a stimulus in skin-nerve recordings. Warm-activated fibers showed spiking responses to this stimulus, reaching a maximum firing rate of ~8 Hz, but only ~ 2 Hz in the first second after stimulus onset (n = 21 warm-activated fibers, **Figure 14E**). Furthermore, reducing the stimulus amplitude led to a reduction in total number of evoked spikes (**Figure 14F**).

Together, this data indicates that forepaw warming activates a subset of C-fiber polymodal afferents.

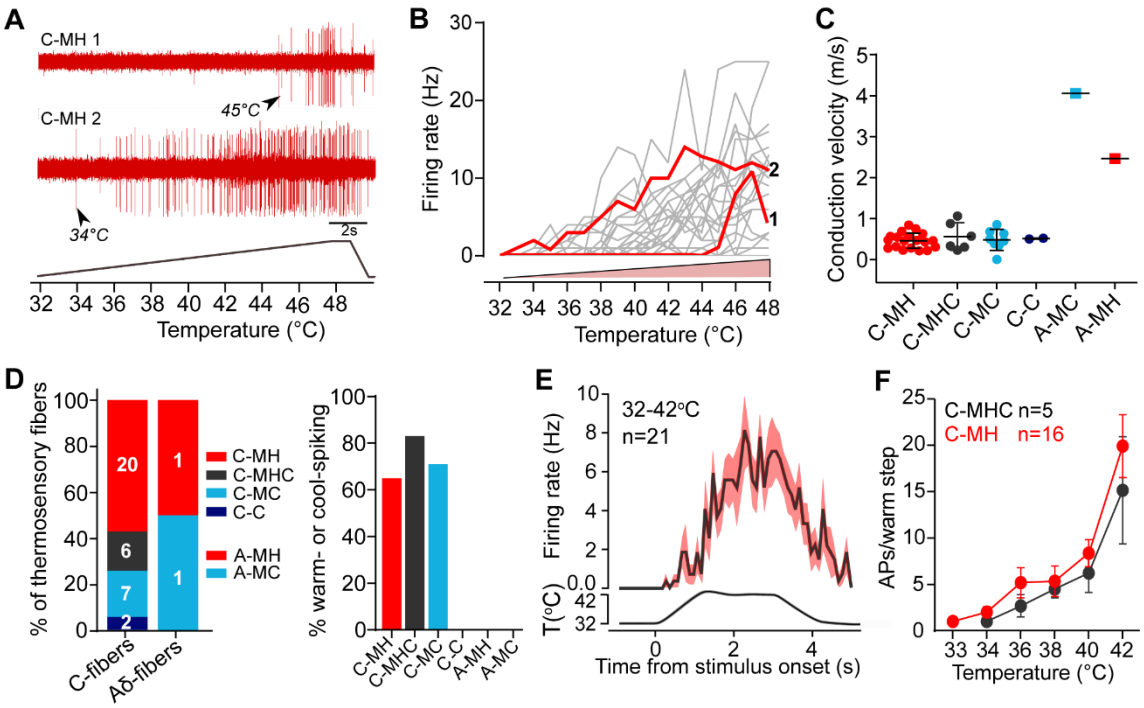

Figure 14. Warming activates C-fiber afferents in the mouse forepaw.

(A) Representative C-MH fibers firing action potentials during a slow (1°C/s) heating ramp of 32-48°C.

(B) Firing rates of all heat-activated fibers during a 1°C/s, 32-48°C heating ramp. Examples from panel (A) are depicted in red.

(C) Conduction velocities were slow (< 2 m/s) in most heat- and cold-spiking thermosensitive fibers identified by cold (1°C/s, 32-12°C) and hot (1°C/s, 32-48°C) ramps, and therefore these units were classified as unmyelinated C-fibers.

(D) (left) Proportions of thermosensitive C- and A-fibers found to fire action potentials during warming (32-42°C) or heating (43-48°C) (C-MH, C-MHC and A-MH), and cooling (32-12°C) (C-MC, C-MHC, C-C and A-MC). (right) Only C-fibers responded to non-noxious warming (32-42°C) or low-intensity (32-22°C) cooling.

(E) PSTH of mean spike rate evoked by brief (3.5 s) warming stimuli of 32-42°C in warming-excited afferent fibers (n = 21).

(F) Mean action potentials evoked by different warming stimuli was similar in C-MH (n = 16) and C-MHC (n = 5) fibers.

3.2.2 Warming silences C-fibers with cool-driven ongoing activity

The skin-nerve afferent recordings shown previously were performed with a buffer at 32ºC to preserve the tissue. This temperature of choice has been widely used in studies of rodent afferents (Koltzenburg et al., 1997; Lynn & Carpenter, 1982; Zimmermann et al., 2009), based on the assumption that the rodent forepaw and the human hand have a similar temperature of ~32ºC (Asahina et al., 2013; Ceron et al., 1995). However, I carried out infrared (thermal) imaging of the forepaw of wild type mice, and found that mouse forepaw has a temperature around 27ºC, with the wrist being warmer (~28ºC) than the fingertips (~26ºC) (**Figure 15A**). This finding motivated a further exploration of the warming and cooling encoding in the mouse sensory afferents, given that prior recordings were carried out while preserving the tissue at 32ºC.

Therefore, *ex vivo* skin-nerve afferent recordings of wild type mice were performed again while maintaining the tissue at a more physiological temperature of 27ºC, keeping the same Peltier baseline of 32ºC as in previous recordings (**Figure 15A**).

Firstly, slow (1ºC/s) ramping stimuli were used to screen all thermosensitive units responsive to the 12-48ºC thermal range. The proportion of warm- and heat- as well as cool- and cold-excited units was similar to the previous set of experiments (**Figure 15B**).

Next, short warming (32-42ºC) stimuli of 3.5 s were used to detect sensory fibers that underlie the warming detection behavior. Many afferents were again found to be excited by warming (10/27, 37%). Again, warm-activated afferents were polymodal C-fiber Mechanoheat (C-MH) or Mechanoheatcold (C-MHC) fibers with no activity during baseline. The spiking activity of warm-activated afferents increased as the Peltier temperatures were higher (**Figure 15C**).

Besides warm-activated afferents, surprisingly, a new warming-responding population of afferents was found in these experiments. The new units were polymodal C-Mechanoheatcold (C-MHC) or C-Mechanocold (C-MC) that had spiking activity during baseline (between 0.2 and 6 Hz, **Figure 16A-B**) and were quickly silenced by warming stimuli, mirroring the activity of warm-activated units (**Figure 16C**). In addition, these fibers increased their firing rate upon cooling (**Figure 16D-E**). These sensory afferents were termed warm-silenced.

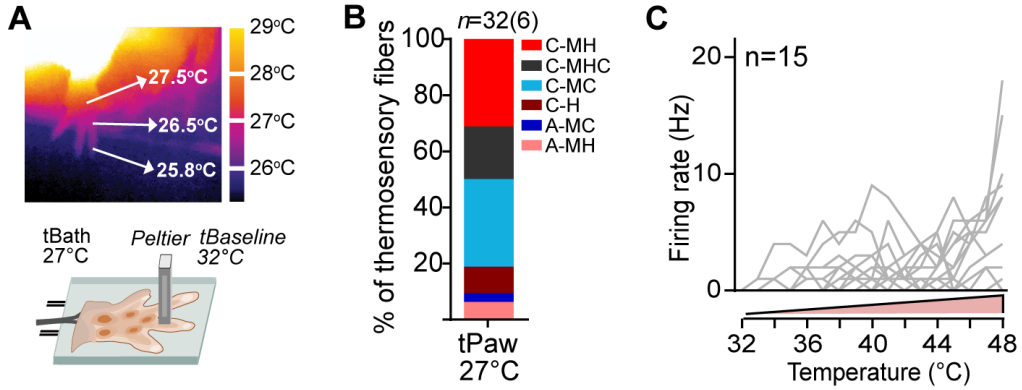

Figure 15. Warming-activated C-fibers at more physiological temperatures (27°C).

These findings indicate that forepaw warming silences a population of polymodal C fibers with cool-driven ongoing firing.

(A) (top) Thermal image of a mouse forepaw at room temperature shows that temperature at the paw is about 26-28°C, with the fingertips being cooler than the wrist. (bottom) Schematic of the skin-nerve single unit recordings, performed with a bath temperature of 27°C but with the Peltier stimulator surface at 32°C during baseline.

(B) Proportions of thermosensitive fibers firing action potentials to a heating ramp (1°C/s, 32-48°C) and cooling ramp (1°C/s, 32-12°C). Again, most thermosensitive units were polymodal C-fibers.

(C) Firing rate evoked by a heating ramp (1°C/s, 32-48°C) in all identified warming- or heating-activated fibers (n = 15).

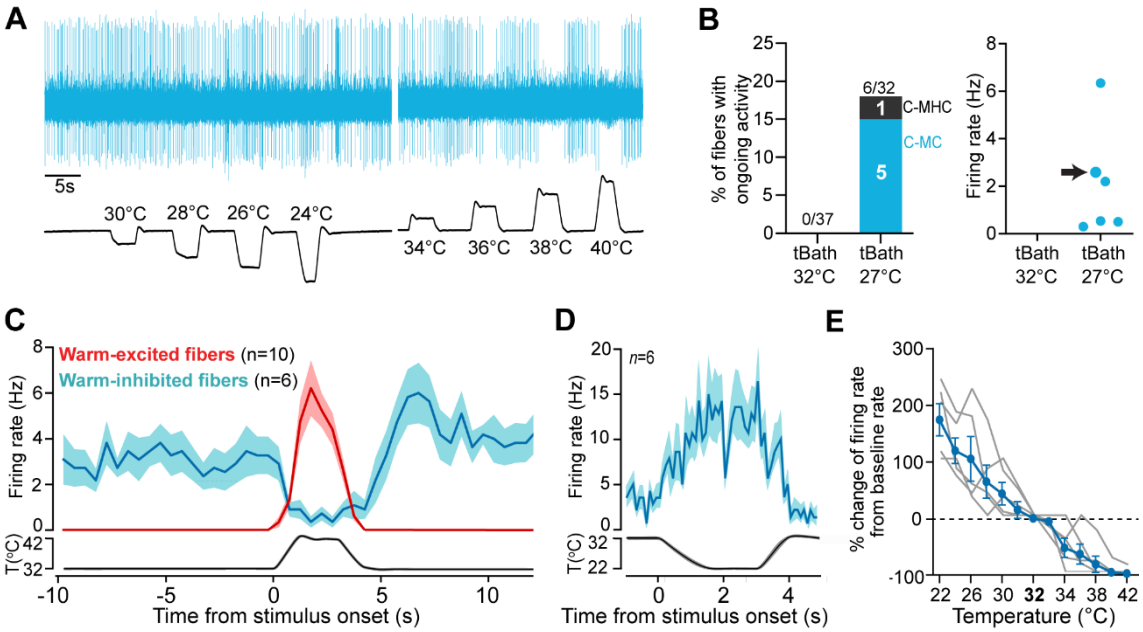

Figure 16. Warming silences a subset of C-fibers.

(A) Example firing activity of a thermosensitive fiber with ongoing firing activity during baseline. Cooling stimuli increased its baseline firing rate, and warming stimuli inhibited it.

(B) (left) Proportions of thermosensitive fibers with ongoing spiking during baseline, found under bath temperature of 32ºC (0/37) and 27ºC (6/32). (right) Firing rate during Peltier baseline of 32ºC of these fibers.

(C) PSTH of the spiking activity of warming-excited fibers (red, n = 10) and warming-inhibited fibers (blue, n = 6) found to respond to a warming stimulus of 32-42ºC under the conditions of buffer bath temperature 27ºC.

(D) PSTH of the spiking activity of the same warming-inhibited fibers, as a response to a cooling stimulus of 32-22ºC (n = 6).

(E) Effect of different temperatures on the firing rate of warming-inhibited fibers. Temperatures above 32ºC suppressed or completely silenced their firing rate, whereas cooling below 32ºC increased it.

3.2.3 Warm-activated responses depend on absolute temperature

Because mice were capable of detecting warming stimuli of 22-32ºC and even had an increased performance compared to sensing 32-42ºC (**Figure 9A,C-F**), the sensory afferent encoding of warming stimuli of 22-32ºC was explored. Therefore, *ex vivo* skin-nerve afferent recordings of the medial and ulnar nerves of wild type mice were carried out, keeping the tissue at the more physiological temperature of 27ºC and the Peltier at 22ºC.

First, slow (1ºC/s) ramps were used to find all thermosensitive afferents responding to stimuli between 12-42ºC, and functional warm- as well as cool-excited units were found (**Figure 17A**). Then, a constant Peltier baseline of 22ºC was set and short (3.5 s) warming stimuli of 22-32ºC were delivered. Interestingly, warming of 22-32ºC elicited very weak responses in warm-activated afferents (**Figure 17B**). This contrasted greatly with the robust responses observed upon warming of 32-42ºC (**Figure 17C**). On the other hand, warm-silenced responses to warming stimuli of 22-32ºC were present (**Figure 17B,D**) and similar to those observed upon warming of 32-42ºC (**Figure 16C**). Interestingly, the firing rate of warm-silenced units during 22ºC baseline was similar to their firing at baseline of 32ºC (**Figure 17D, Figure 16B**). This suggests that warm-silenced afferent neurons have some degree of adaptation.

These findings indicate that, on one hand, warm-activated sensory afferents encode warmth depending on the absolute temperature, with higher temperatures eliciting greater spiking responses. On the other hand, warm-silenced neurons adapt their baseline firing rate, and the inhibition they undergo as a response to warming does not depend on absolute temperature. Furthermore, these findings suggest that the perception of warming steps of 22-32ºC greatly depends on warm-inhibited sensory afferents, with little contribution of warm-activated fibers. On the other hand, warming steps of 32-42ºC elicit robust responses in both warm-activated and warm-inhibited afferents, suggesting that both fiber types contribute to the perception of this stimulus type.

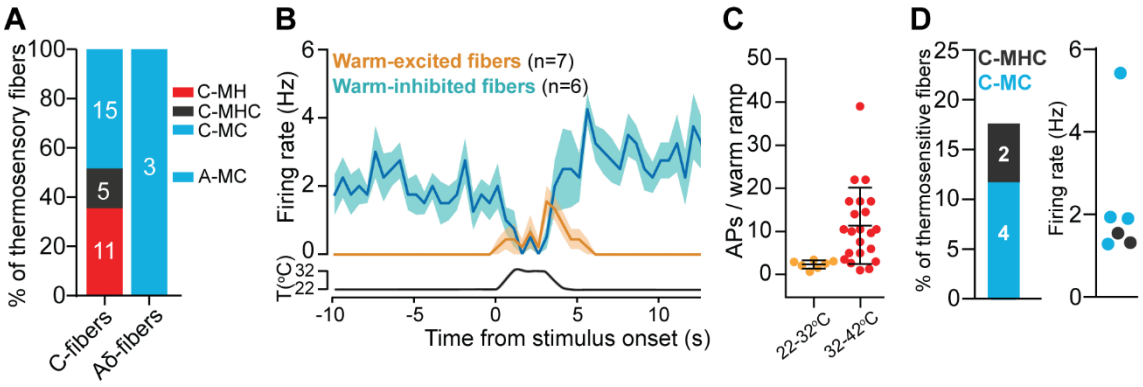

Figure 17. Warming of 22-32ºC is encoded mainly by warming-silenced fibers.

(A) Proportions of thermosensitive afferent fibers found to respond to temperatures between 12ºC and 42ºC, while keeping the tissue at 27ºC buffer temperature. Both cooling- and warming-spiking units were found.

(B) Warming-spiking units (n = 7) were mostly silent during warming steps of 22-32ºC, a step that can be easily detected by mice. On the other hand, warming-inhibited afferents (n = 6) were reliably silenced by these stimuli.

(C) Comparison of the total number of action potentials of warming-excited fibers evoked by warming of 22-32ºC and 32-42ºC.

(D) (left) Proportions and (right) baseline firing rate of warming-inihibited afferents found in experiments with Peltier baseline of 22ºC, and the tissue kept at 27ºC.

3.2.4 Cooling activates a subset of C-fiber afferents

Cooling-spiking C-fibers have been shown to be crucial for cooling perception in mice (Milenkovic et al., 2014). Consistent with this, the skin-nerve recording data presented here shows that non-noxious cooling activated only polymodal C-fibers (**Figure 14D**). To further investigate the sensory afferent encoding of forepaw cooling, brief (3.5 s) stimuli

of 32-22ºC and of 22-12ºC were also delivered during the same skin-nerve recording experiments presented earlier.

In experiments with Peltier baseline of 32ºC, robust cooling-evoked spiking was found in cool-activated C-Mechanocold (C-MC) and C-Mechanoheatcold (C-MHC) fibers (n = 14 fibers, **Figure 14D, Figure 15B**). Cooling of 32-22ºC evoked an average firing rate of up to 10-12 Hz in these units (**Figure 18A**). Similarly, in experiments with Peltier baseline 22ºC, cooling steps of 22-12ºC also elicited strong responses in C-MC and C-MHC fibers (n = 19 fibers, **Figure 17A**). Cooling of 22-12ºC evoked an average firing rate of up to 6-8 Hz, slightly below the spiking driven by cooling at 32-22ºC. Importantly, some of the cooling-spiking units presented in these two datasets are the warming-inhibited fibers identified earlier (n = 6 for 32-22ºC and n = 6 for 22-12ºC, **Figure 18B**).

These results indicate that forepaw cooling activates a subset of polymodal C-fiber afferents, some of which are active during baseline. Consistent with behavioral experiments, both cooling steps of 32-22ºC and 22-12ºC evoke robust spiking activity in cooling-excited sensory afferents.

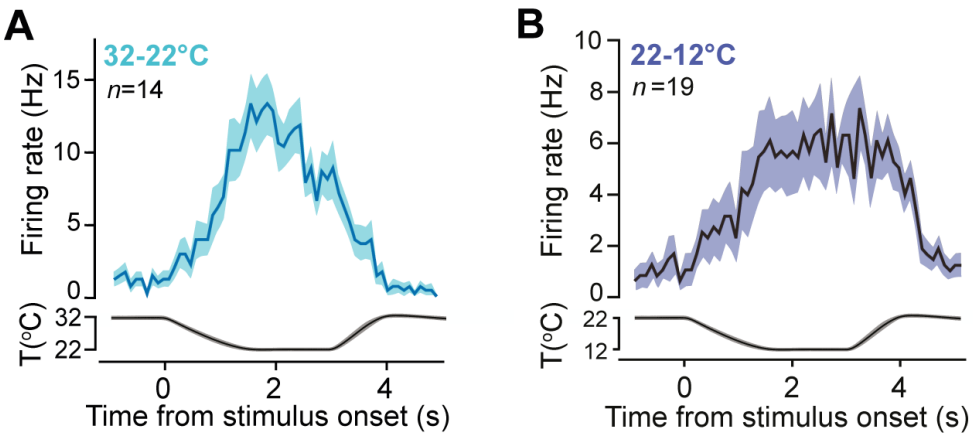

Figure 18. Cooling-spiking fiber responses to cooling at baseline 32ºC and 22ºC.

(A) PSTH of the spiking of cooling fibers (n = 14), evoked by a cooling stimulus of 32-22ºC.

(B) PSTH of the spiking of cooling fibers (n = 19), evoked by a cooling stimulus of 22-12ºC. While the responses of warming-excited fibers depended greatly on absolute temperature, cooling fibers responded similarly to cooling of 32-22ºC and 22-12ºC.

3.3 MOLECULAR TRANSDUCERS OF WARMING

Prior work had identified a family of ion channels involved in thermal sensing: the Transient Receptor Potential (TRP) channels. Their role in the perception of non-painful sensation has been controversial and based on innate, thermal avoidance behavioral tasks (Caterina et al., 2000; S. M. Huang et al., 2011; Tan & McNaughton, 2016; Vandewauw et al., 2018). Having already explored the features of warming and cooling perception in WT mice, as well as their afferent encoding properties of thermal stimuli, I therefore aimed to investigate which of these molecular sensors expressed in warming-encoding afferents play a role in the detection of warming.

3.3.1 Mice lacking TRPV1 detect warming stimuli

Despite initially classified as a noxious heat sensor (Caterina et al., 1997, 2000; Woodbury et al., 2004), the capsaicin (chili) receptor TRPV1 has been recently suggested as a molecular sensor required for the perception and peripheral encoding of warmth in the oral cavity (Yarmolinsky et al., 2016). To assess whether TRPV1 is required for the perception of warming stimuli in the forepaw, I trained mice that constitutively lack the TRPV1 receptor (*trpv1*$^{-/-}$) to report warming stimuli of 32-42^{o}C.

Interestingly, *trpv1*$^{-/-}$ mice could learn the warming detection task after a few sessions, with the hit and false alarm rates showing statistically significant differences (n = 8, **Figure 19A**), and similar latencies to report warming as in wild type mice (as shown by the lick PSTHs, **Figure 22A-B**). I next directly compared the performance of WT and *trpv1*$^{-/-}$ mice throughout the training sessions, and found that the sensitivity (d') of the two mouse groups, despite being lower on the last training session for the *trpv1*$^{-/-}$ mice (n = 12 wild type, n = 8 *trpv1*$^{-/-}$ mice **Figure 22E**), was very similar over the training (**Figure 22F**).

Once *trpv1*$^{-/-}$ mice underwent the warming training, I tested their warming perceptual threshold by reducing the stimulus amplitudes in subsequent sessions. I found that, similar to wild types (**Figure 5D**), *trpv1*$^{-/-}$ mice could report warming stimuli of just 1^{o}C above the baseline of 32^{o}C (**Figure 19B, Figure 22F**).

These findings indicate that the ion channel TRPV1 is not required for forepaw warming perception.

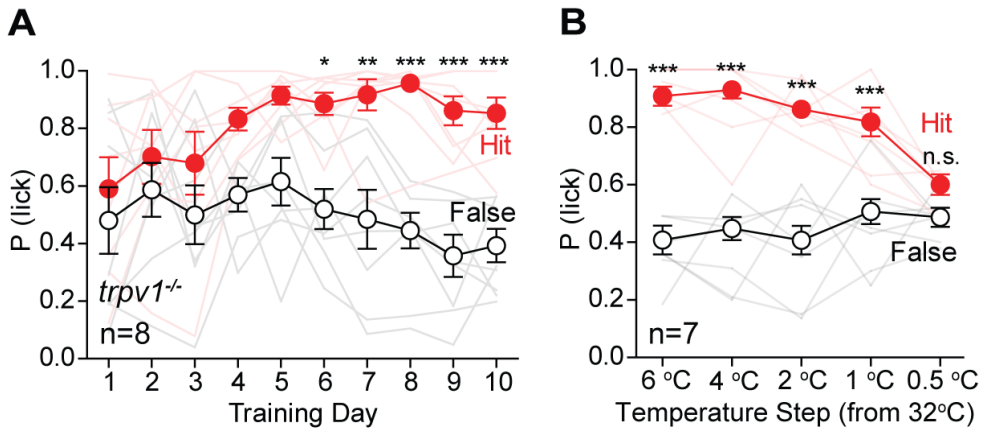

Figure 19. Mice lacking TRPV1 detect forepaw warming.

(A) Mice lacking TRPV1 learnt to report forepaw warming of 32-42°C after a few days of training (n = 8, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(B) Mice lacking TRPV1 were able to report warming steps of as little as 1°C, as indicated by different hit and false alarm rates (n = 8, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

3.3.2 Mice lacking TRPM2 detect warming with blunted sensitivity

The thermally gated ion channel TRPM2 has been recently proposed to play a role in warming perception (Tan & McNaughton, 2016) and in warm core temperature sensing in the hypothalamus (Song et al., 2016). Therefore, I next investigated the contribution of TRPM2 in warming perception by training TRPM2 knockout mice (*trpm2*$^{-/-}$) to report warming stimuli of 32-42°C in the forepaw.

Trpm2$^{-/-}$ mice could learn to report forepaw warming, as shown by statistically significant differences between hit and false alarm rates (n = 6, **Figure 20A**). However, analysis of the lick peristimulus time histograms showed that the average lick response peak during stimulus trials was not as pronounced as in WT mice, suggesting a poorer ability to detect the stimulus (n = 12 WT and n = 6 *trpm2*$^{-/-}$ mice, **Figure 22C**). Similarly, analysis of sensitivity (d') across warming detection sessions revealed that *trpm2*$^{-/-}$ mice detected warming stimuli less accurately than WT mice (**Figure 22E-F**). On the last day of training, *trpm2*$^{-/-}$ mice had an average sensitivity of d' = 1.03 ± 0.29, which was significantly smaller than the sensitivity of WT mice on the same session (d' = 2.45 ± 0.30) (**Figure 22E**).

Next, I tested the perceptual thresholds of forepaw warming in *trpm2*$^{-/-}$ mice. Warming of 2°C was correctly detected by *trpm2*$^{-/-}$ mice when starting from a 32°C baseline. However, the hit and false alarm rates of *trpm2*$^{-/-}$ mice were similar when the temperature amplitude was 1°C (**Figure 20B**), a temperature step that was detected by WT mice. This, together with the lower sensitivity (d') of *trpm2*$^{-/-}$ mice during threshold testing (**Figure 22F**), suggests that *trpm2*$^{-/-}$ mice have a higher perceptual threshold than WT mice.

Together, the warming perceptual abilities of *trpm2*$^{-/-}$ mice suggest that TRPM2 is partially involved but not absolutely required to perceive forepaw warming.

