Hibernation induces pentobarbital insensitivity in medulla but not cortex

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Hibernation induces pentobarbital insensitivity in medulla but not cortex. Am J Physiol Regul Integr Comp Physiol 297: R1028–R1036, 2009. First published August 12, 2009; doi:10.1152/ajpregu.00239.2009.—The 13-lined ground squirrel (Ictidomys tridecemlineatus), a hibernating species, is a natural model of physiological adaption to an extreme environment. During torpor, body temperature drops to 0–4°C, and the cortex is electrically silent, yet the brain stem continues to regulate cardiorespiratory function. The mechanisms underlying selective inhibition in the brain during torpor are not known. To test whether altered GABAergic function is involved in regional and seasonal differences in neuronal activity, cortical and medullary slices from summer-active (SA) and interbout aroused (IBA) squirrels were placed in a standard in vitro recording chamber. Silicon multichannel electrodes were placed in cortex, ventral respiratory column (VRC), and nucleus tractus solitarius (NTS) to record spontaneous neuronal activity. In slices from IBA squirrels, bath-applied pentobarbital sodium (300 μM) nearly abolished cortical neuronal activity, but VRC and NTS neuronal activity was unaltered. In contrast, pentobarbital sodium (300 μM) nearly abolished all spontaneous cortical, VRC, and NTS neuronal activity in slices from SA squirrels. Muscimol (20 μM; GABA_A receptor agonist) abolished all neuronal activity in cortical and medullary slices from both IBA and SA squirrels, thereby demonstrating the presence of functional GABA_A receptors. Pretreatment of cortical slices from IBA squirrels with bicuculline (100 μM; GABA_A receptor antagonist) blocked pentobarbital-dependendent inhibition of spontaneous neuronal activity. We hypothesize that GABA_A receptors undergo a seasonal modification in subunit composition, such that cardiorespiratory neurons are uniquely unaffected by surges of an endogenous positive allosteric modulator.

γ-aminobutyric acid receptors; ventral respiratory groups; nucleus tractus solitarius; respiratory control

DURING WINTER HIBERNATING, mammals enter a state of torpor, defined by dramatically reduced metabolic and physical activity and low body temperature (Tb). The torpid state is interrupted periodically by interbout arousals to euthermia (Tb = 37°C) that generally last less than 24 h (3, 8). During torpor bouts, Tb drops to just above ambient temperature and can approach 0°C, and respiration and heart rate drop as low as 1% of euthemic values (39, 71). Neurons in the cortex, hippocampus, and thalamus are electrically silent in torpid hibernators (14, 67). Despite evidence for global forebrain depression, torpid hibernators maintain a robust, neurally regulated cardiorespiratory output (37, 25, 41), suggesting that respiratory- and cardiovascular-related neurons in the brain stem remain active. The mechanisms that selectively depress the forebrain during hibernation are not well understood and may contribute to hibernators’ unique ability to survive ischemia, hypovolemia, and hypothermia (5, 12, 31, 34, 66).

While euthanizing aroused hibernators for an unrelated study, we noted that pentobarbital sodium, an allosteric modulator of gamma aminobutyric acid type A (GABA_A) receptors, when used at doses sufficient for euthanasia, rapidly immobilized animals yet had no observable effect on respiratory rhythm. Subsequently, we began to investigate the role of GABA and GABAergic synaptic transmission in respiratory control during hibernation. GABA is the primary inhibitory neurotransmitter in the brain, so increased GABA receptor activation is a logical candidate for forebrain neuronal depression during torpor. Activation of synaptic GABA_A receptors produces acute, phasic inhibition, while extrasynaptic GABA_A receptors modulate general network excitability via tonic inhibition through constant activation by ambient GABA (14, 43). Although GABA_A receptors involved in phasic and tonic inhibition are activated by GABA at micromolar concentrations, tonic GABA_A receptors may be particularly sensitive to both GABA and allosteric modulators of the receptor, such as barbiturates, benzodiazepines, ethanol, and neurosteroids (9, 43, 64). Allosteric modulators increase or decrease the flow of chloride ions through GABA_A receptors via actions at sites distinct from the GABA binding site. Pentobarbital sodium, a positive allosteric modulator of GABA_A receptors often used for euthanasia, rapidly induces respiratory depression and sensorimotor loss in mammals at high doses (69), yet the expression of a rare subunit yields a GABA_A receptor insensitive to pentobarbital sodium (23). Allosteric modulation of GABA_A receptors provides the capacity for selective activation of GABA_A receptors in different brain regions. Accordingly, we hypothesized that the cortex of hibernating squirrels expresses GABA_A receptors that are sensitive to positive allosteric modulators of GABA_A receptors, such as pentobarbital, whereas respiratory- and cardiovascular-related neurons in the medulla increase expression of GABA_A receptors that are insensitive to allosteric positive modulators.

