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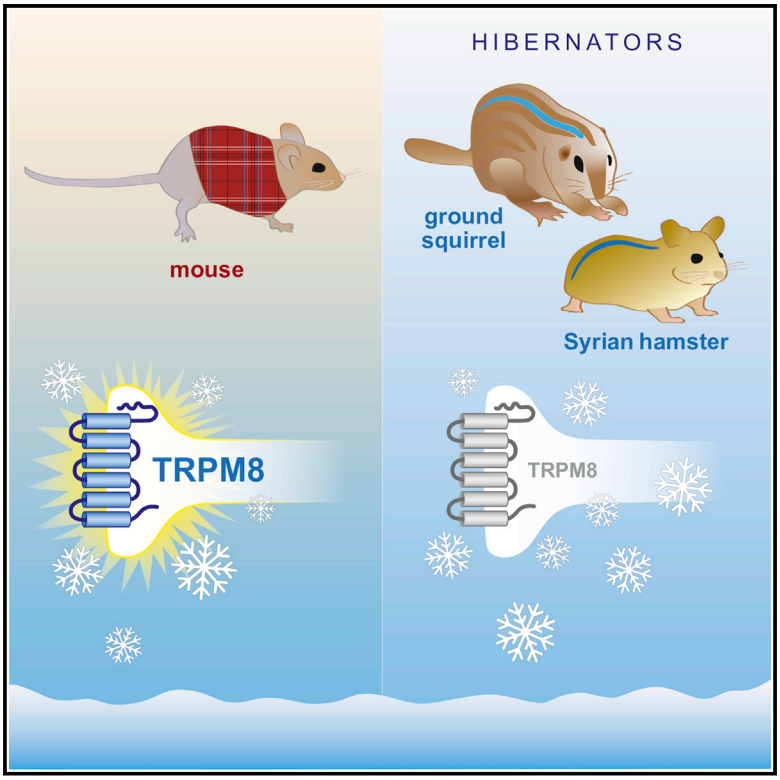
Molecular Prerequisites for Diminished Cold Sensitivity in Ground Squirrels and Hamsters

Graphical Abstract



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In Brief

Matos-Cruz et al. show that ground squirrels and hamsters exhibit cold tolerance even in the active non-hibernating state, partially due to independent modifications in the core transmembrane domain of the cold-sensing channel, TRPM8. The study reveals molecular adaptations that accompany cold tolerance in two species of active mammalian hibernators.

Highlights

- Squirrels and hamsters are cold tolerant even in active, non-hibernating state
- Cold tolerance in hibernators is partially supported by somatosensory system
- Squirrel and hamster somatosensory neurons express cold-insensitive TRPM8
- Cold sensitivity of squirrel TRPM8 channel is back-engineered by six mutations

Data and Software Availability

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Molecular Prerequisites for Diminished Cold Sensitivity in Ground Squirrels and Hamsters

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SUMMARY

Thirteen-lined ground squirrels and Syrian hamsters are known for their ability to withstand cold during hibernation. We found that hibernators exhibit cold tolerance even in the active state. Imaging and electrophysiology of squirrel somatosensory neurons reveal a decrease in cold sensitivity of TRPM8-expressing cells. Characterization of squirrel and hamster TRPM8 showed that the channels are chemically activated but exhibit poor activation by cold. Cold sensitivity can be re-introduced into squirrel and hamster TRPM8 by transferring the transmembrane domain from the cold sensitive rat ortholog. The same can be achieved in squirrel TRPM8 by mutating only six amino acids. Reciprocal mutations suppress cold sensitivity of the rat ortholog, supporting functional significance of these residues. Our results suggest that ground squirrels and hamsters exhibit reduced cold sensitivity, partially due to modifications in the transmembrane domain of TRPM8. Our study reveals molecular adaptations that accompany cold tolerance in two species of mammalian hibernators.

INTRODUCTION

The somatosensory system evolved to accommodate behavioral needs of various species and inhabit a wide spectrum of geographical ranges (Gracheva and Bagriantsev, 2015). Cold sensitivity, a specific aspect of somatosensitivity, is a key physiological capacity pertinent to all vertebrates and invertebrates. In the somatosensory system, temperature changes are detected by the primary afferent of somatosensory neurons localized within trigeminal and dorsal root ganglia (DRG). Cold receptors account for 15%–20% of the total neuronal population in the DRG of mice and many other vertebrates (McKemy, 2013). The molecular mechanism of cold sensitivity involves TRPM8, a cold-activated non-selective cation channel. TRPM8 mediates physiological responses to environmental cold below 26°C and is activated by the same temperature range *in vitro* (Bautista et al., 2007; Dhaka et al., 2007; McKemy et al., 2002; Peier et al., 2002). As a cold sensor, TRPM8 is an essential part of the thermosensory apparatus, which, along with other organs and systems, defines the range of temperature tolerance for a species and, ultimately, the breadth of its geographical habitat. An extreme example of temperature tolerance is demonstrated by mammalian hibernators that can withstand prolonged exposure to cold and extreme hypothermia (Carey et al., 2003). In order to survive harsh environmental conditions, hibernators must have developed adaptations at the molecular level, but most of them, including the suppressed ability to respond to cold, remain unknown. In this study, we explored the contribution of the somatosensory system to cold detection in two species of mammalian hibernators, the thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) and Syrian hamsters (*Mesocricetus auratus*).