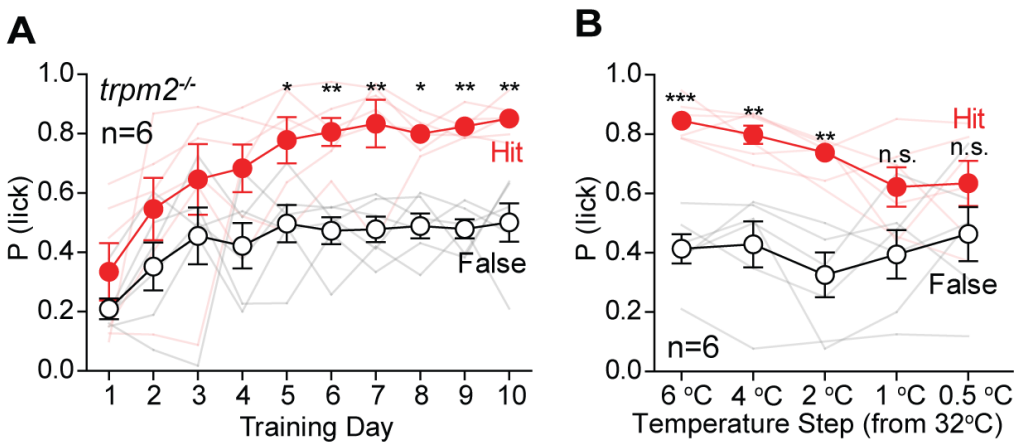

Figure 20. Mice lacking TRPM2 detect forepaw warming.

(A) Mice lacking TRPM2 learnt to report forepaw warming of 32-42°C after a few days of training (n = 6, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(B) Mice lacking TRPM2 were able to report warming steps of as little as 2°C, as indicated by different hit and false alarm rates (n = 6, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

3.3.3 Mice lacking TRPV1, TRPA1 and TRPM3 detect warming

The skin-nerve afferent data showed that the vast majority of warm-activated sensory neurons encode both warming and noxious heat via different spiking rates. This could mean that some alterations in the encoding of warmth may affect heat encoding, and vice-versa. Recently, a combination of three thermally gated TRP (TRPV1, TRPA1 and TRPM3) channels was shown to mediate acute heat perception in mice, and this accompanied a loss of heat-evoked spiking in sensory afferents (Vandewauw et al.,

2018). Given the strong behavioral phenotype (lack of avoidance of harmful temperatures of 50ºC) and the afferent encoding deficit observed in *trpv1:trpa1:trpm3*$^{-/-}$ mice, I aimed to investigate the possible contribution of these three combined channels in warming perception.

Surprisingly, triple knockout *trpv1:trpa1:trpm3*$^{-/-}$ mice learnt to report forepaw warming stimuli of 32-42ºC in a few sessions (n = 10, **Figure 21A**), like wild types. Moreover, the latency of the licks reporting warm stimuli resembled that of WT mice, with most licks happening within the first second after stimulus onset, and a large separation between the hit and false alarm lick distributions (**Figure 22D**). However, comparing the sensitivity (d') between *trpv1:trpa1:trpm3*$^{-/-}$ and WT mice throughout training sessions suggested that combined loss of TRPV1, TRPA1 and TRPM3 had minor effects on warming detection performance (**Figure 22E-F**). On the last training session, *trpv1:trpa1:trpm3*$^{-/-}$ mice had a sensitivity of d' = 1.28 ± 0.20, significantly lower than the WT sensitivity (n = 12 and d' = 2.45 ± 0.30 for WT mice, n = 10 for *trpv1:trpa1:trpm3*$^{-/-}$ mice, $p < 0.01$, **Figure 22E**).

Next, I decreased the stimulus amplitude during warming detection to investigate the warming perceptual thresholds of *trpv1:trpa1:trpm3*$^{-/-}$ mice. Interestingly, I observed statistically significant differences between hit and false alarm rates at all warming amplitudes tested, including 0.5ºC (n = 10, **Figure 21B**). However, the difference between hits and false alarms was smaller than in wild types, as shown by a generally reduced d' during threshold testing (**Figure 22F**).

Furthermore, I tested other features of thermal sensitivity of *trpv1:trpa1:trpm3*$^{-/-}$ mice. I found that *trpv1:trpa1:trpm3*$^{-/-}$ mice could detect cooling stimuli, also with small amplitudes (n = 6, **Figure 23A,C**); and warming stimuli of 22-32ºC (n = 10, **Figure 23B,D**).

Together, these results indicate that mice can sense warming and cooling stimuli without TRPV1, TRPA1 and TRPM3 receptors. However, the minor sensitivity (d') deficits observed in *trpv1:trpa1:trpm3*$^{-/-}$ mice may indicate that these channels play a limited role in warming perception. Thus, importantly, noxious heat perception can be disabled in mice without major alterations in non-noxious thermal perception.

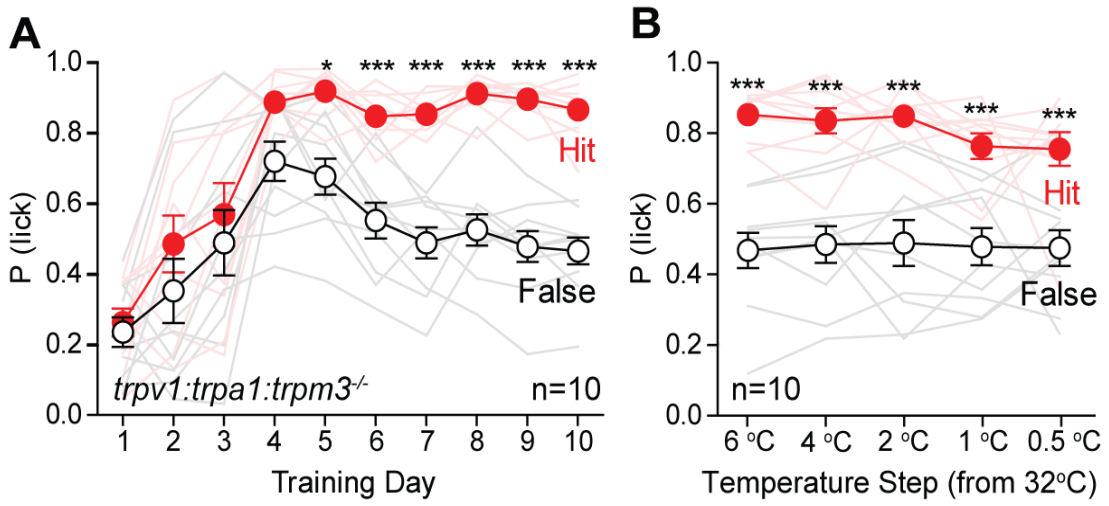

Figure 21. Mice lacking TRPV1, TRPA1 and TRPM3 detect forepaw warming.

(A) Mice lacking TRPV1, TRPA1 and TRPM3 learnt to report forepaw warming of 32-42°C after a few days of training (n = 10, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(B) Mice lacking TRPV1 were able to report very small warming steps, as indicated by different hit and false alarm rates (n = 10, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

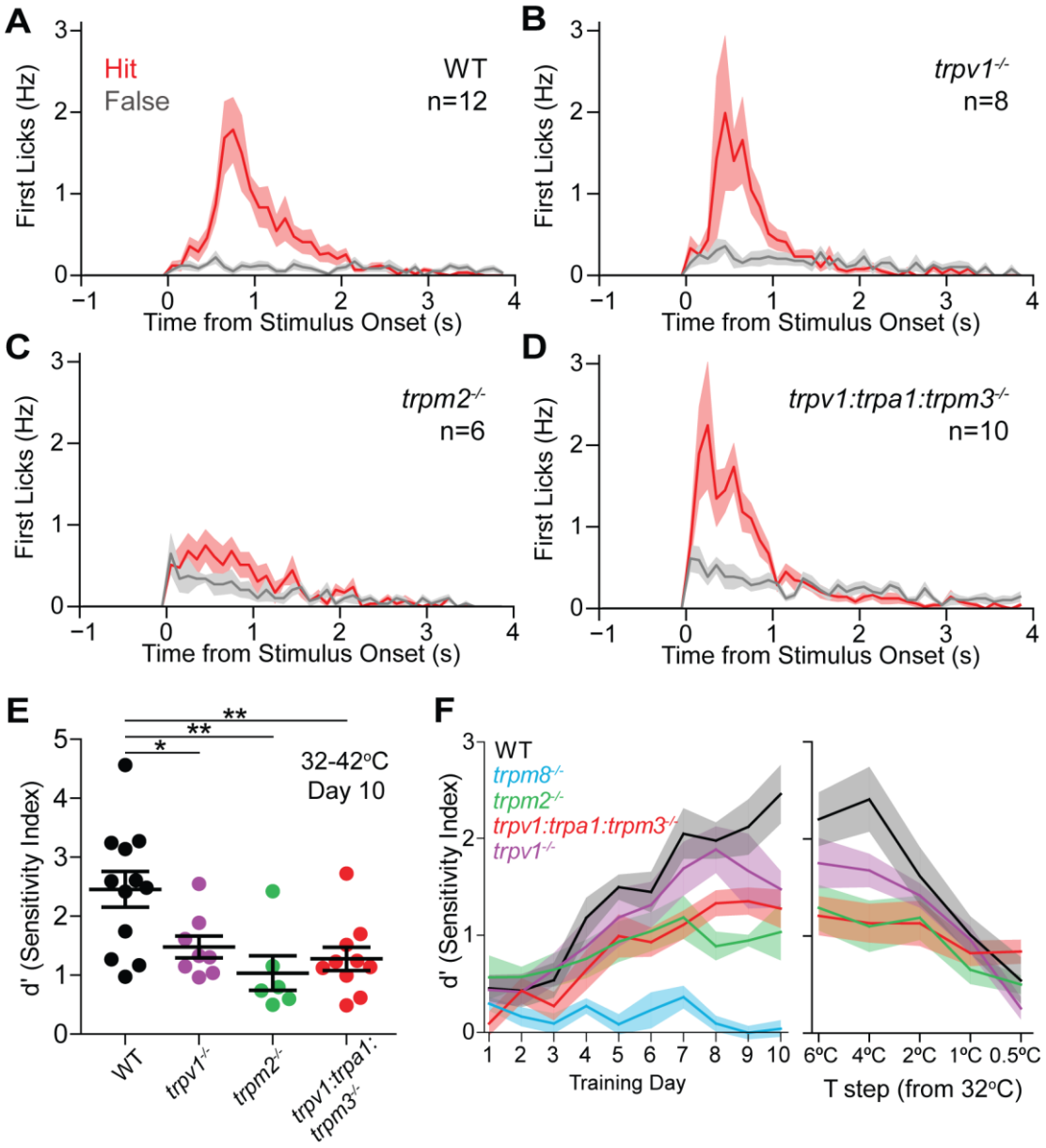

Figure 22. Warming perception comparison between WT and mice lacking TRP channels.

(A) PSTH of first licks to report warming of 32-42°C from all WT mice at day 10 (n = 12).

(B) PSTH of first licks to report warming of 32-42°C from all *trpv1*$^{-/-}$ mice at day 10 (n = 8).

(C) PSTH of first licks to report warming of 32-42°C from all *trpm2*$^{-/-}$ mice at day 10 (n = 6).

(D) PSTH of first licks to report warming of 32-42°C from all *trpv1:trpa1:trpm3*$^{-/-}$ mice at day 10 (n = 10).

(E) Sensitivity (d') to report forepaw warming (32-42°C) showed significant differences between WT mice (n = 12) and *trpv1*$^{-/-}$ (n = 8, $p < 0.05$), *trpm2*$^{-/-}$ (n = 6, $p < 0.01$), and *trpv1:trpa1:trpm3*$^{-/-}$ (n = 10, $p < 0.01$) (unpaired t tests).

(F) Sensitivity (d') to report forepaw warming (32-42°C) throughout learning (left) and threshold tests (right), in all genotypes tested. While *trpv1*$^{-/-}$ (n = 8), *trpm2*$^{-/-}$ (n = 6) and *trpv1:trpa1:trpm3*$^{-/-}$

(n = 10) mice appear to have partial deficits in reporting forepaw warming, only *trpm8*$^{-/-}$ mice (n = 10) had a performance of near chance level (d' = 0) throughout the learning.

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

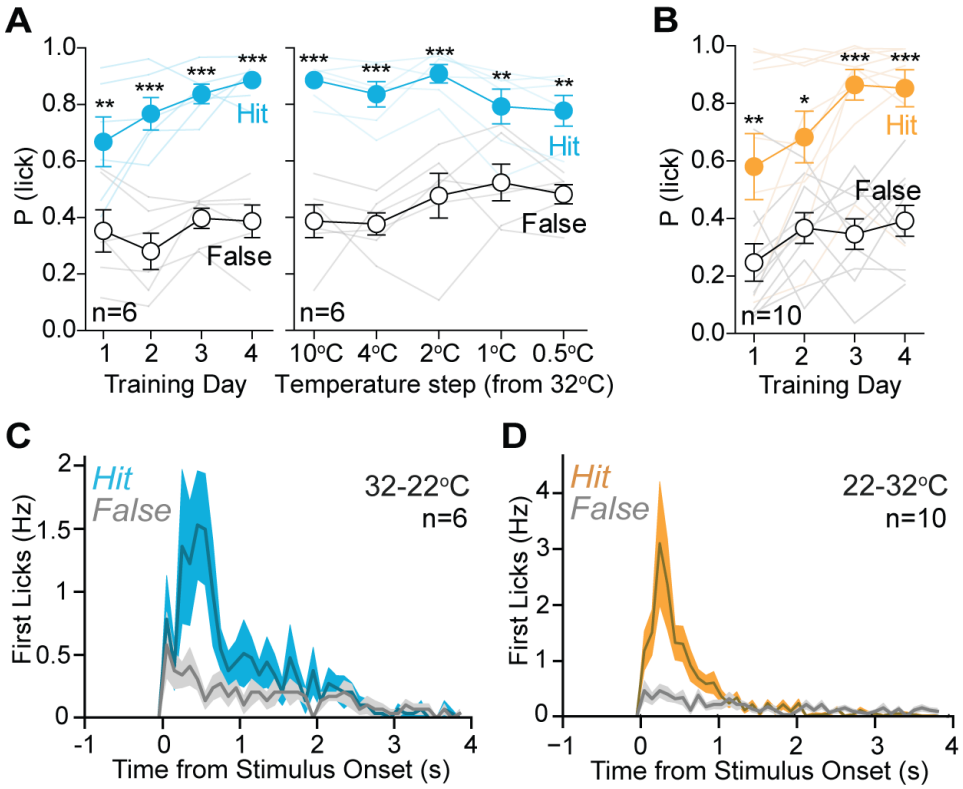

Figure 23. Mice lacking TRPV1, TRPA1 and TRPM3 detect forepaw cooling (32-22°C) and warming from a lower baseline (22°C).

(A) (left) Learning curve to report cooling (32-22°C) and (right) threshold testing of *trpv1:trpa1:trpm3*$^{-/-}$ mice shows that these mice sense cooling (n = 6, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(B) Warming of 22-32°C was also detected by *trpv1:trpa1:trpm3*$^{-/-}$ mice (n = 10, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(C) PSTH of first licks to report forepaw cooling (32-22°C) of *trpv1:trpa1:trpm3*$^{-/-}$ mice (n = 6).

(D) PSTH of first licks to report forepaw warming of 22-32°C of *trpv1:trpa1:trpm3*$^{-/-}$ mice (n = 10).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

3.3.4 Mice lacking the cooling sensor TRPM8 cannot sense warming

Because the skin-nerve recordings show that warming is partially encoded by warm-inhibited neurons that are cooling-sensitive, I hypothesized that warming perception may be indirectly affected by the ability to sense cooling. Since the main molecular transducer of cooling is TRPM8 (Dhaka et al., 2007; McKemy et al., 2002), and we had previously shown that TRPM8 is required for cooling perception (Milenkovic et al., 2014), I tested the ability of TRPM8-null mice (*trpm8*$^{-/-}$) to perceive forepaw warming.

Unexpectedly, *trpm8*$^{-/-}$ mice were completely unable to sense forepaw warming of 32-42^{o}C throughout the 10 warming detection training sessions, as indicated by equal hit and false alarm rates (n = 10, **Figure 24A**). On the last training session, *trpm8*$^{-/-}$ mice had a sensitivity (d') of ~0, indicating chance level performance (d' = 0.04 ± 0.09, n = 10). This was significantly different from wild type mice (d' = 2.45 ± 0.30, n = 12, **Figure 24B**). Moreover, analysis of the *trpm8*$^{-/-}$ lick PSTHs revealed that the lick distributions were very similar between hit and false alarm trials (n = 10, **Figure 24C**), further indicating a major stimulus detection deficit. To rule out the possibility that *trpm8*$^{-/-}$ mice have a cognitive defect that impairs general learning, I trained *trpm8*$^{-/-}$ mice again in the same task, but replacing the warming stimulus by a sound cue of low volume (~40 dB SPL, sound pressure level), and the mice started learning this task (n = 5, **Figure 25A**). Later, I trained the same *trpm8*$^{-/-}$ mice to detect a tactile stimulus delivered to the forepaw (ramp and hold stimulus lasting as long as the thermal stimuli in the previous task, 3.5 s). Unlike with warming, *trpm8*$^{-/-}$ mice were able to quickly report the tactile stimulus, as shown by robust differences between hit and false alarm rates (n = 5, $p < 0.0001$, **Figure 25B**). Moreover, the latencies to report mechanical stimuli were very quick and constant, as shown by the sharp PSTH shape (n = 5, **Figure 25C**).

This data indicates that mice lacking the cooling sensor TRPM8 are unable to detect quick (3.5 s) warming stimuli in the forepaw. However, they are capable of detecting tactile or acoustic stimuli.

Besides warming, mice lacking TRPM8 are unable to detect cooling of 32-22^{o}C when delivered to the forepaw in a small (3 x 3 mm stimulator) area (Milenkovic et al., 2014). Because I found that a larger stimulation area helps in the detection of warming, I investigated whether *trpm8*$^{-/-}$ mice have improved sensitivity to cooling when delivered to the glabrous skin of the whole forepaw. Despite the increase in surface of stimulation, however, *trpm8*$^{-/-}$ mice were unable to detect cooling of 32-22^{o}C delivered to the whole forepaw (n = 4, 7 cooling training sessions, **Figure 25D**). This further confirms that

TRPM8-null mice are unable to sense forepaw cooling, even when the area stimulated is the whole forepaw.

Together, these results indicate that the cooling sensor TRPM8 plays a surprising role in warming perception.

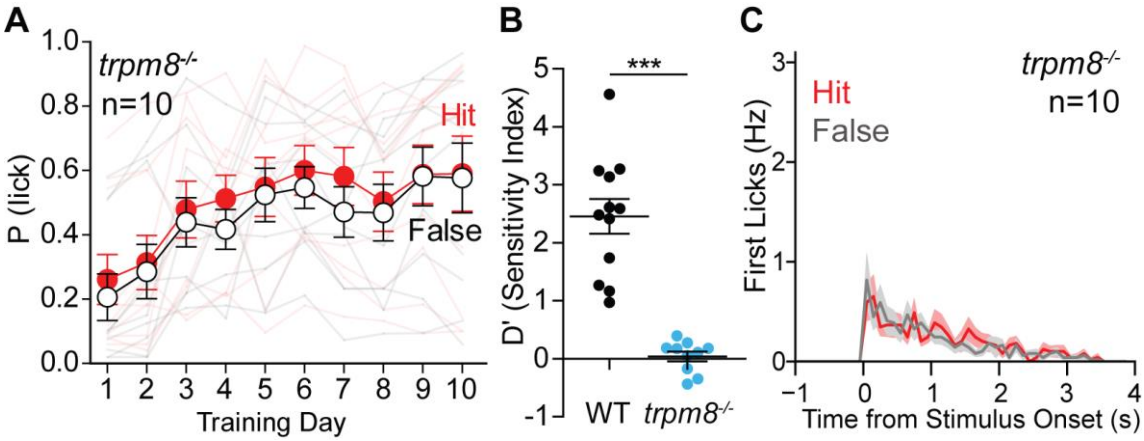

Figure 24. Mice lacking TRPM8 are unable to report forepaw warming.

(A) Forepaw warming (32-42°C) was not detected by *trpm8*$^{-/-}$ mice throughout 10 training sessions (n = 10, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(B) Sensitivity (d') to report forepaw warming at day 10 was near chance level in *trpm8*$^{-/-}$ mice (n = 10), as opposed to WT mice (n = 12, $p < 0.001$, unpaired t test).

(C) PSTH of first licks to report warming in *trpm8*$^{-/-}$ mice at day 10 show that the lick distributions were very similar during stimulus and no stimulus trials (n = 10).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data = mean ± SEM.

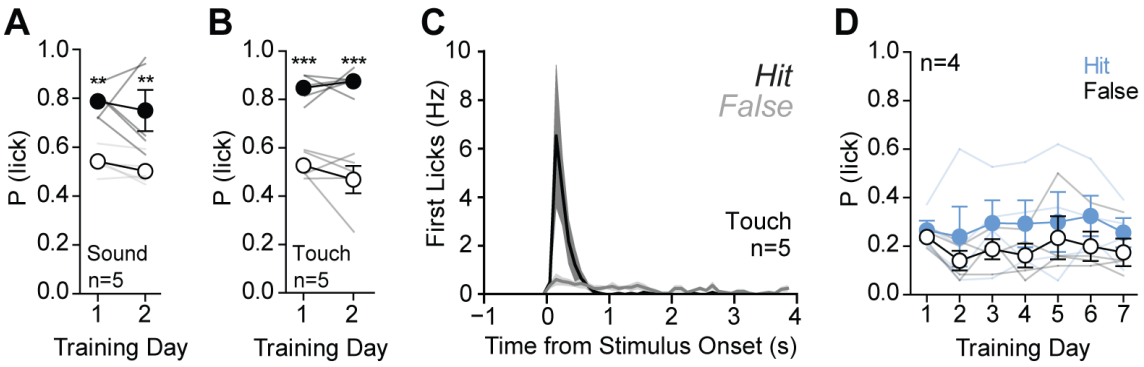

Figure 25. Mice lacking TRPM8 report touch but not cooling in the forepaw.

(A) Following unsuccessful warming training, *trpm8*$^{-/-}$ underwent training to report an acoustic cue. Hits and false alarms were in this case significantly different (n = 5, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(B) Tactile stimuli in the forepaw were successfully reported by *trpm8*$^{-/-}$ mice (n = 5, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(C) PSTH of first licks to report tactile stimuli in *trpm8*$^{-/-}$ mice at day 2 show that the lick distributions are very different during stimulus and no stimulus trials. Hit licks were very locked to stimulus onset (n = 5).

(D) As expected, *trpm8*$^{-/-}$ mice were unable to report forepaw cooling delivered with an 8 x 8 mm Peltier element (n = 4).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

3.3.5 Pharmacological inhibition of TRPM8 impairs warm perception

The TRPM8-null mice used here are constitutive knockouts, so they lack functional TRPM8 channels since early developmental stages. Given that early sensory deprivation has been shown to impact the development of sensory circuits (Merabet & Pascual-Leone, 2010; Ueno et al., 2015), one possibility that could explain why *trpm8*$^{-/-}$ mice cannot sense warming is that cooling signaling may be required at some developmental stage/s for the normal development of the thermosensory system. Another possibility is that TRPM8 is acutely needed for warming perception. To test whether functional TRPM8 is acutely and locally required for warming perception, I performed transdermal injections (**Figure 26A**) of PBMC (1-Phenylethyl-(2-aminoethyl)[4-(benzyloxy)-3-methoxybenzyl]carbamate), an antagonist of TRPM8, in the forepaw of warming-trained wild type mice and assessed whether this impairs warming perception. PBMC has been previously used in rodents *in vitro* and *in vivo* (Gardiner et al., 2014; González et al., 2017; Knowlton et al., 2011; Yudin et al., 2016).

Despite mice injected with PBMC still had significantly different hit and false alarm rates (n = 5, **Figure 26B-C**), acute pharmacological blockade of TRPM8 significantly impaired the perception of 32-42ºC forepaw warming, as shown by a lower sensitivity (d') than when injected with DMSO vehicle (n = 5, **Figure 26D**). 24 hours after treatment, PBMC-injected mice recovered and showed again a higher sensitivity to forepaw warming (**Figure 26C-D**).

To ensure that the observed effects are specific for thermosensation and general nerve transmission is not impaired upon PMBC injection, I trained wild type mice to report a tactile stimulus. In this case, injection of PBMC did not impair the sensitivity of mice to tactile stimuli (n = 6, **Figure 26E**).

Together, these results indicate that TRPM8 is required acutely and locally for the perception of forepaw warming.

