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Diminished Cold Avoidance Behaviours after Chronic Cold Exposure – Potential Involvement of TRPM8

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Abstract—Ambient temperature changes trigger plastic biological responses. Cold temperature is detected by the somatosensory system and evokes perception of cold together with adaptive physiological responses. We addressed whether chronic cold exposure induces adaptive adjustments of (1) thermosensory behaviours, and (2) the principle molecular cold sensor in the transduction machinery, transient receptor potential melastatin subtype 8 (TRPM8). Mice in two groups were exposed to either cold (6 °C) or thermoneutral (27 °C) ambient temperatures for 4 weeks and subjected to thermosensory behavioural testing. Cold group mice behaved different from Thermoneutral group in the Thermal Gradient Test: the former occupied a wider temperature range and was less cold avoidant. Furthermore, subcutaneous injection of the TRPM8 agonist icilin, enhanced cold avoidance in both groups in the Thermal Gradient Test, but Cold group mice were significantly less affected by icilin. Primary sensory neuron soma are located in dorsal root ganglia (DRGs), and western blotting showed diminished TRPM8 levels in DRGs of Cold group mice, as compared to the Thermoneutral group. We conclude that acclimation to chronic cold altered thermosensory behaviours, so that mice appeared less cold sensitive, and potentially, TRPM8 is involved.

Key words: cold exposure, cold acclimation, dorsal root ganglion, TRPM8, icilin, mice.

INTRODUCTION

In many parts of the world, enduring cold exposure is an inevitable part of life while working, commuting and taking part in outdoor physical activity (Mäkinen et al., 2006; Mäkinen and Hassi, 2009; Sue-Chu, 2012). The peripheral nervous system (PNS) encompasses primary sensory neurons with a capacity to detect ambient cold and transduce this information through conversion of channel-activating thermal signals to electrical activity and firing of higher order neurons in the central nervous system (CNS) (McKemy, 2013, 2018; Vriens et al., 2014; Gracheva and Bagriantsev, 2015). The PNS conveys thermal information needed for triggering the behavioural and physiological responses that facilitate cold adaption and tolerance (Castellani and Young, 2016). The transient receptor potential melastatin subtype 8 (TRPM8) ion channel is an essential component of the thermosensory apparatus, and is substantially involved in the mechanisms of cold sensation (McKemy, 2013). TRPM8 channels are expressed in primary somatosensory neurons (Takashima et al., 2007), localized in the dorsal root ganglion (DRG) and trigeminal ganglion (TG), and detect temperatures below 28 °C (González-Muñiz et al., 2019). TRPM8 is also required for cold pain sensation and noxious cold (< 15 °C) (Knowlton et al., 2010, 2011) and warm perception (Keller et al., 2020); and has a role in thermoregulation (Reimúndez et al., 2018).

In evolutionary terms, the thermosensory system adapts, for example, by changing ion channel expression levels or by altering their molecular structures (Bagriantsev and Gracheva, 2015; Gracheva and Bagriantsev, 2015; Matos-Cruz et al., 2017; Hoffstaetter et al., 2018). In a recent publication, comparative genomics of tropical and arctic animals revealed that the protein structure of the TRPM8 pore domain influences cold sensitivity, so that mice engineered to express penguin TRPM8 show tolerance to cold (Yang et al., 2020). While genetics may allow a species to inhabit new thermal niches, the capacity to adapt through acclimatization as thermal exposures vary, may be an equally important contributor to fitness.

Changes in ambient temperature (T_a) beyond the body temperature range of homeotherms trigger behavioural and physiological responses to maintain the defended core body temperature (T_c) (Castellani and Young, 2016; Tan and Knight, 2018). Acute cold expo-

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Abbreviations: BAT, brown adipose tissue; DRGs, dorsal root ganglia; PNS, peripheral nervous system; TG, trigeminal ganglion; TRPM8, transient receptor potential melastatin subtype 8; UCP1, uncoupling protein 1; VAMP7, vesicle-associated membrane protein 7; WDSs, wet dog shakes.

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sure induces shivering thermogenesis in skeletal muscles (4). Extended or repeated cold exposure leads to habituation or alleviated responses to cold; including blunted shivering and cutaneous vasoconstriction (Castellani and Young, 2016). Chronic cold exposure evokes non-shivering thermoregulatory adaptations in brown adipose tissue (BAT) and skeletal muscle (Townsend and Tseng, 2012; Betz and Enerbäck, 2018), helping to maintain T_c and energy homeostasis at low temperature (Tan and Knightv, 2018; Yu et al., 2018). Perhaps the best-described physiological response to cold exposure in rodents is the upregulation in BAT of Uncoupling protein 1 (UCP1), a mitochondrial proton carrier and critical effector of adaptive thermogenesis (Townsend and Tseng, 2012; Nedergaard and Cannon, 2018).

As the physiological adaptive responses to cold exposure are established, it is reasonable to hypothesize that the thermosensory system also adjusts its phenotype through plastic responses to persistent cold stimuli. The data we present here adds to the current knowledge of physiological adaptations to cold exposure. Knowledge of environmental factors that shape bodily functions, including the somatosensory system, are central to determining risk factors of health (Farbu et al., 2019). Acclimation dramatically improves physiological function in the cold range of thermal conditions, whereas it may have the opposite effect once conditions extend outside this range (Kristensen et al., 2008). Although *in vivo* evidence is limited, adaptation to cold exposure may shape the somatosensory system, and affect its function after acute and intermittent cold exposure (Montserrat-de la Paz et al., 2015). Our fundamental question was whether chronic cold acclimation alters thermal preference, and if this corresponds with changes in TRPM8 expression. To answer this question, we developed a four-week acclimation protocol for mice in which housing temperature was either lowered down to 6 °C, gradually, to prevent severe stress, or increased to 27 °C to attain thermoneutral conditions for controls (Fig. 1). Acclimated mice were tested for thermal preference and tissues were sampled. The main finding was that cold acclimation leads to less cold avoidance behaviour. In parallel, our data suggest that cold acclimation reduces both peripheral cold sensor TRPM8 expression and response to TRPM8 agonist (icilin). To our knowledge, this shows for the first time that chronic cold exposure can change thermosensory behaviours in association with diminished TRPM8 responses.

EXPERIMENTAL PROCEDURES

Animals

A total of 66 male C57BL/6 mice were used in this study. Animal experimental protocols (i.e. importing, housing, lab-procedures, -personnel and -facilities) were pre-approved by Institutional Animal Care and Use Committees (IACUCs) at the National Institute of Occupational Health (STAMI, Oslo, Norway), and then methodologically and ethically approved at the Norwegian Food and Safety Authority (NFSA) according to the national regulations (Approval ID # 11816 and # 17007). All the experiments were performed in accordance with relevant guidelines and regulations approved by IACUCs and NFSA, under supervision of a local veterinarian and animal welfare bodies at STAMI. Efforts were made to minimize animal discomfort or suffering, and the number of mice used.