To address these hypotheses, multichannel recordings of spontaneous neuronal activity were performed in vitro in medullary and cortical brain slices from 13-lined ground squirrels (Ictidomys tridecemlineatus). In medullary slices, neuronal activity in the ventral respiratory column (VRC) and the nucleus tractus solitarius (NTS) was measured before and after bath application of pentobarbital. The VRC is an elongated rostrocaudal column of respiratory-related neurons that contains the Bo¨tzinger region, pre-Bo¨tzinger complex (preBo¨tC), and rostral and caudal ventral respiratory groups (rVRG; cVRG; 40). The NTS receives sensoryafferent inputs from chemoreceptors and baroreceptors, as well as lung mechanoceptors (2, 30). In our experiments, VRC neurons were sampled from a rostrocaudal area that contained all of these groups except the Bo¨tzinger region, and NTS neurons were...
sampled from medial, ventromedial, and ventral NTS (Fig. 1).
To test for regional differences in allosteric modulation of
GABA\(_{A}\) receptors, neuronal activity was also recorded in the
primary motor and somatosensory regions of cortical slices
(Fig. 1). To test for seasonal differences, recordings were
performed in slices isolated from hibernating and summer-
active (SA) ground squirrels. To rule out temperature-depend-
et effects, slices from hibernating squirrels were taken during
interbout arousals (IBA), when squirrel Tb is similar to that
of SA squirrels. Preliminary results were published in abstract
form (17–19).

METHODS

Experimental animals. All experimental procedures were in accord-
ance with the National Institutes of Health’s guidelines and approved
by the University of Wisconsin-Madison Institutional Animal Care
and Use Committee. Thirteen-lined ground squirrels (I. tridecen-
lineatus) were trapped in and around Madison, WI, between May
and September. Animals were housed individually with access to
food and water ad libitum. From May through September, animals
were maintained at an ambient temperature of 22°C with a 12:12-h
light-dark cycle. In September through February, animals were
housed in a dark room maintained at 4°C to facilitate hibernation.
From May through September, animals were maintained at an ambient temperature of 22°C with a 12:12-h
light-dark cycle. In September through February, animals were
housed in a dark room maintained at 4°C to facilitate hibernation.
Food and water were removed after ~2 wk of torpor/arousal
cycles. All hibernating animals completed at least four full torpor
bouts prior to being used in experiments. Torpor bouts were
monitored daily by the sawdust method (4). Experiments were
conducted during naturally occurring interbout arousals (n = 37
squirrels; November to February) or during the SA months (n = 15
animals; May to July). Tb of IBA hibernators was between 35°C
and 38°C and SA animals between 36°C and 38°C.