RESULTS

Squirrel TRPM8$^+$ Receptors Are Poorly Sensitive to Cold

We characterized the temperature sensitivity of active ground squirrels and hamsters (Figure 1A) using a two-plate temperature preference test (Laursen et al., 2016). We quantified the time spent by the animals on a reference plate set at 30°C or a test plate set to a temperature ranging from 0°C to 30°C. Consistent with earlier studies, mice strongly prefer 30°C over cooler temperatures and completely avoid temperatures below 10°C (Bautista et al., 2007; Dhaka et al., 2007). Squirrels and hamsters, on the other hand, exhibited a significant preference to the 30°C plate only when the test plate reached 5°C and 10°C, respectively, and failed to show a complete avoidance even at 0°C (Figures 1B and 1C). The behavior exhibited by squirrels and hamsters is remarkably similar to that reported for mice with genomic ablation of TRPM8, a cold-activated ion channel (Figure 1D) (Bautista et al., 2007; Dhaka et al., 2007). We hypothesized that the apparent cold tolerance exhibited by squirrels and hamsters could be caused, at least partially, by either the decreased abundance of TRPM8 neurons or their diminished cold sensitivity. Using RNA *in situ* hybridization, we estimated that TRPM8 was expressed in 9.7 ± 0.5%, 9.4 ± 0.7%, and 9.8 ± 0.9% (mean ± SEM; n = 1,912–2,870 cells) of neurons

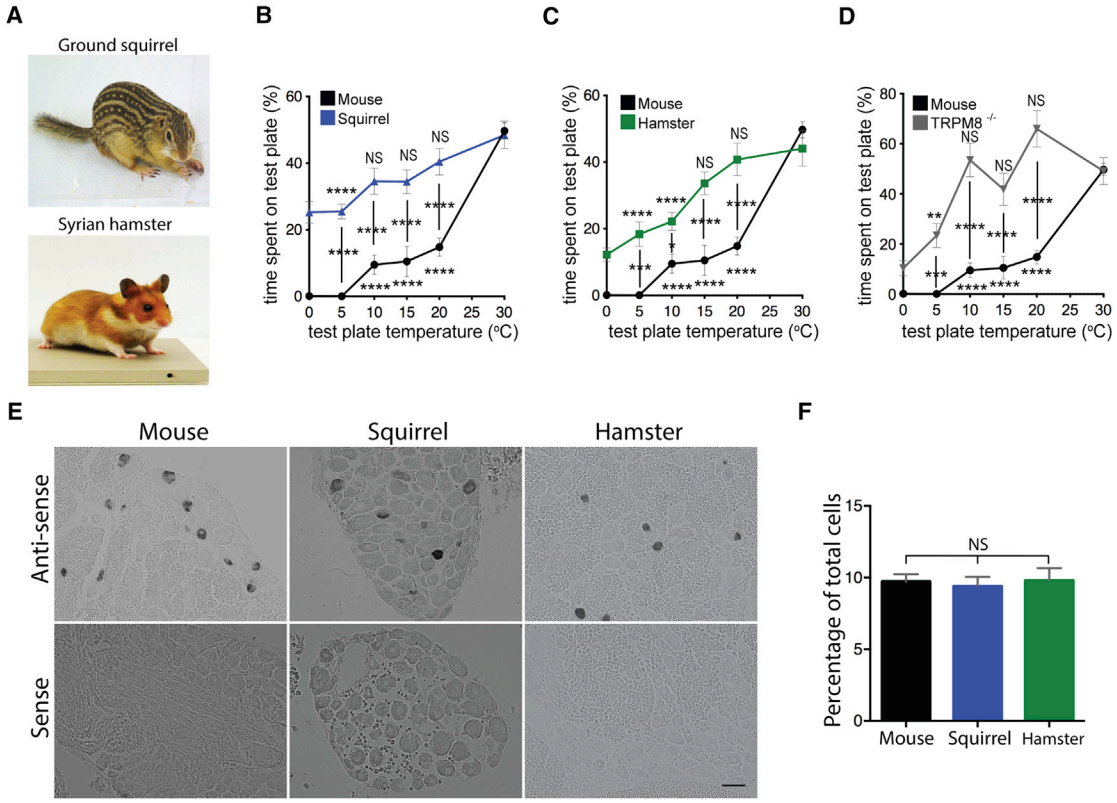

Figure 1. Squirrel and Hamster TRPM8 Have Diminished Cold Sensitivity

(A) Image of a thirteen-lined ground squirrel and Syrian hamster (courtesy of the Gracheva lab).

(B–D) Quantification of animal behavior in temperature preference tests shows the percentage of time spent by wild-type mice (B–D), squirrels (B), hamsters (C), and TRPM8$^{-/-}$ mice (D) on the test (0°C–30°C) versus control (30°C) plate over 5 min. Data were collected from 20 wild-type mice, 8 TRPM8$^{-/-}$ mice, 19 squirrels, and 19 hamsters for each temperature point. NS, not significant, $p \geq 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$, ordinary two-way ANOVA ($p < 0.0001$ for species effect) with Tukey post hoc test. Statistical comparisons are shown between species for each temperature (vertical lines) and within species versus the reference 30°C plate (shown above or below symbols).

(E) RNA *in situ* hybridization images showing expression of Trpm8 transcripts in tissue sections from mouse, squirrel, and hamster DRG (scale bar, 50 μm).

