Somatosensory Neurons Enter a State of Altered Excitability during Hibernation

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SUMMARY

Hibernation in mammals involves prolonged periods of inactivity, hypothermia, hypometabolism, and decreased somatosensation. Peripheral somatosensory neurons play an essential role in the detection and transmission of sensory information to CNS and in the generation of adaptive responses. During hibernation, when body temperature drops to as low as 2°C, animals dramatically reduce their sensitivity to physical cues [1, 2]. It is well established that, in non-hibernators, cold exposure suppresses energy production, leading to dissipation of the ionic and electrical gradients across the plasma membrane and, in the case of neurons, inhibiting the generation of action potentials [3]. Conceivably, such cold-induced elimination of electrogenesis could be part of a general mechanism that inhibits sensory abilities in hibernators. However, when hibernators become active, the bodily functions—including the ability to sense environmental cues—return to normal within hours, suggesting the existence of mechanisms supporting basal functionality of cells during torpor and rapid restoration of activity upon arousal. We tested this by comparing properties of somatosensory neurons from active and torpid thirteen-lined ground squirrels (Ictidomys tridecemlineatus). We found that torpid neurons can compensate for cold-induced functional deficits, resulting in unaltered resting potential, input resistance, and rheobase. Torpid neurons can generate action potentials but manifest markedly altered firing patterns, partially due to decreased activity of voltage-gated sodium channels. Our results provide insights into the mechanism that preserves somatosensory neurons in a semi-active state, enabling fast restoration of sensory function upon arousal. These findings contribute to the development of strategies enabling therapeutic hypothermia and hypometabolism.

RESULTS AND DISCUSSION

Somatosensory Neurons Retain Action Potential Electrogenesis during Hibernation

We analyzed the electrical properties of dorsal root ganglion (DRG) neurons isolated from active and torpid squirrels (Figure 1A). We focused on thermoreceptors and nociceptors, which in squirrels and other rodents have a soma diameter of less than 30 μm (Figure 1B) [4–6]. To preserve state-specific properties, neurons were incubated at their respective physiological temperatures (37°C for active and 10°C for torpid neurons) prior to patch-clamp recordings at 20°C, the most commonly used temperature for electrophysiological recordings from DRG. Strikingly, we found that, even though squirrels spent 1 or 2 weeks in torpor with body temperature below 10°C, the resting membrane potential (RMP) and input resistance of torpid neurons remained unchanged compared to active neurons (RMP, mean ± SEM: −56.95 ± 1.06 mV and −59.46 ± 1.24 mV for active and torpid neurons, respectively, p = 0.1302; input resistance: 883.8 ± 112.9 MΩ and 743.1 ± 101.2 MΩ for active and torpid neurons, respectively, p = 0.3399; n ≥ 30 cells; Figures 1C and 1D). This suggests that the machinery that maintains the ionic gradient remained functional throughout the period of prolonged hypothermia. Direct in vivo measurements are needed to confirm this observation in live animals. In contrast, when active squirrel neurons were incubated at 10°C prior to recordings at 20°C, we detected a hyperpolarization of the RMP (mean ± SEM: −56.95 ± 1.06 mV and −61.59 ± 1.15 mV for active and cold-incubated active neurons, respectively; p = 0.006; n ≥ 19 cells) and a two-fold decrease in input resistance (mean ± SEM: 883.8 ± 112.9 MΩ and 398.6 ± 50.7 MΩ for active and cold-incubated active neurons, p = 0.0038, respectively;
Figure 1. Somatosensory Neurons Retain Action Potential Electrogenesis during Hibernation

(A) Thirteen-lined ground squirrel in the active and torpid state (courtesy of the Gracheva lab).

(B) Diameter of DRG neurons from active and torpid squirrels. NS, not significant; p > 0.05; Mann-Whitney U test. Data are shown as mean ± SEM; n ≥ 32 cells.

