Title: Hibernation reduces GABA signaling in the brainstem to enhance motor activity of breathing at cool temperatures

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Abstract:

Background: Neural circuits produce reliable activity patterns despite disturbances in the environment. For this to occur, neurons elicit synaptic plasticity during perturbations. However, recent work suggests that plasticity not only regulates circuit activity during disturbances, but these modifications may also linger to stabilize circuits during future perturbations. The implementation of such a regulation scheme for real-life environmental challenges of animals remains unclear. Amphibians provide insight into this problem in a rather extreme way, as circuits that generate breathing are inactive for several months during underwater hibernation and use compensatory plasticity to promote ventilation upon emergence.

Results: Using *ex vivo* brainstem preparations and electrophysiology, we find that hibernation in American bullfrogs reduces $GABA_A$ receptor ($GABA_AR$) inhibition in respiratory rhythm generating circuits and motor neurons, consistent with a compensatory response to chronic inactivity. Although $GABA_ARs$ are normally critical for breathing, baseline network output at warm temperatures was not affected. However, when assessed across a range of temperatures, hibernators with reduced $GABA_AR$ signaling had greater activity at cooler temperatures, enhancing respiratory motor output under conditions that otherwise strongly depress breathing.

Conclusions: Hibernation reduces $GABA_AR$ signaling to promote robust respiratory output only at cooler temperatures. Although animals do not ventilate lungs during hibernation, we suggest this would be beneficial for stabilizing breathing when the animal passes through a large temperature range during emergence in the spring. More broadly, these results demonstrate that compensatory synaptic plasticity can increase the operating range of circuits in harsh environments, thereby promoting adaptive behavior in conditions that suppress activity.

Main text:

Background

Animals have the mysterious ability to produce reliable behaviors while navigating environments that should otherwise disrupt activity of the nervous system [1]. This is thought to occur, in part, through a set of compensatory mechanisms that sense activity perturbations, and in turn, adjust neuronal properties to counteract the disturbance. For example, if activity falls, neurons increase synaptic excitation, decrease inhibition, and alter ion channel densities to enhance neuronal excitability [2-5]. This framework, termed "homeostatic plasticity," has been foundational for the past 30 years [6], but it has been challenging to link ethologically relevant behaviors in animals, as neurons often need to be perturbed artificially or pathologically to reveal homeostatic responses [7-12].

Certain organisms inhabit natural environments with potent abiotic stressors, such as pH, temperature, and hypoxia, which can cause activity challenges within the nervous system [13]. An extreme example of this problem is encountered by hibernating frogs. Like most vertebrate species, rhythmic neural circuits in the brainstem generate breathing to meet metabolic demands of the organism. However, in the cold hibernation environment frogs may spend several months underwater using only skin for gas exchange [14], while respiratory motor behavior is completely suspended [15]. To successfully emerge following months underwater, frogs employ various compensatory mechanisms to maintain these networks so that they can work effectively when needed. This includes a classic activity-dependent mechanism of compensatory plasticity called "synaptic scaling" at excitatory synapses [16, 17], and also metabolic adjustments that improve performance in hypoxia [18, 19]. As amphibians use synaptic plasticity to overcome this large environmental challenge, they provide an intriguing opportunity to understand how plasticity mechanisms are integrated within animals to promote adaptive behavior.

Compensatory forms of synaptic plasticity are often interpreted as a conceptually simple regulatory regime used to counteract activity disturbances (e.g., when activity goes down, excitatory synaptic strength goes up). However, experimental and modeling work indicates that when circuits are disrupted, compensatory adjustments can also "prime" circuits to perform better during future perturbations and influence the capacity for subsequent plasticity [20-22]. Thus, we hypothesized that the hibernation environment not only triggers synaptic plasticity, but also that it induces network modifications to expand the operating range in challenging environments encountered following the initial activity challenge. To test this in an ethological context, we built upon previous work demonstrating that hibernation drives apparent homeostatic plasticity at excitatory synapses [16, 17] and addressed whether inhibitory synapses contribute as well. We then asked how plasticity through inhibition influences motor function of breathing at colder temperatures, where neuronal activity is otherwise markedly suppressed. Here, we report that hibernation strongly reduced GABAergic inhibition, consistent with a classic response to chronic network inactivity [3, 23]. Despite the critical role of inhibition in this network [24], circuit function appeared surprisingly normal at warm temperatures. However, when we assessed activity across a range of temperatures, $GABA_AR$ plasticity served mainly to offset the depressive effects of acute

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95   cooling, boosting the strength and frequency of motor output at lower temperatures.
96   Given that these animals must pass through a range of temperatures as they
97   reestablish life on land after hibernation [25, 26], network modifications that improve
98   network activity within this range seem to play an adaptive role. Therefore, although
99   GABAergic plasticity has no obvious impact on baseline motor function, it expands the
100 operating range of the respiratory circuit in an otherwise suppressive environment to
101 promote adaptive behavior.
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Results

Respiratory motor output in adult frogs is thought to arise *via* central pattern-generating mechanisms consistent with reciprocal inhibition involving GABA [24]. Rhythmic activity in these premotor neurons is then carried to motor pools through interneuronal pathways that control motor outflow to respiratory muscles [27]. Activity of this network can be assessed in isolated brainstems *in vitro* which produce rhythmic motor output on a variety of cranial nerves including the trigeminal, vagal, and hypoglossal nerve root. Although the activity pattern of these nerves may differ, the output produced is largely synchronous providing a representation of the motor output that resembles lung ventilation in the whole animal [28, 29]. Here, we measured activity of the vagus nerve to monitor respiratory-related motor output from the intact brainstem preparation (cranial nerve X; CNX; schematic in Figure 1).

To investigate plasticity in GABAergic control of the respiratory network, we first bath-applied bicuculline, a $GABA_A$ receptor antagonist, to intact brainstem preparations. Consistent with an important role for $GABA_ARs$ in rhythmic motor activity, bicuculline caused a dose-dependent decrease in burst frequency in control brainstems at room temperature (Baseline, 42.60 ± 16.32 bursts/min; 0.5 µM Bicuculline, 26.32 ± 19.68 bursts/min; 1 µM Bicuculline, 16.86 ± 17.03 bursts/min; 5 µM Bicuculline, 5.66 ± 3.61 bursts/min; n=5, Figure 1A,C), with most having little-to-no activity at the highest dose (10 µM Bicuculline, 1.74 ± 1.10 bursts/min; n=5, Figure 1C). Under the same experimental conditions, hibernators strongly resisted bicuculline (Baseline, 21.12 ± 6.53 bursts/min; 0.5 µM Bicuculline, 23.80 ± 8.72 bursts/min; 1 µM Bicuculline, 21.52 ± 7.71 bursts/min; 5 µM Bicuculline, 14.50 ± 5.54 bursts/min; n=5, Figure 1B,C), with respiratory activity persisting at the highest dose (10 µM Bicuculline, 13.50 ± 4.12 bursts/min; n=5, Figure 1B,C). In regard to motor amplitude, two-way ANOVA revealed a significant main effect of bicuculline on normalized motor amplitude ($F_{(2.055, 16.44)}$ = 4.499, P=0.0269) but not a significant effect of group or an interaction between bicuculline and group as we saw for frequency. However, this bicuculine effect is likely to be minor, as post hoc analysis showed that significantly larger bursts compared to baseline occurred at 5 µM in controls (p=0.010; Holm-Sidak Multiple Comparison's test), while all other comparisons to baseline were not significantly different from baseline.

Loss of $GABA_AR$ signaling appeared to be localized to breathing circuits. In both control and hibernators, non-respiratory motor activity (long duration bursts with qualitatively different shape than respiratory bursts [30, 31]) were rare at baseline with only 1 control preparation containing these bursts. Interestingly, and despite a clear group x bicuculine interaction for respiratory burst frequency, we did not observe this same response for non-respiratory bursts (group x bicuculline interaction in two-way ANOVA; p=0.5373). Non-respiratory bursts emerged similarly in a manner that depended on the dose of bicuculline (Figure 1A-B,D) in both controls (Baseline, 0.04 ± 0.09 bursts/min; 0.5 µM Bicuculline, 0.44 ± 0.39 bursts/min; 1 µM Bicuculline, 1.44 ± 1.05 bursts/min; 5 µM Bicuculline, 3.18 ± 0.81 bursts/min; 10 µM Bicuculline, 3.08 ± 1.01 bursts/min, n=5) and hibernators (Baseline, 0.00 ± 0.00 bursts/min; 0.5 µM Bicuculline, 0.28 ± 0.63 bursts/min; 1 µM Bicuculline, 0.56 ± 0.98 bursts/min; 5 µM Bicuculline, 2.62 ± 1.50 bursts/min; 10 µM Bicuculline, 3.60 ± 1.86 bursts/min, n=5).

Given the difference in how respiratory and non-respiratory bursts alter sensitivity to bicuculline, hibernation strongly reduces the role of GABA$_A$Rs in a way that appears specific to circuits that generate breathing but not non-respiratory behaviors.

The previous experiment demonstrated that GABA$_A$Rs have a strongly reduced role in the production of the respiratory rhythm after hibernation. Next, we assessed whether plasticity influenced the capacity of the respiratory network to be modulated by GABA$_A$R activation. Therefore, we bath-applied muscimol, a GABA$_A$R agonist, to globally activate all GABA$_A$Rs that influence activity. In controls, the lowest dose of muscimol (500 nM) did not alter respiratory burst frequency (Baseline, 8.27 ± 3.7 bursts/min *vs.* 500 nM muscimol, 7.20 ± 4.0 bursts/min; n=5). Raising the concentration to 1 µM caused a significant decline (1 µM muscimol, 3.47 ± 1.9 bursts/min; p= 0.0282; n=5; Figure 2A,C), and exposure to 3 µM silenced all preparations (5 out of 5). In contrast, preparations from hibernators did not significantly alter respiratory burst frequency between baseline and any dose of muscimol (Baseline, 11.00 ± 2.1 bursts/min; 500 nM muscimol, 11.93 ± 1.8 bursts/min; 1 µM muscimol, 14.47 ± 2.8 bursts/min; 3 µM muscimol, 6.47 ± 7.3 bursts/min; n=5; Figure 2B-D), with 5 out of 5 preparations maintaining respiratory bursting at the highest dose. While suppression of network activity with both the antagonist and agonist may seem paradoxical, these results are consistent with the necessity of phasic inhibition for generation of respiratory activity and neuronal silencing through further activation ofGABAergic inhibition, respectively. Indeed, in other circuits that use inhibition for rhythmogenesis, antagonism of GABA$_A$Rs disrupts network dynamics required for network activity [32], while agonism of GABA$_A$Rs also suppresses activity through membrane hyperpolarization [33]. With both agonist and antagonist experiments, these results show that hibernation leads to a large reduction in the ability of GABA$_A$Rs to generate and modulate breathing.

