Changes in CNS responsiveness during hibernation

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THE MANNER IN WHICH ANIMALS enter hibernation makes it evident that the process does not simply involve a passive decline in the activity of physiological systems (23). For example, heart rate and oxygen consumption begin to decline before any significant decrease in body temperature occurs (24) and, as time progresses, periodic increases in heart rate, metabolic rate, and muscle action potentials (24, 39) control and slow the transition from euthermia to hibernation. The spontaneous electroencephalogram (EEG) activity of the central nervous system (CNS) undergoes a decline in amplitude and regularity (32, 38, 39) during entrance into hibernation that can occur in a stepwise fashion from higher to lower levels of the brain (32). Suppression of EEG activity is first evident in the cerebral cortex, followed by decreases in activity in the midbrain reticular formation (MRF), and last, in areas of the limbic system (32, 35). During deep hibernation, EEG activity in the MRF is characterized by small-amplitude waves, occasionally punctuated by spiking, and by periods of almost complete electrical silence (32, 35, 39). However, rostral brainstem and limbic areas, particularly the hippocampus, septal area, and hypothalamus, remain almost continuously active with small-amplitude EEG activity (32, 35, 39).

These characteristics have prompted suggestions that active inhibitory mechanisms, perhaps in the limbic system, play a significant role in producing the onset and maintenance of hibernation (16, 37). Hammel and co-workers (15, 16, 19) have speculated that the inhibition of the brainstem reticular formation, including the MRF, might be particularly important for hibernation because of the loss of facilitation of preoptic/anterior hypothalamic (PO/AH) neurons that control thermogenic responses and, therefore, the decline in body temperature that characterizes entrance into hibernation. This notion is supported by studies in which electrical (11) or thermal (28) stimulation of the MRF influenced the firing of PO/AH thermosensitive neurons in nonhibernators and by studies in which chemical stimulation of the MRF produced increases in body temperature during hibernation and euthermia (2, 3).

If inhibition of the MRF is important for the control of body temperature during hibernation, then we would expect the thermogenic responses produced by MRF stimulation to diminish during entrance into hibernation and remain at a diminished level during deep hibernation. The purpose of the present experiments was to test this assumption. We measured the thermogenic responses produced by acetylcholine (ACh) stimulation of the MRF at successive portions within individual hibernation bouts. ACh was selected as the source of stimulation in these experiments because in earlier studies (2) using norepinephrine and 5-hydroxytryptamine as well, only ACh produced reliable and consistent thermogenic responses in the MRF. We report that a substantial reduction of thermogenic response magnitude was indeed evident during the early portion of the bout, followed by larger responses, including activation of the arousal trigger process, as time in the bout elapsed.

METHODS

During the period from late summer to early fall, 13 male and female California golden-mantled ground squirrels (Citellus lateralis) were anesthetized with Nembutal (75 mg/kg, ip) and stereotaxically implanted under aseptic conditions with a bilateral cannula guide assembly (21-gauge stainless steel tubes). Details of the guide have been described previously (1). The guide tubes were directed into the MRF at coordinates A-P: 3.5 to 4.1, L: ±2.5, and H: ±6.0, as depicted in the atlas of Joseph et al. (21). Procaine penicillin (30,000 U, im) was given preoperatively.

Internal temperature, measured in the midbrain, was
sensed by a calibrated bead-type thermistor (VECO 32A7) that was attached to the tip of one of the cannula guide tubes. The thermistor leads were connected to a miniature Amphenol connector that was anchored during the surgical procedure to the animal's skull with stainless steel screws (size 0-80) and self-curing dental acrylic. A polyethylene (PE-160) thermistor reentrant tube, sealed at one end, was inserted subcutaneously in the interscapular region and the open end was fixed to the acrylic cap.

Within 1 wk following surgery, the animals appeared fully recovered with all normal behavior patterns intact. In October, the animals were transferred from the colony room (ambient temperature: 25 ± 2°C; light/dark cycle synchronized with sunrise/sunset) to a cold room maintained at 5 ± 2°C where they were individually housed until April. Each animal was provided with cotton nesting material, food (sunflower seeds, raisins, and laboratory rat chow), and water ad libitum. Dim illumination was synchronized with sunrise and sunset.