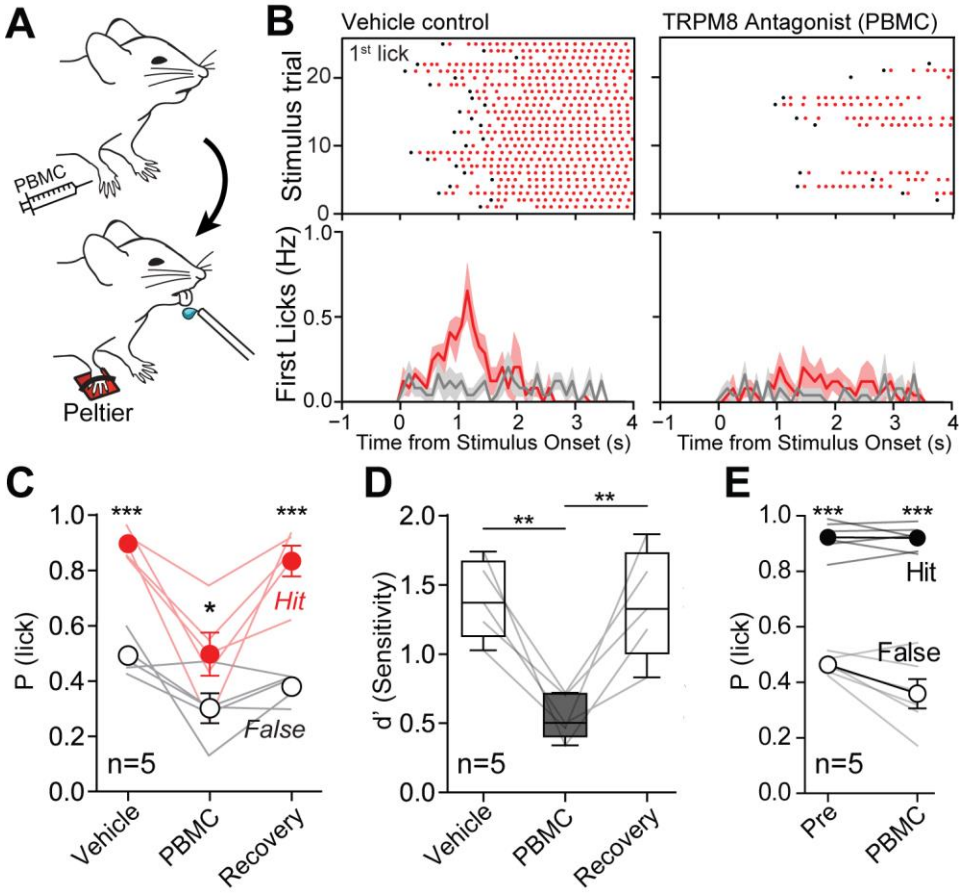

Figure 26. Pharmacological inactivation of TRPM8 impairs warming perception.

(A) Wild type mice were first trained to report forepaw warming (32-42^{o}C). Once they learnt the task (shown by statistically significantly differences between hits and false alarms for 3/4 consecutive days), TRPM8 antagonist PBMC (or DMSO vehicle) was microinjected into the plantar side of the forepaw under brief (< 5 min) isoflurane anesthesia. 20 minutes later, the warming detection abilities were tested.

(B) (top) Raster plots with all licks for 2 example mice injected with vehicle (left) and TRPM8 antagonist PBMC (right). The first lick to report each stimulus is shown in black. (bottom) Average lick PSTHs for mice injected with vehicle (left) and PBMC (right) (n=5 each).

(C) Hits and false alarms while detecting forepaw warming showed statistically significant differences during both vehicle and PBMC injections (n = 5, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(D) However, when measuring performance, mice reported the stimuli with less fidelity when they were injected with TRPM8 blocker PBMC, as shown by a lower sensitivity (d') (n = 5, $p < 0.01$ and $p < 0.01$, paired t tests vehicle vs PBMC, and PBMC vs recovery).

(E) Injection of PBMC did not impair a tactile detection task, as shown by similar hits and false alarms detecting forepaw touch between non-injected and PBMC-injected sessions in the same mice (n = 5, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

3.3.6 Sensory afferents have normal warming-activated responses in the absence of TRPV1, TRPM2, TRPA1, TRPM3 and TRPM8

I have shown that mice lacking the warm/heat activated sensors TRPV1, TRPM2, or the combination of TRPV1, TRPA1 and TRPM3 have different degrees of impairment in the perception of forepaw warming. Moreover, counter-intuitively, the cooling sensor TRPM8 is required for warming sensation. To understand the underlying defects of these TRP-null mice, as well as to elucidate which features of warming encoding at the periphery may be required for warming sensation, I again collaborated with Dr. Frederick Schwaller, who performed *ex vivo* skin-nerve afferent recordings of the paws of TRP mutant mice that had undergone behavioral testing. First, warm-activated fibers of *trpv1*$^{-/-}$, *trpm2*$^{-/-}$, *trpv1:trpa1:trpm3*$^{-/-}$ and *trpm8*$^{-/-}$ mutant mice were investigated.

As with previous experiments in wild types, slow (1ºC/s) ramps were used to screen for all thermosensitive afferents activated by thermal stimuli between 12 and 48ºC. First, sensory afferent recordings of *trpv1*$^{-/-}$ and *trpm2*$^{-/-}$ mice did not show a reduction in the proportion of heat-activated afferents (as shown by a similar number of C-MH and C-MHC fibers, data from hindpaw recordings with buffer temperature at 32ºC of n = 10 WT, n = 9 *trpv1*$^{-/-}$ and n = 6 *trpm2*$^{-/-}$ mice, **Figure 27A**). Interestingly, however, *trpm2*$^{-/-}$ mice had a smaller proportion of cooling-sensitive C-MC fibers. This was unexpected, since TRPM2 has been reported to activate upon warm temperatures (Tan & McNaughton, 2016). Importantly, warm-activated afferents of *trpv1*$^{-/-}$ and *trpm2*$^{-/-}$ mice responded to warming of 32-42ºC with a similar number of spikes as wild type mice (**Figure 27B**). However, the sensory afferents of *trpv1*$^{-/-}$ mice had reduced spiking to heat temperatures beyond the pain threshold (>42ºC, **Figure 27C**), consistent with the idea that TRPV1 is mainly a noxious heat sensor (Caterina et al., 2000). These results indicate that warm-activated afferents do not require TRPV1 or TRPM2 to encode warming.

On the other hand, forepaw afferent recordings of *trpv1:trpa1:trpm3*$^{-/-}$ mice showed a slightly lower amount of heat-activated CMH fibers than wild type mice (buffer temperature at 27ºC, data from n = 6 WT and n = 6 *trpv1:trpa1:trpm3*$^{-/-}$ mice, **Figure 28A**). The number of warm-activated afferents was also slightly reduced in *trpv1:trpa1:trpm3*$^{-/-}$ mice, compared to wild types (**Figure 28B**). Warm-activated sensory

fibers of *trpv1:trpa1:trpm3*$^{-/-}$ mice had a total number of warming-evoked (32-42ºC) spikes that was not significantly different from WT (**Figure 28C**). However, the population spiking PSTH appeared slightly different to that of wild type mice. On average, warm-activated afferents from wild type mice reached firing rates of ~6 Hz during a 32-42ºC warming stimulus, whereas the fibers from *trpv1:trpa1:trpm3*$^{-/-}$ mice reached only ~4 Hz (n = 18 WT and n = 8 *trpv1:trpa1:trpm3*$^{-/-}$ warm-activated fibers, **Figure 28D**). This data indicates that the joint loss of TRPV1, TRPA1 and TRPM3 does not greatly impact warming encoding in warm-activated afferents.

Finally, the primary sensory afferents of *trpm8*$^{-/-}$ mice were investigated. As expected from previous studies (Milenkovic et al., 2014), very few cooling-sensitive units (C-MC or C-MHC fibers) were found in forepaw skin-nerve recordings (buffer temperature of 27ºC, n = 6 WT and n = 7 *trpm8*$^{-/-}$ mice, **Figure 28A**). However, many sensory afferents were activated by warming stimuli of 32-42ºC, and these had normal firing responses to warming (n = 18 WT and n = 20 *trpm8*$^{-/-}$ warm-activated units, **Figure 28B-C**). The firing rate PSTH of warm-activated fibers was comparable to that of WT fibers (**Figure 28D**). These results suggest that TRPM8 is not required for encoding of warming in warm-activated sensory neurons. On the other hand, the low proportion of cooling-spiking units found in these mice confirm that cooling sensitivity at the peripheral nervous system requires TRPM8 channels (Colburn et al., 2007; Dhaka et al., 2007; Milenkovic et al., 2014).

Together, these findings suggest that warm-activated sensory afferents can encode warming (32-42ºC) stimuli in absence of TRPV1, TRPM2, TRPA1, TRPM3 and TRPM8. On one hand, TRPV1, TRPA1 and TRPM3 may play minor contributions to warming encoding, but the observed trend in warming-activated fiber proportions and firing were so small that a greater number of animals should be tested to explore whether these differences reach statistical significance. On the other hand, surprisingly, warm-activated afferents lacking either TRPM2 or TRPM8 showed normal warming encoding. Therefore, the inability of TRPM8-null mice to sense warming is likely not caused by an impairment in warm-activated afferent encoding.

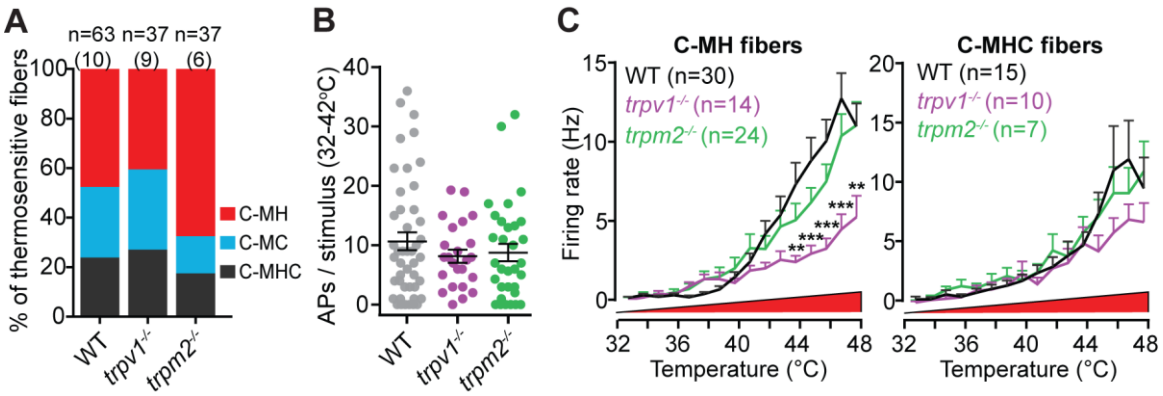

Figure 27. Warming-evoked spiking is normal in *trpv1*$^{-/-}$ and *trpm2*$^{-/-}$ mice.

(A) Thermosensitive fiber proportions found in WT, *trpv1*$^{-/-}$ and *trpm2*$^{-/-}$ mice via hindpaw skin-nerve recordings at buffer temperature 32°C.

(B) Total action potentials recorded as a response to the warming phase (32-42°C) of a heating ramp, in warming-activated afferents from WT, *trpv1*$^{-/-}$ and *trpm2*$^{-/-}$ mice.

(C) Firing rate in warming-activated C-MH (left) and C-MHC (right) fibers of WT (n = 30 and 15 for C-MH and C-MHC fibers, respectively), *trpv1*$^{-/-}$ (n = 14 and 10) and *trpm2*$^{-/-}$ (n = 24 and 7) mice by warm and hot temperatures. *trpv1*$^{-/-}$ C-MH fibers had lower responses to noxious heat (>43°C) than WT fibers (two-way repeated measures ANOVA with Bonferroni post-hoc tests).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

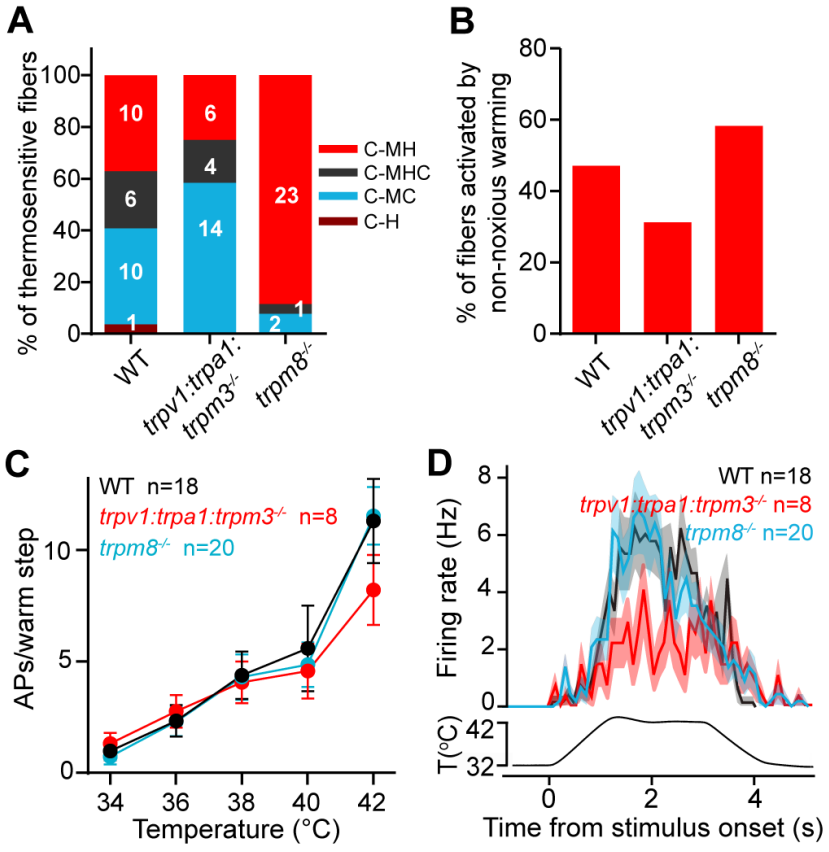

Figure 28. Warming-evoked spiking is normal in *trpv1:trpa1:trpm3*$^{-/-}$ and *trpm8*$^{-/-}$ mice.

(A) Thermosensitive fiber proportions found in WT, *trpv1:trpa1:trpm3*$^{-/-}$ and *trpm8*$^{-/-}$ mice via forepaw skin-nerve recordings at buffer temperature 27ºC. As expected, cooling-spiking fibers were found in a smaller proportion in *trpm8*$^{-/-}$ mice.

(B) Proportion of thermosensitive fibers that fired action potentials to non-noxious warming stimuli (up to 42ºC) in WT (n = 18), *trpv1:trpa1:trpm3*$^{-/-}$ (n = 8) and *trpm8*$^{-/-}$ (n = 20) mice.

(C) Similar firing rate evoked by short (3.5 s) warming steps of different amplitudes in warming-activated fibers from WT (n = 18), *trpv1:trpa1:trpm3*$^{-/-}$ (n = 8) and *trpm8*$^{-/-}$ (n = 20) mice.

(D) PSTHs of spiking activity in warming-activated fibers from WT (n = 18), *trpv1:trpa1:trpm3*$^{-/-}$ (n = 8) and *trpm8*$^{-/-}$ (n = 20) mice, as a response to a warming step of 32-42ºC.

3.3.7 Sensory afferents have ablated warm-inhibited responses in the absence of TRPM8

Despite being warming-insensitive at the perceptual level, *trpm8*$^{-/-}$ mice had normal encoding of warmth in warm-activated afferents. However, besides eliciting spiking in warm-activated fibers, warming suppresses the firing activity of warm-silenced units that are cool-driven (**Figure 16C, Figure 17B**). Therefore, we next examined warm-silenced fibers in *trpm8*$^{-/-}$ mice.

Skin-nerve recordings of the medial and ulnar nerves of *trpm8*$^{-/-}$ mice were carried out, maintaining the tissue in a buffer at 27ºC, to find warm-silenced fibers. Interestingly, no fibers with ongoing spiking activity at 32ºC Peltier baseline were found in these mice (n = 7 mice, **Figure 29A**). Therefore, unlike in WT mice, no warm-silenced responses were observed in *trpm8*$^{-/-}$ mice to 32-42ºC warming stimuli (**Figure 29B-C**). This data indicates that TRPM8 is required for warm-silenced responses to take place.

Finally, to investigate the contribution of TRPV1, TRPA1 and TRPM3 in the warming encoding of warm-silenced fibers, this fiber subtype was investigated in *trpv1:trpa1:trpm3*$^{-/-}$ mice. Medial and ulnar skin-nerve recordings (buffer 27ºC) revealed that fibers with ongoing activity at Peltier baseline 32ºC were present in *trpv1:trpa1:trpm3*$^{-/-}$ mice, and had similar firing rates to those of WT mice (n = 6 fibers in 6 WT mice, n = 5 fibers in 6 *trpv1:trpa1:trpm3*$^{-/-}$ mice, **Figure 29A**). In response to warming of 32-42ºC, these units underwent inhibition of a comparable magnitude to wild type warm-silenced fibers (**Figure 29B-C**). These findings indicate that TRPV1, TRPA1 and TRPM3 do not play a role in warming encoding in warm-silenced fibers.

Together, these data indicate that TRPM8 is required to encode warming at the forepaw via warm-silenced responses. Because mice lacking functional TRPM8 are unable to sense warming, input from warm-silenced C-fibers appears to be required in forepaw warming perception. On the other hand, TRPV1, TRPA1 and TRPM3 do not contribute to warming encoding in warm-silenced fibers.

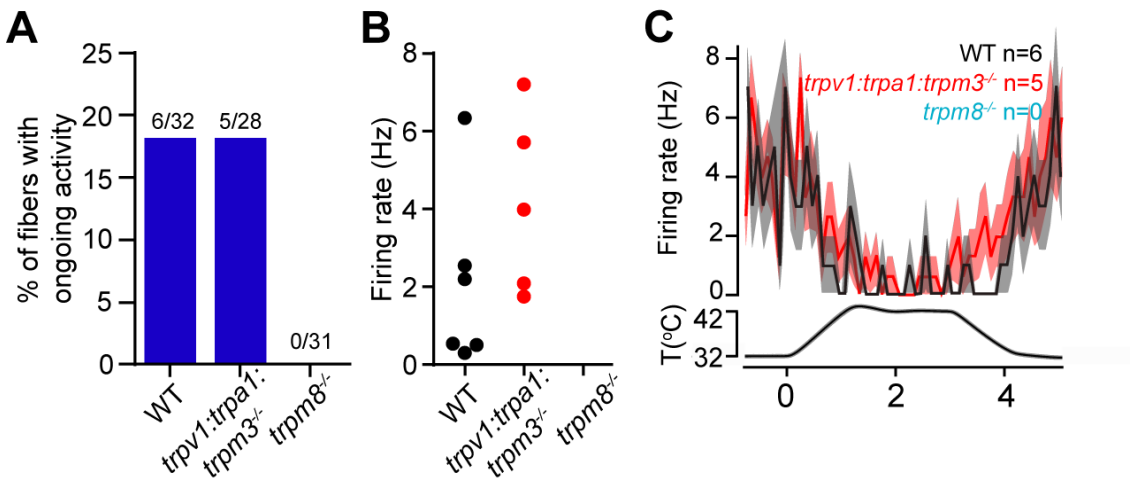

Figure 29. Warm-silenced afferent responses are missing in *trpm8*$^{-/-}$ mice.

(A) Proportion of thermosensitive fibers with ongoing spiking activity (during baseline 32°C, buffer at 27°C), found in WT (6/32), *trpv1:trpa1:trpm3*$^{-/-}$ (5/28) and *trpm8*$^{-/-}$ (0/31).

(B) Firing rates of thermosensitive fibers with firing activity at rest, at Peltier baseline 32°C.

(C) PSTHs of spiking activity in response to warming (32-42°C) shows that fibers with spiking activity during baseline are inhibited by warming. While warming-inhibited fibers of *trpv1:trpa1:trpm3*$^{-/-}$ mice undergo a spiking suppression similar to WT fibers, *trpm8*$^{-/-}$ mice are devoid of this mechanism of warming encoding.

3.3.8 Inactivation of afferents with ongoing activity ablates warming perception

Because mice without TRPM8 are unable to sense warming, and their only warming encoding deficit is the lack warm-silenced sensory afferents, it is likely that these neurons are required for warming sensation. However, testing this hypothesis is challenging because the genetic identity (besides the likelihood that they are TRPM8-positive) of these fibers remains unknown. In the experiments presented so far, I interfered with the TRPM8 signaling in different ways, but this approach also heavily impacts most cooling-sensitive afferents that do not spike during baseline and are not warm-silenced (**Figure 28A**). For this reason, I next aimed to target warm-silenced fibers without impacting the rest of the cooling-spiking neurons to further confirm their role in warming perception.

One tool that has been used to disable specific sensory afferents *in vivo* is the sodium channel blocker QX-314 (N-Ethyllidocaine) chloride. QX-314 is a small (~260 Da), permanently charged molecule that cannot cross the plasma membrane on its own. However, it is thought to enter the cell via open receptors and, once present intracellularly, it blocks sodium channels and subsequently the action potential firing. Application of QX-314 together with an agonist of either TRPV1 or TRPM8 has been shown to inactivate TRPV1- and TRPM8-positive afferents, respectively, which was effective to reduce pain sensation in rodents (Binshtok et al., 2007; Brenneis et al., 2013; Ongun et al., 2018; Ries et al., 2009).

The skin-nerve afferent data presented here show that warm-silenced fibers have ongoing spiking (at Peltier baseline 32°C) that is TRPM8-dependent. In addition, this population is the only thermosensitive fiber type that is active at rest. Therefore, I used forepaw application of QX-314 to selective disable afferent fibers that were active in absence of thermal stimuli, and test whether warm-silenced afferents are required for the perception of warming.

Transdermal injections of QX-314 in the forepaw of warming-trained wild type mice dramatically (d' < 1) decreased warming (32-42°C) detection performance compared to when injected with vehicle (n = 6 mice, $p < 0.0001$, **Figure 30A-B**, behavioral training took place 20' after injection). However, mice treated with QX-314 recovered 24 h later, and their warming detection performance increased (**Figure 30B**). To ensure that the effects of QX-314 were thermal-specific, I trained wild type mice to report tactile stimuli and injected them with QX-314 in the forepaw. In this case, QX-314 transdermal injections did not impair the detection of thermal stimuli (n = 5 mice, **Figure 30C**).

This data suggests that thermosensitive fibers with ongoing activity (e.g. warm-silenced fibers) are required for warming perception.

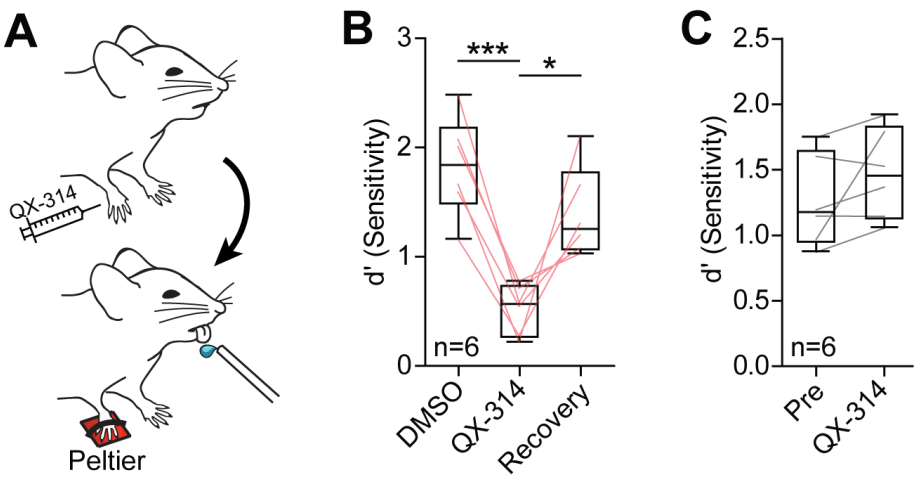

Figure 30. Silencing of afferent baseline activity at the forepaw disrupts warming but not tactile perception.

(A) Warming-trained mice underwent a transdermal injection of QX-314 (or DMSO vehicle control) in the forepaw. Afterwards (15' post injection), the ability of these mice to detect forepaw warming (32-42^{o}C) was tested.

(B) Sensitivity (d') when detecting warming was impaired upon injection of QX-314, in comparison to DMSO control (n = 6, $p < 0.001$ paired t test).

(C) Sensitivity (d') to report forepaw tactile stimuli was similar between mice that did not undergo injection and mice injected with QX-314 (n = 6).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data = mean ± SEM.

3.4 OPTOGENETIC ACTIVATION OF TRPM8+ SENSORY NEURONS TRIGGERS PERCEPTION

The data presented here indicates that afferent neurons that express TRPM8 are the main drivers of quick, non-noxious thermal perception. Therefore, I hypothesized that selective activation of TRPM8-positive afferents would drive a strong behavioral percept that would be reminiscent of cooling sensation in mice. To test this, I performed optogenetic activation of the forepaw afferents of Trpm8-ChR2 mice. These mice (a kind gift from the lab of Professor Ardem Patapoutian) have been generated by crossing Trpm8^{Cre} mice with animals expressing a floxed, GFP-bound channel rhodopsin 2 (ChR2) (Kim et al., 2018).

First, I measured the amount of dorsal root ganglia neurons expressing channel rhodopsin 2 in these mice. To do that, I performed immunohistochemical staining of cryosections (30 µm thick) of DRGs innervating the limbs of Trpm8-ChR2 mice. All Trpm8-ChR2+ DRG neurons found were small in size (>20 µm on average, n = 12 DRG neurons in 2 animals, **Figure 31A-C**). This was expected, as TRPM8+ DRG neurons have been shown to be of small size (Dhaka et al., 2008; L. Su et al., 2011). Furthermore, large DRG neurons are known to have myelinated axons, both shown *in vitro* (Windebank et al., 1985) and *in vivo* (Ruit et al., 1992). Surprisingly, however, the number of Trpm8-ChR2+ DRG neurons was very small in proportion (0.83 % of all DRG neurons, n = 2 animals, **Figure 31A-C**). The proportion of sensory afferents expressing TRPM8 has been previously reported to be ~2% in functional studies (Jankowski et al., 2017), and ~5-8% using *in situ* hybridization (Dhaka et al., 2008; Pogorzala et al., 2013). This suggests that, in this Trpm8-ChR2 mouse line, less than half of TRPM8+ neurons might express ChR2. Future work would require dual TRPM8 antibody and GFP labelling

to confirm ChR2-expressing neurons also express TRPM8. However, to date, highly sensitive and specific TRPM8 antibodies are not available in the market.