The previous experiments identify that hibernation reduces the role of GABA$_A$Rs in generating and modulating the respiratory rhythm. At the level of the motoneuron, an activity-dependent mechanism known as "synaptic upscaling" strengthens excitatory synapses in response to hibernation [16, 17], aligning with classic homeostatic responses to chronic network inactivity [4]. Thus, we tested if hibernation also "downscales" postsynaptic GABA$_A$Rs to reduce GABA$_A$R inhibition. We used patch clamp electrophysiology to record miniature inhibitory postsynaptic currents (mIPSCs) carried by GABA$_A$Rs in identified vagal motoneurons that cause breathing in amphibians. The GABA$_A$R currents were isolated in TTX, strychnine (strych), and DNQX to block presynaptic action potentials, glycine receptors, and AMPA-glutamate receptors, respectively. GABA$_A$R minis were recorded from a holding voltage of -60 mV. The Nernst potential for Cl$^-$ under our experimental conditions was ~5 mV; thus, inward currents in the presence of strychnine and DNQX represent minis that arise from GABA$_A$Rs. This was confirmed in each experiment by applying bicuculline (bic) follow each recording. We did not observe changes in the mIPSC amplitude (p=0.5218; unpaired t test), charge transfer (p=0.4391; unpaired t test), rise time (p=0.1029; unpaired t test), frequency (p=0.5576; unpaired t test), as well as neuronal input resistance after hibernation (p=p=0.3647; unpaired t test) (Figure 3A-C). Therefore,

194 hibernation does not influence postsynaptic $GABA_ARs$ and is unlikely to account for the
195 loss of $GABA_AR$ signaling at the network level.
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197   As the quantal $GABA_AR$ currents were unchanged on the postsynaptic
198 motoneuron, we assessed if loss of the GABAergic tone is associated with reduced
199 network-driven presynaptic input onto motoneurons, like that seen in sensory
200 deprivation models of homeostatic plasticity [10, 11]. Specific respiratory synapses are
201 not yet tractable for stimulation experiments in this system; therefore, we developed an
202 approach to circumvent this issue. We used a novel semi-intact preparation that permits
203 recording of individual motoneurons receiving excitation and inhibition from rhythmic
204 premotor circuits that shape motoneuron firing of breathing (Figure 4A) [34]. As shown
205 in Figure 4A, vagal motor neurons fire bursts of action potentials during respiratory
206 population motor activity, reflecting activity of breathing at a single-cell resolution. Since
207 the postsynaptic receptor density of $GABA_ARs$ does not appear to change within these
208 neurons after hibernation (Fig. 3), any difference in the role of GABAergic synapses for
209 the control of motoneuron firing rate would reflect changes in network-driven
210 presynaptic GABA release. To isolate $GABA_ARs$ that control respiratory-related firing of
211 motoneurons, we focally applied bicuculline to the motoneuron cell body and
212 surrounding region *via* local pressure injection while recording its activity shaped by
213 excitation and inhibition from rhythmic premotor inputs in the intact network. We
214 simultaneously recorded rhythmic vagal motoneuron activity and the trigeminal nerve (a
215 nerve that innerves the buccal floor to drive air into the lungs and activates near-
216 synchronously with the vagal outflow) to monitor respiratory circuit output. We used the
217 trigeminal nerve (cranial nerve V; CN V) in these experiments because physical
218 constraints in the bath and the focal drug delivery system prevented simultaneous
219 recording of the vagus nerve.
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221   Motoneurons from both groups had similar average burst firing rates during
222 breathing and resting membrane potentials in between respiratory bursts (firing rate:
223 control, 45.26±34.8 Hz vs. hibernation, 24.19±23.0 Hz; p=0.133, unpaired t-test;
224 membrane potential: control, -61.52 ± 4.6 mV vs. hibernation, -59.24 ± 6.5 mV,
225 p=0.4094 unpaired t-test). In controls, local application of bicuculline led to a reversible
226 increase in average firing frequency during the respiratory burst (Baseline, 45.26 ± 34.8
227 Hz; Bicuculline, 89.36 ± 61.4 Hz; p<0.0001; Holm-Sidak multiple comparisons test;
228 n=12; Figure 4B,D). This was presumably not due to changes in the chloride gradient,
229 because two neurons in the control group that were recorded in the "loose patch"
230 configuration, which does not disturb intracellular milieu (Fig. 4E shown in orange),
231 more than doubled their firing rates during bicuculline exposure, aligning with data from
232 the whole-cell mode. These results demonstrate that phasic inhibition onto motoneurons
233 dampens firing rate during the respiratory burst. In contrast, local bicuculline had no
234 significant effect on the firing rate of motoneurons from hibernators (Baseline, 24.19 ±
235 23.0 Hz; Bicuculline, 38.75 ± 44.9 Hz; p=0.2629; n=9; Holm-Sidak multiple comparisons
236 test; Figure 4C-E). Additionally, there was no change in the interburst (resting)
237 membrane potential during focal application of bicuculline in either control (Baseline: -
238 61.52 ± 4.6 mV; Bicuculline: -60.92 ± 4.9 mV; n=10) or hibernation groups (Baseline: -
239 59.24 ± 6.5 mV; Bicuculline: -59.53 ± 6.5 mV; n=7), further supporting that phasic

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240 respiratory-related inhibition influences motoneuron firing rate rather than a tonic
241 GABA$_A$R current. Taken together with the mIPSC results, network-driven GABA release
242 appears to be reduced in a way that no longer dampens the firing rate of motoneurons
243 during activity associated with breathing.

245   These results demonstrate a critical role for GABA$_A$Rs in generating and
246 modulating the respiratory rhythm, as well as controlling motoneuron firing rate in
247 control frogs. Therefore, we were surprised that baseline network frequency on average
248 (Fig. 1C, p=0.2021) and motor burst morphology (Figure 5) in hibernators did not
249 change despite a rather dramatic loss of GABA$_A$R signaling in the network. These
250 results mirror ventilation data *in vivo*, whereby breathing frequency and breath volume
251 were the same after hibernation [35]. Despite these similarities at warm temperatures,
252 frogs likely need to restart breathing to some degree at temperatures around 8°C to
253 maintain aerobic metabolism [35, 36] and then produce reliable rhythmic output at
254 temperatures above 13°C to maintain metabolic homeostasis as they reestablish a
255 warmer life on land [25, 26]. Yet, cold temperatures ≤15°C strongly depress the network,
256 making it difficult to generate respiratory activity [15, 37]. Interestingly, cooling does not
257 suppress breathing exclusively through "passive" effects on cellular properties (*e.g.*,
258 slower action potentials, decreased rates of synaptic transmission), but rather, through
259 noradrenergic signaling. Indeed, blocking α adrenergic receptors blunts decreases in
260 activity during cooling, and the actions of norepinephrine on respiratory activity in the
261 frog brainstem are known to act through GABAergic pathways [38, 39]. This led us to
262 test whether decreased GABA$_A$R signaling enhances respiratory motor output at cold
263 temperatures faced by frogs when they need to resuscitate breathing while emerging
264 from hibernation.

266   To address this, we use three groups of *ex vivo* brainstem preparations: controls,
267 hibernators (reduced GABA signaling), and controls with GABA$_A$Rs experimentally
268 reduced with a subsaturating dose of bicuculline (2 μM). We hypothesized that control
269 preparations with impaired GABA$_A$R signaling would have similar responses to cooling
270 that were like hibernators if GABA$_A$R signaling is part of the process by which cold
271 temperatures depress activity of the respiratory network. That is, less GABA$_A$R signaling
272 would cause enhanced activity at colder temperatures. First, we addressed the
273 respiratory frequency. Cooling control brainstems from 20°C to 8°C expectedly reduced
274 the respiratory burst frequency (Figure 6A). Interestingly, the decline in hibernators was
275 less pronounced, with a temperature that produces 50% of baseline burst rate shifting to
276 colder temperatures (Figure 6B). Decreasing GABA$_A$R signaling in controls via
277 subsaturating block of GABA$_A$Rs also produced greater burst frequency at cold
278 temperatures; however, this was more dramatic than hibernators, resulting in some
279 networks with activity at 8°C (Figure 6C). Summary statistics are shown in Figure 6D.
280 Two-way ANOVA revealed a significant temperature, group, and temperature by group
281 interaction effect. These results were driven by several pairwise differences caused by
282 hibernators and controls with bicuculline. Hibernators had statistically greater
283 normalized burst frequency than controls at cooler temperatures (16°C: p=0.0267;
284 Holm-Sidak Multiple Comparisons test, Figure 6D blue). In addition, bicuculline
285 application to controls had significantly greater normalized burst frequency at lower

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temperatures (Figure 6D gray). These results are also summarized as the temperature at which burst frequency decreased by 50% (T_{50}) (Fig 6E). Indeed, hibernators had lower T_{50} than controls, and controls with bicuculline had the lowest T_{50} values. Taken together, the network is more active at cooler temperatures when $GABA_AR$ signaling is reduced naturally by hibernation or by experimentally blocking $GABA_A$Rs.