(C–E) RMP (C), input resistance (D), and current threshold (E). NS, not significant; p > 0.05; unpaired t test (C) and Mann-Whitney U test (D and E). Data are shown as mean ± SEM; n ≥ 30 cells.

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n ≥ 19 cells; Figures S1A and S1B). Thus, the phenotype observed in torpid neurons does not result from cold exposure alone but rather reflects changes in cellular properties associated with hibernation. We next examined action potential generation in torpid neurons in response to current injection. Similar to active state, most torpid neurons generated action potentials (30 out of 32 active neurons and 31 out of 34 torpid neurons). Consistent with unchanged RMP and input resistance, both groups required the same amount of current to generate action potentials (mean ± SEM: 66.33 ± 17.88 pA and 61.61 ± 13.99 pA for active and torpid neurons, respectively; p = 0.3593; n ≥ 15 neurons; Figure 1E). In contrast, the current threshold was significantly increased in cold-incubated active neurons (mean ± SEM: 88.42 ± 20.75 pA; p = 0.0345; n = 19 cells; Figure S1C). Increasing current injection elevated the firing frequency in both groups, but torpid neurons had a two- to three-fold lower firing rate (Figure 1F). Thus, although torpid neurons fire at a reduced frequency relative to active neurons, the machinery that generates action potentials remains intact. Overall, our results show that the functionality of somatosensory neurons is suppressed, but not eliminated, during hibernation.
A similar reduction in firing rate was observed in tonically firing mouse DRG neurons as a result of knockout of Nav1.8, a major voltage-gated sodium channel in C-type nociceptors [7]. Therefore, to understand the molecular basis of the impaired action potential electrogenesis during torpor, we sought to investigate the activity of voltage-gated sodium channels in DRG neurons.

**Somatosensory Neurons from Hibernating Squirrels Have Decreased Nav Activity**

In the majority of thermoreceptors and nociceptors, action potentials are mainly (though not exclusively) generated and propagated by a triad of voltage-gated sodium channels: Nav1.9; Nav1.7; and Na v1.8 [10–19]. Na v1.9 is activated at the most hyperpolarized potentials and generates persistent non-inactivating currents, contributing to setting the RMP and the threshold for action potentials [20–23]. Na v1.7 is responsible for the initial phase of action potential, leading to the rapid depolarizing upstroke produced by Nav1.8 [7, 24–26]. We hypothesized, based on our observation of reduced firing rate and redistribution of firing patterns, that torpid neurons have altered Nav channel function.

Using a protocol developed for recording tetrodotoxin-resistant (TTX-R) Nav1.9 current in rodent DRG [27], we recorded a sodium current with similar characteristics in squirrel DRG neurons, suggesting it is mediated by the squirrel ortholog of Na v1.9. We found no differences in Nav1.9 current density, voltage dependence of activation, and steady-state inactivation between active and torpid neurons (V 50 of activation, mean ± SEM: 70.46 ± 0.83 mV and 70.27 ± 0.73 mV for active and torpid neurons, respectively, p = 0.8642, n ≥ 15 cells; V 50 of inactivation: 69.55 ± 2.31 mV and 77.89 ± 5.71 mV for active and torpid neurons, respectively, p = 0.2042, n ≥ 9 cells; Figure 2). Of note, compared to Nav1.9 orthologs from other mammals, Nav1.9 from active and torpid neurons showed little inactivation, even at positive potentials. Overall, these data are consistent with unchanged RMP and current threshold (Figures 1C and 1E).