Experimental preparation. For electrophysiological recordings,
squirrels were deeply anesthetized with 5% isoflurane and decapi-
tated. Brains were removed and placed in cold artificial cerebrospinal
fluid (aCSF). Medullary and cortical slices (350 μm thick) were cut
with a vibrating microtome (Campden Instruments, Layfayette, IN).
Cortical slices contained primary motor and primary somatosensory
areas (Fig. 1A), while medullary slices contained the NTS and VRC
(Fig. 1B). Slices were placed into an interface recording chamber
(Warner Instruments, Hamden, CT) and subfused with warm aCSF at
a rate of 8 ml/min. Slices were maintained at 37°C (Harvard Appa-
ratus, Holliston, MA). Humidified gas (95% O\(_2\)-5% CO\(_2\)) was
blown across the top of the slices. The composition of the aCSF was
(in mM) 120 NaCl, 26 NaHCO\(_3\), 20 glucose, 2 MgSO\(_4\), 1.0 CaCl\(_2\),
and 1.25 Na\(_2\)HPO\(_4\). To increase the yield of spontaneously active
neurons, aCSF containing 9 mM KCl was used in most experiments.

Experimental protocol. Recordings were made simultaneously
from medullary and cortical slices taken from the same animal. One
silicon 16-channel extracellular electrode (model a 4–3 mm
100–177, Neuronexus, Ann Arbor, MI) was placed in the VRC, one
in the NTS, and two near the midline of the cortical slice, such that all
six cortical layers were spanned (Fig. 1, A and B). Slices were allowed
equilibrate for 120 min. Thereafter, baseline activity was recorded
for 60 min followed by application of pentobarbital sodium (150–300
μM), muscimol (20 μM, GABA\(_{A}\) receptor agonist), or bicuculline
(100 μM, GABA\(_{A}\) receptor antagonist). Pentobarbital was applied to
test properties of allosteric modulation of the GABA\(_{A}\) receptor.
Muscimol was applied to test for the presence and normal function of
GABA\(_{A}\) receptors. Bicuculline was applied to confirm that pentobar-
bital effects depended on GABA\(_{A}\) receptors. Raw data signals were
digitized at 25 kHz (Medusa PreAmp, Tucker-Davis Technologies,
Alachua, FL) and sent by fiber-optic link to a digital signal processor
(Pentusa Base Station, Tucker-Davis Technologies). Data were ini-

Fig. 1. Multichannel recordings of spontaneous neuronal ac-
tivity in cortical and medullary slices from interbout aroused
(IBA) ground squirrels. A: cortical activity was recorded from
neurons in the primary motor (M1), supplementary motor (M2),
and primary somatosensory (S1) areas. Slices were cut approx-
imately −1.30 mm caudal to bregma [anatomical images
adapted from Paxinos and Watson (50)]. Shaded areas indicate
electrode placement. B: medullary activity was recorded from the
nucleus tractus solitarius (NTS) and ventral respiratory
column (VRC). Slices were cut approximately −13.68 mm
caudal to bregma. Shaded areas indicate electrode placement.
C: representative cortical recordings are shown during baseline
(top left) and after 60 min of pentobarbital sodium (300 μM)
treatment (top right). Traces that cross the detection threshold
(dashed white lines) are overlaid (bottom right). D: repre-
sentative recordings from VRC neurons are shown during baseline
(top left) and after 60 min of sodium pentobarbital (300 μM, top right). Traces that cross the detection threshold (dashed
grey lines) are overlaid (bottom right).
tially recorded and displayed by custom software programmed in MATLAB (v. 2006a, The MathWorks, Natick, MA).

Data analysis. Individual neurons were identified and separated on the basis of their spike waveform shapes using principal component analysis (1). To group waveforms associated with an individual neuron, all waveforms were projected into the three-dimensional space spanned by the three eigenvectors with the largest associated eigenvalues. The KlustaKwik unsupervised clustering algorithm (16) was used to identify waveform clusters, assumed to correspond to individual neurons. Neuronal activity was averaged in 1.0-min bins throughout each experiment and normalized to the mean firing rate during a 60-min baseline recording prior to drug application. Individual neurons that were recorded on multiple, adjacent channels were counted only once. Neurons were discarded from analysis if their mean normalized firing rate for cortical (n = 39), VRC (n = 20), and NTS (n = 37) neurons was 100 ± 26%, 137 ± 19%, and 120 ± 20% of baseline, respectively. Likewise, after 2 h, in slices from IBA squirrels (n = 4), the mean normalized firing rate for cortical (n = 22), VRC (n = 40), and NTS (n = 12) neurons was 107 ± 14%, 132 ± 15%, and 115 ± 14% of baseline, respectively. Thus, despite a trend for an increased firing rate in VRC neurons, there were no significant time-dependent changes in spontaneous firing. There were no significant differences between regions or seasons in time-control recordings (P > 0.05). Likewise, there were no significant differences between males (n = 3) and females (n = 4) in response to pentobarbital application (P > 0.05).