Cold acclimation protocol

Upon arrival from a breeding colony (Janvier Labs, France) at age of six weeks, pooled mice were randomly allocated in pairs to each cage (425 × 276 × 153 mm) and provided with standard pellet chow, water and wooden chips bedding, but without nesting material or enrichment devices. After one week at a standard T_a of 21 °C using the Scantainer/Scanclime system (Scanbur Technology, Karlslunde, Denmark), cages were randomly allocated to either the Cold or Thermoneutral groups and placed in ventilated climate chambers (HPP750life, Memmert, Germany) with controlled T_a to induce long-term exposure to cold or thermoneutral temperature. Relative humidity set to 50%, and a 12:12 h dark-light cycle (lights ON at 07:00 AM) was used. For cold acclimation, T_a was decreased 3 °C/day to reach 12 °C. Target T_a for the following 4 weeks were: first two weeks at 12 °C, third week at 9 °C and last week at 6 °C (Fig. 1). For

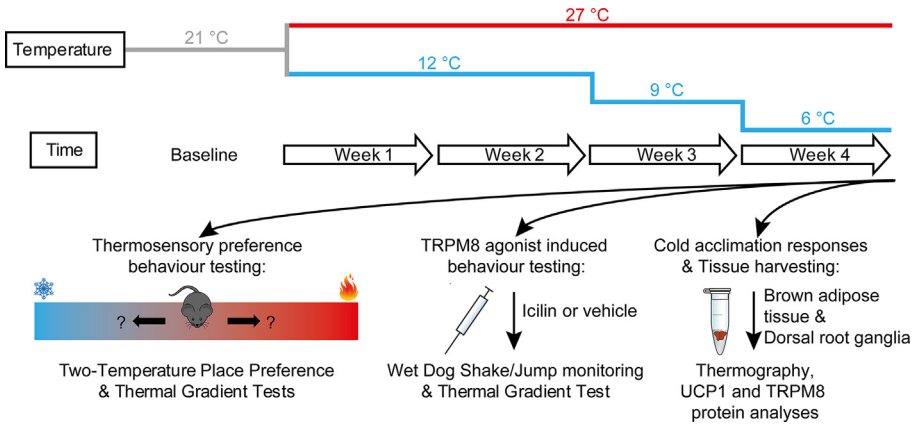


Fig. 1. Schematic summary of experiments. Male C57BL/6 mice were acclimated for four weeks to appropriate thermal conditions in two separate climate chambers by gradually lowering the ambient temperature (T_a) to 6 °C (Cold group; indicated by blue), or increasing to thermoneutral temperature 27 °C (Thermoneutral group; indicated by red). After acclimation, mice were assigned to three different experiments as indicated: First, thermosensory preference behaviour testing using Two Plate Choice Test and Thermal Gradient Test. Second, injections with either TRPM8 agonist icilin or vehicle prior to Thermal Gradient Test, and third, evaluation of cold acclimation responses including thermography imaging and molecular analyses of TRPM8 and UCP1 expression in dorsal root ganglion (DRG) and interscapular brown adipose tissue (iBAT) homogenates, respectively.

acclimation at thermoneutral T_a, the temperature was increased 3 °C/day and the mice were exposed to constant 27 °C and relative humidity of 50% for four weeks. After the acclimation period at either cold or thermoneutral conditions, mice were assigned to different experimental protocols (Fig. 1).

Evaluation of energy and heat homeostasis

Mice were weighed at baseline (at the end of the week before starting the acclimation protocol) and at the end of each week. After every week, the weight of remaining chow in each cage was subtracted from a weighed aliquot at the beginning of the week, and then the daily average caloric intake for each individual mouse was calculated relative to body weight (kcal/g/day). Energy intake was calculated on the basis of 2.6 kcal/g for the chow used (RM1, SDS, UK). After four weeks acclimation to cold or thermoneutral T_a (as described above), we measured skin temperature. Infrared imaging of the dorsal surface was performed immediately after taking the mice out of the climate chambers using a Thermal Imaging Camera Flir T1020 (FLIR Systems, Wilsonville, Oregon, USA), while the mice were in their housing cages. FLIR ResearchIR MAX software (Version 4.40.9.30) was used for analysing the thermographic images. For all measurements, the camera emissivity coefficient was set to 0.95.

Thermosensory preference behaviour testing using Two-Temperature Place Preference Test and Thermal Gradient Test

After four weeks of acclimation to cold or thermoneutral temperature, mice were tested on two thermosensory test stages (Fig. 1). First, thermal preference was tested using Two-Temperature Place Preference Test (Bioseb, France).The apparatus was composed of two adjacent thermoelectric cooling/heating plates.giving the mice a choice of spending time on a test plate or on a neutral reference plate, The small gap between two plates was closed with a non-conductor material, and twin enclosures (area 330 × 80 mm) with non-transparent walls restrained mice to the plates, allowed testing of two mice simultaneously. The mice were taken out of the climate chambers and habituated to the test stage two days beforehand; 5 min/day; while the plates (area 165 × 80 mm each) were set at 10 or 27 °C. On the test day, the temperature of the plates was set at 17 and 27 °C and mice were allowed to move freely on the plates for 15 min with automated video tracking. Place preference was measured using T2CT software (Version 2.2.6). One week later, mice were tested on the Thermal Gradient apparatus (Bioseb, France), which consisted of thermoelectric cooling/heating plates (as above) set to 0 °C and 50 °C, respectively. The plates were spanned by one compartment (1200 × 80 mm) with an aluminium alloy floor to provide a stable temperature gradient for the mice to walk on. The compartment was divided in two parts using a separating wall to provide the opportunity to test two mice at each time. On the test day, the animals were put in the middle of the lanes and allowed to move freely along the device for 30 min with video tracking from above. Using the software, the floor of the gradient was virtually divided into 10 zones. Activity time and time spent in each zone were measured and analysed using Software TGS (Version 1.5). To ensure consistent testing conditions, the border and mid-zone temperatures were measured using an infrared thermometer before and after starting of the experiment on the test day.

Thermosensory preference behaviour testing using Thermal Gradient Test after TRPM8 agonist icilin administration

After four weeks acclimation to cold or thermoneutral T_a (as described above), spontaneous WDSs were counted for 10 min. TRPM8 agonist Icilin (REF#I9532; Merck) was dissolved in vehicle solution (60% DMSO and 40% saline) (REF#196055; MP Biomedicals, LLC) and injected at a volume of 30 μL per 50 g body weight, so that the mice received a dose of 5 mg/kg. Negative control groups were injected with vehicle solution alone. The chemicals were delivered subcutaneously (s.c.), injected with an insulin syringe (1 mL, 30 G) under the loose skin over the back of the neck. The mice were randomly injected with either icilin or vehicle. Afterwards, counting the WDSs and jumping behaviours was done until 30 min after the injection of icilin or vehicle by an experimenter who was blind to the treatment. Mice were then transferred to the Thermal Gradient apparatus and tested for 30 min as before.

Western blotting

Tissues were sampled from naive mice (without previous behavioural testing) that had been acclimated to cold or thermoneutral T_a for four weeks (Fig. 1). Mice were anesthetized via a mask over the nose at 5% isoflurane in 2 L/min oxygen for 3 min. Anaesthesia was maintained at 3% isoflurane in 0.5 L/min oxygen. Thoracotomy and a cardiac puncture was performed to draw blood, by inserting a 22 G syringe into the ventricle. Mice were perfused with ~10 mL PBS buffer (REF#P4417-50TAB, Merck) containing 5000 U/mL heparin (LEO Pharma Norge AS Lysaker, Norway) until the liver bleached. A lumbar laminectomy was performed, and L4 and L5 DRGs (from both Cold and Thermoneutral groups) which innervate and mediate thermosensation in hind paws were dissected out under a stereo microscope. Trigeminal ganglia and liver (left lobe) were dissected from thermoneutral group. Interscapular brown adipose tissue (BAT) was also collected from both groups to study the UCP1 expression. Tissues were transferred immediately into liquid N_2 and stored at −80 °C. The expression of TRPM8 in DRG, TG and liver, and UCP1 in BAT was examined by western blotting. RIPA buffer (150 mM NaCl, 1.0% Igepal CA-630, 0.5% sodium deoxycholate,