Next, to visualize the target of Trpm8-ChR2+ neurons, I performed spinal cord immunohistochemical stainings of Trpm8-ChR2 mice. As expected, the GFP signal labeled only puncta at the superficial layers of the dorsal spinal cord (n = 2 animals, **Figure 31D**). This confirms that the axons of TRPM8+ afferents terminate in the laminae I-II of the spinal cord, which has been shown previously (Dhaka et al., 2008; Wrigley et al., 2009).

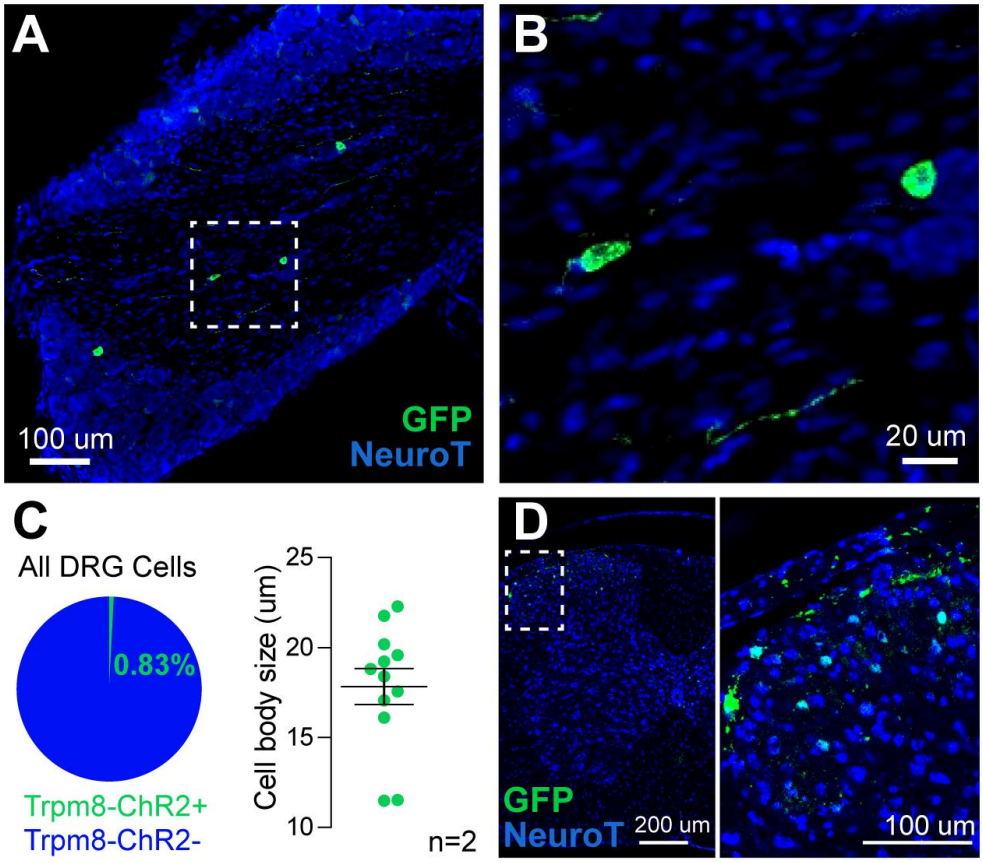

Figure 31. TRPM8-ChR2+ DRG neurons are small caliber neurons that target the dorsal laminae of the spinal cord.

(A) Representative immunohistochemical staining of a dorsal root ganglion (DRG) from a TRPM8-ChR2 mouse. Neurons are labelled in blue (NeuroTrace 435/455, a type of Nissl stain) and TRPM8-CR2+ neurons are shown in green (antibody-amplified signal of ChR2-tagged GFP) (30 µm cryosections).

(B) Magnified inset of (A), showing two TRPM8-ChR2+ cell bodies.

(C) TRPM8-ChR2+ neurons were less than 1% of the total DRG neuronal population (left), and all had small cell body sizes (>25 µm) (n = 2 mice).

(D) Spinal cord immunohistochemical staining showing axonal processes from TRPM8-ChR2+ DRG neurons (green puncta, antibody-amplified signal of ChR2-tagged GFP) in the dorsal laminae.

Data = mean ± SEM.

Next, I aimed to find whether selective activation of TRPM8-ChR2+ sensory afferents drives perception in mice, and if the evoked percept is reminiscent of a thermal sensation. Afferent activation has been achieved via light stimulation in ChR2-expressing transgenic mice (Baumbauer et al., 2015), as well as via virally-induced expression of opsins in wild type animals (Kubota et al., 2019). Here, light stimulation was applied to the glabrous skin of the forepaw of thermally-trained mice. The data presented here was obtained by both Dr. Annapoorani Udhayachandran (cooling-trained, then light-tested dataset) and myself (warming-trained, then light-tested dataset; and also light-trained, then thermal discrimination dataset). I performed the data analysis of all sets of experiments.

3.4.1 Cooling-trained TRPM8-ChR2 mice report forepaw light

Because TRPM8+ neurons are cooling-sensitive and TRPM8 itself is a cooling sensor (Dhaka et al., 2008; Milenkovic et al., 2014; Yarmolinsky et al., 2016), the most straightforward hypothesis is that selective activation of the TRPM8-ChR2+ subset of afferents mimics a cooling stimulus at the perceptual level. To test this, Dr. Udhayachandran trained TRPM8-ChR2 mice to report cooling stimuli (32-22^{o}C, 3.5 s long) when delivered to their forepaw. Once the mice were trained, the cooling stimulus was replaced by LED light application in the same stimulation area (whole forepaw area at the glabrous skin side, 5 mW, 40 Hz, 3.5 s long pulses, **Figure 32A**). The light stimulus was hidden from the animals by using an optical blackout fabric that covered the whole paw and the LED stimulator. This was to prevent mice from visually noticing the light stimulus.

Interestingly, cooling-trained TRPM8-ChR2 mice reported the light stimulus as well (n = 7 animals, **Figure 32B**). Sensitivity (d') was very similar while reporting cooling or light (n = 7, **Figure 32C**). Additionally, latencies to report cooling and light stimuli were indistinguishable (n = 7, **Figure 32D**). This data suggests that selective activation of a subset of TRPM8+ sensory afferent triggers thermal perception robustly. Moreover, cooling-trained Trpm8-ChR2 mice reported the light stimulus since the beginning of the behavior session, suggesting that they perceived light as an equivalent to cooling (**Figure 32D**, **Figure 34A-B**). Together, these data suggest that selective activation of

TRPM8-ChR2+ sensory afferents triggers a very robust percept that may be equivalent to cooling.

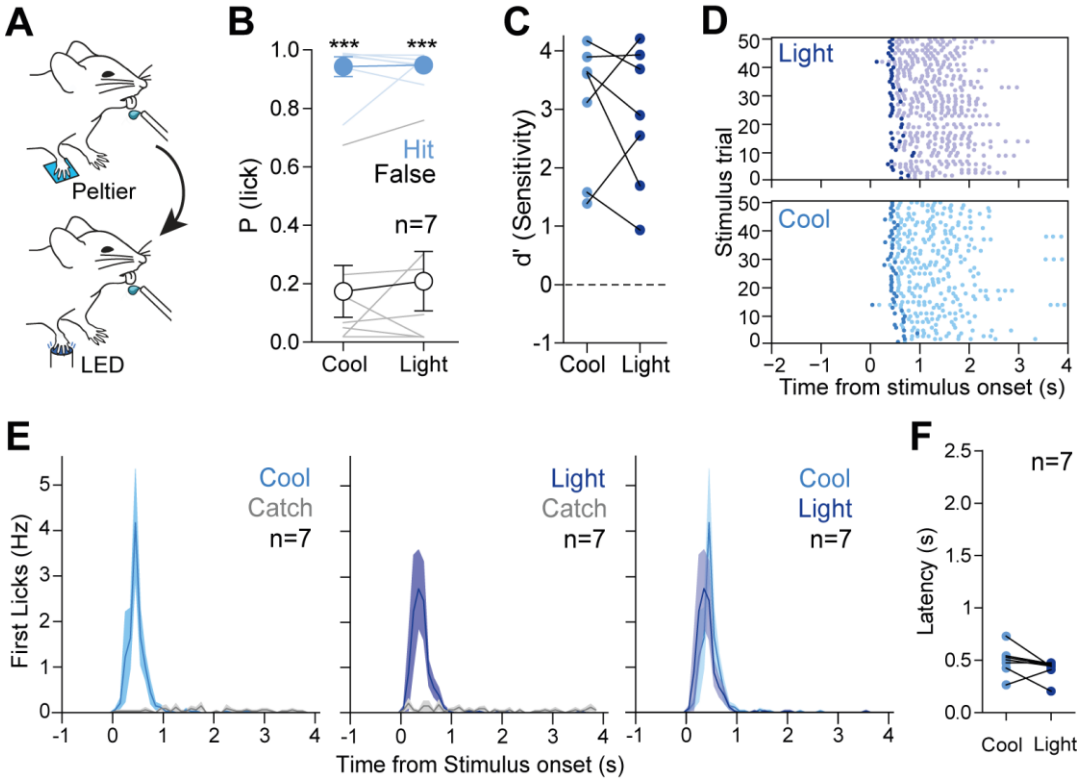

Figure 32. Optogenetic activation of TRPM8-ChR2 neurons drives a perceptual response very similar to cooling.

(A) TRPM8-ChR2 mice were first trained to report forepaw cooling (32-22ºC). Next, the warming stimulus was replaced by optogenetic stimulation of the glabrous skin of the forepaw. In order to prevent mice from visually noticing the light, the animals had the whole forepaw covered during warming and light sessions.

(B) Hit and false alarm rates while reporting forepaw cooling or light. Hit and false alarm rates remained similar and showed statistically significant differences in both sessions (n = 7, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(C) Sensitivity (d') values for cooling and light detection of TRPM8-ChR2 were similar for cooling and light detection sessions, and always above chance (d' = 0) level (n = 7).

(D) Example raster plot of a mouse showing the licks to report cooling (bottom) and light (top), in different sessions. Both the spread and the latency to report cooling and light appeared almost identical.

(E) Lick PSTHs of cooling-trained Trpm8-ChR2 mice to report cooling (left), light (middle) and the overlay of both (right) (n = 7).

(F) Mean individual latency values of cooling-trained Trpm8-ChR2 mice to report cooling and light were very similar (n = 7).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

3.4.2 Warming-trained TRPM8-ChR2 mice report warming and light differently

The afferent data indicates that a significant (~20%) proportion of all thermosensitive afferents classifies as C-MHC (**Figure 14D**, **Figure 15B**) and is therefore activated by both increases and decreases of temperature. Moreover, *trpm8*$^{-/-}$ mice had a robust deficit in C-MHC fibers (**Figure 28A**), suggesting that these are TRPM8-positive. Because activation of this fiber subclass may be interpreted by the central nervous system as a warming or ambiguous thermal stimulus, I considered the possibility that selective activation of TRPM8+ neurons may lead to an unclear thermal percept that mice could associate with a cooling or warming sensation. Therefore, I next tested whether selective activation of TRPM8-positive afferent neurons may trigger a percept that is similar to warming in warming-trained mice.

I trained TRPM8-ChR2 mice to report forepaw warming (32-42ºC, 3.5 s long). After a few sessions, I replaced the warming stimuli by light pulses (whole forepaw area, 5 mW, 40 Hz, 3.5 s long pulses, **Figure 33A**) throughout the session. Interestingly, as a population, the mice reported the light, as shown by differences between hit and false alarm rates (n = 6, **Figure 33B**). However, unlike in cooling-trained mice, I observed that 2 out of 6 mice did not report the light stimulus and had similar hit and false alarm rates (**Figure 33B-C**). Furthermore, the same 2 mice had sensitivity (d') values of around chance level (d' = 0), while the rest of the animals had a good performance (**Figure 33C**).

One hypothesis that could explain this data is that there is inter-individual variability in the expression of ChR2, and light may have triggered a percept in some but not all mice. However, while plausible, I considered this was unlikely because all mice (6/6) from the cooling-trained dataset felt and reported the light. Another possibility would be that warming and light would not be felt by mice in an equivalent manner, but instead mice could learn to report a robust new stimulus throughout the behavior session, especially if this stimulus is related (thermal). To test this hypothesis, I analyzed the performance of these mice in the early phase of the light detection task, when it was less likely the mice had learnt to report the light, if warming and light were not felt equivalently.

When reporting warming, 66.6 % (4/6) of the TRPM8-ChR2 mice had a performance of d' > 1 in the early phase (first third) of the task, whereas only 16.6 % (1/6) had a d' > 1 when reporting light in the early period (n = 6, **Figure 33D**). Moreover, when looking at the individual licks across time, some warming-trained TRPM8-ChR2 mice reported the light since the beginning (fast learners, 2/6), some did it after a significant amount of missed trials (slow learners, 2/6), and some never did it throughout the session (non-learners, 2/6) (**Figure 33E-G**). Next, I analyzed the sensitivity (d') to report the stimulus throughout the last warming session and the light stimulation session. I found that, unlike in the cooling dataset (**Figure 34A-B**), warming-trained mice had a delayed performance when reporting light, compared to reporting warming. Overall, warming-trained mice had sensitivity values below chance level (d'=0) until trial 15 when reporting light, whereas these same mice had an average sensitivity over d' > 0 already since trial 1 when reporting warming (**Figure 34C-D**).

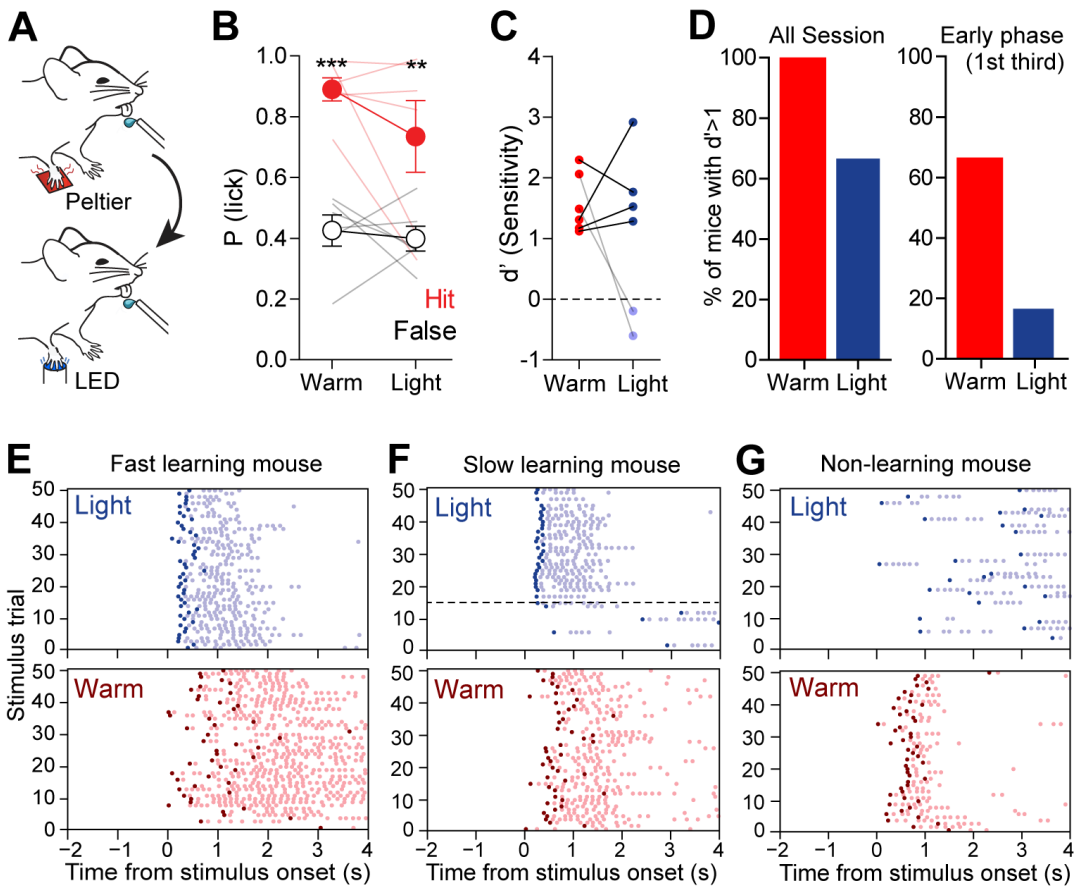

Figure 33. Optogenetic activation of TRPM8-ChR2 afferent neurons drives a perceptual response distinct from warming.

(A) TRPM8-ChR2 mice were first trained to report forepaw warming (32-42ºC). Next, the warming stimulus was replaced by optogenetic stimulation of the glabrous skin of the forepaw. In order to

prevent mice from visually noticing the light, the animals had the whole forepaw covered during warming and light sessions.

(B) Hit and false alarm rates while reporting forepaw warming or light. Hit and false alarm rates showed statistically significant differences in both sessions. However, 2/6 mice did not report the light stimulus, despite reliably reporting warming (n = 6, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(C) Sensitivity (d') values for warming and light detection of TRPM8-ChR2 mice show that 4/6 mice reliably reported the light stimulus and 2/6 had performance near chance level (d' = 0) (n = 6).

(D) Percentage of mice that reported the warming or light stimulus reliably (d' ≥ 1) over the whole session (50 stimulus + 50 catch trials) (left), and over the early phase (1st third of the task) (right). 4/6 mice showed robust warming detection already at the early stage, but only 2/6 mice had such early performance with the light stimulus. This suggested that mice may have learnt to report a new stimulus, instead of confusing light with warming.

(E-G) Raster plots of the licks to report warming (bottom) and light (top) show that: (E) some mice licked to the light stimulus from the start (2/6), (F) some started to lick to the light after a significant amount of missed trials (2/6) and (G) some mice never reported the light reliably (2/6). In mice that reported the light (E and F), the latencies to report light appeared shorter and less spread than latencies to report warming.

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

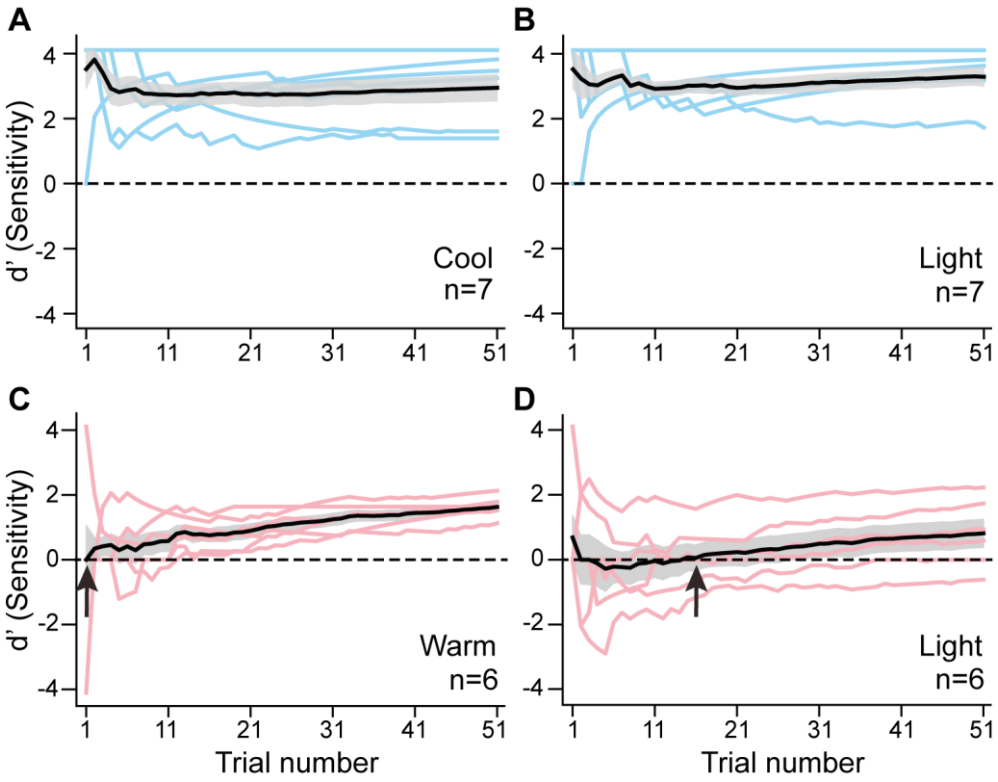

Figure 34. Cooling-trained Trpm8-ChR2 mice report light equally well as cooling, but warming-trained mice need longer to report light than to report warming.

(A) Evolution of cooling-trained Trpm8-ChR2 mice sensitivity (d') across trials on the last cooling detection session (n = 7).

(B) Evolution of cooling-trained Trpm8-ChR2 mice sensitivity (d') across trials on the light detection session (n = 7).

(C) Evolution of warming-trained Trpm8-ChR2 mice sensitivity (d') across trials on the last warming detection session (n = 6). The black arrow points at the last trial number where the average d' was of chance level (d' = 0).

(D) Evolution of warming-trained Trpm8-ChR2 mice sensitivity (d') across trials on the light detection session (n = 6). The black arrow points at the last trial number where the average d' was of chance level (d' = 0).

Finally, when analyzing the latencies to report warming and light, I found that TRPM8-ChR2 mice detected warming (n = 6, mean latency = 0.94 ± 0.23 s) slower than light (n = 6, mean latency 0.43 ± 0.04 s, for mice with d' > 0 when detecting light, **Figure 35A-B**). Interestingly, the comparison of the lick PSTHs of TRPM8-ChR2 as a response to light was reminiscent of the cooling- vs warming-trained latency comparison carried out in wild type mice (**Figure 7C**) (n = 6, **Figure 35A**).

This suggests that the activation of TRPM8-ChR2+ afferents drives a percept that is different from warming sensation. Warming-trained TRPM8-ChR2 mice do not seem to mistake warming with light, but instead likely learn to report a new sensation triggered by the light.

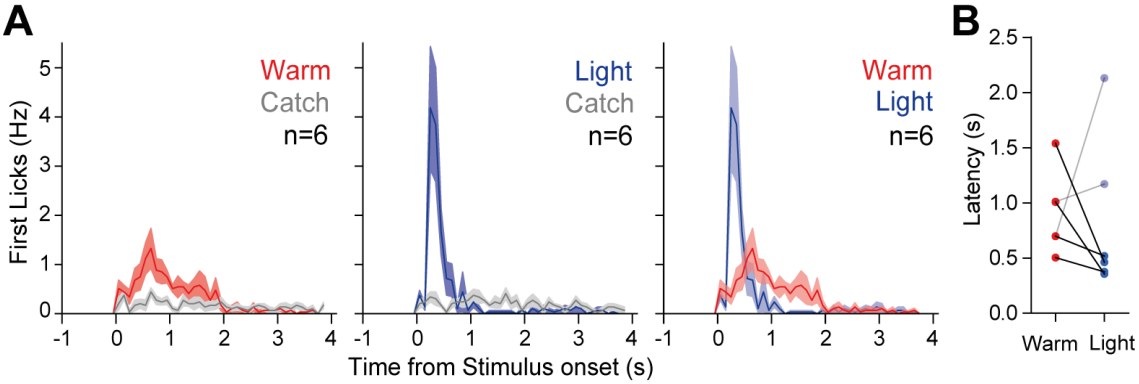

Figure 35. Optogenetic activation of TRPM8-ChR2 afferent neurons is perceived more quickly than a warming stimulus.

(A) (left) PSTHs of first licks to report warming of TRPM8-ChR2 mice (n = 6). Stimulus trials are shown in red, and catch trials are depicted in grey. (middle) PSTHs of first licks to report light of TRPM8-ChR2 mice (n = 6). Stimulus trials are shown in blue, and catch trials are depicted in

grey. (right) Overlay of the warming-detection and light-detection PSTHs. Licks to report light were less spread and faster than licks to report warming.

(B) Session average latency to report warming or light (n = 6).

3.4.3 Optogenetic activation of TRPM8+ sensory neurons triggers cooling perception

The experiments presented above with Trpm8-ChR2 mice suggest that mice may be able to learn to report a stimulus very quickly (a few trials within a session), and that this learning may be a confounding factor when trying to assess whether mice perceive 2 different stimuli as equivalent. For this reason, I next developed a new behavioral training paradigm that assesses whether a new stimulus is perceived as equivalent to a previously learnt one, in the absence of rewards. This way, animals are not motivated to learn to report a new stimulus, but instead will only lick if they mistake the second stimulus with the previously learnt one. In other words, mice will only lick repeated times to an unrewarded stimulus if they already know that such a percept is normally accompanied by a reward.

I used this new paradigm to assess whether selective activation of TRPM8+ afferents elicits a warming, cooling or ambiguous thermal percept. Therefore, Trpm8-ChR2 mice were first trained to report forepaw light stimulation (2.5 s long stimuli of 470 nm, 7 mW, 100 Hz pulses of 5 ms light) in the previously used Go/No-Go training paradigm. Once mice had learnt to report the light, this stimulus was then replaced by interleaved cooling (32-22^{o}C) and warming (32-42^{o}C) stimuli of the same length. This time, however, mice would not get water rewards even if they licked during these stimuli (**Figure 36A**).