(F) Quantification of Trpm8 mRNA expression within mouse, squirrel, and hamster DRG (mean ± SEM from 1,912 neurons, 15 DRG sections for mouse; 2,538 neurons, 20 DRG sections for squirrel; and 2,870 neurons, 20 DRG sections for hamster, from at least two animals for each species). NS, not significant; $p \geq 0.05$, one-way ANOVA with Dunnett's post hoc test).

from, respectively, mouse, squirrel, and hamster DRG, suggesting that the diminished cold sensitivity cannot be explained by a decrease in the number of cold-sensing cells (Figures 1E and 1F). To assess functional properties of neuronal cold receptors, we performed ratiometric calcium imaging of dissociated DRG neurons, focusing on cells activated by icilin, a specific agonist of TRPM8 (McKemy et al., 2002). As expected, all wild-type mouse neurons sensitive to icilin (3.0% of 2,352 neurons) were also sensitive to cold. We also detected robust icilin responses in a subset of squirrel DRG neurons (3.1% of 1,177 neurons), demonstrating the presence of functional TRPM8 (Figures 2A and 2B). However, even though squirrel and mouse cells had identical icilin responses (Figure 2C), and all squirrel icilin-sensitive neurons were activated by cold, the amplitude of cold-evoked response was significantly diminished, compared to mouse cells in the 10°C–25°C range, suggesting that squirrel neurons express TRPM8 with normal icilin but impaired cold sensitivity (Figure 2D). In agreement with this, whole-cell electrophysiological recordings showed that the amplitude of cold-induced current normalized to icilin response is significantly lower in squirrel compared to mouse DRG neurons (Figures 2E and 2F). These data suggest that the apparent cold tolerance of squirrels can be explained, at least partially, by diminished cold sensitivity of TRPM8-expressing neuronal cold receptors.

Squirrel and Hamster TRPM8 Have Diminished Cold Sensitivity

We cloned TRPM8 from squirrel and hamster DRG and analyzed their chemical and temperature sensitivity by two-electrode voltage clamp in *Xenopus* oocytes in comparison with rat TRPM8 (Figure S1A), a well characterized TRPM8 ortholog with properties similar to those of the mouse channel (McKemy et al., 2002; Peier et al., 2002). We found that squirrel and hamster TRPM8 are sensitive to icilin and menthol, with EC_{50} indistinguishable from that of the rat ortholog (icilin half-maximal effective concentration [EC_{50}], mean ± SEM: 0.60 ± 0.12 μM,

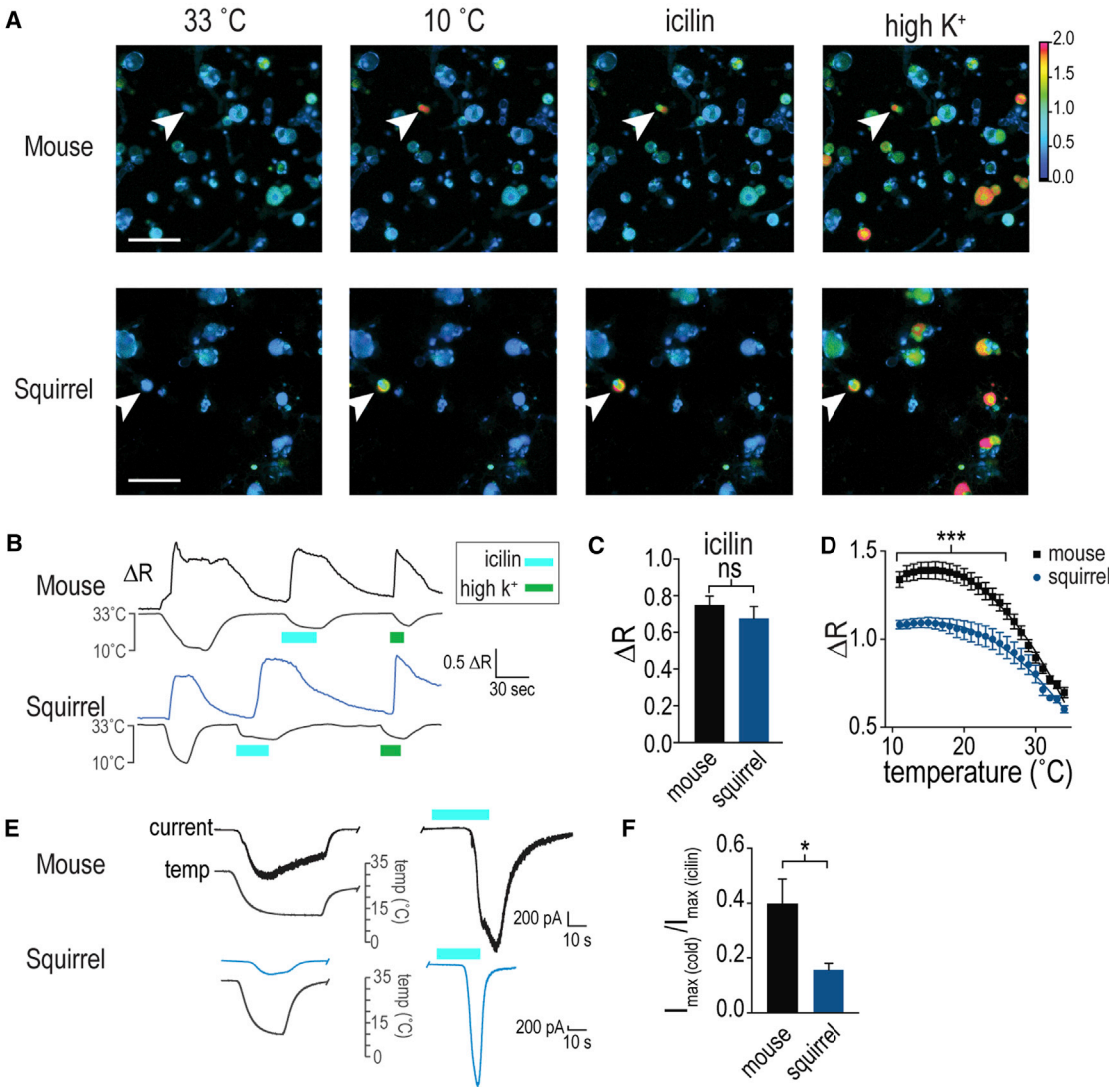

Figure 2. Squirrel TRPM8 Neurons Have Diminished Cold Sensitivity

(A) Representative partial fields of view of Fura-2AM ratiometric calcium imaging in squirrel and mouse dissociated DRG neurons. White arrowheads indicate cold and icilin-responding cells. Color coding denotes lowest and highest ratios from bottom to top. Scale bars, 100 μm.