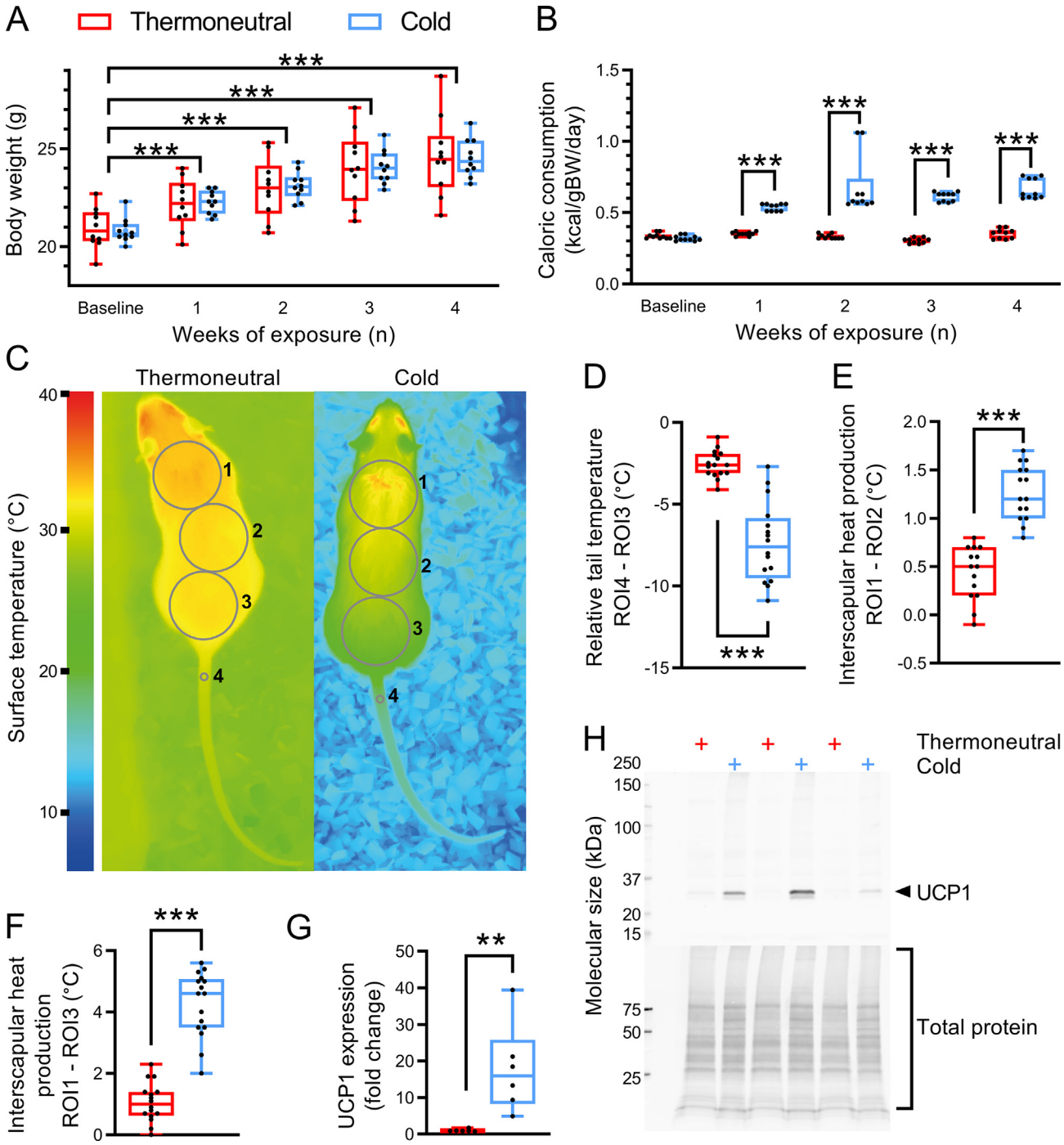


Fig. 2. Thermoregulatory responses in male mice after four weeks acclimation to cold or thermoneutral temperature. **(A)** Weekly body weight measurement showed a significant increase compared to the baseline ($F_{1.7,\ 31.3}$ = 162.669, $P \leq 0.0001$), the week before starting the four-week protocol, but there was no significant effect of ambient temperature (T_a) on body weight between two groups ($F_{1,\ 18}$ = 0.00054, P = 0.9817). **(B)** Caloric consumption was monitored throughout the experimental period (Kcal/gBW/day). Caloric consumption was significantly affected by Ta ($F_{1,\ 18}$ = 317.572, $P \leq 0.0001$) and exposure time ($F_{4,\ 72}$ = 20.6124, $P \leq 0.0001$), and increased significantly from baseline to weeks 1 to 4 in the Cold group, whereas it remained unchanged in the Thermoneutral group (n = 10 in each group). **(C)** Infrared thermography of mice in their cages, immediately after taking them out of the climate chambers set to 6 or 27 °C. The colour of background in each group reflects the housing temperature. The temperature of the dorsal surface was imaged using infrared camera after four weeks acclimation (example images are shown): Thermoneutral group (left) and Cold group (right). Four Regions of Interest (ROI) were defined along the caudal-cranial axis, as indicated by the numbered circles: ROI-1 (interscapular), ROI-2 (thoracic/lumbar), ROI-3 (lumbar/sacral), and ROI-4 (tail, 1 cm from the base). A colour palette ranging from 5 to 40 °C indicates surface temperatures. **(D)** Tail vasoconstriction, represented as the temperature difference between ROI-4 and ROI-3. Relative tail temperature was significantly lower in the Cold group compared to the Thermoneutral group ($t(30)$ = 7.593, $P \leq 0.001$) (n = 16 in each group). **(E, F)** Interscapular heat production is presented as the temperature difference of ROI-1 and ROI-2 ($t(27)$ = 7.97, $P \leq 0.001$) (n = 14–15 in each group); and ROI-1 and ROI-3 ($t(30)$ = 10.36, $P \leq 0.001$) (n = 16 in each group) (Meyer et al. 2017). **(G)** UCP1 expression in interscapular BAT was significantly higher in the Cold group compared to the Thermoneutral group after four weeks acclimation at 6 or 27 °C ($t(10)$ = 3.38, $P \leq 0.01$) (n = 6 in each group). **(H)** Representative western blot of UCP1 expression in the Cold group (lanes 2, 4 and 6) compared to the Thermoneutral group (lanes 1, 3 and 5). Data are expressed as mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to Thermoneutral group.

0.1% Sodium dodecyl sulphate, 50 mM Tris-HCl pH 8.0) with complete Protease Inhibitor Cocktail (Roche Diagnostics Gmbh, Germany) was added to the tissue samples, and tissues were homogenized using Biomasher II Closed System Disposable Tissue Homogenizers (Kimble Chase, USA). Lysates were placed on constant agitation at 4 °C for 2 h, and then centrifuged for 20 min at 55,000*g*. The resulting supernatant was used for further analysis. Protein concentration was measured using DC Protein Assay (REF#500-0116, Bio-Rad Laboratories, USA). For determining the specificity of TRPM8 antibody on DRGs using blocking peptide (NBP1-97311PEP, Novous), 10 μg protein was applied into each well of the gel. TG and liver (8 μg protein) were used as positive and negative controls for TRPM8 antibody, respectively. For BAT, L4 and L5 DRGs, we used 5, 8 and 3–5.5 μg protein, respectively. Samples were mixed with 4× Laemmli Sample Buffer (Bio-Rad Laboratories, USA) and incubated at 100 °C for 5 min under non-reducing conditions. Proteins were separated by SDS-PAGE, performed on a Mini-Protean Tetra Cell Electrophoresis System, using 4–20% Mini-Protean TGX Precast gels and TGS buffer (all from Bio-Rad Laboratories, USA). Precision Plus Protein Western C Blotting Standard (Bio-Rad Laboratories, USA) was used as reference. Proteins were blotted to PVDF membrane using the Trans-Blot Turbo RTA Transfer kit on the Trans-Blot Turbo Transfer System (both from Bio-Rad Laboratories, USA). Total protein was labelled with Invitrogen No-Stain Protein Labelling Reagent (Thermo Fisher Scientific, USA), according to the manufacturer's protocol. Membranes were blocked with Immobilon Block-FL (EMD Millipore Corp., USA) for one hour at room temperature. Then the membranes were incubated at 4 °C over night with a fluorescent antibody against TRPM8 (REF#NBP1-97311C; TRPM8 monoclonal antibody conjugated with Dylight 650 fluorochrome, Novus Biologicals, USA) diluted 1:2000 in Immobilon Block-FL, or with a primary mouse antibody against UCP1 (REF # MAB6158, R&D Systems) diluted 1:1000 in Immobilon Block-FL, which was detected using a 1:5000 dilution of fluorescent Goat anti-Mouse secondary antibody (Alexa Fluor 647, # A32728, Thermo Fisher Scientific, USA). Then, membranes were washed 3 × 10 min in TBST pH 7.6 (Tris-buffered saline, 0.1% Tween 20), rinsed three times in TBS pH 7.6, and then dried before imaging. The blots were imaged and duplex colour signals were collected using the Amersham Imager 600 (GE Healthcare Corp., Japan). The intensity of target protein bands was quantified using the Image Lab analysis software from Bio-Rad (Hercules, USA). A rectangle was drawn around the target protein (TRPM8, UCP1 or total protein bands) in each lane, and the signal intensity inside the rectangle was measured. The background signal of the membrane was subtracted from each band. The target protein/total protein ratio was obtained by dividing the target protein signal on the total protein signal for each lane.