In addition to respiratory frequency, we addressed the amplitude of the motor output, as this variable relates to activation of the respiratory muscles. Like frequency, cooling also depressed motor amplitude in controls, reflecting lowered motoneuron firing or recruitment during the fictive breath (Figure 6A). In hibernators and controls in the presence of bicuculline, burst amplitude appeared to be maintained across the full temperature range (Figure 6B-C). Summary statistics are shown in Figure 6F. Two-way ANOVA revealed a significant temperature, group, and temperature by group interaction effect. These results were driven by several pairwise differences in hibernators and controls with impaired $GABA_A$ signaling. Hibernators and controls with bicuculline had statistically greater burst amplitude than controls at 12°C and 10°C (12°C: hibernator vs. control: p=0.0276; bic vs. control: p=0.0039, 10°C: hibernator vs. control: p=0.0254, bic vs. control: p=0.0254) Holm-Sidak Multiple Comparisons test). Overall, while network output at warm temperatures were similar between controls and hibernators with reduced GABA signaling, these results demonstrate that the downregulation of $GABA_AR$ signaling by hibernation drives more robust respiratory motor activity (frequency and motor amplitude) at cooler temperatures.

Discussion

Neural circuits face constant disturbances from the environment, placing a strong pressure on plasticity mechanisms to regulate activity. Here, we corroborate previous work suggesting $GABA_A$Rs play a role in generating the respiratory motor pattern in adult frogs and identified that they also play a distinct modulatory role to depress activity during cooling. We found that the state of hibernation triggers a downregulation of synaptic inhibition which would have been expected to cause general effects on network activity. Instead, activity of the respiratory network became more resistant to decreases in activity caused by cooling in a way that would help stabilize respiratory output as the brainstem passes through a large temperature range during emergence from the hibernation environment. Therefore, compensatory forms of synaptic plasticity can expand the operating range of circuits in challenging environments without obvious impacts on activity in unstressed conditions.

Reduced GABAergic inhibition at rhythm generating and premotor loci

Given that the respiratory motor system undergoes a massive activity challenge (inactivity during winter [15]), mechanisms consistent with activity-dependent compensation have been predicted to play a role in maintaining network integrity to promote motor behavior in the spring [16, 17, 35]. In addition to upregulation of excitatory synaptic strength on motoneurons, here we found that hibernation strongly reduced $GABA_AR$ signaling throughout the respiratory motor network.

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The present data lead us to suggest GABA$_A$R plasticity at two loci within the network (Fig. 7; top panel labeled "warm"): rhythm generating circuits and motoneurons. First, expression of the respiratory rhythm in this species involves GABA$_A$Rs [24], likely through mechanisms involving reciprocal inhibition. GABA$_A$Rs in premotor rhythm-generating circuits must have a diminished contribution after hibernation because motor frequency in hibernators was strongly resistant to depression caused by GABA$_A$R antagonists or agonists. Although counterintuitive, these opposing pharmacological manipulations (activating and inhibiting the receptors) both lead to suppression of the rhythm. This is likely to occur because too much inhibition depresses activity by hyperpolarizing neurons, and too little prevents phasic inhibition required for rhythmogenesis. This is reminiscent of what occurs in other species where rhythm generation depends on synaptic inhibition [32, 33]. Second, activity of the respiratory rhythm generator is transmitted through excitatory and inhibitory interneuronal pathways to recruit motoneurons that drive breathing. In contrast to inhibition's presumed role in facilitating rhythm generation, inhibition onto motoneurons has a conventional role of constraining the firing rate during respiratory-related bursting, which is reduced after hibernation (Fig. 4). As mIPSCs carried by GABA$_A$Rs on motoneurons were unchanged (Fig. 3), differences in presynaptic properties of inhibitory interneurons within the respiratory network likely account for weakened GABAergic control of firing (Fig. 4). Mechanistically, it is difficult at present to parse out the variables that account for the apparent loss of network-driven presynaptic GABA (*e.g.*, reduced vesicle release probability, decreased excitability of GABAergic interneurons, *etc*.), as respiratory-related synapses and GABAergic interneurons have not yet been identified in this network. Of note, rhythm and pattern generation are thought to arise, in part, from distinct populations within the respiratory central pattern generator of mammals [40]. As such, the differential effects we observed involving loss of inhibition in the respiratory circuit may stem from distinct populations within the central pattern generator itself. Overall, the specific mechanisms and network interactions await further experimentation, but the sum of our data points to the idea that rhythm-generating circuits and premotor neurons rely significantly less on GABA$_A$Rs after hibernation.

Given the profound loss of GABA$_A$R signaling, which is normally required to express the respiratory rhythm, one implication of these results is that some other mechanism must take over to produce breathing after hibernation. These results align well with recent work demonstrating circuits that recently recovered from an activity challenge use a different profile of ion channels to generate similar network output under baseline conditions [20, 21]. This leads us to speculate that plasticity resulting from hibernation may switch the mechanism of rhythmogenesis from one which relies heavily on inhibition to others that use excitatory synapses or intrinsic pacemaker mechanisms [24, 41] (Fig. 7; warm panel highlight shift in rhythm generating mechanisms, circular arrows). Consistent with this idea, the amphibian respiratory network appears to use voltage-dependent pacemaker mechanisms early in development, which then is replaced by network-dependent mechanisms *via* inhibition following metamorphosis [24]. Therefore, hibernation may revert the mechanisms of rhythm generation to that seen in an earlier life stage. Indeed, other aspects of breathing such as modulation of frequency by respiratory gases also revert to a

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378 juvenile-like state following hibernation [42], consistent with the idea that emerging from
379 aquatic hibernation may share similar environmental pressures as developing air-
380 breathing during the shift from aquatic to terrestrial habitats [43].
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382 ***Reduced GABA$_A$R signaling increases the network operating range in the cold***
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384   The most intriguing aspect of these results was that networks operating with less
385 GABA$_A$R signaling after hibernation seemed to perform better at cooler temperatures
386 (Figure 6), despite the fact that activity was largely unchanged at warm temperatures
387 (Figure 1&5). Our data indicate that GABA$_A$R signaling is part of the process by which
388 cold normally depresses the frequency and amplitude of the motor output in control
389 animals (Fig. 7; bottom panel labeled "acute cooling"). Although temperature effects on
390 neural circuits are multifaceted, we draw this conclusion because subsaturating block of
391 GABA$_A$Rs in control animals opposed the typical depressive response to cooling. The
392 mechanisms by which GABA$_A$R signaling normally decreases activity in the cold seems
393 to occur through two different processes. First, at the motoneuron level, cold may
394 facilitate inhibitory GABAergic input that we showed to exist on these neurons (Figure.
395 4) to depress the population motoneuron output (Figure 6). Thus, losing this inhibition
396 during hibernation likely explains maintenance of the burst amplitudes across the full
397 temperature range during acute cooling (Fig. 7, bottom right, less inhibitory input in the
398 cold). Second, our pharmacological data suggest that GABA$_A$R signaling plays a role in
399 slowing breathing frequency during cooling. Therefore, the loss of this inhibition in
400 hibernators accounts for the ability to produce stable rhythmic activity at cooler
401 temperatures (Fig. 6E). The network-level mechanisms remain to be uncovered;
402 however, cold temperatures activate noradrenergic neurons of the locus coeruleus [44,
403 45], which then depress breathing frequency through α-adrenoreceptors [39]. Since
404 noradrenergic signaling depresses respiratory activity mainly through GABAergic
405 pathways [38], modulatory mechanisms involving norepinephrine may be activated by
406 cooling to recruit GABAergic interneurons that depress the breathing frequency at cold
407 temperatures.
408
409   Regardless of the specific mechanism by which cold temperature and GABA$_A$Rs
410 interact, reduced GABA$_A$R signaling in hibernators, therefore, serves to boost
411 respiratory frequency and motor drive at cooler temperatures, with likely relevance for
412 restarting breathing upon exit from hibernation as the animal passes through a range of
413 cool temperatures (Fig. 7; bottom panel labeled "cold"). As such, we interpret plasticity
414 of GABA$_A$R signaling here as "homeostatic," as it is a change in network function that
415 opposes the activity disturbance and enhances network excitability in an environment
416 that otherwise depresses neuronal output. The loss of GABA$_A$R signaling after
417 hibernation seems to explain most of the maintenance of motor amplitude across
418 temperatures, since hibernators and controls with experimentally reduced GABA$_A$R
419 signaling had similar profiles across temperatures. However, despite hibernators having
420 higher activity at lower temperatures than naïve controls, activity still dropped to a lower
421 extent than we observed in controls with GABA$_A$R signaling experimentally reduced
422 (Fig. 6A-D). We speculate the quantitative differences between these two conditions
423 might point to additional plasticity mechanisms that tamp down enhanced excitability at

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424 cold temperatures to prevent ectopic breaths while submerged in cold water. While the
425 mechanisms that serve this purpose are not fully known, we have shown that sensory
426 input from CO_2/pH chemoreceptors that normally stimulate breathing is reduced in
427 hibernators [35, 42], suggesting that hibernation may also reduce excitability in some
428 parts of the network to help keep breathing off until it is needed. The exact "switch" that
429 restarts breathing also remains unclear, but nevertheless, our results suggest that the
430 loss of GABA$_A$R signaling prepares the network to produce strong motor bursting at cool
431 temperatures when it does restart, which would likely contribute to restoring breathing
432 as the animal passes through a range of colder temperature before returning to a
433 warmer body temperature after emergence.