Interestingly, light-trained Trpm8-ChR2 reported cooling but not warming stimuli, as indicated by statistical differences between hit and false alarm rates (n = 5 mice, $p < 0.001$ hit vs false alarm rates while reporting light and cooling, two-way ANOVA with Bonferroni post-hoc, **Figure 36B**) and a similarly high d' value for cooling and light but a d' ~ 0 for warming stimuli (light d' = 2.69 ± 0.39, cooling d' = 2.12 ± 0.24, warming d' = 0.09 ± 0.08, re-cooling d' = 0.31 ± 0.12; $p = 0.0014$ cool vs warm, $p = 0.005$ light vs warm, paired t tests, **Figure 36C**). Reaction times while reporting cooling were slower than reporting light, (mean light latency = 0.32 ± 0.04 s, cooling = 0.95 ± 0.16 s; $p = 0.012$ light vs cool, paired t test, data not shown), but this delay is most likely due to the absence of rewards, which reduce overall motivation in the task. This decrease in motivation can also be observed by a slight reduction in the hit and false alarm rates (**Figure 36B**), and by the fact that mice kept engaged in the task for less trials during the unrewarded

thermal discrimination (Figure 37A) than during the previous (rewarded) light detection session (Figure 38A). Previously I have shown that, when Trpm8-ChR2 mice report rewarded light and cooling stimuli, the latencies are very similar (Figure 32E-F), and the wild type dataset also shows that cooling latencies are much shorter than a second (Figure 7C).

Finally, I tested whether the percept elicited by TRPM8+ afferent activation is different from mechanical touch sensation. This experiment was motivated by two facts: first, cooling and touch both elicit responses in the same (S1) neocortical region (Milenkovic et al., 2014). Second, cooling-activated afferents are also excited by mechanical stimuli (although in the noxious range). Therefore, I tested the licking behavior of light-trained Trpm8-ChR2 mice during an unrewarded detection session where cooling, vibrotactile and multisensory thermo-tactile (simultaneous cool and touch) stimuli were interleaved (equal proportion of stimulus presentations, 1 s long). Mice reported unrewarded cooling and multisensory, but not vibrotactile stimuli (Figure 39A-C). Because mice only licked the sensor when the cooling stimulus was present, this suggests that TRPM8+ afferent activation does not elicit mechanical touch perception.

Together, these data demonstrate that selective activation of TRPM8+ sensory afferent neurons triggers an unequivocal cooling percept. Moreover, optogenetic activation of forepaw somatosensory afferent neurons is a very useful method to dissect the neural mechanisms of sensory perception. With discrimination paradigms such as the one shown here, “artificial” sensory percepts can be triggered by targeting sensory neuron populations and, then, their similarity to natural stimuli can be investigated. This is a very powerful tool to establish causal links between neural activity and sensation.

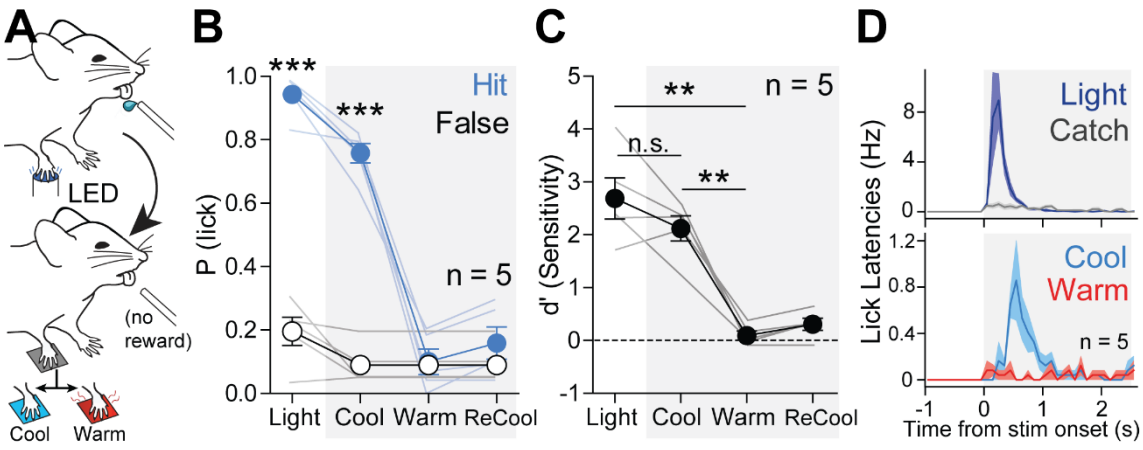

Figure 36. Optogenetic activation of TRPM8+ afferents triggers cooling but not warming perception.

(A) The stimulus discrimination behavioral paradigm consisted in, first, training Trpm8-ChR2 mice to report forepaw light stimulation via LED. Afterwards, the light stimulus was replaced by interleaved cool (32-22ºC) and warm (32-42ºC) stimuli, and licking behavior was monitored. However, during discrimination, mice were not rewarded if they licked to cooling or warming. When licking behavior was being reduced due to lack of rewards, a water droplet was given between stimuli to motivate licking for a few more trials.

(B) Hit and false alarm rates while reporting forepaw light, cooling, warming and re-cooling (post-warm). Hit and false alarm rates showed statistically significant differences for light and cooling trials (n = 5, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(C) Sensitivity (d') while reporting light and interleaved cooling and warming stimuli. Light and cooling was reported with similar accuracy, whereas performance while reporting warming was near chance level (d' ~ 0).

(D) PSTHs of first licks to report light during a rewarded light detection session and while reporting interleaved cooling and warming stimuli during an unrewarded thermal discrimination session.

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

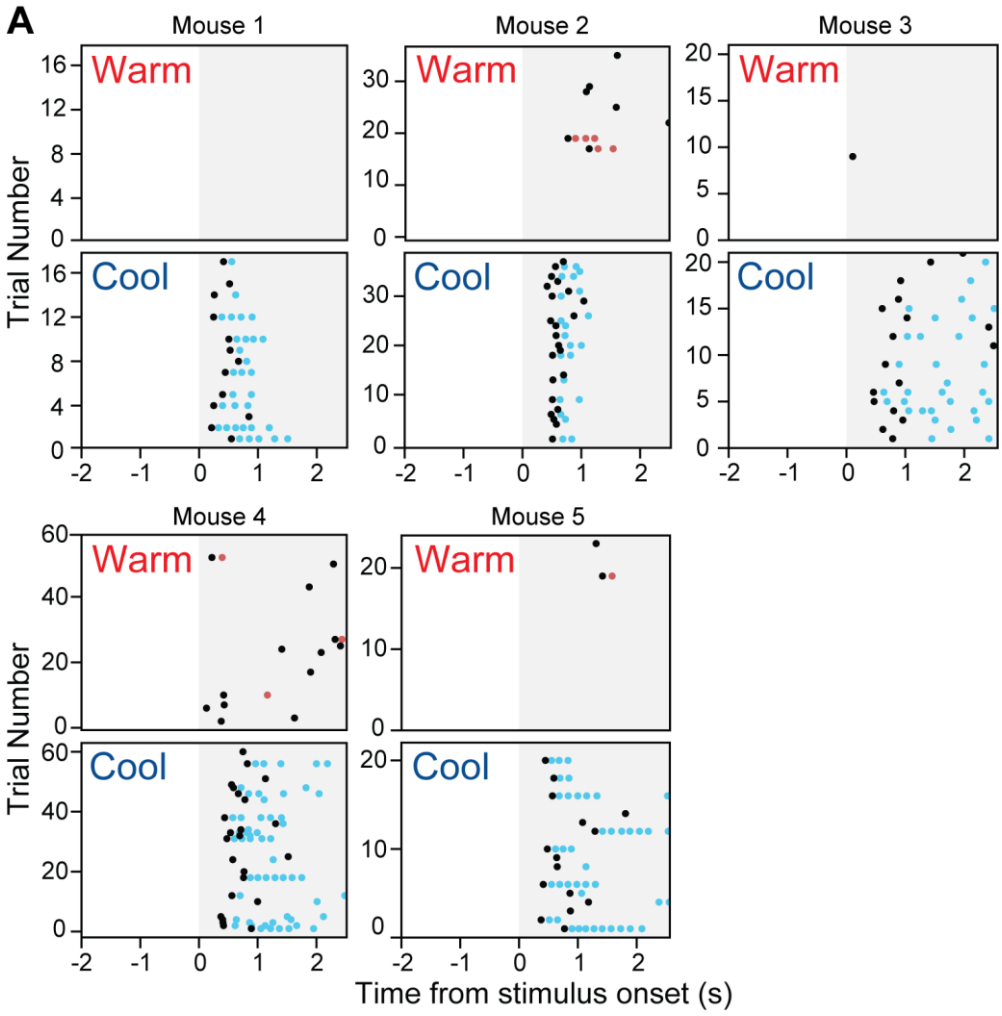

Figure 37. Light-trained Trpm8-ChR2 mice reported unrewarded cooling but not warming.

(A) Individual raster plots of licking behavior of light-trained Trpm8-ChR2 during an unrewarded thermal discrimination task. All 5 mice reported cooling stimuli for a few trials, indicating that they expected to receive rewards. First licks, shown in black, were time locked to cooling but not warming onset. Because the thermal stimuli were unrewarded, most mice stopped licking behavior after 20-30 trials.

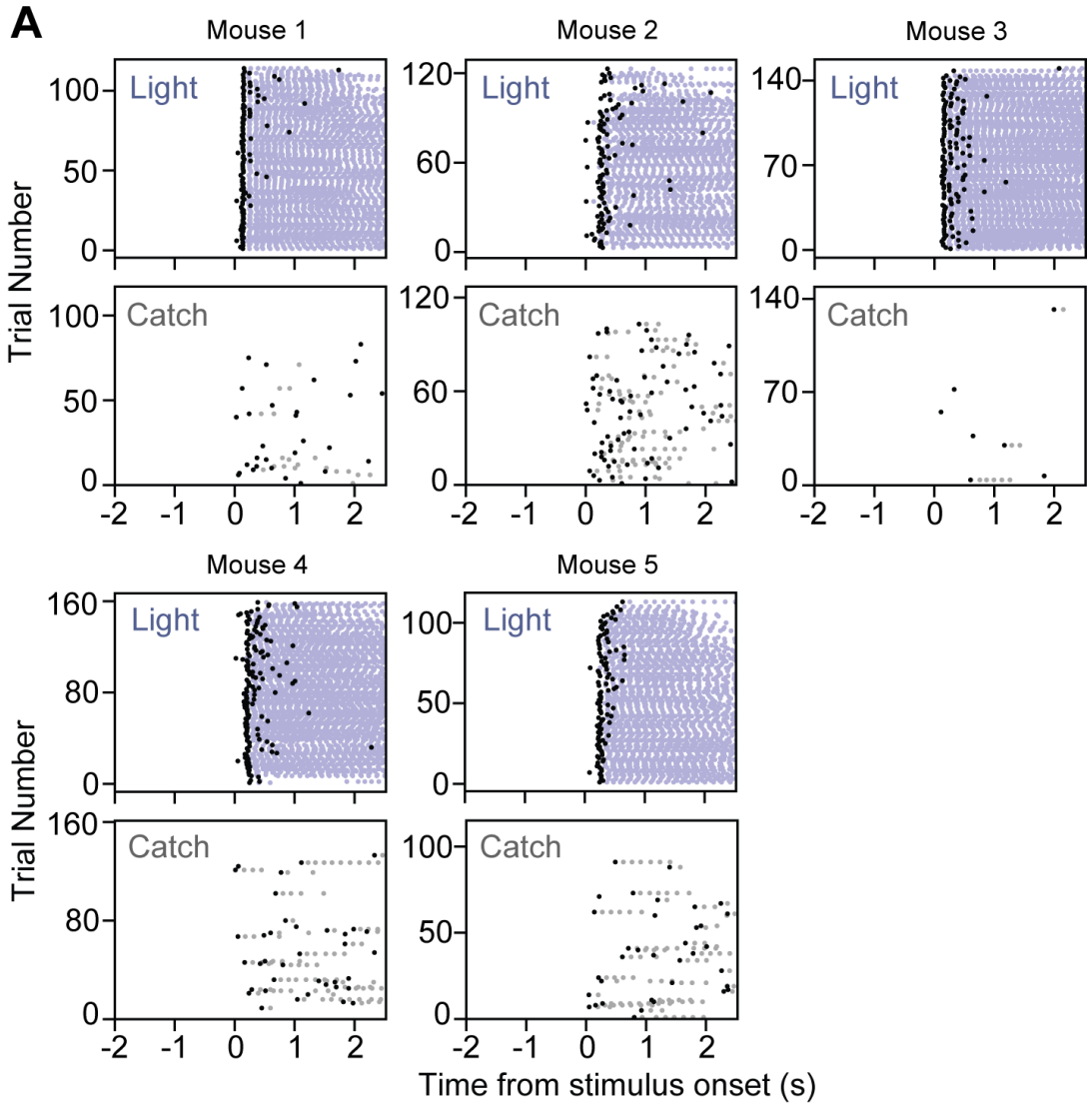

Figure 38. Light-trained Trpm8-ChR2 mice report light very quickly and reliably.

(A) Individual raster plots of licking behavior of light-trained Trpm8-ChR2 during the rewarded light detection task. All 5 mice showed very fast reaction times since stimulus onset, and latencies had low variability. First licks are shown in black. Mice licked to the light stimuli (and sometimes also during catch trials, especially during the first half of the session) for over 100 trials.

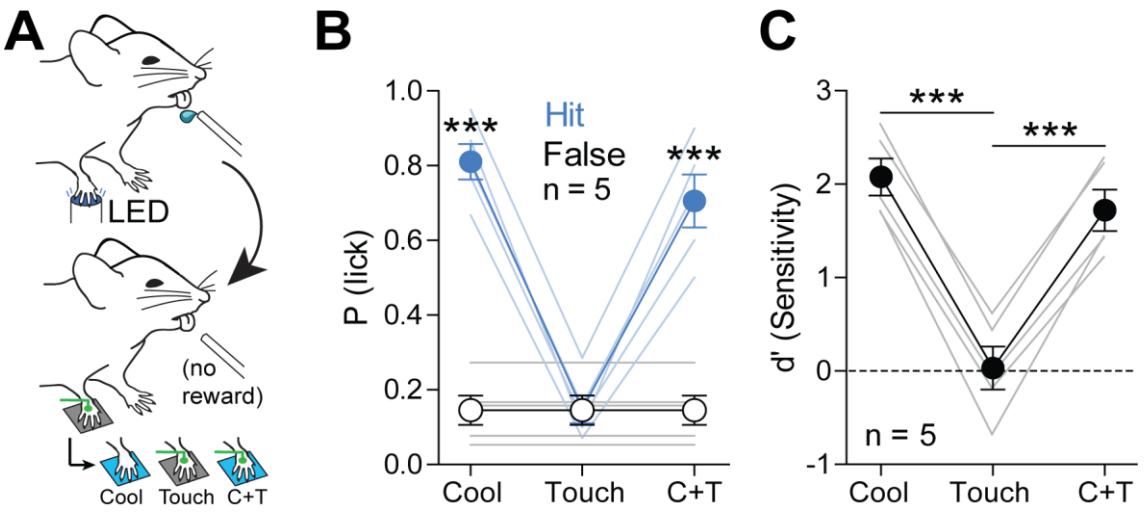

Figure 39. Activation of TRPM8+ afferents does not elicit mechanical touch sensation.

(A) First, Trpm8-ChR2 mice were trained to report light stimulation at the forepaw. Afterwards, mice were exposed to interleaved cooling (32-22^{o}C), vibrotactile and multisensory (simultaneous cooling and vibrotactile) stimulation at the forepaw. During light training, mice were rewarded when the stimuli were correctly reported. However, during exposure to natural stimuli, mice were not rewarded even when reporting the stimuli correctly.

(B) Hit and false alarm rates while reporting (unrewarded, and interleaved) forepaw cooling, vibrotactile and multisensory (simultaneous cooling and touch, C+T) stimuli. Hit and false alarm rates showed statistically significant differences for cool and multisensory trials (n = 5, two-way repeated measures ANOVA with Bonferroni post-hoc tests).

(C) Sensitivity (d') while reporting (unrewarded) cooling vibrotactile and multisensory stimuli. Mice reported cool and multisensory trials with a high sensitivity (d > 1.5), whereas the performance while reporting touch was near chance level (d' ~ 0).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

3.5 PRIMARY SOMATOSENSORY CORTEX AND WARMING PERCEPTION

The primary somatosensory cortex (S1) has been shown to be a relevant brain region for thermal processing in rodents. S1 has been found to respond to forepaw cooling, as shown via intrinsic optical imaging, whole-cell patch clamp recordings (Milenkovic et al., 2014) and increased c-fos reactivity (Beukema et al., 2018). Moreover, pharmacological inhibition of S1 neurons impairs the detection of forepaw cooling (Milenkovic et al., 2014). However, the role of S1 in the perception of warming is unknown.

For this reason, I performed a series of experiments to investigate whether primary somatosensory cortex is required by mice to detect forepaw warming. Finally, I aimed to find S1 population responses to forepaw warming.

3.5.1 Pharmacological inactivation of S1 ablates warming perception

To investigate whether primary somatosensory cortex is required for the perception of warming stimuli, I first used a pharmacological approach to silence S1 activity during warming detection. For that purpose, I performed microinjections of muscimol in the contralateral S1 of WT mice that were trained to report warming. Muscimol is a selective $GABA_A$ agonist that is commonly used to silence specific brain regions during behavior (Hikosaka et al., 1985; Rogers-Carter et al., 2018; Sreenivasan et al., 2016). Injections were performed at forepaw S1, identified in each animal by finding intrinsic optical imaging responses to tactile stimuli.

In warming-trained WT mice, muscimol (30 mM in Ringer's solution, 200 nL distributed across the cortical column in 50 nL doses at cortical depths of 200, 400, 600 and 800 µm) but not vehicle (Ringer's solution) microinjections impaired the perception of warming stimuli of 32-42^{o}C (n = 5, **Figure 40A**). Sensitivity (d') to detect warming stimuli after S1 injection of muscimol was near chance level (~ 0), and significantly lower than after vehicle injections (p = 0.0216, paired t test, n = 5, **Figure 40A**). Furthermore, the lick PSTHs at stimulus and catch trials remained similar upon muscimol but not vehicle (n = 5, **Figure 41A**), and lick latencies to report stimuli were increased after muscimol injections (p = 0.0025, paired t test, n = 5, **Figure 41C**). However, these effects were reversible, as performance returned to baseline 24 h post muscimol (p = 0.0023, paired t test, n = 5, **Figure 40A**).

Next, I tested whether the effects of muscimol in warming perception are specific to S1, and not due to the diffusion of drug into other cortical or subcortical areas. Therefore, I performed muscimol microinjections in primary visual cortex (V1) and investigated whether this affects warming detection. Unlike injections in forepaw S1, muscimol in V1 did not affect warming perception (n = 4, **Figure 40B**).

Because neurons near forepaw S1 have been suggested to mediate licking behavior in mice (Komiyama et al., 2010), I tested whether inactivation of forepaw S1 impairs the general licking ability of mice. Therefore, I trained wild type mice to report a sound cue and found that muscimol injected in forepaw S1 did not impair acoustic detection (n = 3, **Figure 40C**, **Figure 41B,D**).

Together, these results suggest that pharmacological inactivation of primary somatosensory cortex impairs forepaw warming perception.

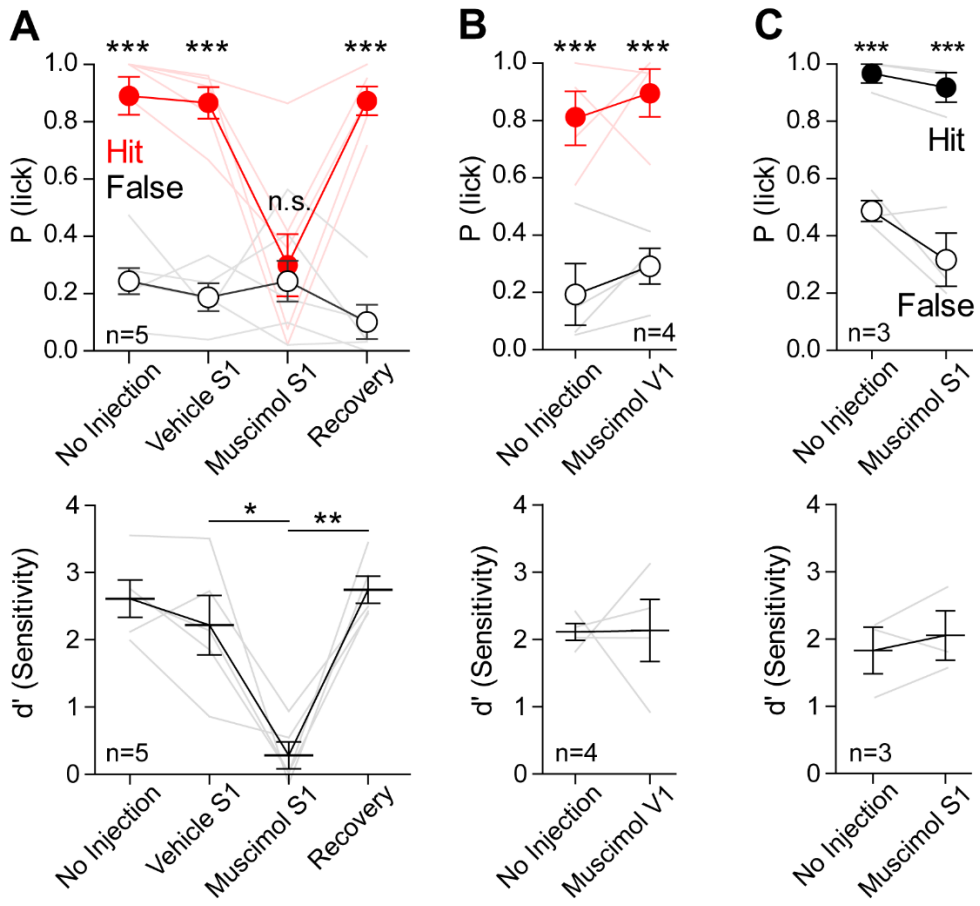

Figure 40. Pharmacological inactivation of S1 impairs warming perception.

(A) (Top) Hit and false alarm rates of warming-trained mice during warming (32-42^{o}C) detection sessions, in conditions of no injection, vehicle (Ringer's solution) and muscimol (30 mM, total 200 µL distributed in four 50 µL injections at cortical depths 200, 400, 600 and 800 µm) S1 microinjections, as well as 24h post muscimol recovery. (Bottom) Sensitivity (d') was significantly lower when muscimol but not vehicle was injected into S1 (n = 5, p = 0.0216, paired t test).

(B) Injecting muscimol in V1 of warming-trained mice did not impair warming detection, as shown in hit and false alarm rates (top) and d' (bottom).

(C) Injecting muscimol in S1 of sound-trained mice did not impair sound detection or licking in the task, as shown by hit and false alarm rates (top) and d' (bottom).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

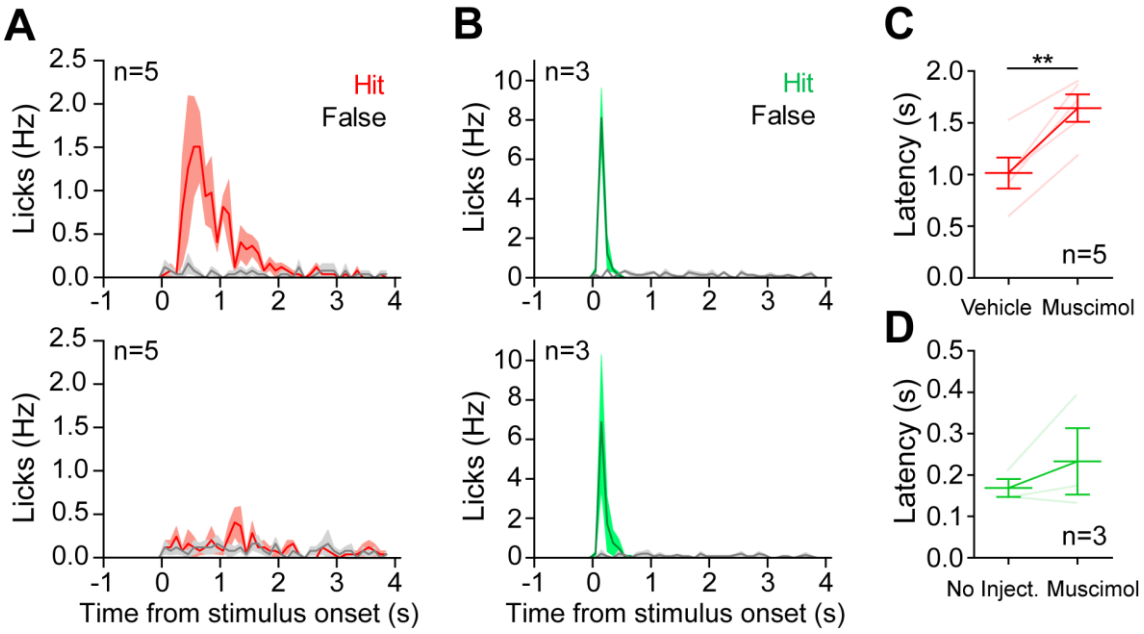

Figure 41. Pharmacological inactivation of S1 impairs warming perception (II).

(A) (Top) Population- and trial-averaged PSTHs of lick latencies from mice reporting warming (32-42ºC), when injected with vehicle (top) and muscimol (bottom). Note that only muscimol microinjection into S1 resulted in very similar hit and false alarm distributions (n = 5).

(B) (Top) PSTHs of lick latencies from mice reporting an acoustic cue were very similar between non-injected (top) and muscimol-injected (S1) conditions (bottom) (n = 3).

(C) Average latency to report warming stimuli was higher when mice where injected with muscimol in S1 than when injected with vehicle (n = 5, p = 0.0025, paired t test).

(D) Average latency to report acoustic stimuli was similar between non-injected and muscimol-injected conditions (n = 3).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

3.5.2 Optogenetic inactivation of S1 impairs warming perception

An alternative approach to disrupt brain cortical function is the use of optogenetics. By this method, specific subsets of neurons can be stimulated with light in genetically modified mice. This can be achieved by controlling the expression of light-sensitive opsins via specific gene promoters (Deubner et al., 2019). This technique has allowed neuroscientists to study the role of defined cell populations and brain regions in specific percepts or behaviors (Babl et al., 2019; Guo et al., 2014; Xu Liu et al., 2012; Mayrhofer et al., 2019).