Data analysis

GraphPad Prism 8 was used for analysing and plotting the data. Sample distributions were tested and normal distribution and equal variances in sampled distributions were found for all data. Pearson test and Shapiro-Wilk test were used for testing the normality of two or more groups of variables, respectively. Comparisons between groups were done using parametric tests Two-way ANOVA with Sidak multiple comparison post hoc test or Student's *t*-test, as appropriate. All quantifications are shown as mean ± standard error of the mean (SEM). Differences with P-values less than or equal to 0.05 were considered statistically significant.

RESULTS

Acclimation of mice to chronic cold exposure

Thermoregulatory adaptations to different T_a were studied in two groups: (1) Cold group; and (2) Thermoneutral group; using measures of energy balance and heat balance. Over four weeks, a stepwise lowering of T_a to 6 °C was imposed on the Cold group, whereas T_a was increased to 27 °C for the Thermoneutral group (Fig. 1). Body weight which was measured weekly, increased in both Cold and Thermoneutral groups with no significant difference between them (Fig. 2A). Caloric consumption in the Thermoneutral group remained unchanged compared to baseline (one week prior to onset of T_a exposures). In contrast, the Cold group showed a 2-fold increase in food intake, compared to its own baseline, and compared to the Thermoneutral group as well (Fig. 2B).

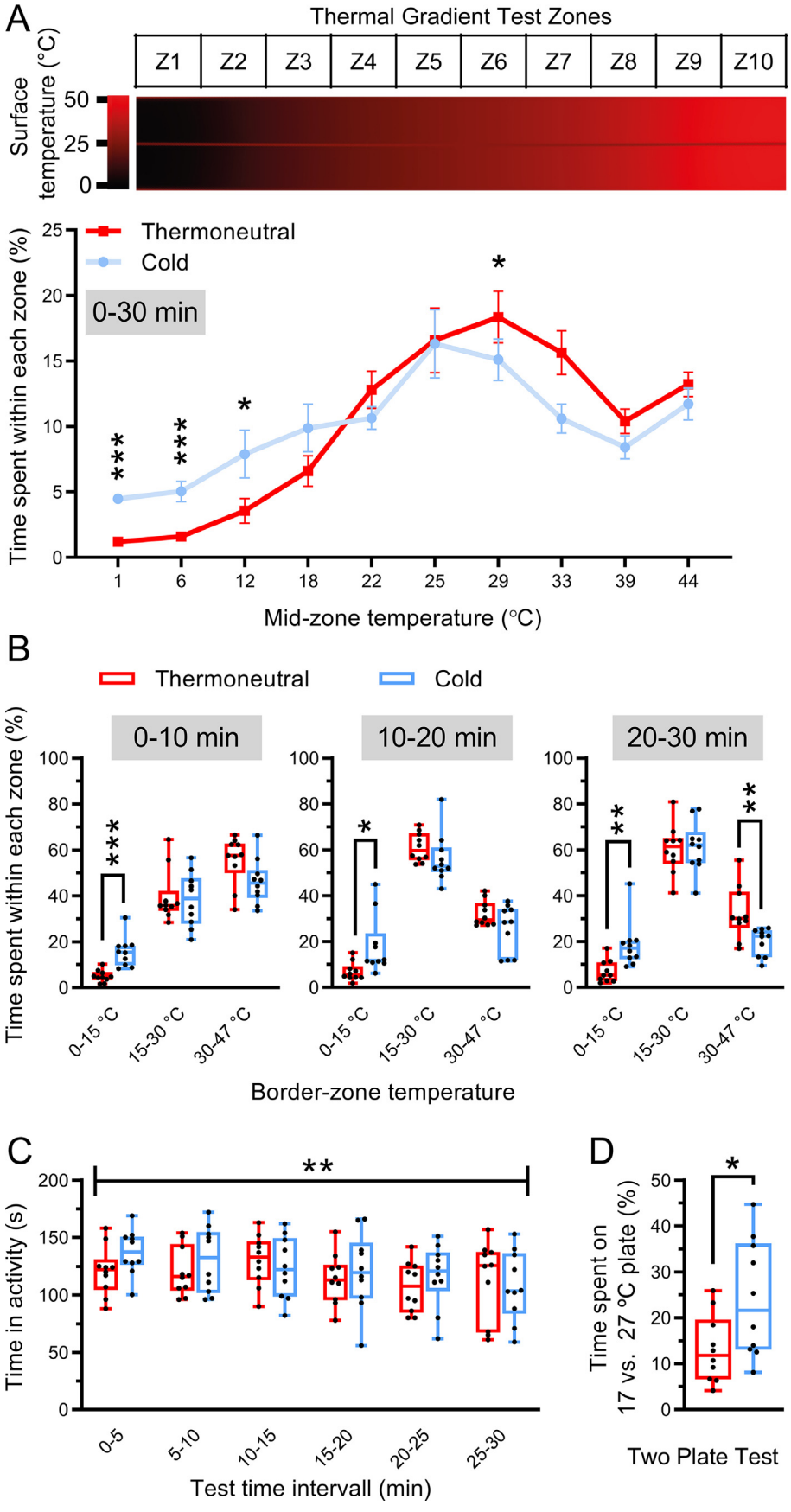
Infrared thermography was used to assess heat dissipation in the interscapular region, a major site of heat producing BAT (ROI-1, Fig. 2C); and the tail, a site of heat loss regulation through vasoactive mechanisms (ROI-4, Fig. 2C). Tail temperature was significantly lower in Cold group, compared to Thermoneutral group (4.8 ± 0.6 °C) (Fig. 2D). Cold group mice also dissipated a relatively higher amount of heat from the interscapular region, displayed by a steeper temperature gradient spanning the dorsal caudal-cranial axis: the temperature of the interscapular region was 0.8 ± 0.1 °C and 3.2 ± 0.3 °C higher than that of the thoracic/lumbar (Fig. 2E) and lumbar/sacral (Fig. 2F) regions, respectively.

In addition, the protein expression level in interscapular BAT of UCP1, was upregulated in the Cold group as compared with the Thermoneutral group (Fig. 2G, H).

We conclude that the Cold group mice showed signs of being cold-acclimated, in terms of energy balance and heat balance, after 4 weeks of continuous cold exposure.

Thermal preference in cold-acclimated mice

After exposure to four weeks of either cold or thermoneutral T_a conditions, we studied thermal preference behaviours using the Thermal Gradient Test, by means of video recording freely moving mice on the



behavioural stage and calculating time spent in ten pre-assigned temperature zones ranging from 0 °C to 50 °C (Fig. 3A). In the Thermal Gradient Test both groups of mice spent the greater part of a total 30 min duration in the intermediate range of temperatures (Fig. 3A). Cold and Thermoneutral groups showed maximum preference for ~25 °C and ~29 °C, respectively (Fig. 3A), and were less prone to stay in the colder and warmer zones. However, a difference was noted in zones below ~15 °C as mice in the Cold group spent ~2.8-fold more time in these zones compared to the Thermoneutral group (P = 0.0004) (Fig. 3A). Correspondingly, mice in the Thermoneutral group spent ~1.5 times more time in the 30–36 °C zones compared to the Cold group (P = 0.0129). Both groups showed similar preference to the warmest zones above 36 °C (Fig. 3A).

Next, we combined zones of the Thermal Gradient Test data into tertiles to evaluate whether accumulated testing time (30 min total) affected mouse behaviour, i.e. if the behaviours changed over the course of the experiment. For the first 10-minute time interval, both Cold and Thermoneutral groups preferred the warm temperature zones (> 30 °C) whereas in the 10–20 and 20–30 min intervals, the intermediate temperatures were preferred (Fig. 3B). Nevertheless, for all 10-minute intervals Cold group mice spent more time (> 1 min) in the cold zones (< 15 °C) compared to the Thermoneutral group (Fig. 3B). The inverse behaviour was noted during the 10 last minutes for the Thermoneutral group which spent more time in the warm zones (> 30 °C) than the Cold group (Fig. 3B).