(B) Example traces from the shown images of squirrel and mouse neurons responding to cold, 10 μM icilin, and 135 mM KCl (high K+).

(C) Baseline-corrected peak calcium responses during icilin application (mean ± SEM; n = 37 squirrel and 75 mouse icilin-sensitive neurons from a total of 1,177 squirrel and 2,532 mouse DRG neurons, obtained from 3 animals for each species; ns, not significant, p = 0.4, Mann-Whitney U test).

(D) Population data of cold responses in icilin-sensitive neurons from squirrel and mouse binned by degrees Celsius (mean ± SEM; two-way ANOVA with Bonferroni correction: $p < 0.0001$; main effect of species, temperature; ***$0.001 < p < 0.05$ for multiple comparisons at temperatures between 10°C and 25°C; not significant ($p \geq 0.05$) between 26°C and 34°C.

(E) Example current traces evoked by cold and 10 μM icilin in dissociated mouse and squirrel DRG neurons held at −60 mV in voltage-clamp mode.

(F) Quantification of maximal inward current evoked in mouse and squirrel DRG neurons by temperature stimulation, normalized to maximal response evoked by 10 μM icilin (mean ± SEM; *$p < 0.05$, Mann-Whitney U test; n = 4 squirrel and 5 mouse DRG neurons from 3 animals for each species).

See also Figure S1.

0.53 ± 0.06 μM, 0.55 ± 0.02 μM for rTRPM8, sqTRPM8, and hamTRPM8, respectively, n = 7–8; menthol EC_{50}: 38.42 ± 4.80 μM, 32.08 ± 3.45 μM, 35.04 ± 4.59 μM for rTRPM8, sqTRPM8, and hamTRPM8, respectively, n = 6; Figures 3A–3C and S2A–S2D). These results agree with the presence of intact putative binding sites for these agonists in both squirrel and hamster TRPM8 (Figure S1A) (Bandell et al., 2006; Chuang et al., 2004). As expected, rat TRPM8 exhibited gradually increasing activity in response to cooling of the extracellular solution from 30°C to 20°C (linear activation slope, k = −0.018 ± 0.000, mean ± SEM; n = 10) and from 20°C to 10°C (k = −0.027 ± 0.000; n = 10), with maximal cold-evoked current amplitude reaching ~51% of that evoked by 1 μM icilin (Figures 3D–3F). In contrast, squirrel and hamster TRPM8 activity only