Here, I used optogenetics to inhibit the S1 cortical network by specifically stimulating GABAergic neurons, whose activity silences pyramids (Guo et al., 2014). Because pharmacological inactivation of S1 disabled warming perception in mice, I hypothesized that optogenetic silencing of forepaw S1 neurons would also impact warming sensation.

First, VGAT-ChR2 mice were trained to report forepaw warming stimuli of near-threshold amplitude (1^{o}C amplitude, 32-33^{o}C steps, 2s long). Once the mice were capable of reliably reporting warming (as shown by statistically significant differences between hit and false alarm rates, as well as sensitivity of d' ~1), the mice were exposed to the same task again but, this time, S1 cortex was stimulated with light during 50% of the trials (473 nm, 5 mW, 100 Hz pulses that lasted for the whole trial and were delivered via a 200 µm diameter LED). To allow access of the light into the brain cortex, I performed a "clear-skull" preparation, in which the scalp is dissected and the skull is lightly polished and covered with transparent dental cement (Guo et al., 2014). To prevent the mice from seeing when S1 light stimulation was taking place, all experiments (as well as the previous training) took place with masking lights randomly flickering near the face.

Light stimulation of S1 in VGAT-ChR2 mice impaired the detection of near-threshold warming stimuli, as shown by a decrease in sensitivity (n = 8, p = 0.0007, paired t test of d' light vs no light trials, **Figure 42A**). Similarly, the lick PSTHs during stimulus and catch trials clearly differed in no-light trials, but not during light trials (n = 8, **Figure 43A**).

Next, the same experiment was repeated with higher warming amplitudes (2^{o}C for 4 mice and 4^{o}C for 2 mice). This time, warming-trained VGAT-ChR2 mice could still report some of the warming stimuli during light trials, as shown by differences between hits and false alarms (n = 6, **Figure 42A**). However, sensitivity (d') was again reduced during light stimulation, compared to no-light trials (n = 6, p = 0.0343, paired t test, d' light vs no light trials, **Figure 42A**).

Similar to the pharmacological experiments, I performed a control experiment where I tested whether optogenetic silencing of V1 impacts warming perception. Light stimulation of V1 did not impact the ability of VGAT-ChR2 to sense near-threshold warming, as shown by similar hit and false alarm rates, as well as d' during light and no-light trials (n = 4, **Figure 42B**).

Finally, I tested whether optogenetic inactivation of S1 impairs the ability of the mice to lick in this task, by training VGAT-ChR2 mice to report sound stimuli. Light stimulation of S1 did not prevent VGAT-ChR2 mice from reporting sound cues with the same accuracy as no-light trials (n = 4, **Figure 42C**).

Together, these results suggest that optogenetic inactivation of primary somatosensory cortex impairs warming perception. Specifically, the detection of forepaw warming of near-threshold amplitude (1°C) was particularly disrupted by light stimulation over S1 of VGAT-ChR2 mice.

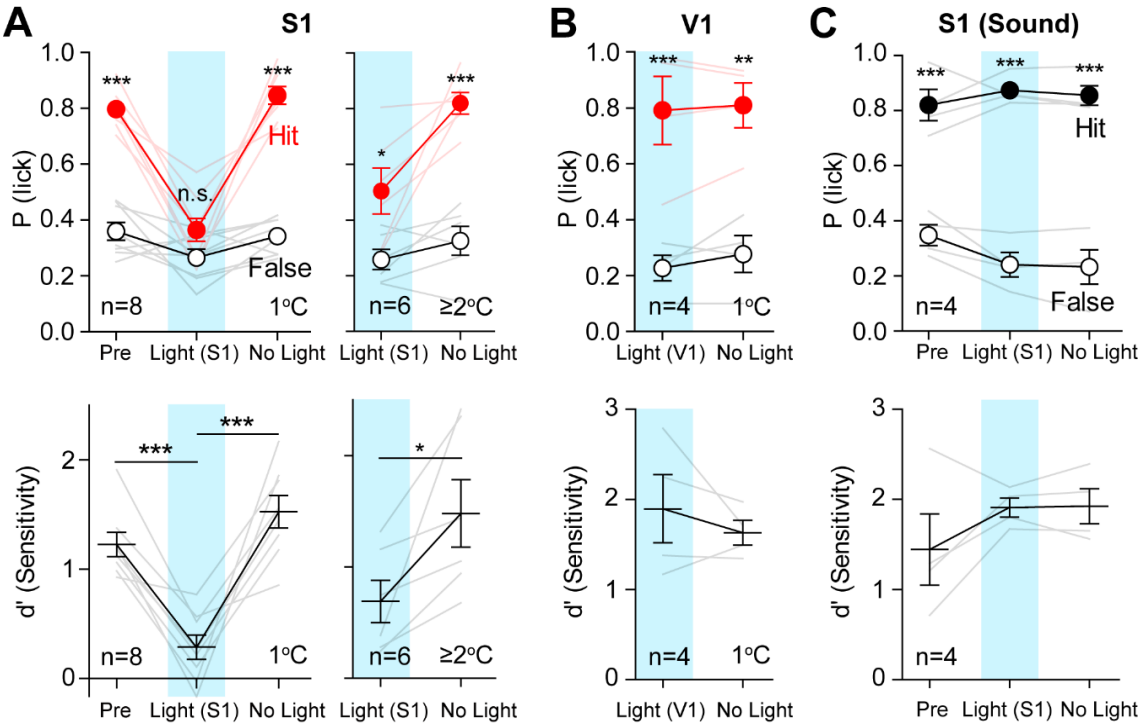

Figure 42. Optogenetic inactivation of S1 impairs warming perception.

(A) Hit and false alarm rates (top) and sensitivity (d', bottom) of warming-trained VGAT-ChR2 mice in low-amplitude warming (1 and ≥2°C, as shown), before ("pre") and during optogenetic silencing. Warming trials with simultaneous light stimulation (data over a blue shade) (100 Hz, 5 mW lasting for the whole trial duration, 2s) over S1 were randomly alternated, in the same session, with trials without light stimulation. Light stimulation of S1 almost entirely disabled the detection of near-threshold warming (1°C) stimuli (n = 8, p = 0.0007, paired t test, d' light vs no light trials) and reduced the ability to detect greater (2 or 4°C) warming stimuli (n = 6, p = 0.0343, paired t test, d' light vs no light trials).

(B) Light stimulation over V1 in VGAT-ChR2 mice did not impair the detection of near-threshold (1°C) warming stimuli, as shown by hit and false alarms (top) and sensitivity (bottom) (n = 4).

(C) Light stimulation over S1 in VGAT-ChR2 mice did not impair the detection of acoustic cues, as shown by hit and false alarms (top) and sensitivity (bottom) (n = 4).

* P < 0.05, ** P < 0.01, *** P < 0.001. Data = mean ± SEM.

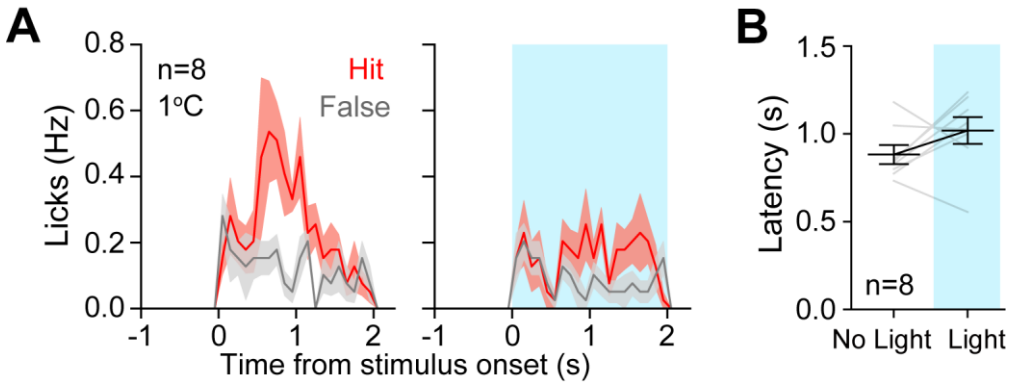

Figure 43. Optogenetic inactivation of S1 impairs warming perception (II).

(A) Population- and trial-averaged PSTHs of VGAT-ChR2 mice during near-threshold warming (1°C, 2 s long) detection. Left shows trials with no light stimulation in S1, and right shows light stimulation trials (n = 8).

(B) Mean hit latency during light and no-light trials (n = 8).

Data = mean ± SEM.

3.5.3 No warming-evoked population response detected in S1

Both pharmacological and optogenetic inactivation of S1 disrupted, to some extent, the perception of forepaw warming in mice. These findings suggest that S1 plays a role in the processing of warming stimuli. Therefore, I investigated the population neural activity in S1 in response to tactile and thermal stimuli delivered to the forepaw via intrinsic optical imaging. Using this technique, population responses to tactile and cooling stimulation of the forepaw have been found in mouse S1 (Milenkovic et al., 2014).

Intrinsic signal optical imaging (ISOI) is an *in vivo* technique used to map sensory responses in superficial brain areas. This method relies on the property that the brain changes the reflection of light depending on its activity (Grinvald et al., 1986). Typically, red light (wavelength of ≥ 600 nm) is shone onto the brain and, depending on its activity, more or less light is reflected from the tissue, which is captured with a camera (Frostig et al., 1990; Nakamichi et al., 2019; Turley et al., 2017). With ISOI, small (~0.1 mm) regions responding to a stimulus can be identified, with a temporal resolution of about 1-2 seconds (Lu et al., 2017). The mechanisms underlying intrinsic optical signals are under debate. One of the proposed generators of intrinsic optical signals are the local hemodynamic changes that occur in the brain and correlate with neural activity (Malonek & Grinvald, 1996; Roy & Sherrington, 1890). In addition, activity-evoked changes in metabolism (Vanzetta, 1999) or oxygenation (Nemoto et al., 1999) have also been proposed to underlie intrinsic signals. More recently, it has been reported that axonal

swelling and not changes in vasculature may underlie intrinsic signals (Vincis et al., 2015). Despite the neuronal basis not being completely clear, ISOI is a widely used and robust technique to identify activated cortical regions (Ferezou et al., 2006; Juavinett et al., 2017; Milenkovic et al., 2014; Morone et al., 2017; Nakamichi et al., 2019).

First, I performed ISOI in WT mice (under isoflurane anesthesia) and looked for forepaw S1 responses to tactile stimulation (4 s long, vibrotactile stimulus delivered to the glabrous skin of the forepaw) (n = 7 WT mice, **Figure 44A**, **Figure 45A**). Next, I exchanged the vibrotactile stimulus by a cooling step of 32-22ºC (4 s long), and again found responses in S1 (n = 6 WT mice, **Figure 44B**, **Figure 45B**). Similar to what has been already reported (Milenkovic et al., 2014), the cooling-responsive area of S1 was partially overlapping with the tactile-responsive area. Finally, I investigated the ISOI S1 responses to warming of 32-42ºC (4 s long). However, surprisingly, no reliable ISOI responses to warming were found near forepaw S1 (n = 7 WT mice, **Figure 44C**, **Figure 45C**).

Together, these data suggest that forepaw warming does not elicit robust population responses in primary somatosensory cortex (S1). Therefore, the previously observed loss in warming perception associated to S1 pharmacological or optogenetic manipulation should be interpreted with caution. One possible explanation for these findings is that S1 is required for warming perception in an indirect manner, similar to the findings observed in mice lacking the cooling sensor TRPM8. Despite warming does not elicit population responses in S1, optogenetic and pharmacological inactivation of S1 disturbs cooling signaling (Milenkovic et al., 2014), which may be necessary for the perception of warm stimuli.

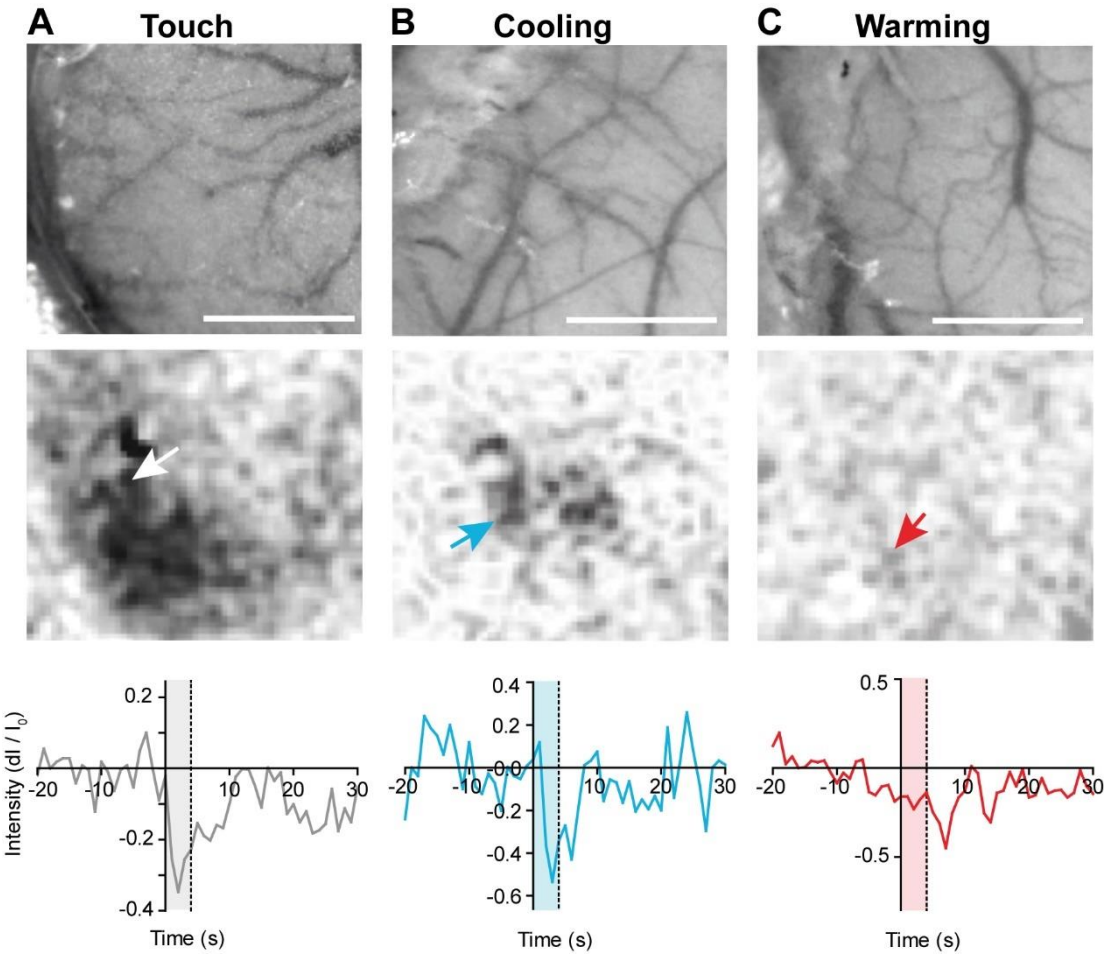

Figure 44. Intrinsic Signal Optical Imaging of S1 during touch, cool and warm stimuli.

(A) Intrinsic Signal Optical Imaging (ISOI) response to tactile stimulation of the contralateral forepaw, recorded in S1. (top) Image showing the blood vessel pattern, obtained with green light. (middle) Image showing the variation in light absorption over the stimulus period (4 s). Darker pixels indicate a larger absorption of light (i.e. less light being reflected from the tissue) than surrounding area, which indicates higher brain activity. The pixel that had the most pronounced stimulus-evoked peak in absorption is marked with an arrow. (bottom) Light reflection data for pixel highlighted in (middle). Data in A, B and C are from different WT mice.

(B) ISOI example S1 response to cooling stimulation (4 s long, 32-22^{o}C ramp) of the contralateral forepaw. (top) Blood vessel pattern. (middle) Variation in light absorption over S1 region, as a response to stimulation. (bottom) Light reflection data for pixel highlighted in (middle).

(C) ISOI example S1 response to warming stimulation (4 s long, 32-42^{o}C ramp) of the contralateral forepaw. (top) Blood vessel pattern. (middle) Variation in light absorption over S1 region, as a response to stimulation. (bottom) Light reflection data for pixel highlighted in (middle). Note that no reliable response was found during warming, as opposed to touch or cooling.

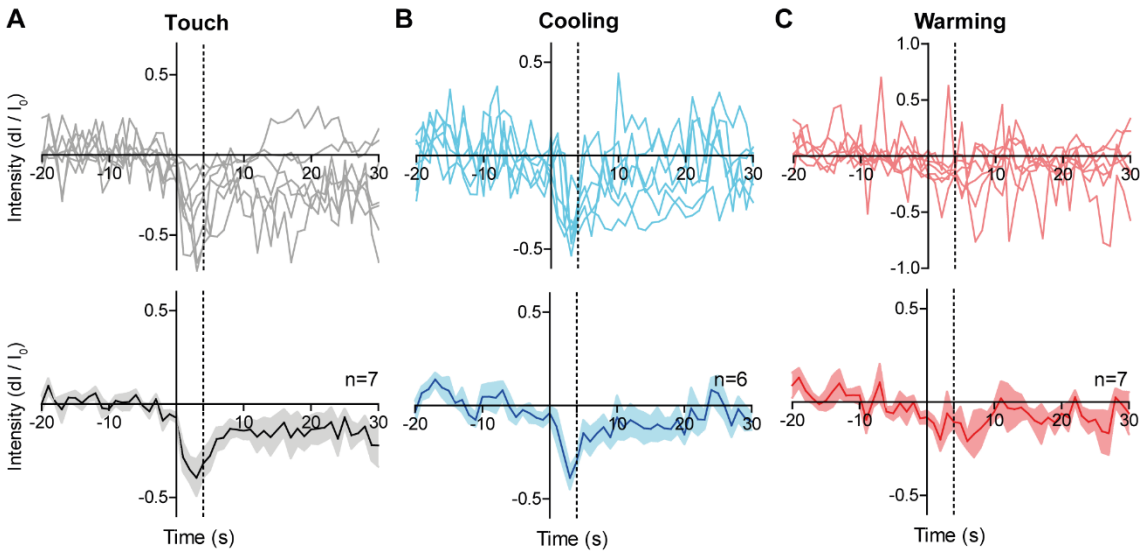

Figure 45. No reliable ISOI warming-evoked responses in S1.

(A) (top) ISOI S1 responses during tactile stimulation of the contralateral forepaw. Each trace corresponds to the averaged (over 5-15 trials) tactile-evoked response of a single mouse. (bottom) ISOI S1 mean (± SEM) population response to contralateral tactile stimulation (n = 7).

(B) (top) ISOI S1 responses during cooling (32-22ºC) stimulation of the contralateral forepaw. Each trace corresponds to the averaged (over 5-15 trials) cooling-evoked response of a single mouse. (bottom) ISOI S1 mean (± SEM) population response to contralateral cooling stimulation (n = 6).

(C) (top) ISOI S1 responses during warming (32-42ºC) stimulation of the contralateral forepaw. Each trace corresponds to the averaged (over 5-15 trials) warming-evoked response of a single mouse. (bottom) ISOI S1 mean (± SEM) population response to contralateral warming stimulation (n = 7).

3.6 LICKING BEHAVIOR THROUGHOUT LEARNING IN A GO/NO-GO DETECTION TASK

For the work presented here, I trained a large number of mice to report thermal stimuli by using a Go/No Go detection paradigm. In total, 31 mice were trained to report cooling or warming (10ºC amplitude, starting from either 22 or 32ºC baseline) for at least 10 consecutive sessions. To better understand licking behavior in mice under this paradigm, and to allow for efficient planning of future experiments, in this section I analyzed how different features of mouse licking behavior changed: i) over trials throughout a session and ii) over learning stages (naïve vs expert).

For this analysis, I included 12 mice trained for 32-42ºC warming, 6 mice trained for 22-32ºC warming, 7 mice trained for 32-22ºC cooling and 6 mice trained for 22-12ºC cooling.

For the naïve dataset, I took the data from the very first behavioral session at which learning was observed in each of these datasets (shown by statistical differences between hits and false alarms). In turn, the expert dataset includes the data from the same mice at the last (10^{th}) behavioral training session. Water reward size was kept constant for each dataset (always between 4-7 µL), and each behavioral session typically consisted of 50 stimulus (and 50 catch) trials, but some mice underwent more trials if they still showed task engagement. Spontaneous licks were only punished by delaying the next stimulus presentation and therefore making the mouse wait longer for the next reward.

First, the effects of training showed, as expected, a clear increase of sensitivity (d') at the expert stage, compared to the novice phase (**Figure 46A**). This increase in sensitivity was caused by both an increase in hit rate (**Figure 46B**) and a decrease in false alarms, which were very high at the novice stage (**Figure 46C**). Overall licking rate remained unchanged (**Figure 47A**) and, interestingly, the reaction time to report the stimuli was faster in expert mice than in novice mice (**Figure 47B**).

These data indicate that, throughout behavioral training, mice miss less stimulus trials, and their licking becomes less spontaneous and more restricted to stimulus presentation. Moreover, mice are slightly faster when reporting the stimulus at the expert stage, which suggests that the speed of sensory perception may be underestimated in experiments with mice that are not optimally trained.

Then, I analyzed the licking behavior throughout the trials of a behavior session, both at the novice and expert stages. As previously reported (Berditchevskaia et al., 2016), sensitivity (d') clearly increased over stimulus trials in novice mice. In expert mice, a similar but smaller trend was observed (**Figure 48A**). This increase in sensitivity was due to a slightly smaller hit rate at the very early trials (**Figure 48B**) and mostly to the decrease in false alarms throughout the task (**Figure 48B**). In expert mice, the hit rate was remarkably constant throughout the session, but the false alarm rate showed a decreasing trend over trials (**Figure 48B**). Moreover, in novice mice, the lick rate decreased over trials (**Figure 49A**), and the reaction times became slower at the latter trials than at the early stage of the session (**Figure 49B**). In expert mice, lick rate and reaction times remained more constant throughout the session. These two parameters suggest that mice endure better at the expert stage than at the novice stage. At the novice stage, mice seem to undergo fatigue or an earlier decrease in motivation, which makes them lick less often and more slowly at the latter trials within a session.

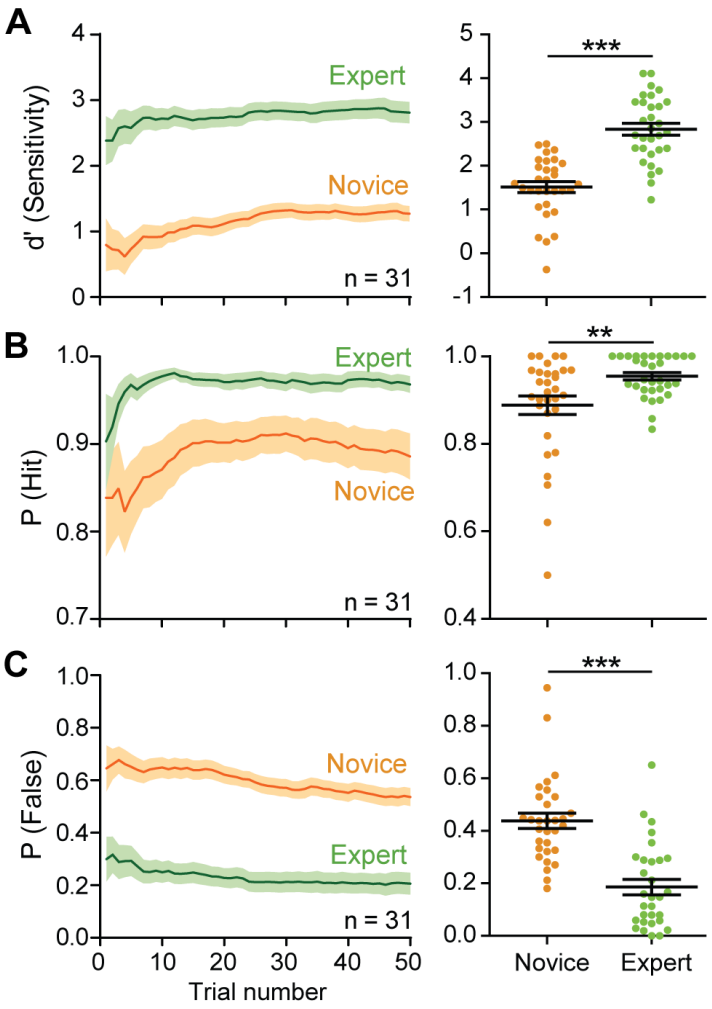

Figure 46. Sensitivity (d') and hit and false alarm rates in the thermal Go/No Go detection task at naïve and expert stages.

On the left panels, group averages ± SEM over trials; on the right, session average for each individual mouse (dots) are shown on the right.

(A) d' (sensitivity) while detecting thermal stimuli was higher at the expert stage, compared to the novice stage ($p < 0.0001$, $n = 31$, Wilcoxon signed-ranked test).

(B) The percentage of correctly reported stimuli (hits) was higher for expert mice than novice mice, despite in both cases it was generally high (> 80%) at both stages ($p = 0.0019$, $n = 31$, Wilcoxon signed-ranked test).

(C) Training reduced false alarm rates, an indicator of spontaneous licking, as seen by statistically significant differences between novice and expert stages ($p < 0.0001$, $n = 30$, paired t test).