Changes in spontaneous neuronal firing rate induced by bath-applied pentobarbital (300 μM) differed by region (cortex vs. VRC vs. NTS) and by season (SA vs. IBA). In slices from SA animals (n = 6), pentobarbital reduced the firing rate of cortical (n = 77), VRC (n = 24), and NTS (n = 52) neurons to 18 ± 7%, 24 ± 9%, and 43 ± 7% of baseline, respectively, after a 60-min drug exposure (Fig. 2; P < 0.001 for all comparisons between SA time controls and treatment). In slices from IBA animals (n = 7), pentobarbital sodium reduced the firing rate of cortical (n = 79) neurons to 11 ± 1% after a 60-min drug exposure (P < 0.001 compared with time-controls; Fig. 2A). In contrast, the mean normalized firing rate of VRC (n = 54) and NTS (n = 37) neurons was 137 ± 20%
are similar at lower [KCl].

The distribution of mean normalized firing rates after the 60-min pentobarbital sodium exposure for cortical, VRC, and NTS neurons in slices from IBA and SA squirrels is shown in Fig. 3. Most mean normalized firing rates for cortical neurons were less than 0.25 of baseline in slices from IBA (71/78 neurons <0.25) and SA squirrels (65/77 neurons <0.25) (Fig. 3, A and B). For VRC neurons in slices from IBA squirrels, mean normalized firing rates were less than 0.25 (18/29 neurons <0.25) with 11/29 neurons distributed between 0.25 and 1.25; no neurons were found above 1.25 (Fig. 3D). For NTS neurons in slices from IBA squirrels, mean normalized firing rates in 23/37 neurons were distributed between 0.25 and 1.75 with one neuron >2.5 (Fig. 3E). For NTS neurons in slices from SA squirrels, mean normalized firings in 41/52 neurons were less than 0.5 with 10/52 neurons distributed between 0.5 and 1.75 (Fig. 3F).

Pentobarbital-dependent alterations in neuronal firing rates are similar at lower [KCl]. In the previous experiments, the [KCl] in the aCSF was 9.0 mM to increase the number of actively firing neurons and increase their spontaneous firing rate. To test whether similar results could be obtained at physiological [KCl], cortical and medullary slices from IBA squirrels (n = 4) were exposed to pentobarbital sodium (150 μM) for 60 min with aCSF [KCl] at 5.0 mM. The lower concentration of pentobarbital was used to compensate for the decreased level of excitation in the tissue associated with 5.0 mM KCl. Similar to the responses observed at 9.0 mM KCl, pentobarbital reduced the mean normalized firing rate of cortical neurons (n = 64) to 21 ± 6% after a 60-min drug exposure (P > 0.05 compared with firing rate at 9.0 mM [KCl]; Fig. 4A). In contrast, the mean normalized firing rate of VRC (n = 25) and NTS (n = 44) neurons was 147 ± 51% (P > 0.05) and 38 ± 13% (P > 0.05) of baseline, respectively, after a 60-min drug exposure (Fig. 4A). These results suggest that medullary insensitivity to pentobarbital during hibernation is observed at a more physiologically relevant [KCl].