435 ***Conclusions: What does it mean for plasticity to be "homeostatic?"***

437   Since its inception [6], compensatory mechanisms within the theme of
438 homeostatic regulation are most often portrayed as a conceptually simple (but
439 mechanistically complex) counterbalancing of altered excitability. Our results provide
440 evidence that mechanisms consistent with homeostatic plasticity can increase the
441 operating range of circuits in harsh environments that otherwise disrupt activity. We
442 acknowledge that a strict definition of "homeostatic plasticity" might not encompass the
443 plasticity we observed. Through a homeostatic lens, one might expect network activity
444 that drives lung ventilation to restart as compensation enhances network excitability.
445 However, breathing does not restart during hibernation [15], which in itself seems
446 important for animal survival as restarting breathing prematurely could be detrimental
447 underwater. In addition, the integrated output of the entire respiratory network does not
448 appear obviously hyperexcitable after emergence, as breathing in vivo is comparable
449 between controls and hibernators, which could be interpreted as inconsistent with
450 homeostatic theory [35]. While our observations deviate from traditional definitions of
451 negative feedback homeostasis on which the field was founded [4, 6, 46], reduced
452 GABA$_A$R signaling indeed increases the strength and frequency of respiratory motor
453 activity in cool conditions that must be passed through to reestablish life on land after
454 hibernation, thereby widening the circuit's operating range across temperatures.
455 Intriguingly, hibernation also leads to enhanced desensitization and reduced Ca^{2+}
456 permeability of NMDA receptors that serves to constrain excitability of this network
457 during severe hypoxia that occurs as the animal emerges, with no impact on baseline
458 activity of the network [47]. Therefore, multiple forms of synaptic plasticity appear to be
459 masked at baseline, but ultimately increase the operating range for motor activity under
460 the variety of stressors that occur during emergence from the hibernation environment.
461 Overall, these results support the view that circuits may implement compensatory
462 mechanisms in ways that enhance activity under specific sets of environmental
463 constraints. These findings may have important implications for diverse neural systems,
464 as the nervous system of most animals operate homeostatically over a range of abiotic
465 conditions, such as changes in local tissue temperature [48], pH [49, 50], oxygen levels
466 [47], hormonal state, and other factors that have the potential to disrupt network activity
467 on acute timescales. By linking plasticity to a challenging environment in vivo, our

468 results lead us to emphasize that frameworks seeking to link compensation to behavior
469 must ultimately incorporate how plasticity is integrated over the range of environments
470 that neural circuits may encounter. Understanding how the nervous system is tuned to
471 survive extreme conditions in intact animals will continue to provide new insights along
472 this path.

473

Methods

475 **Animals** All experiments performed were approved by the Animal Care and Use
476 Committee (ACUC) at the University of Missouri (protocol #39264). Adult Female
477 American Bullfrogs (~100 g weight, n= 62), *Lithobates catesbeianus*, were purchased
478 from Rana Ranch (Twin Falls, Idaho) and were housed in 20-gallon plastic tanks
479 containing dechlorinated water at room temperature bubbled with room air. Control frogs
480 were acclimated for 1 week following arrival before experiments. Frogs had access to
481 wet and dry areas. Frogs were maintained on a 12-hour light/dark cycle and fed once
482 per week. Water was cleaned daily and changed weekly. Hibernated frogs were kept in
483 plastic tanks under the same conditions (exception of food being withheld) as control
484 frogs for > 1 week before temperature was gradually lowered to 4°C over 10 days in a
485 walk-in temperature-controlled environmental chamber. Once water temperature
486 reached 4°C, air access was blocked using a plastic screen placed in the tank. After 4
487 weeks of submergence, experiments commenced.

488

Drugs

490 Strychnine hydrochloride was from Sigma-Aldrich (St Louis, MO, USA). (-)-Bicuculline
491 methiodide, tetrodotoxin citrate, DNQX disodium salt, and Muscimol were from Hello Bio
492 (Princeton, NJ, USA).

493

Brainstem-spinal cord preparation

495 Brainstem-spinal cord preparations were generated as previously described [16]. Briefly,
496 frogs were deeply anesthetized with isoflurane until visible respirations had ceased and
497 response to foot pinch was absent. Frogs were then decapitated with a guillotine. The
498 head was submerged in ice-cold bullfrog artificial cerebrospinal fluid (aCSF;
499 concentrations in [mM]: 104 NaCl, 4 KCl, 1.4 $MgCl_2$, 7.5 glucose, 40 $NaHCO_3$, 2.5
500 $CaCl_2$ and 1 NaH_2PO_4, and bubbled with 98.5% O_2, 1.5% CO_2; pH = 7.85) and the
501 forebrain was pithed. The brainstem-spinal cord was then carefully removed, keeping
502 nerve roots intact. Following the dissection, the brainstem was transferred to a chamber
503 superfused with oxygenated aCSF. In a subset of experiments where bicuculline was
504 bath applied to the *in vitro* preparation (described below), brainstems were transected at
505 the spinomedullary junction before recording to avoid the contribution of spinal motor
506 populations to non-respiratory motor activity induced through disinhibition [51]. Cranial
507 nerve X (vagus) activity was recorded using glass suction electrodes immediately
508 following dissection. Recordings were AC amplified (1000x, A–M Systems Model 1700,
509 A–M Systems, Carlsborg, WA, USA), filtered (10 Hz – 5 kHz), and digitized (Powerlab
510 8/35 ADInstruments, Colorado Springs, CO, USA). Nerve activity was rectified and

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511   integrated (time constant =100 ms) online to allow for visualization of nerve output
512   pattern.
513
514   Vagus nerve output was then allowed to stabilize for ≥ 1 hour before drug application. In
515   a subset of experiments (n=10 bullfrogs, 5 per group), various does of bicuculline (500
516   nM, 1 µM, 5 µM, 10 µM), a GABA$_A$ receptor antagonist, were superperfused for 10
517   minutes in order of increasing concentration and then washed out. In a different subset
518   of experiments (n=10 bullfrogs, 5 per group), various doses (500 nM, 1 µM, 3 µM) of
519   muscimol, a GABA$_A$ receptor agonist, were added to the perfusate. Each dose was
520   superperfused for 10 minutes in order of increasing concentration. Bicuculline (10 µM)
521   was added to the perfusate following muscimol to reverse muscimol's effects serving as
522   a control that output depression was not due to some kind of drift over time, but rather
523   activation of GABA$_A$ receptors.
524
525   For temperature experiments, preparations were allowed to recover for ~1 hr at 22°C
526   before exposure to the temperature ramp from 20° to 8°C. Preparations from controls
527   (n=5) and hibernators (n=6) were cooled to each temperature for 10 minutes and then
528   the temperature was lowered by 2°C. Following cooling, preparations were rewarmed
529   on a continuous ramp that lasted approximately 5 min. Separate control preparations
530   (n=5) were treated with 2 µM bicuculine for 30 minutes prior to starting the cooling
531   ramp. 2 µM was selected as it was the highest dose where respiratory-related bursts
532   were prominent in controls. The temperature was controlled by a Warner Instruments
533   bipolar in-line temperature controller (model CL-100; Hamden, CT), and chamber
534   temperature was monitored directly next to the brainstem.
535

536   **Dye labeling of vagal motor neurons**
537   Brainstems were isolated as described above. In a different group of experiments,
538   following isolation, brainstems were transferred to a chamber superfused with
539   oxygenated aCSF. The 4th branch of the vagal nerve root, which primarily innervates the
540   glottal dilator [52] was then isolated and suctioned into a glass pipette (bilaterally).
541   Fluorescent dextran dye dissolved in PBS (tetramethylrhodamine 3000 MW lysine
542   fixable dye; Invitrogen – Thermo Fisher, Waltham, MA, USA) was then backfilled into
543   both pipettes and left to diffuse into the nerve root for 2 hours. This generated robust
544   labeling of vagal motor neurons as previously described [16].
545

546   **Semi-intact brainstem-spinal cord preparation**
547   Following dye labeling, in a subset of experiments (n=15 bullfrogs) the brainstem was
548   embedded into agarose and sliced, similar to previously described [33] with some
549   modifications. Briefly, partially cooled, but not solidified, agarose was pipetted into a thin
550   layer onto a scored (with fine forceps) agar block. The brainstem was then dragged onto
551   the agarose-covered agar block, laying horizontally dorsal side up. Agarose (~57° C)
552   was pipetted onto the top of the brainstem caudal to obex. The agar block was then
553   mounted on a vibrating microtome plate (Campden Vibrating Microtome 7000smz,
554   Campden Instruments; Lafayette, IN, USA) and covered in ice-cold bubbled aCSF. The
555   blade was zeroed at the top of the brainstem, then slices were taken caudal to rostral,
556   stopping shortly rostral to obex which was approximately 1/3 of the distance from the

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hypoglossal root to the vagal root and spanned a fraction of the vagal motor pool extent. The blade was removed after each slice and the resulting attached slice was trimmed off the brainstem with fine spring scissors. Approximately 650 μm were removed from the dorsal surface in total, providing access to labeled motor neurons for recording. The brainstem was removed from the remaining agar and transferred to a 35 mm glass bottom sylgard-coated dish with a stainless-steel mesh insert. The brainstem was then pinned dorsal side up. The dish was placed in a QE-1 platform (Warner Instruments, Holliston, MA, USA) that was mounted on a fixed stage microscope (FN1, Nikon Instruments Inc., Melville, NY, USA). Once on the rig, the preparation was superperfused with bubbled aCSF either using gravity or with a peristaltic pump (Rainin Rabbit). The trigeminal nerve (CN V) was recorded using a glass suction electrode to monitor preparation output over time. Nerve recordings were AC amplified (1000x, A–M Systems Model 1700, A–M Systems, Carlsborg, WA, USA), filtered (10 Hz – 5 kHz), and digitized (Powerlab 8/35 ADInstruments, Colorado Springs, CO, USA). The trigeminal nerve was recorded instead of the vagal nerve root because of physical constraints in the bath with the patch pipette and picospritzer pipette used in experiments described below. The trigeminal provides a sufficient surrogate for efferent respiratory motor output [29, 53], as respiratory activity of the trigeminal and vagus nerves activate near-synchronously *in vitro*. Labeled motor neurons were then located and visualized using an imaging camera (Hamamatsu ORCA Flash 4.0LT sCMOS, Hamamatsu Photonics, Hamamatsu City, Japan) coupled to Nikon imaging software (NIS elements).