Data are mean ± SEM. Statistically significant differences are displayed with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

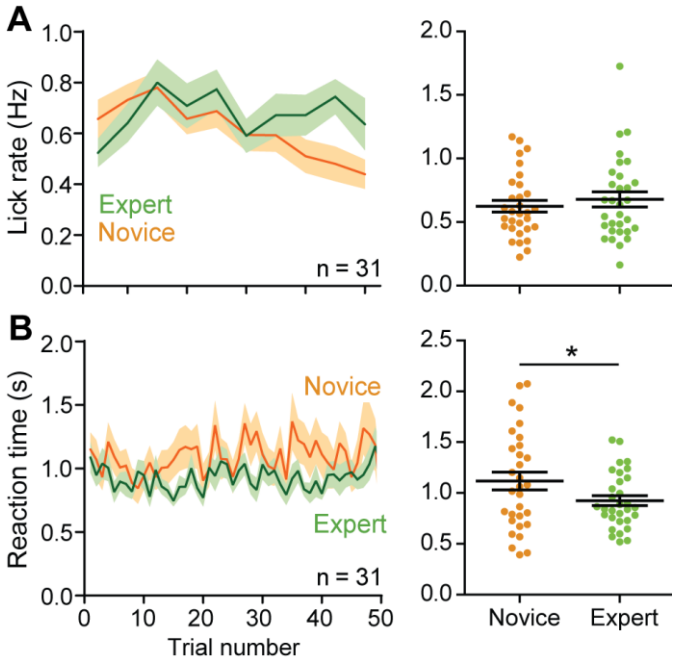

Figure 47. Lick rate and reaction time in the thermal Go/No Go detection task at naïve and expert stages.

On the left panels, group averages ± SEM over trials; on the right, session average for each individual mouse (dots) are shown on the right.

(A) Lick rate did not differ between novice and expert stages of behavior. Lick rate is shown in Hz, and was measured between the onset of one stimulus trial and the onset of the next. Note that reward-triggered licks are not excluded from analysis.

(B) Training slightly reduced the average reaction time to report the stimulus ($p < 0.0153$, $n = 31$, paired t test).

Data are mean ± SEM. Statistically significant differences are displayed with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Overall, these data indicate that training enhances performance in Go/No Go behavioral tasks over days by reducing misses and spontaneous licking, and reaction times become faster with training. Moreover, expert mice endure better throughout the task, displaying a more constant performance; whereas novice mice lick less often and more slowly by the end of the session.

These results suggest that the performance in this task greatly depended on the ability of mice to restrict their licking to the stimulus, but also on their resistance to fatigue or their ability to remain attentive and engaged. Future experiments aiming to maximize d' values should consider training mice for long time, and during a larger amount of trials. Including a stricter punishment upon spontaneous licks could also be an option (*e.g.* air puff instead of a delayed waiting period). However, it is possible that reaction times are

underestimated with longer training paradigms, as mice may get progressively slower and less attentive or motivated throughout the session.

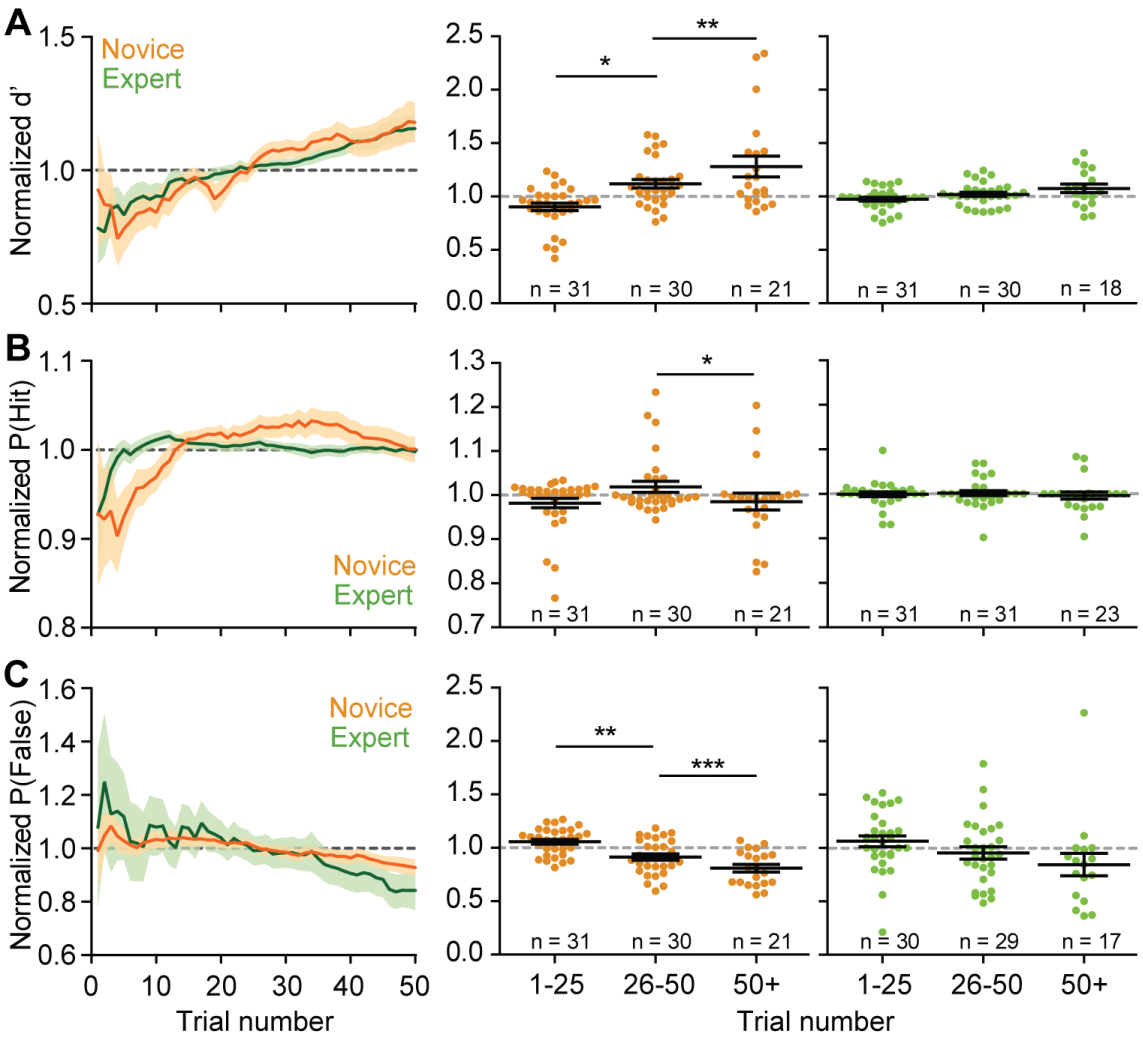

Figure 48. Fluctuation of sensitivity (d'), hit and false alarm rates across session trials in a thermal Go/No Go detection task.

On the left panels, group averages ± SEM over trials; middle and right, session split average for each individual mouse (dots) are shown on the right.

(A) Sensitivity (d') increased over trials within a session in novice mice ($p = 0.0114$, $n = 30$, for 1-25 vs 26-50 trials, Wilcoxon signed-ranked test; $p = 0.0083$, $n = 21$ for 26-50 vs 50+ trials, paired t test). In expert mice, a similar but reduced (and statistically non-significant) trend was observed.

(B) The hit rate remained similar throughout the session for both novice and expert mice, however, the lick probability is slightly lower at the very early trials (0-10) in both conditions, suggesting that some mice need a few stimulations or rewards to start performing optimally. At the novice stage, the hit rate was reduced during the last trials, indicating a reduction of engagement in the task ($p = 0.0263$, $n = 21$, Wilcoxon signed-ranked test, trial windows of 26-50 vs +50).

(C) The false alarm rate decreased over trials at the novice stage (p = 0.0081, n = 30 for 1-25 vs 26-50 comparison; and p < 0.0001, n = 21 for 26-50 vs 50+ comparison, paired t tests), and showed a similar but statistically non-significant trend at the expert stage.

Data are mean ± SEM. Statistically significant differences are displayed with asterisks (* p < 0.05, ** p < 0.01, *** p < 0.001).

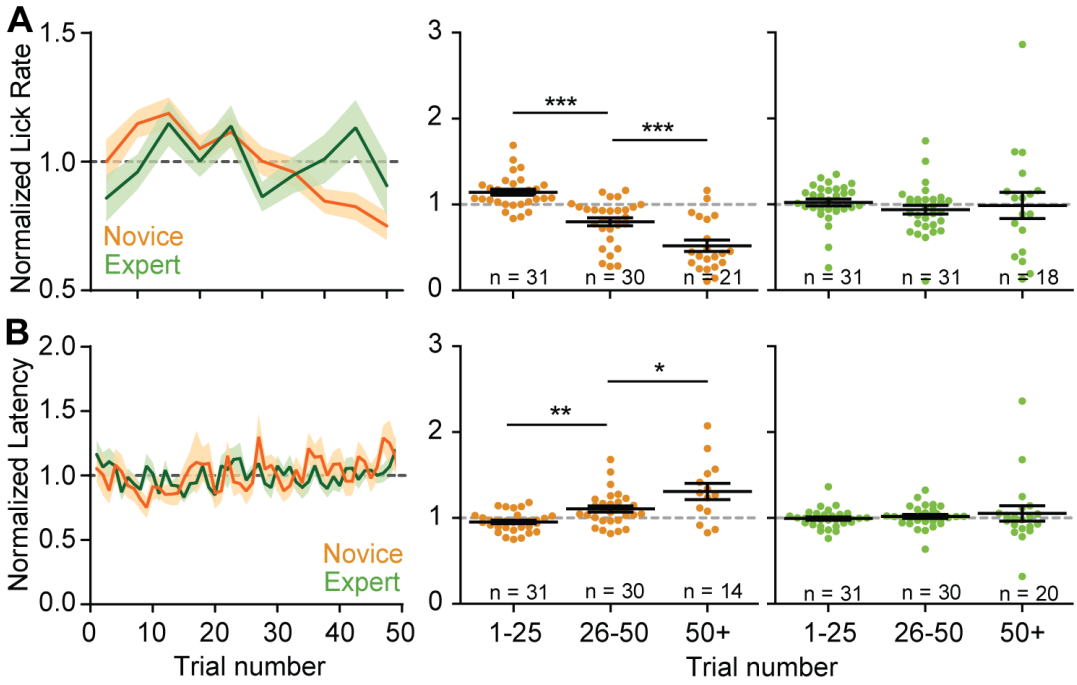

Figure 49. Fluctuation of lick rate and reaction times across session trials in a thermal Go/No Go detection task.

On the left panels, group averages ± SEM over trials; middle and right, session split average for each individual mouse (dots) are shown on the right.

(A) The lick rate decreased over trials in novice mice (p < 0.0001, n = 30 for 1-25 vs 26-50 comparison, paired t test; and p = 0.0005, n = 21 for 26-50 vs 50+ comparison, Wilcoxon signed-ranked test), whereas it remained more stable at the expert stage.

(B) Reaction time (latency) increased over trials at the novice stage (p = 0.0069, n = 30 for 1-25 vs 26-50 comparison; and p = 0.0118, n = 14 for 26-50 vs 50+ comparison, paired t tests), but at the expert stage this trend was reduced and lick latencies were more consistent throughout the session.

Data are mean ± SEM. Statistically significant differences are displayed with asterisks (* p < 0.05, ** p < 0.01, *** p < 0.001).

4. DISCUSSION

4.1. MOUSE THERMAL PERCEPTION

4.1.1 Mice and humans have similar thermal detection capacity

While it was known that mice and humans have similar thermal pain thresholds, the sensitivity of mice to detect tiny (< 2ºC) changes in skin temperature was unknown and, therefore, it was unclear whether non-noxious thermal perception was also similar between rodents and humans. The experiments presented here show: i) mice can reliably detect forepaw cooling and warming, ii) they sense cooling better than warming (amplitude threshold is 0.5ºC and 1ºC, respectively, at 32ºC baseline); iii) mice can discriminate warming from cooling; iv) mice need a longer time window to perceive warming than cooling.

These findings are in line with human psychophysical literature. Humans also discriminate warm from cool, and the detection thresholds for both are also remarkably small, of < 1ºC in many body regions. Interestingly, cooling is also perceived better than warming in humans (Stevens & Choo, 1998). Therefore, taken together, the results presented here indicate that humans and mice have highly comparable perceptual acuity for non-painful temperature. This suggests that the biological mechanisms underlying thermal perception are similar across these two species. Therefore, mice are a useful experimental model to investigate the mechanisms underlying thermal perception.

4.1.2 Warming is sensed more slowly than cooling

One interesting finding of murine thermal perception is that warming is sensed significantly more slowly than cooling (**Figure 7**).

One straightforward explanation could be that warm sensory afferents have a slower conduction velocity than cooling fibers. Indeed, in humans, cool-activated sensory afferents are thought to be either thinly myelinated (Aβ-type) or unmyelinated (C-type), whereas warm afferents are believed to be only unmyelinated (Vriens et al., 2014). However, in mice, almost the entirety of thermosensitive afferents found were unmyelinated (**Figure 14**), therefore the conduction velocity for both warm- and cool-activated fibers is similar. Thus, the longer perceptual latencies likely have a different mechanism.

Another cause for this delay could be that warming-activated or warming-inhibited responses may have a longer latency than cooling-activated responses. Comparing the afferent spike PSTH data upon warming and cooling does not suggest an obvious latency difference in warming responses, however, we did not test this in detail due to low numbers of thermosensitive afferents, and the *ex vivo* nature of these experiments. In fact, it is feasible that warming signaling is slower at the afferent encoding level. There are studies suggesting that keratinocytes may mediate activate primary sensory afferents during warming (Baumbauer et al., 2015; Chung et al., 2004; Sadler et al., 2020). This idea is motivated by the fact that some warm/hot TRP sensors are expressed not in sensory fibers but instead they are keratinocyte-specific (Chung et al., 2004; Xiaoling Liu et al., 2019; Moqrich et al., 2005; Peier, Reeve, et al., 2002; Sondersorg et al., 2014). If warming-evoked spikes (or warming inhibition) at the sensory afferents depend to some degree on keratinocyte signaling, this could potentially explain the few hundred additional milliseconds that are required for warming perception, in comparison to cooling sensing.

Another possible explanation may lie behind the features of warming and cooling processing in the central nervous system. At the afferent level, warmth is encoded via 2 populations of afferents: warm-activated and warm-inhibited units. Cooling, however, simply elicits excitatory responses in cool-sensitive fibers. The central integration of warm-activated and inhibited signals may require a longer time than the processing of cooling-evoked spikes. Furthermore, the data presented here suggests that warming perception relies on warm-evoked inhibition in cool afferents. It is plausible that the central nervous system processes a signal that is encoded via firing rate increase faster (*e.g.* cooling) than via firing rate decrease (*e.g.* warming).

Additionally, at the brain level, it is likely that warming- and cooling-evoked responses follow partially segregated pathways. One piece of evidence suggesting this idea is that cooling-responding neurons have been found in the primary somatosensory cortex (S1) (Milenkovic et al., 2014), but warming responses are not found in that cortical area (as shown by a lack of intrinsic imaging responses to warmth, **Figure 44C**, **Figure 45C**, and other unpublished data from our lab). S1 neurons, however, appear to be involved in a warming detection task nevertheless (pharmacological and optogenetic inhibition of S1 impaired warming detection, **Figure 40**-**Figure 42**). This suggests that there may be cross-talk between cooling and warming neural pathways at the highest levels of processing, or even that some important aspect of warming perception is extrapolated from cooling-activated neurons in the central nervous system. Interestingly, such a mechanism in which central cooling responses are utilized for warming sensation has

been shown in fruit flies (Greppi et al., 2020; Liu et al., 2015), so a similar mechanism could exist in mammals.

In summary, the longer time needed to sense warming as opposed to cooling does not seem to depend on the afferent axonal speed in mice. Instead, initiation of afferent responses or the central processing of temperature may be behind this phenomenon.

4.1.3 Perception of warming and cooling is modulated by baseline temperature

In order to link specific subsets of thermosensitive afferents with perceptual outcome, I carried out behavioral experiments using different baseline temperatures. Surprisingly, mice were better at detecting warming stimuli when they took place at a lower (22ºC) baseline than at a thermoneutral one (32ºC). This is shown by lower perceptual thresholds, shorter latencies and enhanced task performance. These data highlight the importance of warming-inhibited afferents in warming perception, since warming-activated fibers fired very sparsely to 22-32ºC stimuli.

The reason why low baseline warming is sensed better than warming at higher baseline is unclear. In our hands, the activity of warming-silenced fibers was similar during these two stimulus types. However, because warming-silenced fibers are cool-activated, it is possible that, at a lower baseline, they could be slightly more active in magnitude (number of spikes per afferent) or as a population (number of afferents active). This could explain the greater sensitivity of mice to detect warming at a lower baseline, and it could have gone unseen with our relatively small sample size (~30 thermosensitive fibers per dataset). Future experiments that allow for larger sample sizes, like calcium imaging of forepaw DRG populations, could test these hypotheses.

For cooling, however, perceptual abilities were similar at baselines of 22 and 32ºC. The only difference observed was that cooling was perceived more slowly when delivered at a low baseline. Cooling-activated afferents seem to show a great degree of adaptation, and the spike PSTHs during cooling at low and thermoneutral baselines seem quite similar. However, we observed a slightly lower amount of spikes driven by the stimulus at the 22-12ºC step, but more neurons activated by it, than during cooling during a thermoneutral baseline. The lower spikes per afferent triggered by the low baseline cooling may underlie this tiny decrease in perceptual ability. However, cooling perception at a low baseline was also very good, as the mice reliably reported cooling steps of 0.5ºC (as they did during a thermoneutral baseline).

In summary, warming perception is better at lower baseline (22ºC) than at a thermoneutral one (32ºC), which highlights the importance of warming-silenced afferent fibers in perception of warming. Cooling perception, in turn, is quite similar at these two baselines, with reaction times being the only observed difference. To better understand the importance of baseline temperature in thermal perception, future studies should investigate how afferents behave at different baselines *in vivo* and how relative thermal changes modulate such background activity (at both the single afferent and populational levels). Moreover, testing multiple baselines on single experiments should allow us to understand better the time-dependent adaptation of afferent activity.

4.2. MOUSE AFFERENT ENCODING OF TEMPERATURE

4.2.1 Warming is encoded by two populations of sensory afferents

In the *ex vivo* afferent recording experiments carried by my collaborator Dr. Fred Schwaller in the Gary Lewin lab (Max Delbruck Center, Berlin), warming stimuli elicited two types of responses in sensory fibers: i) spiking activity in warm-activated fibers that were silent during baseline, and ii) inhibition of spiking activity in warm-silenced fibers that had ongoing action potentials during baseline. Importantly, all warming-evoked responses in primary sensory afferents occurred in polymodal C-fibers (*i.e.* unmyelinated fibers that were sensitive to noxious mechanical stimuli). In experiments with a thermoneutral baseline (32ºC), warm-activated units represented ~30% of all thermosensitive afferents, whereas warm-inhibited fibers were slightly below 20%.

Warm-activated fibers did not specifically respond only in the non-noxious range, but also fired action potentials beyond the pain threshold (>43ºC). This suggests that thermosensitive afferent neurons may play a role in sensing both non-noxious temperature and pain. Interestingly, some of them (~20% of all thermosensitive afferents) also were cool-activated (C-MHC fibers). Because they signal both warming and cooling in the same way (*i.e.* increase in firing) to the target neurons in the spinal cord, perhaps the role of C-MHC fibers is to encode amplitude of thermal stimuli, instead of direction of change. Warm-activated afferents responded more vigorously as the absolute temperature increased: they responded very sparsely between 22 and 32ºC, had a stronger increase between 32-42ºC and highly robust firing between 42-48ºC. This finding is consistent with the encoding of temperature increases observed in the spinal cord (Ran et al., 2016) and hindpaw DRGs (Wang et al., 2018).

Warm-silenced fibers, on the other hand, had ongoing spiking activity at baseline temperatures (22 or 32ºC) and would cease firing upon warming. Upon cooling, their firing rate was enhanced. Warm-silenced fibers showed a similar degree of inhibition evoked by warming steps of 22-32 and 32-42ºC, suggesting that they encode relative warming rather than absolute temperature. Likewise, their baseline firing at 22 and 32ºC was of similar magnitude.

Cooling, as reported previously (Milenkovic et al., 2014), was encoded by polymodal C-fibers that fired with decreasing temperatures. We tested their responsiveness to 10 degrees of cooling at two different baselines, 22 and 32ºC, and saw comparable spiking responses. This suggests that, as opposed to warming-excited neurons, forepaw afferents encode cooling in a relative way; as previously described in the spinal cord (Ran et al., 2016) and hindpaw DRGs (Wang et al., 2018).

In conclusion, these findings suggest that: first, forepaw warming and cooling are mainly encoded by polymodal C-fibers; second, warm-activated afferents encode non-noxious warmth (and heat) via action potential firing that is increasing with absolute temperature; third, warm-silenced neurons have high spontaneous rates and encode warming via firing rate suppression, which seems to depend on a relative change in temperature; finally, cooling is encoded by an increase in firing rate that appears to depend on the relative change in temperature. When comparing these data to primate studies, the warm-activated C-MH and C-MHC fibers are reminiscent of, respectively, the monotonic and bimodal fibers observed in primates, which are activated by both warmth and heat (**Figure 2**). The warm-inhibited C-fibers described here were either C-MC or C-MHC, and show similar behavior to primate cool Aδ or C-fibers (**Figure 2A**) or bimodal C-fibers (**Figure 2C**), respectively. Here, all warm-responding fibers were found to be polymodal and no warm-tuned afferents were found.

4.3 MOLECULAR TRANSDUCERS OF WARMING PERCEPTION

4.3.1 TRPV1, TRPM2, TRPM3 and TRPA1 are dispensable for warming perception

Previous studies have linked warm- or heat-activated TRP channels to thermal behaviors in the warming or heating range. Blockade of TRPV1 partially disrupted performance in a thermal discrimination task (Yarmolinsky et al., 2016), ablating TRPM2 altered thermal

preference (Tan & McNaughton, 2016) and triple ablation of TRPV1, TRPA1 and TRPM3 eliminated behavioral responses to noxious heat (Vandewauw et al., 2018).

In the experiments presented here, I tested the ability of these mouse mutants in sensing warming stimuli delivered to their forepaw. Overall, mice could report warming, with different degrees of impairment depending on which TRP channel was ablated (**Figure 22**). Interestingly, from these warm- or heat-activated TRPs, loss of TRPM2 appeared to cause the largest, partial deficit; and this mouse line had normal warm-activated, but a slight loss of cooling-activated afferents (**Figure 27A**). This latter result seems rather counter-intuitive, since TRPM2 has been shown to be a warm-activated channel (Tan & McNaughton, 2016). However, this could mean that TRPM2 is expressed in cooling-activated afferents and is involved in warm-inhibitory responses. Unfortunately, skin-nerve afferent recordings in mice lacking TRPM2 (and TRPV1 alone) were carried out whilst keeping the dissected tissue at 32^{o}C buffer (and not at the more physiological temperature of 27^{o}C), which masks the presence warm-silenced afferents; and therefore this fiber population was not investigated. Future experiments investigating warming-silenced afferent neurons could test this hypothesis. Finally, it is also worth noting that TRPM2 is a major warmth sensor at the preoptic area of the hypothalamus. Therefore, its constitutive ablation could alter hypothalamic processing of temperature and this could perhaps affect the ability to detect thermal stimuli (Song et al., 2016).

On the other hand, I observed only very minor effects in warming detection when deleting TRPV1, TRPM3 and TRPA1 simultaneously. Skin-nerve data also suggested that the warming encoding at the afferent level was relatively normal in these mice, but only heat encoding was impaired, as previously reported (Vandewauw et al., 2018). These findings suggest that these three channels play a role in nociception but not in non-noxious warming. Interestingly, this mouse line had particularly high false alarms.

One limitation of this work is that the role of TRPV1, TRPM2, TRPA1 and TRPM3 was only tested using genetic knockouts. It has been observed that genetic ablation can lead sometimes to the increase in expression of other related genes. This genetic compensation has been observed in mice (El-Brolosy & Stainier, 2017). Therefore, it cannot be ruled out that some other thermal sensors may be expressed in larger or lesser amounts in the genetic knockouts tested, perhaps masking larger warming perception deficits. Additionally, genetic mutations may alter the normal behavior or cognition of mice. Therefore, future experiments could test specific antagonists for these TRP channels in WT mice, to confirm whether they are dispensable for warming perception in normal conditions.

4.3.2 TRPM8 is required to perceive warming

Possibly the most surprising finding of this work is that, unlike deletion of warm- or heat-activated TRP channels, ablation of cooling sensor TRPM8 completely disabled quick (3.5 s) warming (32-42ºC) perception in mice. Additionally, pharmacological blockade of TRPM8 at the forepaw suppressed the sensation of warming stimuli in WT mice.

Combining the behavioral and afferent finding, however, it seems that the role of TRPM8 in warming perception is an indirect one. TRPM8-deficient mice have normal warm-activated responses but a loss of cold-activated afferents and, as a consequence, a lack of warm-silenced responses. Therefore, TRPM8 likely drives baseline spiking in warm-silenced fibers, which is shut down by warming in normal conditions (**Figure 16C**, **Figure 17B**). Importantly, it has been shown that some cultured cool-sensitive neurons *in vitro* show ongoing spiking activity at room temperature, and that this feature is dependent on both TRPM8 and the voltage-gated sodium channel NaV1.1 (Griffith et al., 2019). That finding is consistent with TRPM8 being required for afferent spontaneous activity during baseline. The finding that optogenetic activation of TRPM8+ afferents triggers an unambiguous cooling percept (**Figure 36**, **Figure 39**) also indicates that the role of TRPM8 in warming perception is indirect.