Pentobarbital-dependent alterations are not dose-dependent in VRC and NTS. Because of pentobarbital’s potential for non-GABAergic actions, we sought to determine whether the insensitivity of medullary neurons was dose dependent. To test whether VRC and NTS neuron resistance to pentobarbital was dose-dependent, pentobarbital (100 or 200 μM) was applied for 60-min to cortical and medullary slices from IBA squirrels (n = 3 squirrels for each concentration). The mean normalized firing rate for VRC and NTS neurons was unaltered, while pentobarbital-dependent depression of cortical neuron firing was dose dependent. For VRC neurons, the mean normalized firing rate was 98 ± 25% (n = 48; P > 0.05) and 142 ± 39% (n = 53; P > 0.05) of baseline after exposure to 200 and 100 μM pentobarbital, respectively (Fig. 4B). Likewise, for NTS neurons, the mean normalized firing rate was 57 ± 11% (n = 14; P > 0.05) and 57 ± 13% (n = 13; P > 0.05) of baseline, respectively (Fig. 4B). An ANOVA revealed a significant difference when the control traces and dose responses were compared (P < 0.05), yet the post hoc analysis indicated no significant individual differences. For cortical neurons, the mean normalized firing rate was 97 ± 20% (n = 46; P > 0.05) when exposed to 100 μM but reduced to 22 ± 6% (n = 43; P < 0.001) when exposed to 200 μM (Fig. 4B). Statistical comparisons are made between a given value and the corre-

![Fig. 3. Variability in neuronal activity at 60 min following bath-applied pentobarbital sodium. The activity of single neurons was normalized to the mean baseline activity for that neuron. Neurons were binned according to their normalized firing rates during the last 5 min of pentobarbital sodium (300 μM) application. A and B: number of neurons vs. mean normalized firing rate is shown for cortical neurons recorded in slices from IBA (n = 6; n = 79 neurons) and SA squirrels (n = 6; n = 77 neurons). C and D: number of neurons is shown for VRC neurons in brain stem slices from IBA (n = 7; n = 54 neurons) and SA squirrels (n = 6; n = 21 neurons). E and F: number of neurons is shown for NTS neurons in brain stem slices from IBA (n = 7; n = 37 neurons) and SA squirrels (n = 6; n = 21 neurons).]
**GABA<sub>A</sub> receptor activation with muscimol depresses cortical, VRC, and NTS neurons.** To determine whether the inability of pentobarbital to affect neuronal activity in the VRC during hibernation could be explained by a lack of GABA<sub>A</sub> receptors, we applied muscimol, a direct agonist of the GABA<sub>A</sub> receptor. In cortical and medullary slices from IBA (n = 4) and SA squirrels (n = 4), bath application of muscimol (20 μM; 60-min exposure) nearly abolished spontaneous neuronal firing (Fig. 5). In slices from SA squirrels, muscimol reduced the mean normalized firing rate to 0.01 ± 0.2%, 6 ± 3%, and 6 ± 2% in cortical (n = 73), VRC (n = 41), and NTS (n = 54) neurons, respectively. These data demonstrate that functional GABA<sub>A</sub> receptors are present in the VRC of ground squirrels during summer and hibernation.

Because pentobarbital, like other barbiturates, is known to have non-GABAergic actions at high concentrations (70), we pretreated slices with bicuculline to determine the contribution of GABA<sub>A</sub> receptors in the cortical response to pentobarbital.
during hibernation. Bicuculline (100 μM; GABA<sub>A</sub> receptor antagonist) was bath applied for 30 min prior to simultaneous bath application of bicuculline (100 μM) and pentobarbital (300 μM) for 30 min to cortical slices from IBA squirrels (n = 4) (Fig. 6). The mean normalized firing rate in cortical neurons (n = 22) was not significantly increased at 145 ± 24% of baseline (P > 0.05) by bicuculline alone, and increased, not significantly, to 182 ± 33% of baseline (P > 0.05) with bicuculline and pentobarbital coapplication. Similarly, in slices from IBA squirrels (n = 2), the mean normalized firing rate of NTS neurons (n = 12) was 121 ± 23% of baseline (P > 0.05) with application of bicuculline alone, and 119 ± 25% of baseline (P > 0.05) with bicuculline and pentobarbital (data not shown). These data suggest that the observable actions of pentobarbital on the spontaneous neuronal activity in the cortex and NTS were exclusively at GABA<sub>A</sub> receptors.