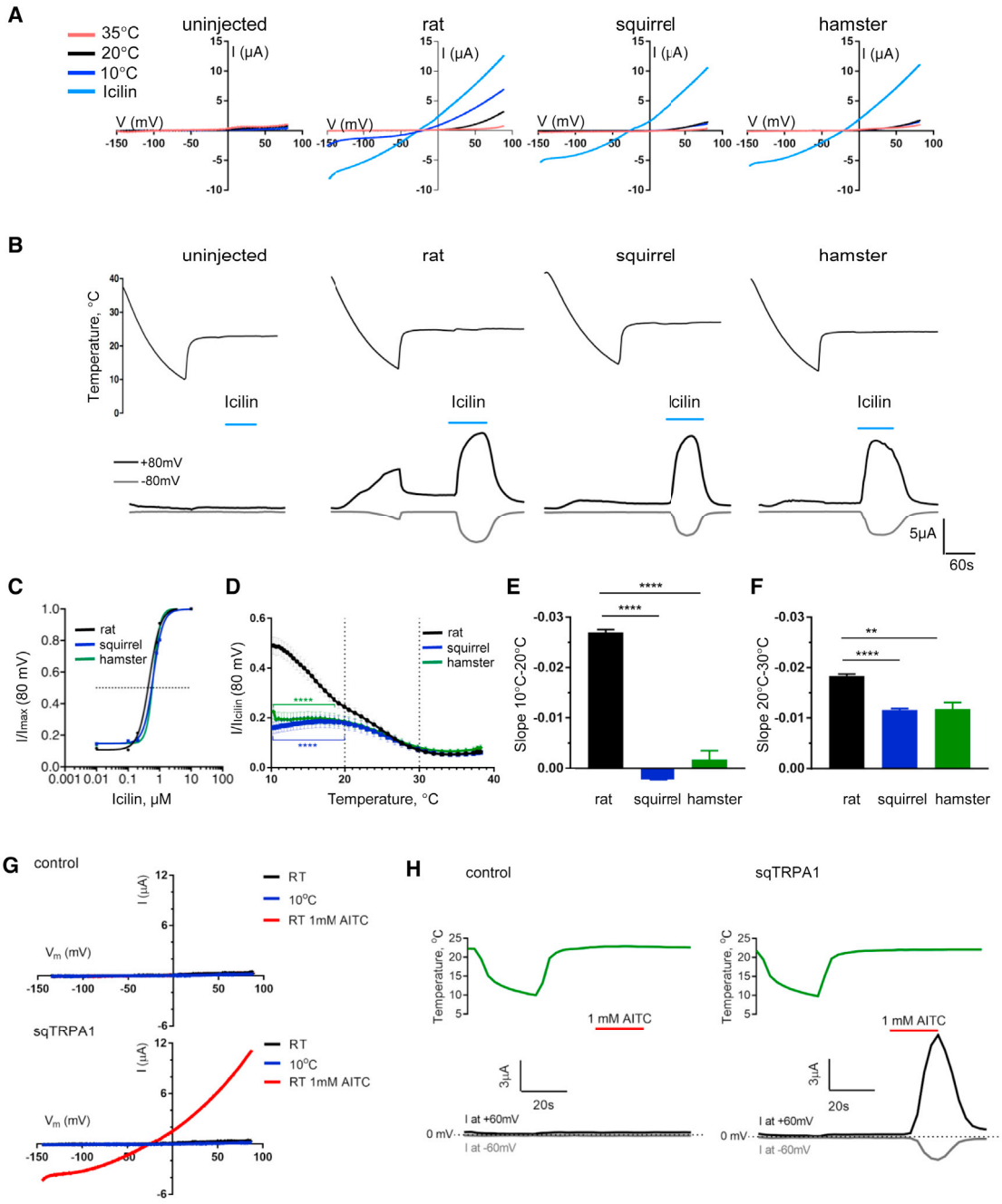

Figure 3. Squirrel and Hamster TRPM8 Have Diminished Cold Sensitivity

(A and B) Exemplar current-voltage plots (A) and traces (B) of responses to temperature ramps (35°C–10°C) and 1 μM icilin obtained by two-electrode voltage clamp in *Xenopus* oocytes expressing rat TRPM8, squirrel TRPM8, or hamster TRPM8.

(C) Icilin dose-response curves for rat, squirrel, and hamster TRPM8 orthologs (mean ± SEM; the error bars are smaller than symbols, $n \geq 5$ for each point).

(D) Temperature-response profiles for TRPM8 orthologs normalized to the maximum icilin response. Data are indicated as mean ± SEM; n = 5–10. ****$0.0001 < p < 0.05$ versus rTRPM8 in the range indicated by brackets; not significant ($p \geq 0.05$) outside this range (two-way ANOVA with Dunnett's post hoc test, $p < 0.0001$ for species effect).

(E and F) Quantification of temperature response steepness (slope) obtained by fitting the data for the (E) 10°C–20°C and (F) 20°C–30°C segments in (D) to the linear equation (mean ± SEM; n = 5–10; **$p < 0.01$; ****$p < 0.0001$, one-way ANOVA with Dunnett's post hoc test, $p < 0.0001$ for both panels).

(G and H) Exemplar current-voltage plots (G) and traces (H) of responses to temperature ramps from room temperature (RT; 22°C) to 10°C, and 1 mM allyl isothiocyanate (AITC) obtained by two-electrode voltage clamp in water-injected *Xenopus* oocytes (control) or oocytes expressing squirrel TRPA1. The images are representative of >10 cells from 2 independent experiments.

See also Figure S2.

slightly increased in the 30°C–20°C segment (k = −0.012 ± 0.000, n = 10; and −0.012 ± 0.001, n = 5, for sqTRPM8 and hamTRPM8, respectively) but remained virtually unchanged upon further cooling from 20°C to 10°C (k = 0.002 ± 0.000 and −0.002 ± 0.002 for sqTRPM8 and hamTRPM8, respectively Figures 3D–3F). The maximal normalized cold-evoked amplitude for both orthologs was diminished to ~18% of that evoked by 1 μM icilin (Figure 3D). Overall, cold responses of squirrel and hamster TRPM8 were significantly reduced compared to that of rat TRPM8 in, respectively, the 10°C–20°C range and the 10°C–18.5°C range (Figure 3D). TRPM8 is known to undergo desensitization due to depletion of phosphatidylinositol 4,5-bisphosphate (PIP_2) by calcium-activated phospholipase C (Liu and Qin, 2005; Rohács et al., 2005). This mechanism is unlikely to be the cause for the observed diminution of cold responses, since squirrel and hamster TRPM8 retain the putative PIP_2-binding site in the C terminus (Figure S1A) (Rohács et al., 2005), and the removal of extracellular calcium failed to potentiate cold responses (Figures S2E and S2F). Thus, squirrel and hamster TRPM8 have diminished overall cold sensitivity and cannot track temperature changes in the 10°C–20°C range *in vitro*, consistent with the reduced cold responses of dissociated neurons and behavioral data.

We wondered whether the remaining cold sensitivity in squirrels is dictated by TRPA1, a polymodal ion channel that is expressed in a neuronal population distinct from TRPM8 and that was proposed to contribute to cold responses (del Camino et al., 2010; Memon et al., 2017). We therefore cloned TRPA1 from squirrel DRG and tested its temperature and chemical sensitivity. We found that squirrel TRPA1 is not activated by cooling to 10°C, even though the channel is activated by the specific agonist allyl isothiocyanate (Figures 3G and 3H). These data suggest that the residual responses to cold in squirrel neurons and in behavioral tests are mediated by other mechanisms.

Transmembrane Core Domain Plays an Essential Role in Dictating Cold Sensitivity of TRPM8

Even though the squirrel and hamster channels are highly homologous to the rat ortholog and display around 90% amino-acid identity, they contain a number of amino-acid substitutions in the putative intracellular, core transmembrane, and extracellular regions (Figure S1A). To identify structural elements that underlie the diminished cold sensitivity in squirrel and hamster TRPM8, we generated chimeric channels with the robustly cold-sensitive rat ortholog and tested them by two-electrode voltage clamp in *Xenopus* oocytes. The substitution of both N- and C-terminal domains in squirrel TRPM8 with homologous domains from the rat channel (chimera RSR; Figure 4A) increased a maximal normalized cold-evoked amplitude from 18% to 28%, while individual N- and C-terminal domains (chimeras RSS and SSR) had no effect (Figures 4B, 4G, and 4H). Even though the potentiation of cold responses in the RSR chimera was substantial, it remained significantly lower than in the rat channel in the 10°–17°C range, prompting us to test the transmembrane core domain (Figure 4B). Strikingly, the substitution of the core transmembrane domain of squirrel TRPM8 with the rat homolog (chimera SRS; Figure 4A) conferred cold sensitivity to the extent indistinguishable from that of the rat channel in the 10°C–30°C range (Figure 4C). Analogously, the transposition of the rat transmembrane core increased cold-evoked amplitude sensitivity of hamster TRPM8 (chimera HRH) in a broad range of temperatures except 10°C–13°C (Figure 4D). All the chimeras had unchanged sensitivity to icilin (Figures S2D and S3A). These data show that, while N and C termini play a modulatory role (Tsuruda et al., 2006; Phelps and Gaudet, 2007), the transmembrane domain alone can dictate cold responses of TRPM8.