Whole-cell recordings were made from labeled vagal motor neurons with an Axopatch 200B amplifier (Molecular Devices) in current-clamp mode (I=0). Cells were selected based on firing pattern, that is only cells that fired spontaneous bursts of action potentials or increased firing frequency (respiratory modulated) during respiratory motor bursts were sampled, as these neurons likely innervate the glottal dilator of bullfrogs [54], which is critical to permit airflow into the lungs of bullfrogs during ventilation and as such remains inactive during hibernation. Accordingly, neurons with subthreshold respiratory input or neurons that were active only during the interburst interval were excluded from testing. Glass pipettes (~4-7 MΩ) were filled with a solution containing (in mmol): 110 potassium gluconate, 2 $MgCl_2$, 10 Hepes, 1 Na_2-ATP, 0.1 Na_2-GTP and 2.5 EGTA, pH ~ 7.2 with KOH. Data were acquired in pClamp 11 software using an Axopatch 200B amplifier and Axon Digidata 1550B digitizer (Molecular Devices, San Jose, CA, USA). To determine inhibitory tone onto respiratory motor neurons, bicuculline (50 μM) was focally applied using a Picospritzer II (General Valve Corporation, Fairfield, NJ, USA) to the recorded cell following a baseline period (≥10 respiratory bursts). Bicuculline was applied using a 5 sec pulse, every 10 secs for 5-10 minutes with the picospritzer pipette slightly larger (~2 μm diameter) than typical for focal application and placed approximately 15 μm away from the cell body. This approach was taken to saturate the cell and surrounding area with the $GABA_AR$ antagonist. Importantly, this approach did not recapitulate systemic effects of bicuculline on the vagal motor pool as the population spans nearly 2.5mm in the rostrocaudal axis in ranid frogs [55] and only a subset of the vagal motor pool was exposed in the preparation as described above. A washout period was used to verify changes in cell firing frequency were due to drug application and not cell drift. Most patched cells were

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603 near the surface of the tissue as the thickness of the preparation hindered cell
604 visualization. As a result, changes in firing frequency during bicuculline application and
605 wash were rapid, suggesting the area was saturated with drug upon administration and
606 then quickly washed. In a subset of cells where a gigaohm seal was not formed,
607 recordings of actional potentials were performed in the loose-patch configuration
608 (V_{hold}=0 mV; n=2 in control, and n=2 in hibernation). Regardless of recording
609 configuration, firing rate data from recorded neurons were combined in group analysis
610 of firing frequency, as data from loose-patch aligned with data from whole cell
611 configuration. All voltages from current-clamp experiments were corrected for a liquid
612 junction potential of 12 mV.
613
614 **Brain Slice electrophysiology**
615 Following dye labeling, in a subset of experiments brainstem slices (300 μm) containing
616 labeled motor neurons were obtained (n=11 bullfrogs). Whole-cell recordings were
617 made from labeled motor neurons with an Axopatch 200B amplifier (Molecular Devices)
618 in voltage clamp (V_{hold}=-60 mV) mode. Glass pipettes (2.6-4 MΩ) were filled with a
619 solution containing (in mM): 95 CsCl, 2 $MgCl_2$, 10 Hepes, 1 Na_2-ATP, 0.1 Na_2-GTP, 10
620 EGTA, 1 $CaCl_2$ and 10 tetraethylammonium-Cl (TEA), pH ~ 7.2 using CsOH. Data were
621 acquired in pClamp 11 software using an Axopatch 200B amplifier and Axon Digidata
622 1550B digitizer (Molecular Devices, San Jose, CA, USA). To examine miniature
623 inhibitory postsynaptic currents (mIPSCs), tetrodotoxin (TTX; 250 nM) and DNQX (10
624 μM) were bath applied for 3 min to block voltage-gated Na^+ channels and AMPA
625 receptors. Strychnine (5 μM) was then bath applied in combination with TTX and DNQX
626 for 2 min to block glycinergic mIPSCs and isolate GABAergic mIPSCs which were
627 recorded for 1 min. All GABAergic mIPSCs were blocked following perfusion of
628 bicuculline (50 μM) confirming their isolation. A 10 mV step was performed at the
629 beginning of each recording and before each solution change to monitor series
630 resistance (R_s; estimated from the peak of the capacitive transient) and input resistance
631 (R_{IN}; estimated from the steady-state current). R_s was not compensated but remained
632 <25 MΩ for inclusion.
633
634 **Data Analysis**
635 For experiments using the intact brainstem preparation, respiratory burst frequency was
636 determined from integrated vagus nerve signals using the peak analysis function in
637 LabChart 8 (ADInstruments Inc., Colorado Springs, CO, USA). Respiratory bursts were
638 identified based on standard metrics used in the field such as ~1 s duration [56, 57].
639 Burst start and stop time points were defined as 5% of the height from baseline. In
640 experiments where muscimol was systemically applied, data were sampled from the last
641 3 minutes of each condition (baseline, 500 nM, 1 μM, and 3 μM). In experiments where
642 bicuculline was systemically applied, data were sampled for 10 minutes during each
643 condition (baseline, 500 nM, 1 μM, 5 μM, 10 μM). In systemic bicuculline experiments
644 where non-respiratory bursting was quantified, non-respiratory were identified by their
645 long duration and qualitatively different shape than respiratory bursts and were counted
646 manually. Non-respiratory bursts appeared in a variety of output patterns as previously
647 characterized *in vitro* (Reid and Milsom, 1998). Non-respiratory bursts were not
648 quantified during systemic muscimol exposure as they were largely absent from

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649 baseline recordings and only emerged with notable frequency following disinhibition with
650 bicuculline. For temperature experiments, burst frequency was averaged in the last 5
651 minutes at each temperature, and burst amplitudes were sampled in the last minute or
652 in the case of the failure temperature, bursts were sampled directly before failure. To
653 compare respiratory burst amplitudes in the cold, we analyzed the change from baseline
654 at the coldest temperature the preparation could produce activity. Across control and
655 hibernation groups this typically occurred at 8-10°C.

657 For experiments using the semi-intact preparation, action potentials were detected with
658 the peak analysis function in LabChart 8 (ADInstruments Inc., Colorado Springs, CO,
659 USA). Following detection of action potentials, a channel was created that plotted firing
660 frequency over time. Average firing frequency per respiratory burst was determined via
661 the mean firing frequency from neuronal burst start to stop. Average firing frequency
662 was sampled and averaged from 10 respiratory bursts in each condition (baseline,
663 bicuculline, and wash).

665 For voltage-clamp experiments in brainstem slices, average amplitude (current
666 measurement from baseline to peak), charge transfer (integral of the mIPSC), rise time
667 (time from baseline to peak), and frequency of GABAergic mIPSCs (mIPSC/sec) were
668 analyzed from one minute of gap-free recording following two minutes of exposure to
669 TTX/DNQX/Strychnine (as described above) using the peak analysis function in
670 LabChart 8 (ADInstruments Inc., Colorado Springs, CO, USA). Events below 7.5 pA
671 were excluded. Events were inspected manually to ensure the accurate detection of
672 mIPSCs. For construction of the cumulative probability histogram, the first 50 mIPSCs
673 from the minute of data obtained from each neuron were sampled.

675 **Statistics**
676 Data are presented as mean ± s.d. unless otherwise stated or shown as individual
677 points and means to highlight individual responses. When two groups of dependent
678 samples were compared ("before-after" experiments), a two-tailed paired t-test was
679 used. When two groups of independent samples were compared, a two-tailed unpaired
680 t-test was used. Non-parametric versions of these tests were used if data sets were not
681 normally distributed. In experiments with one main effect, a one-way ANOVA was used
682 to test for the main effect and the interaction effect. One-way ANOVA was followed up
683 with Holm-Sidak multiple comparisons test. In experiments with two main effects, a two-
684 way ANOVA was used to test for the two main effects and the interaction effect. Two-
685 way ANOVA was followed up with Holm-Sidak multiple comparisons test unless
686 otherwise specified. Cumulative distributions were compared with the Kolmogorov-
687 Smirnov test. Significance was accepted when $P < 0.05$. All analyses were performed
688 using GraphPad Prism (v9.4.1, San Diego, CA, USA).

691 **Declarations**

693 Ethics approval and consent to participate: Animal experiments were approved by
694 University of Missouri Animal Care and Use Committee protocol #39264

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Figures Legends:

Fig. 1 GABAergic tone that controls respiratory burst frequency strongly decreases following hibernation. Integrated vagus nerve activity (CN X) was rhythmic in fully intact brainstem preparations (top right box) from control and hibernated animals. A) Top, continuous recording of CNX during perfusion of increasing doses of bicuculline in a control preparation (black). Bottom, zoomed in view of epochs (orange boxes) indicated in continuous trace directly above. In control preparations, bath application of bicuculline led to a dose dependent decrease in respiratory burst frequency and dose-dependent increase in non-respiratory burst frequency. B) Top, continuous recording of CNX during perfusion of increasing doses of bicuculline in a preparation from a hibernated animal (blue). Bottom, zoomed in view of epochs (orange boxes) indicated in continuous trace above. Respiratory output from hibernated preparations was insensitive to systemic application of bicuculline, however non-respiratory motor bursting increased in a dose-dependent manner like controls. C) Summary of bicuculline-mediated changes in respiratory burst frequency. There was a significant interaction between drug and group by two-way ANOVA ($F_{(4,32)}$=6.741; p=0.0005). Additionally, at 10 µM bicuculline, respiratory burst frequency from controls was significantly lower than hibernated preparations by Holm-Sidak's post-hoc test (p=0.0111; n=5/group). D) Summary of bicuculline-mediated changes in non-respiratory burst frequency. Bicuculline mediated similar increases in non-respiratory bursting in control and hibernated groups. There was no significant interaction between drug and group by two-way ANOVA ($F_{(4,32)}$=0.7948; p=0.5373; n=5/group). a.u., arbitrary units. In summary plots, thick lines represent the group average and thin lines are responses of individual preparations.