Locomotion decreased during the 30 min the Thermal Gradient Test lasted, suggesting a drop in exploration activity throughout, but this trend was similar between the Cold and Thermoneutral groups at different phases of the test (Fig. 3C).

Thermal preference was also studied using an alternative method, Two-Temperature Place Preference Test. Two adjacent temperature regulated plates keeping constant temperatures of 27 °C and 17 °C were applied to assess the dichotomy of warm versus cold preference. Faced with the choice of which plate to stay on top, Cold group mice stayed 11% longer on the cold test plate (17 °C) compared to the Thermoneutral group (Fig. 3D).

The behaviour data suggest that the Cold group mice had developed cold tolerance, and thus would stay longer on cold surfaces.

Effect of cold acclimation on behaviours induced by TRPM8 agonist

At the molecular level, cold is detected by nonselective cation channel TRPM8 expressed by a subset of peripheral sensory neurons. TRPM8 has a pivotal role in cold tolerance and thermotaxis behaviours, i.e. locomotion along a temperature gradient (Colburn et al., 2007; Dhaka et al., 2007; Tajino et al., 2011). Icilin is an established TRPM8 agonist, and by injection, it elicits some stereotypic behaviours: wet dog shakes (WDSs), jumping and cold avoidance/heat seeking behaviours (Werkheiser et al., 2006). We hypothesized that cold acclimation would lower the behavioural responsiveness of the mice to TRPM8 agonist icilin. Mice were acclimated to cold or thermoneutral T_a for four weeks, as before. Spontaneous WDSs were counted for 10 min before injection. Then, they were s.c. injected with either icilin or vehicle (DMSO/saline). Four experimental groups were defined: (1) Cold + Vehicle; (2) Cold + Icilin; (3) Thermoneutral + Vehicle; and (4) Thermoneutral + Ici lin. WDSs and jumping behaviours were counted for thirty minutes in a blinded fashion. Icilin induced WDSs significantly ($P < 0.001$) whereas vehicle injection did not induce WDSs (Fig. 4A). Furthermore, no significant effect of acclimatization on WDSs was found as the Cold + Icilin and Thermoneutral + Icilin groups were similar. Jumping behaviours were also registered, and similarly, there was no significant difference in the number of jumps between Cold + Icilin and Thermoneutral + Ici lin groups after injection (Fig. 4B). Cold + vehicle and Thermoneutral + vehicle mice didn't show jumping behaviours.

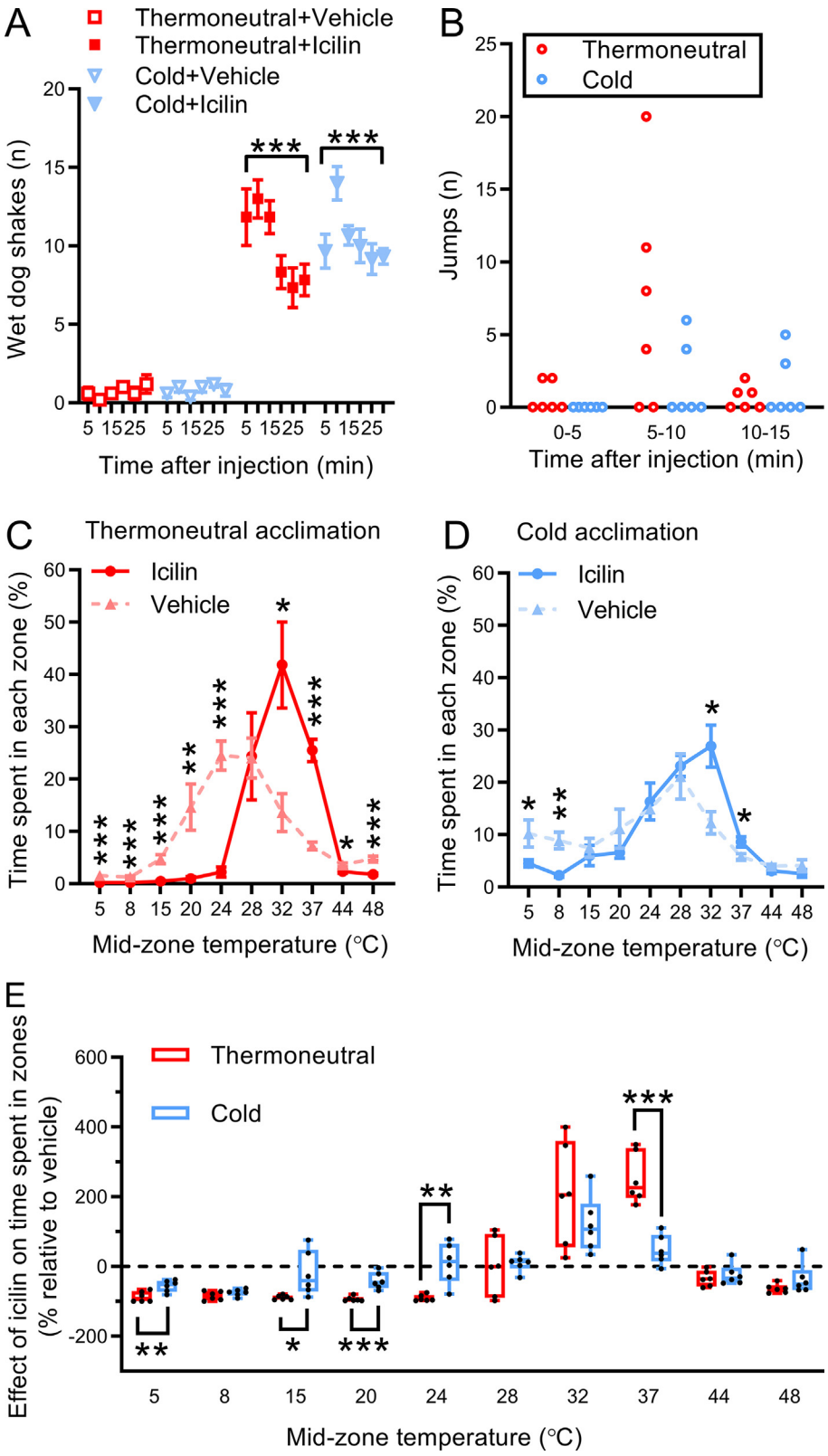
Thirty minutes after icilin or vehicle injection, thermal preference behaviours were assessed in the Thermal Gradient Test. Vehicle injected mice reproduced our previous result (Fig. 3A), indicating less cold avoidance in mice acclimated to cold rather than thermoneutral conditions (Fig. 4C, D). Injections of icilin enhanced cold avoidance behaviours compared to vehicle, and this effect of icilin was evident in mice acclimated to thermoneutral conditions (Fig. 4C) as well as in mice acclimated to cold conditions (Fig. 4D) T_a. However, icilin imposed a significantly larger change to thermal preference in mice acclimated to thermoneutral conditions than in mice acclimated to the cold (Fig. 4E). The data suggest that cold acclimation has dampened thermosensory behaviours induced by icilin, an agonist of TRPM8.

The effect of cold acclimation on cold sensor TRPM8 expression in DRG

As reductions in both cold avoidance behaviours and sensitivity towards TRPM8 agonist coincided in cold acclimated mice, it was essential to assess the potential link. We hypothesized that a reduction of TRPM8 protein expression in peripheral sensory neurons would accompany behavioural insensitivity towards low surface temperatures in Cold group mice. Accordingly, intracellular TRPM8 protein level was assessed by western blotting of the L4 and L5 DRG that contain the somas of the first-order sensory neurons innervating the hind paws, and thus needed for somatosensation of surface temperature.