The transmembrane domains of squirrel and rat TRPM8 differ by only 15 amino acids (Figures 4E and S1A). To delineate molecular determinants of cold sensitivity, we subdivided the core transmembrane domain of TRPM8 into three blocks, each encompassing two transmembrane helices and containing, respectively, five, four, and six amino acid differences between squirrel and rat TRPM8 (Figures S1A and S3B). Interestingly, transposition of any two of the three blocks (chimeras $SR_{1–4}S$, $SR_{3–6}S$, and $SR_{1–2,5–6}S$) or just the transmembrane domains 5 and 6 (chimera $SR_{5–6}S$) from rat to squirrel TRPM8 was not sufficient to confer cold sensitivity to the same extent as transposition of the whole transmembrane domain (chimera SRS), suggesting that the functional amino acids are spread throughout the transmembrane core (Figures S3A–S3C, S3F, and S3G).

By systematically replacing individual amino acids, we identified six residues in squirrel TRPM8 core that, when replaced by homologous residues from the rat channel (sqTRPM8^{6m}: H726Y, A762S, P819S, A927S, H946Y, and S947N; Figures 4E and S1B), conferred robust cold sensitivity in the 10°C–20°C range (Figures 4F and S3A) without affecting icilin response (Figure S2D). Mutating any one of the six amino acids in sqTRPM8^{6m} back to the original squirrel residues (sqTRPM8^{5m}; Figure S3D) either abolished cold sensitivity (Figure S3E) or resulted in non-functional channels, as assessed by the absence of both icilin and cold responses. Conversely, six reciprocal point mutations in rat TRPM8 (rTRPM8^{6m}) significantly reduced cold responses (Figures 4F–4H and S3A) without altering chemical sensitivity (Figure S2D). Thus, we conclude that these six amino acids in the transmembrane core are necessary for cold responses of squirrel and rat TRPM8.

DISCUSSION

Here, we show that animals from two different Rodentia families, thirteen-lined ground squirrels (Sciuridae) and Syrian hamsters (Cricetidae), do not avoid cold as strongly as mice (Muridae) when given a choice between two plates with different temperatures. The apparent cold tolerance exhibited by squirrels and hamsters could be explained by a number of scenarios, including, but not limited to, the reduced ability to perceive cold by the somatosensory afferents or the suppression of cold avoidance at the level of the CNS. Here, we specifically focused on the somatosensory component and analyzed the abundance and functional properties of TRPM8-expressing neuronal cold receptors. In mice, this population of neurons is responsible for the detection of a wide range of temperatures (0°C–26°C) via a mechanism that includes activation of the cold-gated ion channel TRPM8 (Bautista et al., 2007; Dhaka et al., 2007; McKemy et al., 2002; Peier et al., 2002; Pogorzala

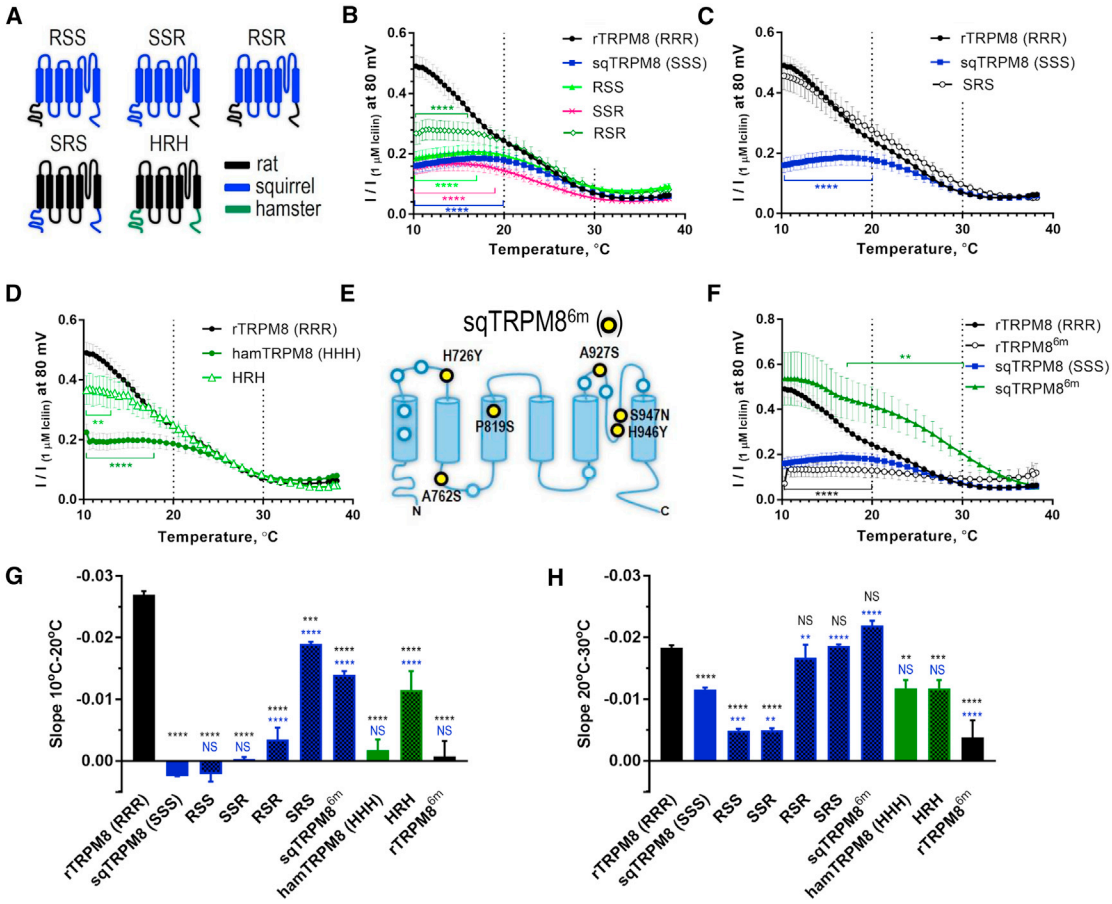

Figure 4. Modulation of Temperature Sensitivity in Squirrel and Hamster TRPM8 Orthologs

(A) Topology diagram of TRPM8 chimeric channels.