Nav1.7 is a tetrodotoxin-sensitive (TTX-S) channel that contributes to ~70% of the total TTX-S current in mouse DRG [28–30]. The voltage-dependence and kinetics of Na v1.7 enable the channel to amplify subthreshold depolarization and set the
gain on the sensitivity of somatosensory neurons [31]. We found a ~50% decrease of TTX-S current density in torpid neurons compared to active (Figures 3A and 3B). We also detected a small depolarizing shift in the voltage dependence of activation but no change in steady-state fast inactivation (Figures 3C–3E). As a result of the change in V_{50} of activation, we also detected a significant prolongation of the rate of Na\textsubscript{v}1.8 current decay (Figures 4F and S4B). The depolarizing shift in activation of Na\textsubscript{v}1.8 is expected to suppress the probability of firing in torpid neurons, and slower inactivation might contribute to increased action potential width.

Cumulatively, 95% of active and torpid DRG neurons contained both TTX-S and TTX-R currents, demonstrating that Na\textsubscript{v}1.7 and Na\textsubscript{v}1.8 are present in the majority of neurons from both groups (Figure S4C). The decrease in the amplitude of the two currents in torpid neurons could be due to diminished gene expression. However, we found only a small (~20%) reduction in Na\textsubscript{v}1.7 message in torpid neurons and no change in Na\textsubscript{v}1.8 message (Figures S4D and S4E), suggesting other regulatory mechanisms, which remain to be determined.

**Conclusions**

Our study revealed several unexpected findings: (1) during torpor, somatosensory neurons preserve general electrical properties—RMP, input resistance, and rheobase—indistinguishable...
from the active state; (2) electrical properties of torpid neurons reflect complex changes associated with hibernation and cannot be recapitulated by merely exposing active neurons to cold; (3) torpid neurons retain the action-potential-generating machinery; and (4) firing of torpid neurons shifts from tonic to irregular and single, suggesting suppressed action potential generation, likely due to the observed changes in functional properties of the Na+
which could arise from alternative splicing, posttranslational modifications, interaction with auxiliary proteins, and/or changes in the plasma membrane. We do not rule out potential contribution from voltage-gated potassium channels, which remains to be determined.

Our results show that the somatosensory system does not undergo complete shutdown during hibernation. Instead, somatosensory neurons employ cold-resistant machineries to maintain ionic gradients and electrical potentials similar to the active state. These processes are energetically expensive, underscoring the notion that torpor is an active physiological process that requires molecular fine-tuning. Indeed, recent studies in hibernating rodents revealed profound genomically encoded changes in temperature sensors, mitochondrial function, protein control machineries, and cytoskeletal integrity [5, 6, 33]. We suggest the existence of other such modifications in various groups of enzymes, ion channels, and transporters implicated in energy homeostasis and ionic balance regulation.

The preservation of action-potential-generating machinery in the somatosensory system during torpor may be beneficial for the animal as it emerges from hibernation and re-establishes the essential means of communication with its environment. The quick restoration of sensory abilities requires such machinery to be in a functional or semi-functional state rather than de novo synthesis and assembly. It appears possible that similar principles could be at work in other systems and organs of mammalian hibernators.

STAR Methods

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Animals
  - Primary neuron dissociation
  - Patch-clamp electrophysiology
  - qPCR
- QUANTIFICATION AND STATISTICAL ANALYSIS

Supplemental Information

Supplemental Information includes four figures and can be found with this article online at https://doi.org/10.1016/j.cub.2018.07.020.

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Author Contributions

L.J.H., M.M., E.O.G., and S.N.B. designed and performed experiments and collected and analyzed data. D.K.M. supplied squirrels and provided advice on animal husbandry. S.D.D.-H. and S.G.W. provided guidance for data analysis. L.J.H., S.N.B., and E.O.G. wrote the manuscript with contributions from all authors. 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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References


STAR METHODS

KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to Elena O. Gracheva (elena.gracheva@yale.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

**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 2018-11497). Thirteen-lined ground squirrels were maintained on a diet of dog food (Iams) supplemented with sunflower seeds, superworms, and fresh vegetables. During the summer active period, squirrels were individually housed on a 12-h light/dark cycle with ad libitum access to food and water. During the winter torpid period, squirrels were individually housed in a specialized facility at 4°C ambient temperature and constant dark.