The mechanisms underlying warm inhibitory responses in TRPM8+ afferents are unknown. Because many TRP channels co-express in sensory neurons (Tan & McNaughton, 2016; Vandewauw et al., 2018), one possibility is that additional thermally gated channels co-express with TRPM8 in warm-silenced sensory afferents. The role of these unknown channels would be to inhibit the (cooling) TRPM8-driven firing. Such a mechanism is reminiscent of the warm-activated TREK-1, TREK-2 and TRAAK channels. For instance, activation of these hyperpolarizing potassium channels has been shown to shut down the action potential firing of thermosensitive neurons where they are expressed (Noël et al., 2009). Because these channels partially co-localize with TRPM8 in sensory neurons (Y. Yamamoto et al., 2009), such a mechanism could be behind the warm-evoked inhibition of TRPM8. On the other hand, another possibility is that TRPM8 alone – or an associated molecule – is responsible for the firing increases and decreases in warming-silenced afferent neurons. Warming steps may close the channel temporarily, until TRPM8 adapts and starts again driving spiking activity.

Interestingly, thermal preference studies in TRPM8-null mice show that mice lacking this channel show some degree of warm avoidance within the non-noxious range, suggesting that they can sense it to some degree (Pogorzala et al., 2013). The main differences between that study and the one presented here are the area of stimulation (whole

forepaw vs whole body), the time of stimulation (3.5 s vs several minutes), and the perceptual feature of interest (detection vs preference). It is possible that TRPM8-null mice can sense warming but need a longer time to do so, or that they can sense it in another body region that is not the forepaw alone. Moreover, another possibility is that thermal preference behaviors can occur in absence of perception. A recent study showed that thermal preference is impaired by inactivation of lateral parabrachial nucleus (LPN), but not VPL thalamic nucleus (Yahiro et al., 2017). This could mean that the spinothalamic pathway – ending in neocortex, and believed to mediate conscious perception – may not be responsible for thermal preference. Instead, the parabrachial stream ultimately reaching the hypothalamus may underlie this behavior. S1 cortex, however, is important for cooling (Milenkovic et al., 2014) and warming detection (**Figure 40**, **Figure 42**).

These hypotheses will have to be tested in future studies, and mouse mutants for different combinations of thermally gated channels will likely continue to be very useful to elucidate the intricacies of the thermosensory system.

4.3.3 Activation of TRPM8+ afferents mimics the perception of cooling

The behavioral and afferent findings indicate that TRPM8 is required for non-noxious thermal perception. To test whether activation of TRPM8+ sensory neurons is also sufficient to trigger a thermal percept, blue light stimulation was used to excite TRPM8+ afferents expressing Channelrhodopsin-2 in thermally-trained Trpm8-ChR2 mice. Interestingly, all cooling-trained mice reported the light with the same acuity and speed as they reported the cooling stimulus (32-22ºC, reaction time was of ~0.5s for both cooling and light). This suggested that light activation of TRPM8+ afferents mimics a cooling percept.

Warming-trained mice, however, responded differently to the light: only some animals learnt to report the light throughout the task. Moreover, those that licked during light stimulation, did so much more quickly than when reporting warming (32-42ºC, reaction time was of ~0.5s while reporting light but ~1s for warming). This, together with the fact that some warming-trained mice started reporting the light in the middle of the session - but not at the beginning - suggested that this was a newly learnt behavior, with mice learning to report the light during the training session rather than stimulation of TRPM8+ afferents mimicking a percept of warmth.

In light of that slightly ambiguous data, I developed a thermal discrimination task that would test this hypothesis. To test whether selective TRPM8+ afferent activation triggers

a cooling, warming or thermally ambiguous percept, I initially trained mice to report selective activation of TRPM8+ afferents (Trpm8-ChR2 mice that reported blue light). These mice, which had never been exposed to natural cooling or warming stimuli during their training, unequivocally reported the cooling stimuli and withheld licking during warming. Because thermal stimuli were never rewarded even when repeatedly reported during the discrimination task, these findings indicate that activation of TRPM8+ afferents elicits a percept of cooling and not warming. Later, I performed a very similar experiment where the unrewarded natural stimuli were cooling, touch and simultaneous cooling and touch. Light-trained Trpm8-ChR2 mice only licked to trials where cooling was present, supporting that TRPM8+ afferent activation triggers an unequivocal cooling percept.

Interestingly, however, these mice rarely licked to the re-cooling phase after warming (which was also unrewarded). This differed from the discrimination data obtained in cool-trained wild types, which licked to rewarded cooling (32-22^{o}C) and unrewarded transitions between 42-32^{o}C that happened after warming (**Figure 8D**). I speculate that mice can distinguish these two cooling steps (based on absolute temperature), and Trpm8-ChR2 mice undergoing unrewarded behavior prioritize licking to the baseline-to-cool transition, because it is likely perceived as equivalent to the OFF-ON transition (or, in the case of putative spontaneously active afferents, baseline-ON) of the TRPM8+ afferents during light training. However, it is also possible that light-trained mice do not lick to the re-cooling because they are confused, surprised or startled, by the warming stimulus presented beforehand. This could be because, at the time of the discrimination, they are exposed to natural cooling and warming for the first time. Because the previous light training evokes cooling percepts in these mice, they are likely familiar with cooling but not warming sensations during the task. This could have made the mice withhold licking to the re-cooling, happening immediately after the warm (perceived as novel) stimulus.

Overall, these results suggest that selective activation of TRPM8+ sensory afferents is sufficient to trigger a robust and rapid percept of cooling.

4.4 MODEL OF AFFERENT ENCODING OF PERCEIVED WARMING

Altogether, the data presented here suggests that two types of sensory afferent responses underlie the perception of warming. On one hand, warm-activated fibers signal absolute temperature and likely contribute to interpret whether the skin is above thermoneutrality or not. On the other hand, warm-silenced afferents signal relative

warming changes, and this fiber type is absolutely required for quick (< 3.5 seconds) perception of forepaw warming (**Figure 50**). Mouse knockout data suggests that TRPV1, TRPM3 and TRPA1 are functional sensors in warm-activated fibers, although their role is mostly restricted to heat nociception. TRPM8, in turn, seems to be expressed in warm-silenced afferents, where it allows for warming encoding.

Because all the behavioral experiments presented here had a focus on rapid stimulus detection (and therefore, fast thermal transitions such as those that mice may encounter during haptic exploration), it is possible that the contribution of warming-activated afferent neurons in warming perception is underestimated. Future studies could aim to test the role of warm-activated responses in other perceptual features, such as slower discrimination between two absolute temperatures, or preference/valence (*e.g.* the pleasure or aversion evoked by warmth or coolness, depending on core temperature). For instance, a lot of research has focused on slow thermal preference tasks in mice, which normally spend longer periods of time (minutes) at temperatures slightly above paw thermoneutrality (Pogorzala et al., 2013; Tan & McNaughton, 2016). Because warming-activated fibers signal absolute temperatures of warmth or heat, I speculate they play a larger role in such perceptual features.

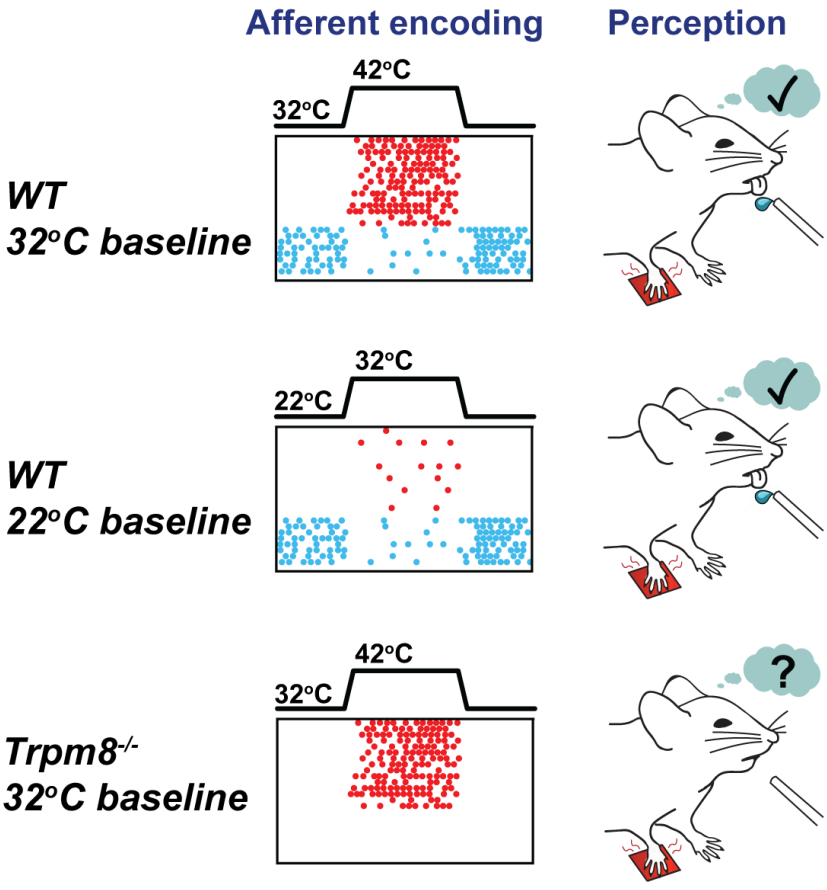

Figure 50. Model of sensory afferent encoding of warming perception.

Wild type mice can perceive warming stimuli of 32-42ºC at their forepaw, and this stimulus elicits responses in two populations of afferents: some are silent at rest and become activated by warmth (warming-activated, in red) and others are ongoing firing activity at baseline and become silenced by warming (warming-silenced, in blue) (top). If warming occurs at a lower baseline (22-32ºC), mice still very reliably detect the stimuli. At the afferent level, warming-silenced responses are present but warming-activated spikes are very sparse and rare (middle). Finally, mice lacking the cooling sensor TRPM8 are unable to sense warming (32-42ºC), despite having normal warming-activated responses. In these mice, warming-inhibited responses are absent (bottom). Altogether, these data indicate that warming-silenced responses require TRPM8 and are necessary for warming perception.

4.5 THE ROLE OF S1 IN WARMING PERCEPTION

S1 neurons activate upon cooling in mice, and blockade of this cortical region is required for cooling detection (Milenkovic et al., 2014). Here, I tested whether inactivation of S1 is also needed for the detection of warming stimuli. Interestingly, both pharmacological and optogenetic inactivation of S1 resulted in different degrees of warm detection impairment.

Muscimol microinjections impaired the detection of high amplitude (10ºC) warming stimuli, whereas optogenetic silencing of S1 in VGAT-ChR2 mice had major effects only when the warming stimuli were near-threshold (1ºC), but less dramatic in more salient stimuli (2ºC and 4ºC). It is possible that the technical limitations of the “clear-skull” technique are behind this difference. Both the skull, which is left almost entirely intact, and the dental cement likely limit the amount of light that reaches the cortex with this set-up. If the stimulation of VGAT-ChR2 neurons is weak, one may expect that the effects of such manipulation are most visible when the task is more challenging, i.e. when the stimulus to be detected is as small as possible.

Because S1 inactivation impaired the detection of forepaw warming, I hypothesized that S1 would exhibit robust neural responses upon warming. Surprisingly, however, only cooling and tactile stimulation elicited strong population responses measured with intrinsic signal optical imaging. This suggests that warming responses, if present, are very small within S1 and undetectable with this technique.

These findings on the role of S1 in warming perception seem, in principle, contradictory. However, they are also reminiscent of the previous experiments in this work: they may support that warming perception requires cooling input. It is possible that S1 cortex,

which encodes both tactile and cooling information (Milenkovic et al., 2014), may be needed indirectly to sense, locate or contextualize warming stimuli. This could be similar to the afferent encoding, where, in order to perceive warming, baseline firing is needed in a subset of cool-sensitive fibers.

Nevertheless, bulk inactivation of sensory areas during perception should be interpreted with caution. It is likely that pharmacological or optogenetic inhibition of S1 is perceived by mice in some way, and this may distract or confuse them during the task, impairing their performance even when the inactivated areas are not strictly required for the task. Here, I tried addressing this confounding factor by assessing that the mice can report a sound cue when S1 is inhibited, as well as by excluding mice that did not show any licking behavior at all.

Therefore, the data presented here suggests that forepaw warming perception requires the primary somatosensory cortex, even if S1 does not seem to main cortical area dedicated to warm encoding. Previous research suggests that, in addition to S1, other cortical regions such as S2 or the insular cortex may be involved in processing of warming (Brooks et al., 2002; Craig et al., 2000; Egan et al., 2005; Guest et al., 2007; Porter et al., 1993; Reimann et al., 2016).

4.6 LABELED LINES AND PATTERN THEORY

A labeled line view on somatosensation implies that subsets of primary sensory afferents are specialized to encode a single stimulus modality. Pattern theory, however, postulates that sensory afferents that respond to diverse modalities are recruited by specific stimuli (Ma, 2010).

The findings presented here suggest that the majority of thermosensitive afferent neurons in the mouse are polymodal and can signal different modalities depending on context. For instance, all sensory afferents that responded to non-noxious warming or cooling also responded to noxious mechanical stimulation. Moreover, the thermal responsiveness of these afferents was not specific to the non-noxious range, as they fired more strongly to noxious cold or heat. Finally, these experiments indicate that at least some cooling-activated afferents are required to sense warming, and warming-activated activity is not sufficient for stimulus detection. Altogether, these data suggest that mouse thermal perception relies on patterned input from diverse sensory afferent populations.

However, the sensitivity of afferent neurons to diverse stimuli ultimately depends on the expression of specific molecular transducers, which will transform natural, physical stimuli from the environment into an electrical signal. For temperature, TRPM8 mediates the encoding of cooling in most afferent neurons, so it is reasonable to refer to TRPM8+ afferents as a labeled line for coolness. In this sense, afferent neurons are indeed "labeled" by specific molecular transducers, and this directly links them to the modalities they encode. However, when taking into account the broad spectrum of natural stimuli, it makes sense – in my view – to describe somatosensory afferent encoding as patterned: i) first, because more than one transducer can be needed for a single percept (as it is for warming); therefore, convergence of signals is needed for some percepts; ii) second, because single sensory afferents express multiple molecular transducers and can signal more than one modality depending on which of these transducers is activated (Tan & McNaughton, 2016; Vandewauw et al., 2018; Wang et al., 2018); therefore, there is divergence of signals; and iii) third, because afferents labeled with a given sensor are not homogeneous (*i.e.* there is molecular heterogeneity and different responsiveness to stimuli within afferents expressing a given sensor). Importantly, the expression of molecules that are not sensors *per se* might as well influence the pattern of activation or the sensitivity to stimuli of thermosensitive neurons (Griffith et al., 2019; Rohács et al., 2005).

For instance, the TRPM8+ afferent optogenetic experiments I conducted indicate that simultaneous activation of these neurons specifically triggers a cooling percept. However, the afferent population being recruited by these experiments is likely heterogeneous, and only a subset of these sensory afferents may encode warming (either by warm-inhibition or warm-activation). Finally, these afferents could possibly signal mechanical or thermal pain if they fired vigorously, and had its activity combined with other pain-encoding afferents.

In summary, the findings presented here suggest that the encoding of temperature relies on neurons that are not stimulus-specific. Instead, neurons with different sensitivities to cooling and warming (and other stimuli) are differently recruited by natural stimuli.

4.7 BEHAVIORAL FEATURES OF GO/NO-GO SENSORY DETECTION TASK

4.7.1 Effect on training over licking behavior

Here, I used a Go/No-Go sensory detection task in a large number of head-restrained, water-restricted mice that had to report different thermal stimuli. Because I realized that different performance metrics changed significantly over consecutive training sessions, I performed an analysis to compare the different metrics before and after mice reached an expert level. This data may be useful not only to better understand Go/No-Go sensory detection metrics, but also to plan behavioral paradigms that aim to answer specific questions in sensory perception.

Comparing early (novice) and late (expert) learning stages in the same mice, mice showed a slight increase in the hit rate and a robust decrease in false alarm rate. This translated in better sensitivity (d') values. Interestingly, the reaction times to report stimuli also became faster with training. This is consistent with data from other somatosensory tasks (Milenkovic et al., 2014; Sachidhanandam et al., 2013).

When comparing licking behavior metrics throughout the same detection session, sensitivity (d') increased over trials, mostly caused by a decrease in false alarms over trials. However, the hit rate remained stable until the mice were completely satiated, a point at which the task was ended. This indicates that, for the task used here, mice become more selective over trials, and prioritize sensitivity (proportion of positives/stimuli correctly identified) over specificity (proportion of negatives correctly identified). This is likely because the punishment for licking outside the stimulus window is only an additional waiting period until the next stimulus. To design tasks where specificity is more relevant for the experimenter, stricter punishments could be used (longer waiting periods, air puffs after each false alarm) (O'Connor et al., 2010). Moreover, water-restricted mice have been shown to have a high licking rate at the beginning of goal-driven tasks. These initial licks are highly impulsive, and including them in the overall analysis may underestimate the real sensitivity of the mice (Berditchevskaia et al., 2016).

Besides the lick specificity, novice mice showed signs of fatigue by reducing overall lick rate throughout trials, as well as longer reaction times. Expert mice, however, had stable lick rates and reaction times throughout trials. This suggests that expert mice could maintain an optimal performance during a larger amount of trials than novice mice.

Overall, these data suggest that many performance metrics in Go/No-Go detection tasks are improved by training, although task design likely plays a large role as well. In order to reduce false alarms in mouse experiments, stricter punishments for spontaneous licking could be used. Other alternatives are excluding the initial part of the session from analysis, when mice are most impulsive; or limiting the overall motivation of the animals by reducing thirst before the task. These measures, however, will likely have a negative impact on hit rates, especially if the stimulus saliency is not high, meaning a lack of trials and poor statistics. Reaction times could also be expected to be longer, and the mice may remain engaged for a shorter time. In addition, learning periods may be longer as tasks become more complex. Using an auditory cue before stimulus/catch presentation may help the animals concentrate over the period of interest (Komiyama et al., 2010), but then a punishment needs to be used to ensure mice will not lick after the cue.

4.7.2 Threshold testing via single amplitude presentation

When trained to detect stimuli in a Go/No-Go behavioral task, one common way of testing whether rodents are able to sense a specific stimulus amplitude is via the use of psychometric curves (Busse et al., 2011; Carandini & Churchland, 2013; O'Connor et al., 2010). To generate them, the animals need to undergo one or more detection sessions where multiple amplitudes are presented to them. The main advantage of psychometric curves is that they allow a categorization of different stimulus strengths: the animals lick more often during the stimuli they sense better, and lick less to the amplitudes that are more challenging to perceive.

In the experiments presented here, however, I did not use psychometric curves with multiple amplitudes tested on the same session. Instead, I performed multiple detection sessions with only one stimulus amplitude in each, in order to find which steps of warming and cooling mice could or could not detect (always comparing to the baseline licking, measured in the catch trials). The reason behind that choice is that rodents have been shown to adapt their performance in detection tasks when multiple amplitudes are presented (Waiblinger et al., 2019). This modulation has been reported to depend on different factors. One of them is the range of stimulus amplitudes presented in the same session: when presenting a broad range of amplitudes, the animals show a bias towards the most salient amplitudes and report less often the low amplitude stimuli. When the highest amplitudes are removed from the task, then the rodents detect better those subtle stimuli. Moreover, the frequency at which high and low amplitude stimuli are presented also plays a role in performance, with better outcomes when high amplitudes

are presented less often (Waiblinger et al., 2019). The reason for this shift in performance seems to be dependent on the animal's decision-making. Rodents in Go/No-Go tasks employ a strategy in which they ensure obtaining a high amount of rewards at the minimum cost. If the animals are often presented with stimuli that are very salient, they appear not to have the same level of engagement when reporting smaller amplitudes.

Therefore, it is possible that the data obtained by presenting several thermal amplitudes in the same session may have underestimated the animals' ability to sense the smallest amplitudes. Because I was particularly interested in the ability of mice to detect very small thermal amplitudes - in order to relate it to human data – I did not employ single session testing with multiple amplitudes. Instead, I trained the mice to detect one given amplitude per session. This way, all rewards obtained by the mice would only come from correctly reported stimuli of the amplitude of choice, ensuring a high level of task engagement for each amplitude.

4.8 CONCLUSIONS

This work is the first detailed characterization of warming perception in mice and their neurobiological encoding mechanisms. By using a rapid goal-directed behavioral task, I show that mice are very sensitive to tiny (≥ 0.5-1^{o}C) warming stimuli and they sense cooling better and they discriminate between the two. I conclude that mouse thermal perception closely resembles human thermal perception, allowing future experiments to investigate the mechanisms of thermal perception using this model.

Mice encode warming at the peripheral nervous system via two populations of polymodal sensory afferents: warm-activated and warm-silenced. The first encode absolute warmth or heat and are not completely needed for warming detection. However, the latter are required for quick stimulus detection, and are adapting, cool-sensitive afferents that become suppressed by relative warming. Future work should investigate afferent encoding *in vivo*.

Mice encode and sense warming without the molecular sensors TRPV1, TRPA1, TRPM3 and TRPM2; but, surprisingly, not without the cooling ion channel TRPM8. TRPM8 drives cooling perception but is also required by warm-silenced afferents to be able to encode warming.

Altogether, this work suggests that input from both cooling and warming sensory pathways is required for warming perception, and has general repercussions on the way we understand how signals are encoded in somatosensation.

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DECLARATION OF INDEPENDENCE

I, Ricardo Paricio Montesinos, confirm that the work presented here is my own. Information or data derived from other sources is cited or acknowledged as such in the text. This dissertation has been composed by myself and any data generated in collaboration with others is specified in the text and indicated in the Statement of Contributions.

The work for this thesis was carried out at the Neural Circuits and Behavior lab from the Max Delbrück Center for Molecular Medicine, under the supervision of Prof. Dr. James Poulet.

This dissertation has not been submitted for any other degree or qualification at any University or similar institution.

STATEMENT OF CONTRIBUTIONS

Because of the interdisciplinary nature of this project, some of the data presented here was obtained by collaborators, to whom I am very grateful. All experiments performed by other people are stated throughout the text in the Results chapter, and here I indicate them as well:

- In sections **3.2** (from **3.2.1** to **3.2.4**), **3.3.6** and **3.3.7**, **Dr. Frederick Schwaller** performed all *ex vivo* skin-nerve afferent recordings, and analyzed all the afferent data except for the PSTH spike rate analyses, which I generated. Dr. Schwaller carried out all his experiments under the supervision of **Prof. Dr. Gary Lewin**.

- In sections **3.1.2**, **Dr. Annapoorani Udhayachandran** performed the cooling training (32-22^{o}C dataset, n=7) of wild type mice, and I carried out the analyses of the data. In section **3.4.1**, she also conducted the experiment where Trpm8-ChR2 mice were trained for light detection and later cooling detection. In those experiments, I also performed the analyses of the data.

All experiments in this thesis were conceived and designed by my PhD supervisor, **Prof. Dr. James Poulet**, and myself, with additional input from the other investigators for the experiments they contributed to.

6. APPENDIX

ABBREVIATIONS

A-MC	A-*delta*-type Mechanocold fiber
A-MH	A-*delta*-type Mechanoheatcold fiber
ANO1	Anoctamin 1
AP	Action Potential
ChR2	Channel Rhodopsin 2
C-C	C-type Cold fiber
C-H	C-type Heat fiber
C-MC	C-type Mechanocold fiber
C-MH	C-type Mechanoheat fiber
C-MHC	C-type Mechanoheatcold fiber
DMH	Dorsomedial Hypothalamus
DMSO	Dimethyl Sulfoxide
DRG	Dorsal Root Ganglia
GC-G	Guanylyl Cyclase G (GC-G)
GluK2	Glutamate receptor, Kainate type 2
IC	Insular Cortex
ISOI	Intrinsic Signal Optical Imaging
LED	Light-emitting Diode
LPN	Lateral Parabrachial Nucleus
PBMC	1-Phenylethyl-(2-aminoethyl)[4-(benzyloxy)-3-methoxybenzyl]carbamate
P(lick)	Percentage of licked trials
POA	Preoptic Area of the hypothalamus
POm	Posterior medial thalamic nucleus

PoT	Posterior Triangular thalamic nucleus
PSTH	Peri-stimulus Time Histogram
S1	Primary Somatosensory Cortex
S2	Secondary Somatosensory Cortex
SPL	Sound Pressure Level
STIM1	Stromal Interaction Molecule 1
TRAAK	TWIK (Tandem of P-domains in a Weak Inwardly rectifying Potassium channel) - Related Arachidonic acid Activated Potassium channel
TREK	TWIK-Related Potassium channel 1
TRP	Transient Receptor Potential (ion channel)
TRPA	Transient Receptor Potential Ankyrin
TRPC	Transient Receptor Potential Canonical
TRPM	Transient Receptor Potential Melastatin
TRPV	Transient Receptor Potential Vanilloid
V1	Primary Visual Cortex
VB	Ventro-Basal complex
VMpo	Ventro-Medial posterior thalamic nucleus
VGAT	Vesicular GABA (*gamma*-Aminobutyric Acid) Transporter
VPL	Ventral Postero-Lateral thalamic nucleus
WT	Wild Type

INDEX OF FIGURES

PUBLICATIONS

Paricio-Montesinos R., Schwaller, F., Udhayachandran, A., Rau, F., Walcher, J., Evangelista, R., Vriens, J., Voets, T., Poulet, JFA., Lewin, GR. (2020) The Sensory Coding of Warm Perception. Neuron 106(5): 830-841.e3. doi: 10.1016/j.neuron.2020.02.035

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