Fig. 2 Hibernation decreases sensitivity to GABA$_A$R agonist (muscimol)-mediated respiratory frequency decline. Integrated vagus nerve activity (CN X) was rhythmic in fully intact brainstem preparations (top left box) from control and hibernated animals. A) Continuous recording of CNX during perfusion of increasing doses of muscimol in a control preparation (black). In control preparations, respiratory output was unchanged by bath application of 500nM muscimol but decreased following elevation to 1µM muscimol and ultimately stopped following further elevation to 3µM muscimol. Preparations were recovered by exposure to bicuculine, demonstrating specificity of the GABA$_A$ receptor agonist. B) Continuous recording of CNX during perfusion of increasing doses of muscimol in a preparation from a hibernated animal (blue). Preparations from hibernated animals were relatively insensitive to muscimol mediated changes in respiratory burst frequency compared to controls. C) Summary of muscimol-mediated changes in respiratory burst frequency. There was a significant interaction between drug and group by two-way ANOVA($F_{(3,24)}$=4.045; p=0.0184). Additionally, at 1 µM muscimol, respiratory burst frequency from controls was significantly lower than hibernated preparations by Holm-Šidák's post-hoc test (p=0.0007, n=5/group). D) Summary of muscimol-mediated changes in respiratory burst frequency relative to baseline. There was a significant interaction between drug and group by two-way ANOVA($F_{(3,24)}$=7.184; p=0.0013). Additionally, at 1 µM muscimol, respiratory burst frequency from controls

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945 was significantly lower than hibernated preparations relative to baseline by Holm-
946 Sidak's post-hoc test (p=0.0030, n=5 per group). In summary plots, thick lines represent
947 the group average and thin lines are individual preparations.

948 **Fig. 3 Postsynaptic GABAergic currents in motoneurons are unchanged following**
949 **hibernation.** A) Example voltage clamp traces of GABAergic miniature inhibitory
950 postsynaptic currents (mIPSCs; holding potential = -60 mV; 250 nM TTX, 10 μM DNQX,
951 5 μM strychnine) from control (black) and hibernated (blue) identified vagal motor
952 neurons in brain slice (middle right box). Addition of 50 μM bicuculline (Bic) to the bath
953 eliminated mIPSCs in both groups, confirming GABAergic identity. B) Cumulative
954 probability histograms of mIPSC amplitudes from control neurons (black line),
955 hibernated neurons (blue line) appeared nearly identical. Accordingly, there was no
956 statistical difference between distributions from control and hibernated cells by
957 Kolomogorov-Smirnov test (p = 0.5041). C) Summary data. There was no statistical
958 difference in mIPSC frequency, average amplitude, rise time, charge transfer, and
959 neuronal input resistance by unpaired t-test (p > 0.05; n= 16 control cells; n=16
960 hibernated cells). Error bars are standard deviation (SD).

961

962 **Fig. 4 Hibernation decreases the influence of presynaptic GABA on motoneuron**
963 **firing.** A) Integrated network output recorded on the trigeminal nerve (CN V) and
964 simultaneous whole-cell current clamp recordings of vagal motor neurons (CN X MN)
965 driven by the respiratory circuit in the semi-intact brainstem preparation (top left box). A
966 pipette was used to focally apply bicuculline onto the recorded vagal motor neuron to
967 isolate the GABAergic tone on individual neurons. An example respiratory neuron (CN X
968 MN, gray, top right) fired rhythmic bursts, characterized by transient increases in firing
969 frequency (Firing Freq, light gray, middle top right) during respiratory motor output
970 (Black, bottom top right, CN V). B) Example respiratory motor activity in a control
971 preparation (black). Example vagal motor neuron (CN X MN) fires bursts of action
972 potentials during the respiratory burst (CN V). Focal application of bicuculline (gray box)
973 to the motor neuron increases neuronal burst firing frequency (Firing Freq). C) Example
974 respiratory motor activity in a preparation from a hibernated animal (blue). Example
975 vagal motor neuron (CN X MN) also fired bursts of action potentials during the
976 respiratory burst (CN V). Focal application of bicuculline (gray box) to the motor neuron
977 increased neuronal burst firing frequency (Firing Freq, Δ~47Hz), but not to the same
978 extent as the control neuron (Firing Freq, Δ~74Hz). D) Summary of bicuculline-mediated
979 increases in motor neuron firing frequency. There was a significant interaction between
980 drug and group by two-way ANOVA ($F_{(2,38)}$=6.391; p=0.0041). Furthermore, there was a
981 significant increase in burst firing frequency following focal application of bicuculline in
982 control but not hibernated cells by Holm-Sidak's post-hoc test (***p<0.0001; n= 12
983 control cells; n=9 hibernated cells). E) Summary of bicuculline-mediated fold change in
984 burst firing frequency from baseline. Control cells had a significantly larger bicuculline-
985 mediated fold change in burst firing frequency than hibernated cells by unpaired t-test
986 (p=0.036; n= 12 control cells; n=9 hibernated cells). Orange cells were recorded in

24

987 loose-patch configuration. Box plots represent interquartile range and whiskers
988 represent the minimum to maximum values. a.u., arbitrary units. Error bars are
989 standard deviation (SD).

990 **Fig. 5 Respiratory burst morphology after hibernation closely matches controls at**
991 **room temperature.** Integrated vagus motor bursts (CN X) in preparations from 5
992 controls (red) and 5 hibernators (blue). Overlayed bursts (n=50) from individual
993 preparations are represented in different shades from top to bottom. Overlaying all
994 bursts from all animals from both groups demonstrates a close matching between
995 controls and hibernators, indicating the loss of GABA$_A$ receptors has no obvious impact
996 on motor burst morphology after hibernation.