**Primary neuron dissociation**

Primary neurons were dissected from dorsal root ganglia of active and torpid squirrels (~1 year old). Active animals were euthanized via CO₂ inhalation followed by decapitation. Dorsal root ganglia were dissected into ice-cold HBSS. Tissue was then briefly treated with collagenase P (1mg/mL in HBSS, 15 min, 37°C) followed by 0.25% trypsin (10 min, 37°C). Finally, tissue was suspended in Hibernate-A media (with custom osmolarity at 280 mOsm) supplemented with B-27 supplement and glutaMAX (GIBCO) according to the manufacturer’s recommendation (adjusted to pH 8.0 with NaOH). Hibernate-A media is designed to have a stable pH at ambient levels of CO₂. Tissue was mechanically dissociated using a plastic tipped pipette before being plated on poly-D-lysine coated coverslips (Coming) at room temperature for 30 s and immediately placed at 37°C in 0.3% CO₂, or at 10°C in 0.3% CO₂ for cold incubation. Torpid animals were euthanized by decapitation. Torpid dorsal root ganglia were dissociated identically to active tissues, but were subsequently incubated at 10°C in 0.3% CO₂. Small diameter neurons (< 30 μm) were selected for recording after becoming adherent (~2hrs), for up to 36 hr after plating.

**Patch-clamp electrophysiology**

Solutions and protocols were based on methods previously described [27]. Data were acquired on a Zeiss Axio-Examiner with an Orca flash 4.0 camera (Hamamatsu) and an Axopatch 200-B amplifier/Digidata 1440 digitizer (Molecular Devices). Recordings were acquired using pCLAMP software (Molecular Devices) sampled at 20-50 kHz and low-pass filtered at 2–10 kHz. Experiments were carried out at a room temperature of 22–25°C. Coverslips that were incubated at 37°C were placed into the recording chamber...
with bath solutions at room temperature, and several neurons were patched on the same coverslip for up to 1 hr. In order to minimize effects of acute warming of cold-incubated cells, coverslips that were incubated at 10°C were placed into the recording chamber with bath solutions cooled to 10°C. Only after a suitable neuron was identified was the bath solution warmed to room temperature, the cell was patched within 5 min of warming and was kept only for the duration of the recording protocol, in some cases up to 30 min.

Current-clamp experiments were performed with DRG neurons collected from summer active squirrels between end of May to beginning of August (n = 8), or from winter torpid squirrels between October and March (n = 5). The internal pipette solution consisted of (in mM): 140 KCl, 0.5 EGTA, 5 HEPES, and 3 Mg-ATP (adjusted to pH 7.3 with KOH, osmolarity adjusted to 300 mOsm with dextrose). External solution contained (in mM): 140 NaCl, 3 KCl, 2 MgCl₂, and 10 HEPES (adjusted to pH 7.3 with NaOH, osmolarity adjusted to 320 mOsm with dextrose). Patch pipettes of borosilicate glass with an o.d. of 1.5 mm (Warner Instruments no. G150F-3) were pulled and polished to a tip resistance of 1–2 MΩ.