(B–D and F) Normalized temperature response profiles for the indicated wild-type and chimeric TRPM8 channels between squirrel, hamster, and rat TRPM8 (mean ± SEM; n = 5–10; **0.01 < p < 0.05; ****0.0001 < p < 0.05 for data of the same color versus rat TRPM8, in the range indicated by brackets; not significant ($p \geq 0.05$) outside this range (two-way ANOVA with Dunnett's post hoc test, $p < 0.0001$ for species effect).

(E) Topology diagram depicting the locations of the 15 non-conserved amino acids in the transmembrane core of rat and squirrel TRPM8 (blue and yellow circles) and the six mutations that confer cold sensitivity to sqTRPM8 (yellow circles; sqTRPM8^{6m}).

(G and H) Quantification of temperature-response steepness (slope) obtained by fitting the data for the (G) 10°C–20°C and (H) 20°C–30°C segments in (B–D and F) to the linear equation (mean ± SEM; n = 5–10). NS, not significant, $p \geq 0.05$; **p < 0.01; ***p < 0.001; ****p < 0.0001 versus rTRPM8 (denoted by black symbols) or sqTRPM8 (denoted by blue symbols), one-way ANOVA ($p < 0.0001$ for both panels) with Dunnett's post hoc test.

See also Figure S3.

et al., 2013). Consistently, TRPM8-deficient mice show a significant reduction, but not a complete elimination, of cold-evoked responses at the behavioral, cellular, and nerve fiber levels (Bautista et al., 2007; Dhaka et al., 2007; Milenkovic et al., 2014). The residual cold responses are attributed to the presence of additional cold-activated ion channels in TRPM8 neurons, as well as non-TRPM8 cold receptors (Knowlton et al., 2013; Memon et al., 2017; Pogorzala et al., 2013; Zimmermann et al., 2007; Lolignier et al., 2015). We focused on TRPM8-expressing neurons and show that these cells are present in squirrel and hamster DRG at proportions identical to that of mice, ruling out an insufficient number of cold receptors as the cause of the observed behavioral phenotype. Functionally, however, squirrel neurons exhibit significantly reduced cold sensitivity compared to mouse cells, when assessed by ratiometric calcium imaging and electrophysiology. These data are consistent with the idea that the diminished cold sensitivity of peripheral cold receptors may contribute to the species-specific cold tolerance that we observed in the temperature preference test.

Since TRPM8 is a major cold-activated excitatory conduit in neuronal cold receptors, we cloned this channel from squirrel and hamster DRG and characterized its functional properties side by side with the rat ortholog in *Xenopus* oocytes. Consistent with earlier data, rat TRPM8 exhibited a progressive activation in response to gradual temperature decrease from 30°C to 10°C (McKemy et al., 2002). In striking contrast, we found that squirrel and hamster channels were significantly less sensitive to cold, exhibiting almost no change in activity below 20°C. At the same time, both orthologs retained sensitivity to icilin and menthol with an EC_{50} identical to that of rat TRPM8, demonstrating that

the functional deficiencies in squirrel and hamster TRPM8 are modality specific. Thus, the reduced cold sensitivity of TRPM8 explains, at least partially, the apparent cold-tolerant phenotype exhibited by squirrels and hamsters in a two-plate preference test in the 10°C–30°C temperature range. Squirrels and hamsters remain sensitive to cooling below 10°C, suggesting the presence of a TRPM8-independent mechanism of cold detection. A number of molecules were suggested for this role, including TRPA1 and the voltage-gated sodium channels $Na_v1.8$ and $Na_v1.9$ (Lolignier et al., 2015; Memon et al., 2017; Zimmermann et al., 2007). Our data show that squirrel TRPA1 is not activated by temperature within the 10°C–22°C range, arguing against its role in cold detection in squirrels, although we were not able to test its activity at temperatures below 10°C.

In contrast to mice or rats, ground squirrels and hamsters can undergo prolonged periods of hibernation, during which their core body temperature drops to ambient and can be as low as 2°C–7°C (Bouma et al., 2011; Merriman et al., 2016; Carey et al., 2003; Tupone et al., 2017). While the mechanism of hibernation is complex and poorly understood, it seems clear that it involves significant modifications to the animal's thermoregulatory responses, which normally rely on the integration and processing of inputs from both peripheral and internal thermosensory systems (Almeida et al., 2012; Weidler et al., 1974; Heller and Colliver, 1974). Accordingly, pharmacological suppression of cold sensitivity in peripheral neurons via inhibition of TRPM8 triggers a complex systemic response, involving an increase in heat dissipation, decreased thermogenesis, and, ultimately, decreased core body temperature (Feketa et al., 2013; Feketa and Marrelli 2015; Almeida et al., 2012). While not very many TRPM8 orthologs have been described, it is interesting to see that the cold sensitivity of TRPM8 seems to follow the species' core body temperature, being the lowest in cold-blooded frogs and highest in birds (Chuang et al., 2004; Gracheva and Bagriantsev 2015; Myers et al., 2009), whose body temperature is above that of rodents or humans. The squirrel and hamster TRPM8 orthologs described here are out of trend, prompting us to speculate that the apparent cold tolerance exhibited by squirrels and hamsters has evolved as a part of a complex physiological mechanism that supports hibernation. Conceivably, a suppressed sensitivity to environmental cold could be essential for both enduring the hibernation as well as entering it. To test this hypothesis would require the generation of transgenic animals expressing a robustly cold-sensitive ortholog of TRPM8 from the native locus—an experiment that appears possible in a few years' time.