997

998 **Fig. 6 Lowering GABA$_A$R signaling enhances motor performance of breathing at**
999 **cold temperatures.** Integrated vagus nerve activity (CN X) from intact brainstem
1000 preparations (bottom right box) showing the response of control (A, black), hibernators
1001 (B, blue), and controls treated with 2 µM bicuculline (C, gray) during cooling ramps from
1002 20°C to 8°C. Zoomed-in traces below the compressed recordings illustrate the effects of
1003 cooling on burst amplitude. Arrows indicate the temperature at 50% of baseline burst
1004 frequency. (D) Mean data showing average temperature responses of normalized burst
1005 frequency among groups (two-way ANOVA interaction of group x temperature,
1006 p<0.0001). (E) Mean data showing a significant difference in the temperature at 50% of
1007 baseline burst rate between control, hibernators, and controls+bicuculline (one-way
1008 ANOVA; p<0.0001) (F) Mean data showing change in burst amplitude from baseline in
1009 control and hibernators during temperature changes. Controls undergo a decrease in
1010 burst amplitude during cooling, while hibernators and controls+bicuculline maintain burst
1011 amplitude throughout the cooling ramp (two-way ANOVA, p=0.0001 group x
1012 temperature interaction); * represents p<0.05, **<0.01, ***<0.001 in Holm-Sidak multiple
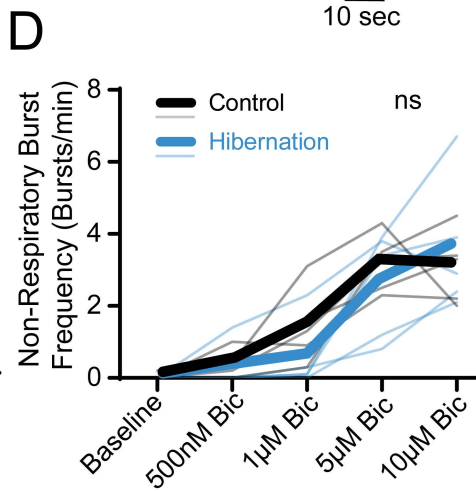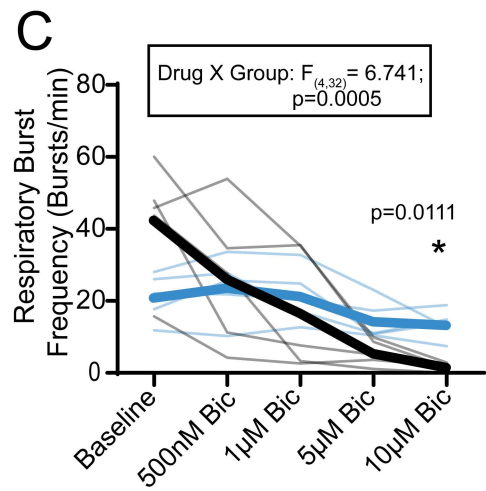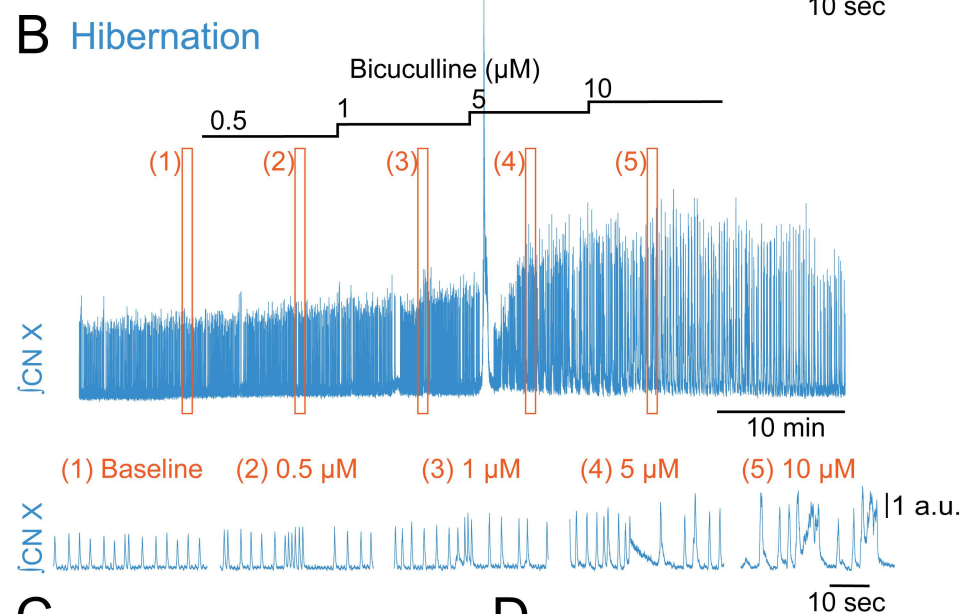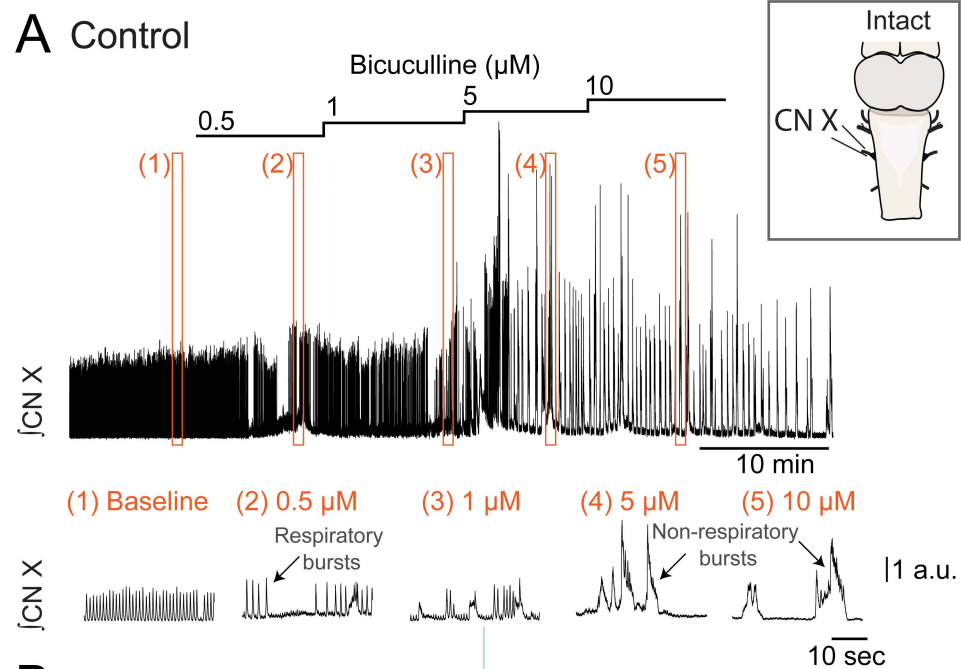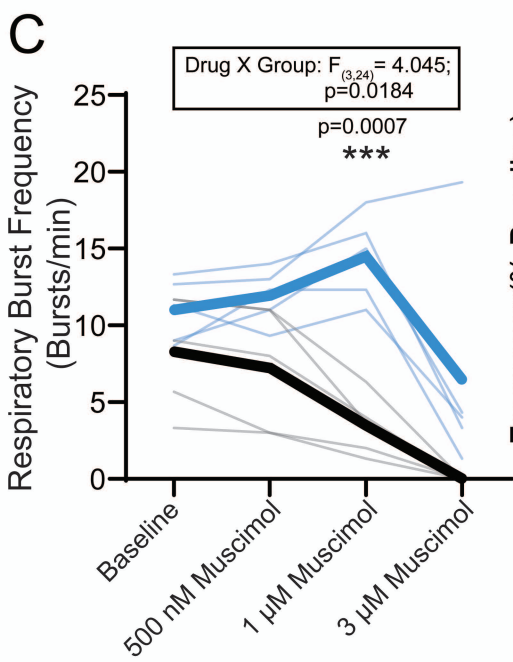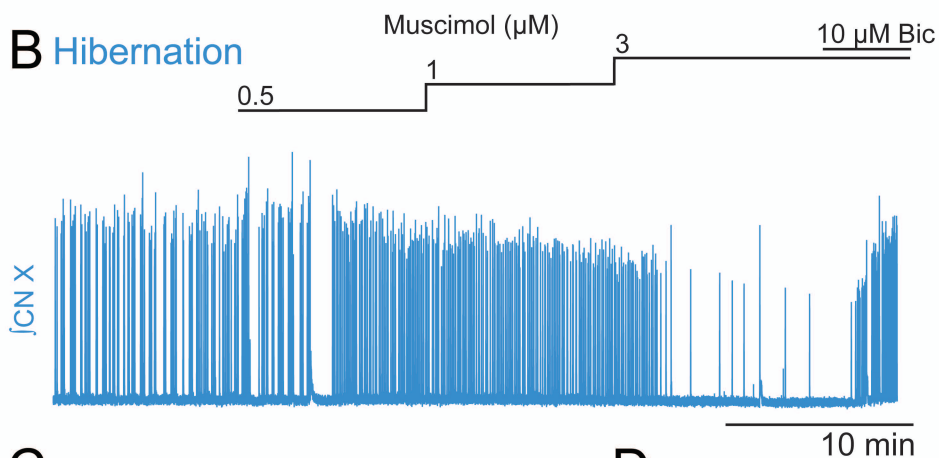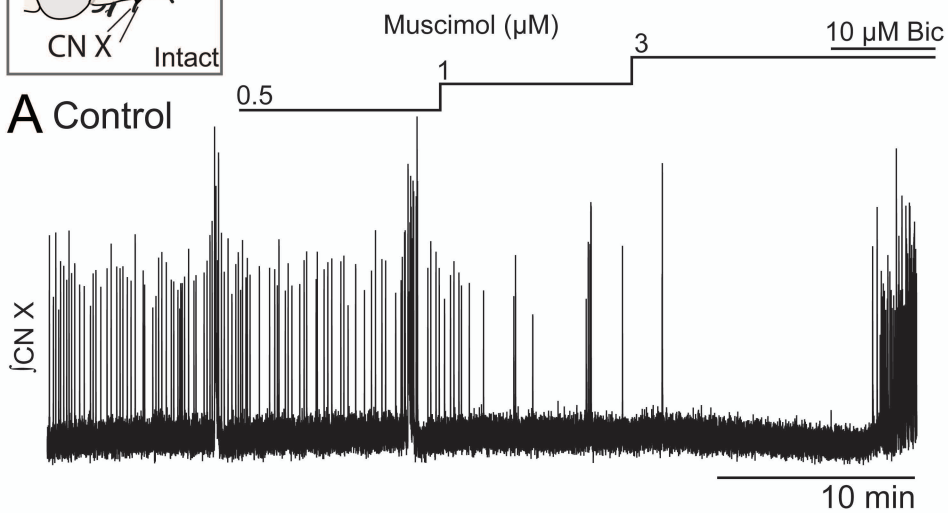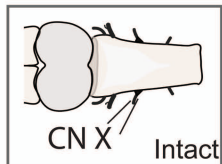1013 comparisons test following one or two-way ANOVAs.