Voltage-clamp experiments were performed with DRG neurons collected from active squirrels (n = 8) or torpid squirrels (n = 6) as described above. The internal pipette solution consisted of (in mM): 140 CsF, 10 NaCl, 2 MgCl₂, 0.1 CaCl₂, 1.1 EGTA, 10 HEPES (adjusted to pH 7.2 with CsOH, osmolarity adjusted to 310 mOsm with dextrose). External solution contained (in mM): 140 NaCl, 3 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, 10 dextrose (adjusted to pH 7.3 with NaOH, osmolarity adjusted to 320 mOsm with dextrose). To isolate sodium currents, 20 mM TEA-Cl (blocks voltage-gated K⁺ channels), and 0.1 mM CdCl₂ (blocks voltage-gated Ca²⁺ channels) were added to the external solution. Due to very large TTX-S currents produced by squirrel neurons, recordings of TTX-S and Nav.1.8 were performed with a low-sodium external solution in which the NaCl concentration was replaced with 35 mM NaCl and 105 mM Choline-Cl. Patch pipettes were pulled and polished to a tip resistance of 1–2 MΩ. Cells with a seal > 1 GΩ and a leak current < 200 pA were used for recordings. 85% series resistance compensation was used to minimize voltage errors. Linear leak subtraction was applied to all recordings, using P/6 (Nav.1.9 recordings) or P/4 (TTX-S and Nav.1.8 recordings) hyperpolarizing control pulses applied after the depolarizing test stimuli. Nav.1.9 currents were isolated by adding 250 nM tetrodotoxin (TTX) to the external solution and holding at hyperpolarized potentials (−120 mV). Only the persistent currents of the resulting recordings were extracted, to avoid the Nav.1.8-mediated component. The peak persistent current was measured from a visually determined window where the non-persistent current has decayed while the persistent current remains constant. For activation, 200-ms pulses were used from −100 mV to +15 mV, in 5 mV steps. For steady-state fast inactivation, 200-ms prepulses from −140 mV to +10 mV, in 10 mV steps were used, followed by a 150-ms test pulse to 0 mV. Measurements of Nav.1.9 current were corrected for liquid junction potentials offline (9 mV).

TTX-S currents were isolated by performing sequential recordings in the absence and in the presence of 250 nM TTX. The TTX-R residual current was subtracted from the total sodium current to obtain the TTX-S component. Holding potential was −100 mV. For activation, 100-ms prepulses were used from −80 mV to +40 mV, in 5 mV steps. For steady-state fast inactivation 100-ms prepulses from −140 mV to 0 mV, in 10 mV steps were used, followed by a 100-ms test pulse to −10 mV.

Nav.1.8 currents were isolated by applying 250 nM TTX and holding at depolarized potentials (−80 mV), at which Nav.1.9 in the presence of CsF becomes inactivated. These recordings were performed in sequence with the recording of TTX-S currents, making it possible to evaluate the co-distribution of Nav.1.8 and TTX-S. For activation, 100-ms pulses were used from −80 mV to +40 mV, in 5 mV steps. For steady-state fast inactivation, 100-ms prepulses from −140 mV to 0 mV, in 10 mV steps were used, followed by a 100-ms test pulse to −10 mV. Measurements of TTX-S, and Nav.1.8 were corrected for liquid junction potentials (8 mV) online.

qPCR
Total RNA was extracted from squirrel DRG using the TRIzol reagent (Invitrogen). One microgram of total RNA was used to synthesize cDNA using the qScript cDNA Supermix (95048; Quanta Biosciences). qPCR calibrations and normalizations were performed using the Illumina Eco System machine in accordance with the manufacturer’s instructions. Each reaction was run in triplicate, using the Hprt1 gene as the reference [34]. Amplification primer pairs (5‘−3‘) were Scn9a: ATCCCAAGCCTCAGTGACAG; CACTCAGAGGAATCTCAATCGGC (product size 133 bp); Scn10a: CTGCAGCAAGTCGGGAGGTA; TGCAGAATCGGAGGACG (product size 109 bp).