Validation showed that the TRPM8 antibody detected a ~140 kDa band in protein extracts obtained from TRPM8-positive tissues (DRGs and trigeminal ganglia) but not in TRPM8-negative tissue (liver) (Fig. 5A). Protein glycosylation may be the reason for identifying the TRPM8 band at ~140 kDa, since unmodified TRPM8 is expected to migrate with an apparent molecular mass of ~127 kDa (Dragoni et al., 2006; Pertusa et al., 2012). In addition, the ~140 kDa TRPM8 band was blocked with epitope containing peptide (Fig. 5B). L4 and L5 DRG tis-

Fig. 3. Thermal place preference in cold-acclimated mice. **(A)** Schematic representation (upper panel) and thermographic imaging (lower panel) of the Thermal Gradient Test stage showing ten predefined temperature zones indicated as Z1 to Z10, and temperature gradient as it develops when the two ends were thermally regulated by cooling and heating plates at 0 and 50 °C, respectively. Acclimated mice from the Cold and Thermoneutral groups were allowed to move freely on the test stage for 30 min while behaviours were registered using video tracking. Reduced cold avoidance or enhanced preference to the cold zones (Zone 1 with mid-zone temperature 1 °C: ($t(18) = 9.78$, $P \leq 0.001$); Zone 2 with mid-zone temperature 6 °C: ($t(18) = 4.338$, $P \leq 0.001$) and Zone 3 with mid-zone temperature 12 °C: ($t(18) = 2.208$, $P = 0.04$)) was observed for the Cold group mice compared to the Thermoneutral group ($n = 10$ in each group). Cold group also showed more preference to the zone 29 °C ($t(18) = 2.721$, $P = 0.0140$). **(B)** Alternatively, the 30 min of behaviour data was split up into three consecutive 10-minute intervals, whereas the zones were stacked into three (Z1-3, Z4-7 and Z8-10). Mice in Cold group stayed more in zones colder than 15 C (Z1-3) at different 10-minute time intervals ($t(18) = 4.775$, $P = 0.0002$; $t(18) = 2.685$, $P = 0.0151$ and $t(18) = 3.265$, $P = 0.0043$, respectively). Mice in Thermoneutral group spent more time in zones warmer than 30 °C (Z8-10) compared to Cold group. ($t(18) = 3.041$, $P = 0.0070$) during the last 10 min. **(C)** Video analyses from the Thermal Gradient Test showed no significant effect of ambient temperature (T_a) on locomotion ($F_{1, 9} = 0.39$; $P = 0.55$). However, locomotion decreased in both groups as the test progressed ($F_{5, 45} = 4.155$, $P = 0.0035$). **(D)** Two-Temperature Place Preference Test showed that Cold group mice spent significantly more time (of 15 min total) on the cold plate (17 °C) than the thermoneutral group mice ($t(18) = 2.36$, $P \leq 0.05$) ($n = 10$ in each group). Data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared to Thermoneutral group.



sues were isolated from acclimated mice. western blot analysis of the DRG tissue homogenates showed a significant reduction in TRPM8 levels in Cold group compared to Thermoneutral group (13 and 21% reduction in the Cold group, respectively) (Fig. 5C, D).

DISCUSSION

After four weeks of cold exposure, mice not only became acclimated in terms of energy balance and heat balance, but also in terms of thermosensation. We found that cold avoidance behaviours were diminished in mice acclimated to the cold, as compared to controls acclimated at thermoneutral temperature. A functional link between reduced cold avoidance and the cold sensor TRPM8 was tested by injecting acclimated mice with icilin, an agonist of TRPM8, and monitoring thermal preference. We also showed that chronic cold exposure induces decreased levels of TRPM8 in DRG. Our results suggest that after prolonged cold exposure, changes in thermal preference behaviour occur, i.e. reduction in cold avoidance, that might be associated with reduced expression of TRPM8.

In this study, we aimed to explore effects of chronic cold exposure on plasticity of thermosensory behaviours and the peripheral somatosensory system in mice. To be able to assess effects of cold acclimation (Cold group), control housing conditions were set to minimize any triggering of cold adaptation (Thermoneutral group). Both groups were in energy homeostasis, to avoid bias owing to differences in body weight and stress of being in negative energy balance. To this end, we established a thermal exposure protocol, modified from Keipert et al. (2017). For the four-week cold acclimation, target housing temperature was 6 °C. This is a temperature that in itself could generate a noxious cold stimulus from the paws of a non-acclimatized mouse (Wang et al., 2018). Attempting to optimize animal welfare and to moderate cold stress, temperature was lowered

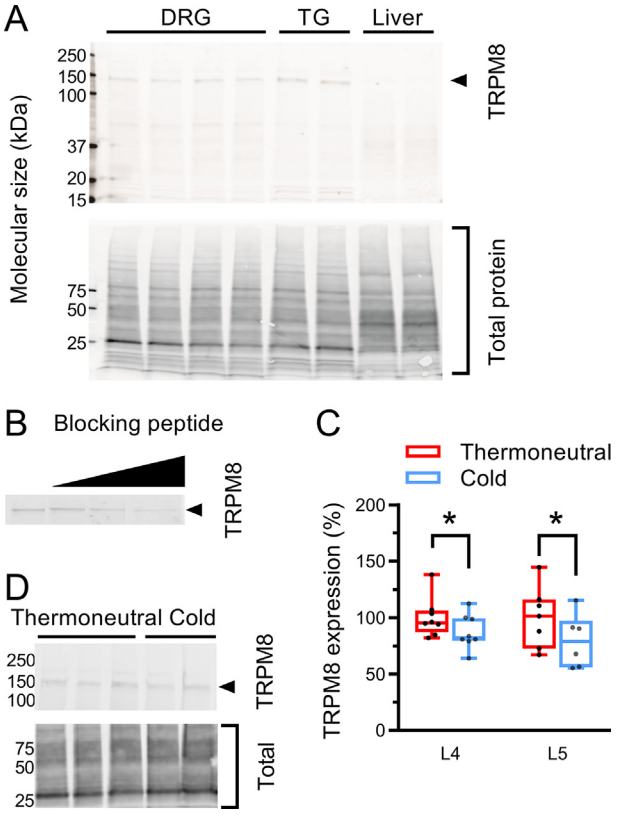


Fig. 5. The effect of cold acclimation on levels of TRPM8 cold sensor in DRG. **(A)** western blot shows TRPM8 band (~140 kDa) detected in tissue homogenates of dorsal root ganglion (DRG) and TG as a positive control, but not liver as a negative control. Total protein stain was used to control gel loading. **(B)** Blocking peptide (the amino acid sequence corresponding to the antibody TRPM8 epitope) dose dependently blocked immune detection of TRPM8 in DRG homogenates. From left to right: unblocked control (1:2000 TRPM8 antibody); + 1:2000 blocking peptide; + 1:400 blocking peptide; and + 1:200 blocking peptide. Each dilution of blocking peptide was run on different gels and blotted separately. Then, one lane from each blot were cut, put together and imaged **(C)** western blot analyses of L4 and L5 DRG tissue homogenates. TRPM8 levels were significantly higher in the Thermoneutral group compared to the Cold group ($F_{1, 25} = 4.548$, $P = 0.0430$) ($n = 6$–9 in each group). **(D)** Representative western blot of TRPM8 expression L5 DRG in the Cold group compared to the Thermoneutral group.

gradually (Montserrat-de la Paz et al., 2015). Thermoneutral conditions, on the other hand, are typically defined as the T_a where metabolic rate is at a minimum. However, the optimal approach is still a matter of discussion (Škop et al., 2020). In our experiments, Thermoneutral group controls were acclimated at 27 °C to minimize cold adaptation seen at typical housing conditions of 20–24 °C (Gordon, 1993), but at the same time avoiding potential heat-stress at higher temperatures.

Our data suggest that mice in both groups attained energy balance to their respective T_a conditions. From week one of the acclimation protocol, food consumption increased in the Cold group, compared to the Thermoneutral group. At the same time, both groups steadily gained weight throughout the experiment, and there was no significant difference between the body weight of Cold and Thermoneutral groups. This can be explained by the fact that increment in energy demand at low temperature is largely compensated by food intake (Cannon and Nedergaard, 2009; Abreu-Vieira et al., 2015; Yu et al., 2018). Although mice of both groups appeared to be in energy balance after four weeks of acclimation, they were different in aspects pertaining to heat balance. Increment in sympathetic tone reflected by tail vasoconstriction and reduction in tail temperature on one hand, and higher heat production and UCP1 expression in the interscapular BAT on the other hand, imply activation of heat preserving and cold-induced thermogenesis in the cold.