**DISCUSSION**

In this study, we found that during hibernation, aroused ground squirrels express pentobarbital-insensitive GABA<sub>A</sub> receptors on medullary neurons. In contrast, cortical neurons are highly sensitive to pentobarbital modulation throughout the year. Since medullary neurons in SA squirrels are pentobarbital sensitive, it appears that allosteric modulation of GABA<sub>A</sub> receptors on medullary neurons. In contrast, cortical neurons are highly sensitive to pentobarbital modulation throughout the year. Since medullary neurons in SA squirrels are pentobarbital sensitive, it appears that allosteric modulation of GABA<sub>A</sub> receptors on medullary neurons may play a critical role in preserving cardiorespiratory function during the extreme changes in arousal state that define mammalian hibernation.

**Relationship of GABA<sub>A</sub> receptor activation to neuronal firing.** In this study, we made extracellular multiunit recordings of neuronal activity in response to bath-applied pentobarbital. GABA<sub>A</sub> receptor-dependent currents in single neurons were not measured directly. Spontaneous neuronal activity is determined by factors such as membrane potential, intrinsic membrane properties, ionic conductances, and synaptic inputs. In addition, we cannot rule out that some of our results were due to complex network interactions (e.g., a neuron expressing GABA<sub>A</sub> receptors that inhibits a non-GABAergic neuron that inhibits a VRC neuron). However, GABA<sub>A</sub> receptors are expressed in VRC and NTS neurons (72, 73), and consistent effects with muscimol were observed in hundreds of neurons in thin slices that limit extensive synaptic connectivity. Thus, it is probable that most of the observed effects of GABAergic drugs on neuronal activity were due to interaction with GABA<sub>A</sub> receptors expressed on VRC, NTS, and cortical neurons.

High concentrations of pentobarbital (100–1,000 μM) can inhibit non-GABAergic receptors and block some ionic conductances in a manner that could reduce spontaneous neuronal activity (70). For example, pentobarbital inhibits AMPA receptors (27), suppresses sodium channels (68), and opens potassium channels (11). To rule out any non-GABAergic effects of pentobarbital in our studies, cortical slices were pretreated with bicuculline. In the presence of the antagonist, neurons were not responsive to subsequent bath-applied pentobarbital, suggesting that the inhibitory effects of sodium pentobarbital were due to GABA<sub>A</sub> receptor activation (Fig. 6).

**GABAergic function during high vs. normal bath [KCl].** Most recordings of spontaneous neuronal activity in slices were performed with aCSF potassium at 9 mM, while serum and CSF potassium levels in rodents are typically ~3.0 mM (44). Although CSF potassium levels in hibernating and SA squirrels are not known, our experimental conditions at 9 mM KCl are likely hyperkalemic. Accordingly, intrinsic membrane properties, neuronal firing properties, and synaptic interactions were altered in our slices and may have affected GABA<sub>A</sub> receptor responses to drugs. To address this potential limitation of our experimental design, similar experiments were performed with aCSF potassium at 5 mM (Fig. 4). The main findings from experiments at 9 and 5 mM were essentially equivalent, suggesting that GABA<sub>A</sub> receptor responses to pentobarbital are not dependent on extracellular potassium levels. The advantage of increasing aCSF potassium to 9 mM is the large increase in viable recordings from cortical and medullary slices.