Functional analysis of chimeras between TRPM8 orthologs showed that cold sensitivity of the squirrel and hamster channels can be restored if their transmembrane cores are replaced with the homologous domain from rat TRPM8, strongly supporting the idea that the transmembrane core is a key determinant of cold sensitivity. Whether the core domain senses temperature directly, or whether it allosterically responds to a discrete temperature sensor located elsewhere in the channel (Arrigoni et al., 2016), remains to be determined. The squirrel's transmembrane core differs from that of rat by 15 amino acids, and transposition of only six of them is sufficient to restore cold sensitivity. Conversely, changing the six amino acids in the rat channel to their squirrel analogs significantly diminishes cold sensitivity, demonstrating that these sites are crucial for cold responses of both channels. None of these residues, which are scattered throughout the core without forming an obvious cluster, have been implicated in the chemical or voltage sensitivity of TRPM8 (Bandell et al., 2006; Chuang et al., 2004; Voets et al., 2007). This indicates that the changes that occurred in squirrel TRPM8 structure have been selected in evolution to specifically suppress cold responses. Interestingly, the six residues are not conserved between squirrel and hamster TRPM8 (Figure S1B). Moreover, of the six residues, four are identical between hamster and rat TRPM8. Together with the observation that the transposition of the rat transmembrane core onto hamster TRPM8 confers cold sensitivity, our findings strongly support the idea that squirrel and rat channels have lost sensitivity to cold via non-identical changes in the transmembrane domain.

Recently, we reported that ground squirrels are tolerant to noxious heat, partially due to diminished heat sensitivity of the TRPV1 channel in peripheral nociceptors (Laursen et al., 2016). Similar to TRPM8, the suppression of temperature sensitivity in squirrel TRPV1 is specific, as the channel remains sensitive to chemical agonists, such as protons and capsaicin, preserving its role in inflammation. The modality-specific diminution of temperature responses, rather than a complete obliteration of the functional gene, suggest that squirrel and hamster TRPM8 may retain other important physiological functions, which currently remain obscure.

EXPERIMENTAL PROCEDURES

Further details and outlines of resources used in this work can be found in the Supplemental Experimental Procedures.

Animals

Animals were housed in a pathogen-free facility at Yale University. All animal procedures were performed in compliance with the Office of Animal Research Support of Yale University (protocol 2015-11497). Summer active squirrels, hamsters, and mice were housed on a 12-hr/12-hr light/dark cycle under standard laboratory conditions with *ad libitum* access to food and water. Thirteen-lined ground squirrels were maintained on a diet of dog food (Iams) supplemented with sunflower seeds, superworms, and fresh vegetables.

Temperature Preference Assay

Behavioral experiments on mice (8- to 14-week-old male C57BL/6 mice and $TRPM8^{-/-}$ in C57BL/6 background; approximate weight, 25 g), active squirrels (1- to 1.5-year-old males; approximate weight, 200 g; of note, squirrels become sexually mature at 8–12 months, and their lifespan is 8–10 years), and hamsters (8- to 14-week-old males; approximate weight, 120 g) were performed in May–July. For the two-plate temperature preference/aversion assay, animals were placed into a chamber containing one floor plate set to a control temperature of 30°C and the other set to a test temperature between 30°C and 0°C (T2CT, Bioseb, Vitrolles, France). Animals were placed onto the control plate and recorded as they freely explored both sides of the chamber, for a total of 5 min. Plate order was reversed between groups and test days. The animal was considered to cross from one plate to the other plate when the animal's four paws crossed into the new plate, even though all animals used for analysis touched the experimental plate at least one time with their front paws.

Tissue Collection

Whole DRG were fixed in 4% paraformaldehyde for RNA *in situ* hybridization or homogenized in the TRIzol reagent for RNA extraction. For functional analyses

of dissociated neurons, DRG were treated with collagenase P, followed by 0.25% trypsin, and suspended in DMEM media supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin.

Statistical Analysis

Data were obtained from at least two independent experiments and analyzed with GraphPad Prism 6.0 (GraphPad Software). Sample size and statistical tests are reported in the figure legends. The Mann-Whitney U test was used for pairwise comparisons, and an ordinary one- or two-way ANOVA with post hoc correction was used for multiple comparisons. Statistical tests were chosen based on the normality of distributions and variance equality, or lack thereof, and the number of samples. Unless indicated otherwise, data were reported as mean ± SEM, and significance is displayed in the figures as not significant (NS), $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$.

DATA AND SOFTWARE AVAILABILITY

The accession numbers for the thirteen-lined ground squirrel TRPM8, the thirteen-lined ground squirrel TRPA1, and the Syrian hamster TRPM8 are GenBank: MF285605, MG012465, and MF285606, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.11.083.

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AUTHOR CONTRIBUTIONS

V.M.-C., E.R.S., M.M., E.O.G., and S.N.B. designed and performed experiments. V.M.-C., E.R.S., M.M., E.O.G. and S.N.B. collected and analyzed data. D.K.M. supplied squirrels and advised on behavioral experiments. V.M.-C., E.R.S., S.N.B., and E.O.G. wrote the manuscript, with contributions from M.M., and D.K.M. E.O.G. and S.N.B. conceived the study and provided guidance and supervision throughout the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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