QUANTIFICATION AND STATISTICAL ANALYSIS
Data were obtained from at least two independent experiments and analyzed with GraphPad Prism 7.0 (GraphPad Software), and the number of experimental points is indicated in each figure. Activation conductance gₘ was calculated from the equation
\[ g_m = I_{Na}/(E_m - E_{rev}), \]
where \( I_{Na} \) is the peak current, \( E_m \) is the corresponding voltage, and \( E_{rev} \) is the estimated reversal potential.
To calculate the midpoints ($V_{50}$) of activation and steady-state fast inactivation, activation conductance was normalized and plotted against the corresponding voltage step, and test-pulse-evoked inactivation current was normalized and plotted against the corresponding conditioning pulse. The resulting curves were fit by a Boltzmann function in Prism 7 (GraphPad). The inactivation rate (tau of decay) was calculated by fitting the decaying component of the current at each voltage to a single-exponential equation ($I = \Delta I \cdot \exp(-t/t_{\text{inact}})$, where $\Delta I$ is the difference between peak sodium current and baseline, $t$ is the time from the peak current, the start of the fit, and $t_{\text{inact}}$ is the decay constant) using MATLAB (MathWorks). Only curves fit with an $R^2 > 0.95$ were included.

Statistical tests were chosen based on the distribution of the data: unpaired Student’s t test for normally distributed data (D’Agostino & Pearson normality test), with Welch’s correction for data with statistically different variances (F test), Mann-Whitney U test for not normally distributed data. Regular 2-way ANOVAs were corrected for multiple comparisons using Bonferroni correction.
Somatosensory Neurons Enter a State of Altered Excitability during Hibernation

Highlights
- Hibernating DRG neurons have unchanged resting potential, resistance, and rheobase
- Hibernating DRG neurons retain electrogenicity, with decreased firing rate
- Properties of hibernating neurons are innate and not triggered by cold alone
- Properties of hibernating neurons are dictated by altered Na\textsubscript{v} channel function

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In Brief
Hoffstaetter et al. show that somatosensory neurons from hibernating squirrels exist in a semi-functional state, with decreased ability to generate action potential, partially due to altered activity of Na\textsubscript{v} channels. This state reflects complex changes associated with hibernation and cannot be recapitulated by merely exposing active neurons to cold.
Supplemental Information

Somatosensory Neurons Enter a State of Altered Excitability during Hibernation

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Figure S1. Cold incubation of active neurons changes their electogenic properties. Related to Figure 1. (A-D) Resting membrane potential (A), input resistance (B), current threshold (C) and action potential parameters (D) obtained from active neurons incubated at 37°C (active) or at 10°C (active cold incubation), *p<0.05, **p<0.01, ****p<0.0001, unpaired t-test (A, D) and Mann-Whitney U test (B-D). Data shown as mean ± s.e.m, n≥19 neurons.
Figure S2. Firing rate of neurons with irregular, phasic, and single firing patterns. Related to Figure 1. Action potential (AP) firing rate at increasing current injections from 0-100 pA. Ordinary two-way ANOVA with Bonferroni correction, NS, not significant; p>0.05. Data shown as mean ± s.e.m; n≥8 irregular, n≥3 phasic, n≥4 single.
Figure S3. Most action potential properties are unchanged during hibernation. Related to Figure 1. (A-D) Action potential properties grouped by firing pattern: peak maximum (A), amplitude (B), minimum of afterhyperpolarization (C), rise time from 20-80% of peak (D). NS,
not significant; $p>0.05$, regular two-way ANOVA with Bonferroni correction. Data shown as mean ± s.e.m; $n\geq 7$ tonic, $n\geq 8$ irregular, $n\geq 3$ phasic, $n\geq 4$ single.
Figure S4. TTX-S and Nav1.8 decay kinetics and expression. Related to Figures 3-4. (A and B) Inactivation rate (tau of decay), calculated from a single-exponential curve fit to the decay of the current at each voltage, and corrected for the indicated difference in $V_{50}$ between conditions. (C) Distribution of Nav1.8 and TTX-S currents in active and torpid DRG neurons. (D, E) Quantitative PCR of Scn9a (gene name for Nav1.7) (D) and Scn10a (gene name for Nav1.8)
transcripts (E) from mRNA isolated from active and torpid squirrel DRG. NS, not significant; p>0.05, *p<0.05, unpaired t-test. Data shown as mean ± s.e.m, n=6-7 squirrels.