**Effects of temperature on drug delivery.** The effect of in vivo pentobarbital in hamsters varies depending on activity state, T<sub>b</sub>, and route of administration (42). Survival time following a lethal dose of pentobarbital delivered intraperitoneally to torpid hamsters is greater compared with active (euthermic) hamsters, and survival in torpid animals is greater when the drug is administered by intraperitoneal compared with intracerebroventricular injection. However, survival time after intracerebroventricular injection is similar in torpid and artificially hypothermic hamsters, suggesting no differences in pentobarbital sensitivity between the torpid and deeply hypothermic states (42). In our study, we eliminated the effect of T<sub>b</sub> on central nervous system (CNS) activity by using IBA hibernators, whose T<sub>b</sub> was similar to those of SA animals. Similarly, seasonal effects on peripheral drug metabolism and delivery to the brain are absent in slice recordings, as the flow rate and drug concentrations are held constant. Under these conditions, differences between SA and IBA squirrels in pentobarbital sensitivity of cortical and medullary neurons were readily apparent.
Identity of recorded neurons. The cell types within the VRC, NTS, and the cortex are heterogeneous (29, 24, 40). Because our recordings were performed in slices, we cannot correlate neuronal recordings and drug responses with specific cell types. However, because of the nature of multichannel probes, we recorded from large numbers of neurons in each region. In the cortex, nearly all neurons were silenced by pentobarbital in slices from IBA and SA squirrels. In the VRC of slices from IBA squirrels, the spontaneous firing rate of the majority of neurons was not decreased by pentobarbital. Thus, this heterogeneous population of neurons behaved very similarly. In contrast, the spontaneous firing rate of some neurons in NTS in slices from IBA squirrels was decreased, whereas others were unaffected by pentobarbital. Thus, it is possible that different cell types in the NTS respond differently to pentobarbital (e.g., cardiovascular vs. respiratory).

The heterogeneous population of neurons within the NTS may have contributed to the trend toward significant differences between control and all doses of pentobarbital tested (Fig. 4B). Although the ANOVA was significant (P = 0.029), a Bonferroni post hoc test revealed individual differences that did not attain significance. Additionally, there were relatively few neurons recorded for this dose response compared with the number of cells analyzed for the principle findings, which may have contributed to this effect.

Role of GABA_A receptors in respiratory control. GABAergic synaptic transmission in the VRC and NTS plays a critical role in respiratory motor control with respect to rhythm generation, pattern formation, and chemosensory responses. For example, “network models” for adult mammals postulate that respiratory rhythm generation is due to GABA_A-dependent reciprocal inhibition between specific groups of respiratory-related VRC neurons (54, 58, 59). Likewise, GABA_A receptors modulate the magnitude and timing of bulbospinal premotoneuron activity in the rVRG and cVRG (73). Tonic GABA_A receptor-dependent inhibition constrains bulbospinal VRG neurons to within 35–50% of their maximal discharge rate, which provides a wide dynamic range for neuronal discharge and allows the respiratory control system to adapt to different physiological conditions (73). Finally, the hypoxic ventilatory response in adult mammals is biphasic with ventilation initially increasing, and then decreasing (termed “hypoxic ventilatory decline”; 52). During hypoxia, GABA_A is released in both the NTS (65) and VRC (57), consistent with the hypothesis that GABA_A receptor activation in NTS and VRC is involved in the hypoxic ventilatory decline (65). Also, GABA_A receptor activation modulates excitatory synaptic inputs onto NTS neurons (6, 33, 72), suggesting that GABA_A receptors are involved in processing respiratory-related sensory afferent information in NTS during normoxia. Because arterial blood gases are well regulated in ground squirrels during hibernation (46), we hypothesize that sufficient GABAergic function in the respiratory control system is maintained during torpor.

Role of GABAergic system during hibernation. Ligand-driven mechanisms controlling hibernation have been investigated for more than 30 years (22, 38, 48). Some of the proposed molecules include circulating hormones such as vasopressin (21), and neurotransmitters, such as serotonin (45), histamine (61), and GABA (49). The GABAergic system is a logical candidate for the induction and maintenance of systemwide CNS inhibition during torpor. However, the role of GABA in hibernation is unclear because GABA levels in torpid animals were reported to either increase by 135% in cortex using proton nuclear magnetic resonance spectroscopy (20), or decrease by 50% in the striatum using microdialysis (49). Because there is no observable neuronal activity in the forebrain at low temperatures during torpor (67, 13), it is unlikely that synaptically released GABA is playing a prominent role in neuronal silencing during torpor.