1014

1015 **Fig. 7 Schematic illustrating how reducing inhibition may promote breathing in**
1016 **the cold.** In controls at warm temperatures (orange left), inhibition (red, minus symbol)
1017 plays an important role in generating the respiratory rhythm (circular arrows) and
1018 controlling the excitability to motoneurons. Following hibernation (orange right), the
1019 respiratory network decreases reliance on inhibition and potentially increases reliance
1020 on mechanisms that involve excitation (dark blue, plus symbol) for respiratory
1021 rhythmogenesis (circular arrows). Nonetheless, there is no discernable change in
1022 network motor output at warm temperatures following hibernation compared to controls.
1023 During acute cooling, inhibitory neurotransmission also plays a modulatory role and is
1024 increased. As a result, controls (blue left) have marked reduction in respiratory
1025 frequency and motor output at colder temperatures due to elevated GABAergic
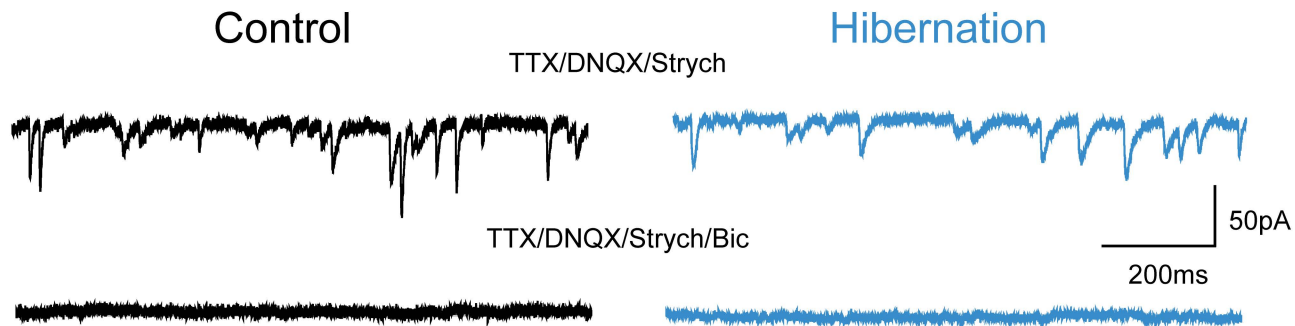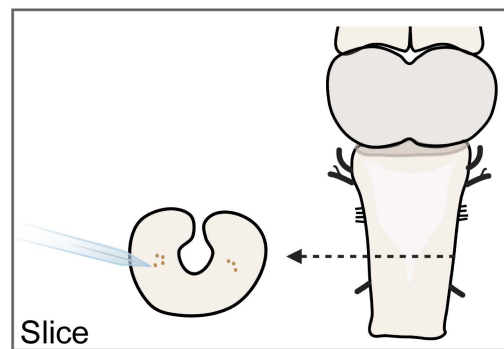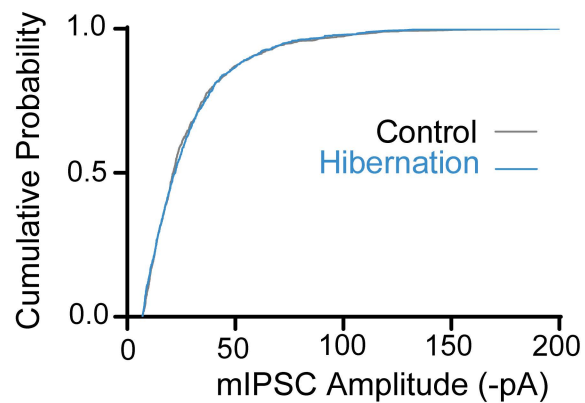1026 inhibition. In contrast, output following hibernation is resistant to depressive effects of
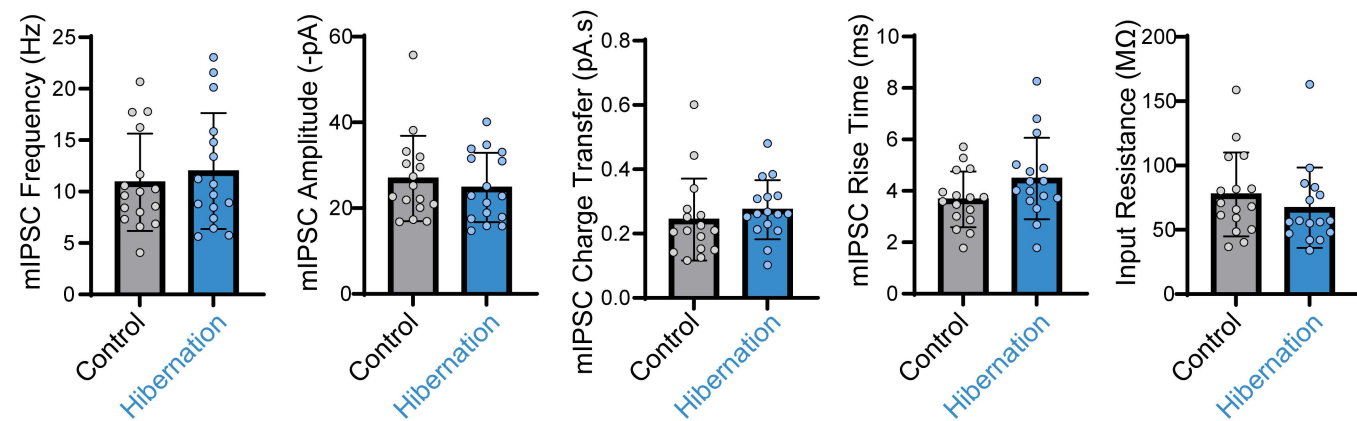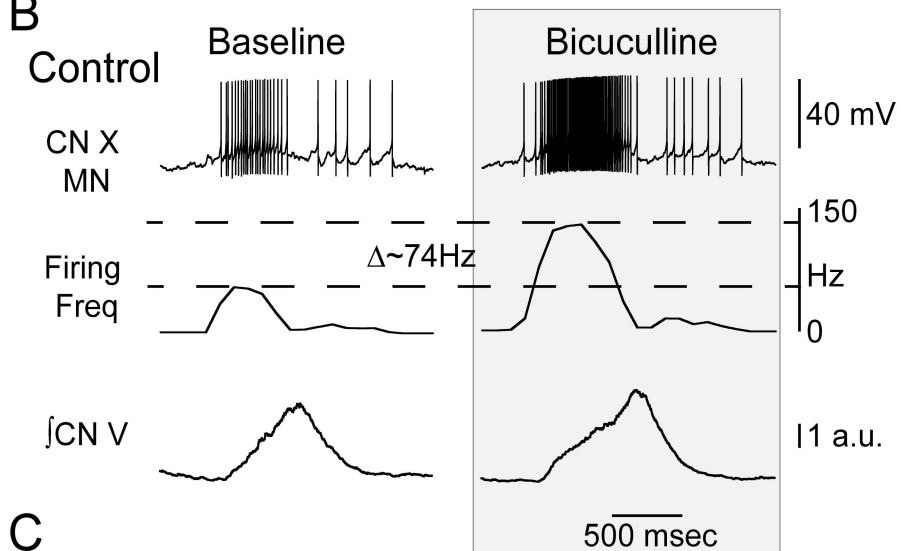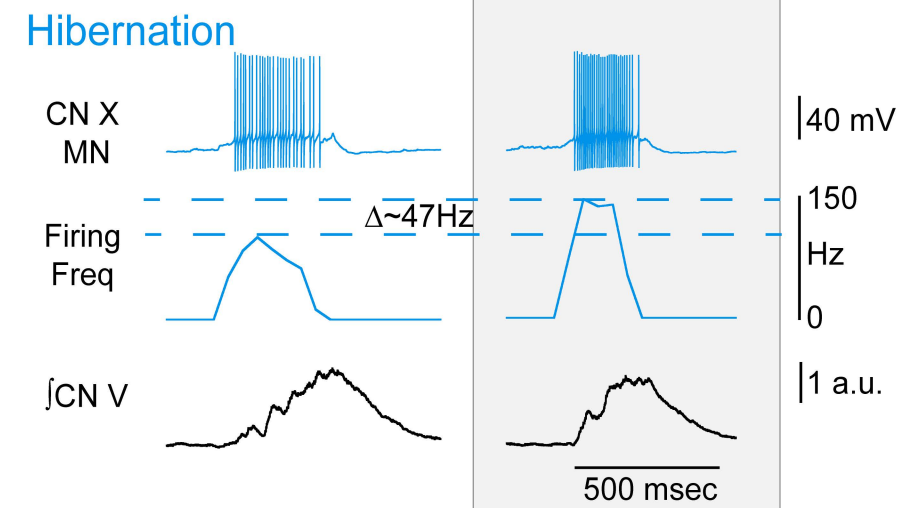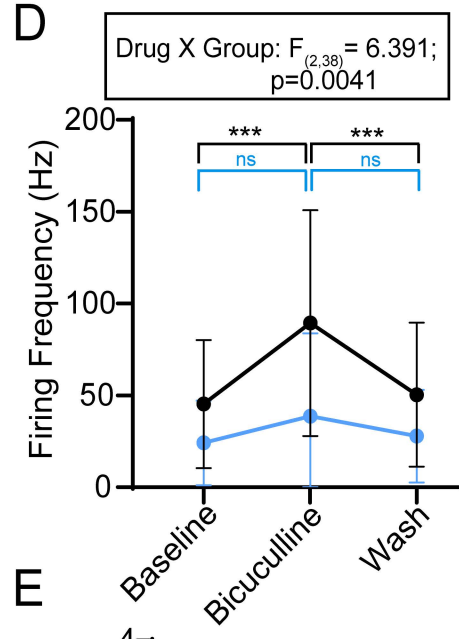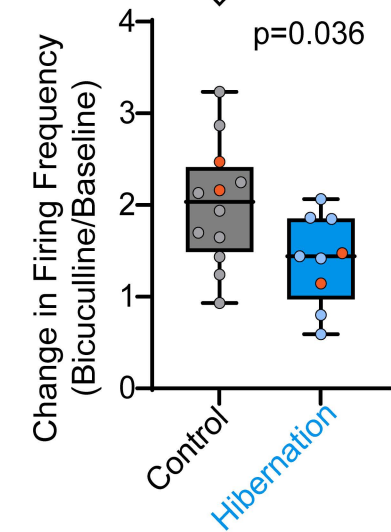
25

1027   the cold, likely with a contribution resulting from the decreased reliance on inhibition that
1028   normally slows activity and depresses motor output. Thus, output following hibernation
1029   is more robust at cooler temperatures, in part, because inhibitory neurotransmission is
1030   decreased in rhythm-generating and interneuronal pathways.

A Control

Bicuculline (µM)

0.5   1   5   10

(1) (2) (3) (4) (5)

∫CN X

10 min

(1) Baseline   (2) 0.5 µM   (3) 1 µM   (4) 5 µM   (5) 10 µM

∫CN X

Respiratory bursts

Non-respiratory bursts

|1 a.u.

10 sec

Intact

CN X

B Hibernation

Bicuculline (µM)

0.5   1   5   10

(1) (2) (3) (4) (5)

∫CN X

10 min

(1) Baseline   (2) 0.5 µM   (3) 1 µM   (4) 5 µM   (5) 10 µM

∫CN X

|1 a.u.

10 sec

C

Respiratory Burst Frequency (Bursts/min)

Drug X Group: $F_{(4,32)}$ = 6.741; p=0.0005

p=0.0111

*

Baseline   500nM Bic   1µM Bic   5µM Bic   10µM Bic

D

Non-Respiratory Burst Frequency (Bursts/min)

ns

— Control
— Hibernation

Baseline   500nM Bic   1µM Bic   5µM Bic   10µM Bic

A Control

B Hibernation

C Drug X Group: $F_{(3,24)} = 4.045$; p=0.0184
p=0.0007
***

D Drug X Group: $F_{(3,24)} = 7.184$; p=0.0013
p=0.0030
*

A Control   Hibernation

TTX/DNQX/Strych

TTX/DNQX/Strych/Bic

50pA

200ms

B

Cumulative Probability

Control
Hibernation

mIPSC Amplitude (-pA)

Slice

C

mIPSC Frequency (Hz)

mIPSC Amplitude (-pA)

mIPSC Charge Transfer (pA.s)

mIPSC Rise Time (ms)

Input Resistance (MΩ)

Control   Hibernation

A

CN X MN

50mV

Firing Freq — 100 Hz / 0

∫CN V — 1 a.u.

10 sec

20mV / 100ms

Inset: CN V, CN X MN, Picospritzer, Semi-Intact

● Control
● Hibernation

B Control

Baseline — Bicuculline

CN X MN — 40 mV

Firing Freq — 150 Hz / 0 — Δ~74Hz

∫CN V — 1 a.u.

500 msec

C Hibernation

Baseline — Bicuculline

CN X MN — 40 mV

Firing Freq — 150 Hz / 0 — Δ~47Hz

∫CN V — 1 a.u.

500 msec

D

Drug X Group: $F_{(2,38)} = 6.391$; p=0.0041

*** ns

Firing Frequency (Hz)

Baseline — Bicuculline — Wash

E

p=0.036

Change in Firing Frequency (Bicuculline/Baseline)

Control — Hibernation

Control

Hibernation

-1s　0　+1s

Control Overlayed

+

-1s　0　+1s

Hibernation Overlayed

-1s　0　+1s

Control + Hibernation
Overlayed

A Control **B** Hibernation **C** Control Bic-Treated

Temp (°C)

Burst Frequency (Bursts/min)

∫CN X

10 min

20°C 8°C

20°C 8°C

20°C 8°C

0.5 sec

D

temp: p<0.0001
group: 0.0047
temp x group: p<0.0001

Burst Frequency (Relative to 20°C)

Temperature (°C)

E

Temp at 50% of baseline (°C)

Control   Hibernation   Control+Bic

F

temp: p=0.0002
group: 0.0070
temp x group: p=0.0001

Burst Amplitude (Relative to 20°C)

Temperature (°C)

● Control (n=5)
● Hibernation (n=6)
● Control Bic-Treated (n=5)

Intact

CN X