Thermosensory preference behaviour testing performed after four weeks of acclimation showed that Cold group mice spent more time in the cold test zones (< 15 °C) compared to the Thermoneutral group. Because mice in Thermal Gradient Test are confined to the test arena for the whole duration of the test, increased presence in cold temperature zones inevitably leads to less presence in other zones. The main driver of Cold group behaviours could be indifference to cold, but enhanced warmth aversion might yield a similar result. The finding that thermal behaviour was significantly different at 17 °C in Two-Temperature Place Preference but not in Zone 4 (range 15–20 °C) of the Thermal Gradient Test, can be explained as follows: first, mice in Thermal Gradient apparatus have more thermal choices and more space to move; and second, the high difference between the mid-zone temperatures of two adjacent zones and small width of each zone (10 cm) makes differentiating between similar temperatures difficult. The connection between long-term cold exposure and changes in thermal preference has, to our knowledge, not been reported before. The concept that physiological conditions alter cold stimuli induced behaviours has been investigated in a few studies earlier. For example, localized plantar

Fig. 4. The effect of TRPM8 agonist icilin on thermal preference of cold-acclimated mice. Mice were acclimated at cold or thermoneutral temperature for four weeks, and then injected with icilin or vehicle (DMSO and saline). **(A)** WDS behaviours were counted for 30 min after the injection. The results are plotted at 5-minute time intervals. There was no significant difference between the Cold + Icilin and Thermoneutral + Icilin groups in the number of WDSs at any time-point ($n = 6$ in each group). Icilin injection increased WDSs in both groups, whereas vehicle had no effect ($n = 6$ in icilin- and $n = 5$ in vehicle-injected groups; $^{***}P < 0.001$). **(B)** In parallel, jumping behaviours were registered. There was no significant difference between the two groups in the number of jumps after icilin injection ($F_{1, 5} = 1.594$, $P = 0.2625$) ($n = 6$ in each group). The points represent jumping behaviours 0–15 min after icilin injection, as they ceased completely after 15 min. **(C, D)** Thermal place preference behaviours were assessed using the Thermal Gradient Test defined by ten virtual zones (mid-zone temperature is indicated). Mice in the Cold + Vehicle group showed less cold avoidance compared with the mice in the Thermoneutral + Vehicle group ($n = 5$ in each group; $^{*}P < 0.05$ and $^{**}P < 0.01$). Icilin injection enhanced the cold avoidance behaviours of Thermoneutral + Icilin and Cold + Icilin group ($n = 6$ in each group; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the vehicle-injected groups). **(E)** The effect of icilin in different test zones is displayed relative to the vehicle alone (baseline or 0%). Thermoneutral + Icilin group showed a significantly greater change in cold avoidance after icilin injection than the Cold + Icilin group. ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$). Data are expressed as mean ± SEM.

inflammation using CFA injections and nerve ligation induced neuropathy, which sensitize mice behaviourally to sensory modalities like heat and mechanical force, also enhances sensitivity to cold stimulation (Brenner et al., 2014). Furthermore, mild burn injury of the paw has been shown to enhance behavioural sensitivity to cold stimulus, proposedly linked to a functional transformation in cold-responding neurons (Yarmolinsky et al., 2016). However, we are aware of only one previous investigation of how cold exposure *in vivo* leads to thermosensory adaptation. Brenner et al. showed that by acclimating mice acutely at different baseline temperatures, 12–30 °C for < 30 min, paw withdrawals were altered in response to a cooling temperature ramp; withdrawal were not provoked at a fixed temperature threshold but rather to a constant temperature deviation from baseline, suggesting that the sensation of cold temperature is dynamic and that acclimation can be acute (Brenner et al., 2014). Warmth sensation, on the other hand, was less dynamic and rather fixed to a specific threshold (Brenner et al., 2014). In our study, we did not test directly whether our observations of long-term acclimation on thermosensation are of a different nature than acute acclimation. However, the Thermal Gradient Test lasted for 30 min, the same time-period used for acute acclimation in the Brenner et al. paper, and so it seems probable that acute and chronic cold acclimation are essentially different. When our Thermal Gradient Test data were broken into smaller time-segments, both Thermoneutral and Cold group showed an augmented preference to the intermediate temperatures 15–30 °C (Z4-7) throughout, seemingly avoiding the warmer zones more and more as the test proceeded. Lack of adaptation in responses to heat, has been speculated to be protective against potential tissue damage (Ran et al., 2016). Although warmth avoidance was augmented in our Thermal Gradient Test experiments, cold avoidance was stable throughout the whole test period and, importantly, diminished in Cold group in all three 10 min segments of the test, suggesting that acute acclimation (< 30 min) is not affecting cold sensitivity in Thermal Gradient Test. This suggests that 4 weeks of chronic cold exposure sets about a cold acclimation process that appears different from acute thermal exposures in the range of 30 min. When that is said, direct comparisons are complicated since methodology in the Brenner et al. publication is quite different from ours; Brenner et al. achieved acute cold acclimation by cooling the surface (floor) temperature down to a minimum of 12 °C, we used a climate chamber and 6 °C; and they used a cold-nociception variant (paw withdrawal) of the plantar test, whereas we used a place preference model. A possible time-dependency of cold acclimation could also be mapped in detail in future studies.

Cold is detected by the nonselective cation channel TRPM8 expressed by a subset of the primary sensory neurons that transduce thermal stimuli into electrical activity (Dhaka et al., 2007; McKemy, 2013). Our next step was to test directly whether cold acclimation altered TRPM8-mediated thermosensation. Since primary sensory neurons which are sensitive to icilin, are also sensitive to cold (Matos-Cruz et al., 2017), this was achieved by injections of the TRPM8 agonist icilin to activate TRPM8 and monitor behaviours. With the Thermal Gradient Test, we were able to reproduce (in the Vehicle groups) our previous finding that cold-acclimated mice had impaired cold avoidance behaviour, relative to their counterparts acclimated at thermoneutral temperature. Our hypothesis, that icilin-induced behaviours would reflect functional changes due to cold acclimation, was tested. We found that icilin injections induced cold aversion in both groups acclimated at cold or thermoneutral temperatures, suggesting functional TRPM8 to be present (Colburn et al., 2007; Dhaka et al., 2007; Tajino et al., 2011). Our foremost discovery was that the effect of icilin on cold avoidance behaviour was more profound in mice acclimated at thermoneutral temperature, and less in cold-acclimated mice. This is consistent with involvement of TRPM8 levels in DRG neurons in determining thermal preference behaviours in cold-acclimated mice. Vesicle-associated membrane protein 7 (VAMP7), mediates fusion of TRPM8 bearing vesicles with the plasma membrane (Ghosh et al., 2016). In this respect, it is shown that VAMP7-deficient mice exhibit reduced functional expression of TRPM8 in sensory neurons and concomitant deficits in cold avoidance and icilin-induced cold hypersensitivity (Ghosh et al., 2016). Accordingly, icilin-induced cold aversion may be partially ascribed to the recruitment of an intracellular pool of TRPM8 channels in response to icilin (Toro and Brauchi, 2015) which may be mediated by VAMP7 (Ghosh et al., 2016). In addition to cold aversion, icilin evoked jumping and WDS behaviours. Unlike the results obtained from the Thermal Gradient Test, Cold and Thermoneutral groups were indistinguishable in jumping and WDS responses to icilin. In addition to inadequate cold avoidance, TRPM8$^{-/-}$ mice do not display icilin-induced WDSs and jumping behaviours (Colburn et al., 2007; Dhaka et al., 2007), suggesting that these behaviours collectively are mediated via TRPM8. However, it seems that the changes in TRPM8 expression in our study after cold exposure was not enough to affect the WDSs and Jumping behaviours. Perhaps more prominent effects on TRPM8 levels are required.