GABA_A receptors can actively elicit long-lasting, rapidly reversible, tonic inhibition of neuronal activity via positive allosteric modulation (32). Barbiturates, such as pentobarbital, can bind to GABA_A receptors at a site distinct from the GABA binding site and potentiate the effects of GABA (47, 62). It can also directly activate GABA_A receptors (28, 47). Positive allosteric modulators can achieve neuronal selectivity by binding to some, but not all, GABA_A receptors. Binding selectivity of allosteric modulators is determined by the specific isofoms of GABA_A receptors (15). Our data suggest that during hibernation, medullary GABA_A receptors with specific properties are inserted into neurons such that normal, ligand-activated function is maintained, while the response to allosteric modulators is altered.

One potential mechanism for seasonal regional selectivity is a change in the pentameric combination of subunits in a GABA_A receptor. The predominant synaptic GABA_A receptor isoform is composed of two α1, two β2, and a γ2 subunits. Upon deletion of the γ2 subunit and the inclusion of αα, αδ, αε, or β subunits, GABA_A receptors have an altered affinity for GABA and allosteric modulators, such as pentobarbital (7, 10, 23, 53). We speculate that altered subunit stoichiometry provides a plausible mechanism for the pentobarbital insensitivity described here. Different GABA_A receptor isoforms could permit active and inactive zones in the brain during hibernation in the face of a seasonally produced endogenous modulator of GABAergic function.

We speculate that this sustained change in medullary GABAergic function during hibernation may allow intermittent surges (e.g., at the end of an IBA period) of an endogenous pentobarbital-like ligand to silence forebrain neurons yet permit continued medullary regulation of cardiorespiratory function. Neurosteroids are pentobarbital-like ligands that may fulfill such a role. Allopregnanolone, a neurosteroid, is a metabolite of progesterone, can bind to GABA_A receptors at a site distinct from the GABA-binding site and, like pentobarbital, can potentiate the effects of GABA, as well as directly activate the receptor (36). Preliminary data suggest that allopregnanolone is more effective than pentobarbital at inhibiting spontaneous neuronal activity in the cortex of IBA squirrels, and actually increases spontaneous activity in NTS and VRG neurons at the same dose (Hengen KB, Behan M, Johnson SM, unpublished observations). While allosteric GABA_A modulation is a plausible mechanism for arousal-state control, other neurotransmitter systems may be involved, and clearly many other physiological systems contribute to the regulation of hibernation.

Perspectives and Significance

Hibernation is a unique physiological state that allows mammals to survive extreme conditions of cold, decreased metabolism, and decreased blood flow to tissues, but the underlying
mechanisms are not well understood. One explanation is that torpor may be regulated, in part, by endogenous ligands with actions similar to pentobarbital that silence neuronal activity in the forebrain yet leave cardiorespiratory neurons unaffected. We hypothesize that 13-lined ground squirrels selectively express ε subunit-containing GABAA receptors in cardiorespiratory brain stem neurons during hibernation. The gene for the ε subunit, whose protein is associated with pentobarbital insensitivity, appears to be highly conserved in all mammals, but there is no evidence for its natural expression and physiological function. Seasonal expression of ε subunit-containing GABAA receptors on cardiorespiratory neurons may be an elegant and simple mechanism for conferring resistance to increased central concentrations of endogenous ligands that act as positive allosteric modulators of GABAA receptors.

Determining the hibernator’s specific manipulation of GABAA receptors on respiratory neurons will promote exploration of related protection in nonhibernating mammals. Although interesting and worthy of study in its own right, understanding the mechanisms that allow tissues to survive torpor can also lead to novel biomedical applications, such as improved methods for organ preservation (35) and protection of the brain during ischemia or cardiac arrest (8). In this study, we characterized a natural, novel GABAA receptor phenotype, in which the GABAA receptor is fully functional but resistant to allosteric modulation. Further understanding of the mechanisms by which pentobarbital-resistant GABAA receptors are induced and controlled in the CNS may allow for the development of anesthetics that reduce cortical activity yet allow normal cardiorespiratory homeostatic mechanisms to function. An anesthetic with these properties could eliminate some of the major deleterious side effects of anesthetics in critically ill or aged patients.

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