Reduced cold sensitivity, similar to behaviours we observed in the Cold group, has been reported for TRPM8$^{-/-}$ mice, which notably exhibit reduced avoidance of cold temperatures in the Two-Temperature Place Preference and Thermal Gradient Test compared to wild-type mice (Colburn et al., 2007; Dhaka et al., 2007; Tajino et al., 2011). In fact, our thermal preference data from Cold group mice suggest that the behavioural changes after cold acclimation match the behavioural deficits that have been reported for TRPM8$^{-/-}$ vs. wild-type mice (Dhaka et al., 2007). Our finding that cold acclimation mirrored thermosensory behaviours of TRPM8 null mice led us to hypothesize that TRPM8 expression was affected. Increase in TRPM8 expression levels in DRG as a means of remodelling cold sensitivity, has been demonstrated after administration of platinum-based chemotherapeutic agents (Descoeur et al., 2011; Chukyo et al., 2018). Cold hypersensitivity is one of the several adverse neurotoxic effects in cancer treatment

with platinum drugs, which is associated with a reversible increase in the expression of TRPM8 in medium-sized DRG neurons (Kato et al., 2014; Chukyo et al., 2018). Our idea was that long-lasting stimulation of cold sensors during our experiments would lead to adaptive protein expression in the peripheral sensory neurons (Flavell and Greenberg, 2008). The neuronal soma contained in DRG produce proteins needed for function, and so we dissected DRG tissue from acclimated mice and analysed TRPM8 expression. Our results suggest that TRPM8 protein expression is downregulated in L4 and L5 DRG of Cold group. In line with our results, although with opposite direction, it has been reported that corneal injury which increased tear production, also enhanced TRPM8 function and agonist sensitivity (Piña et al., 2019).

Overall, peripheral fine-tuning of TRPM8 levels seems like a possible mechanism balancing cold-avoidance/warmth-seeking behaviours. An interpretation of this result is that cold-acclimated mice are functionally less responsive to both cold temperature as well as chemical agonists, which coincides with reduced TRPM8 levels in DRG neurons. Ultimately, our data suggest that cold exposure brings change to the somatosensory PNS thought TRPM8 levels, perhaps as a way to fine-tune thermosensation. According to the thermoregulatory responses, it might be suggested that central brain substrates and mechanisms at least at the level of the preoptic area (POA) and its downstream effectors like BAT, are involved in adaptive thermogenic metabolic responses. Peripheral somatosensory system is the major site of TRPM8 expression, and DRG neurons are the principle site of icilin action (Werkheiser et al., 2006; Ahimsadasan and Kumar, 2019). However, low expression of TRPM8 in various brain regions has also been reported (Ordás et al., 2021). It is therefore possible that a central mechanism of thermosensory acclimation would work in conjunction with PNS responses. Mechanistically, the POA of hypothalamus receives thermosensory input, modulates homeostatic responses to changes in ambient temperature, and has a central role in coordination of food intake, body weight and homeostasis of core body temperature through downstream sympathetic effectors like BAT, skeletal muscles and cutaneous blood vessels (Tan and Knight, 2018; Yu et al., 2018). Additionally, some evidence suggests adaptive kinetics of neurons to the cold stimuli at the level of spinal cord (Brock and McAllen, 2016; Ran et al., 2016). So, although icilin directly affects cold sensitive primary afferents (Werkheiser et al., 2006; Ahimsadasan and Kumar, 2019), different icilin-related behaviours may arise in separate sites of the nervous system, and this awaits further research.

Effects on cold sensitivity by conditional ablation of TRPM8-lineage neurons have also been reported as more pronounced than in null mice (Knowlton et al., 2013; Pogorzala et al., 2013). Based on studies of sensory neurons in response to thermal stimulation, it seems that there are several subpopulations of TRPM8-expressing neurons responding at different thermal levels of cold stimulation, and the pattern of activated neurons by cold stimuli at the population level could be important for encoding thermosensory input (Yarmolinsky et al., 2016; Wang et al., 2018). Thus, it is possible that downregulation of TRPM8 protein in targeted populations of DRG neurons has a disproportionate effect on behaviour.

Our data suggest that long-term cold acclimation involves regulation of TRPM8 activity partially through DRG protein expression, an *in vivo* mechanism that has been investigated in other settings (Chukyo et al., 2018). *In vitro*, alternative mechanisms have been studied with acute cold exposures, suggesting that Ca^{2+} influx through activated TRPM8 activates a $Ca2^{+}$ sensitive PLC isoform, whereas the concurrent decrease in Phosphatidylinositol 4,5-bisphosphate (PIP2) levels result in feed-back to TRPM8 and desensitizes it (Rohács et al., 2005; Daniels et al., 2009; Yudin et al., 2016). In this way, ambient temperature may affect the temperature threshold for TRPM8 activation via PIP2 (Fujita et al., 2013). Recent data suggest a mechanism whereby local concentrations of PIP2 and its access to TRPM8 is regulated (Sisco et al., 2019). It is possible that these mechanisms are working in conjunction with others during long-term cold acclimation, since one study also supports this notion *in vivo* (Brenner et al., 2014).

Other channels than TRPM8 are potentially involved in the modulation of thermosensory behaviors. For instance, cold hypersensitivity induced by platinum-based chemotherapeutic agents has been related to a remodeling of other channels (potassium- and non-selective) in TRPM8 expressing neurons (Descoeur et al., 2011). Studying the effect of cold acclimation on kinetics and modulation of other components of the thermosensory system involved in detection of temperature, initiation, propagation and transduction of action potentials and regulation of the membrane potential at the rest and excitation status (like sodium and potassium channels), at different levels of nervous system (Tajino et al., 2011; McKemy, 2013; Kiss et al., 2014; Gracheva and Bagriantsev, 2015) will increase our understanding of development of cold acclimation. So that, it has already been demonstrated that Nav1.7, Nav1.8 and Nav1.9 have a role in cold sensation (Zimmermann et al., 2007; Minett et al., 2012; Lolignier et al., 2015) and activation of potassium channels TREK-1 and TRAAK lowers the threshold for neural activation by cold (Noël et al., 2009). The role of TRPA1 in cold sensation was debated (Knowlton et al., 2013b; Pogorzala et al., 2013b; Winter et al., 2017; MacDonald et al., 2020), but recently, calcium imaging analyses have shown that more than 95% of TRPA1-positive neurons respond to heat rather than cold stimulus (Yarmolinsky et al., 2016). TRPA1-expressing neurons are a subset of TRPV1-expressing neurons (Pogorzala et al., 2013b). Owing to this, TRPA1-dependent activation of TRPV1 neurons may be perceived as a burning sensation in icilin-injected mice and potentially lead to a pattern of cold-seeking behaviour. Taken together, the icilin-induced cold sensation – via TRPM8 activation – which enforces cold avoidance and the likely reciprocal sensitization to heat – due to TRPA1 activation – should both be taken into consideration as underlying mechanisms determining patterns of thermal preference in experimental settings. In a recent study, it has been shown that

warm perception requires TRPM8 channels in mice (Paricio-Montesinos et al., 2020). This may explain to some extent why cold-acclimated mice which express less TRPM8, spend more time in the warm zones compared to the Thermoneutral group.

We demonstrated that long-term cold acclimation induced cold tolerance behaviours and induced phenotypical changes in TRPM8 levels in DRGs. In our study, time span of cold avoidance behaviour and the role of TRPM8 in cutaneous nerve endings, which seem to be involved in modulatory mechanisms of thermoregulatory behaviours like warmth-seeking behaviour in response to cold T_a (Kobayashi, 2015), were not analysed. Our results do not prove that down-regulation of TRPM8 in cold acclimation masks the sensation of cold, but nor do our results rule it out. We do propose that cold acclimation of the sensory system is directly or indirectly linked to TRPM8 activity, since its agonist icilin less potently elicits cold avoidance behaviors in cold acclimated mice.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Somayeh Ezzatpanah: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Mina Baarnes Eriksen:** Investigation, Writing - original draft, Writing - review & editing. **Anne-Mari Gjestvang Moe:** Investigation, Writing - original draft, Writing - review & editing. **Fred Haugen:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing - original draft, Writing - review & editing.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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