Prior to each experiment, the animal was transferred in its nest to the laboratory via a thermally insulated carrier (air temperature: approx. 5°C) and placed in a test chamber maintained at 5 ± 25°C. Flexible leads were connected to the skull-mounted Amphenol connector and fed into a Wheatstone bridge input of a recording potentiometer for continuous recording of midbrain temperature (Tmb). Another calibrated thermistor was inserted into the interscapular reentrant tube and similarly connected for recording temperature changes produced by interscapular or axillary brown fat thermogenesis (Tfl), an important source of heat production during the early phase of arousal from hibernation (18, 33). Stainless steel stylets, used to seal the guide tubes between experiments, were removed and bilateral microinjection cannulas, connected to two Hamilton microliter syringes via lengths of PE-20 polyethylene tubing, were inserted into the guide assembly. In this manner, microinjections were delivered during the experiment without handling or otherwise disturbing the animal.

The manipulations inherent in connecting the recording leads and inserting the microinjection cannulas sometimes resulted in a rise in body temperature of 2–3°C. However, patience and careful handling most often prevented the occurrence of full arousal and the animal returned to a steady state of hibernation. At least 1 h of stable base-line recording, or a slow (0.005°C/min) drift toward base line, was observed before the microinjection was administered. If the microinjection resulted in full arousal, the animal was returned to the cold room and permitted to reenter hibernation, which usually occurred within 24 h. When no response or a thermogenic response that was confined to the hibernation state ensued, the animal was sometimes left in the test chamber overnight and microinjected with the same concentration of fresh ACh on the following day or a few days later, depending on the length of the animal's normal bout at that time of the season and the particular day in hibernation.

Although the duration of a bout for a given animal remained relatively constant, bout length across animals varied from 4 to 10 days. To standardize the data obtained from ground squirrels with different bout durations, microinjection schedules were determined according to the percent of elapsed time in the animal's current hibernation bout. This procedure has been used by others (40, 41). Data points were then categorized within four quarters of the total current expected bout duration, which was determined by spacing bouts containing no experiments throughout the season. Up to three experiments on a given animal could have been conducted during an average bout of 7 days. Although no adverse effects were noted as a consequence of this microinjection schedule, usually only one experiment was performed on an animal during a single bout.

Thermogenic responses that were confined to the hibernation state were classified according to the characteristics of their rising phase, as described earlier (3). Responses with slow (<0.05°C/min) and variable rising phases were classified as type I responses, and those with a smooth and rapidly increasing (≥0.05°C/min) rising phase were classified as type II responses (3).

Experiments on six euthermic ground squirrels implanted with an MRF cannula guide in early spring were carried out from late spring through early fall. The animals were housed in the colony room and tested unrestrained in a constantly illuminated, sound-attenuated chamber maintained at 25 ± 2°C. Prior to each experiment, the thermistor leads and microinjection cannulas were connected to the skull-mounted assembly. Base-line levels of Tmb were recorded for not less than 30 min prior to microinjection of ACh and continued afterward until temperature returned to base-line levels.

Acetylcholine chloride (ACh) was freshly prepared prior to each experiment on hibernating and euthermic animals in sterile isotonic saline (0.9% NaCl) at its normal pH (4.2–4.5). Doses of 50, 100, and 200 μg/μl (calculated as the salt) were microinjected in a volume of 1 μl per side over a period of 1–2 min. Control microinjections of isotonic saline were adjusted with 0.1 N HCl to the pH range of the ACh solutions and were administered in the same manner in bilateral 1-μl volumes.

When all experiments were completed, the animals were anesthetized with Nembutal (75 mg/kg, ip) and were killed by perfusing 0.9% NaCl followed by 10% Formalin through the ascending aorta. Their brains were histologically prepared either by frozen section technique or celloidin embedding, cut at 60 μm and stained with cresyl violet. Mounted sections containing the cannula guide tracts were examined to determine the location of the cannula tips.

RESULTS

These results are based on 45 experiments that were performed within each of the four quarters of the individual hibernation bouts of 13 animals (Table 1). The mean (±SE) Tmb at time of microinjection was 5.8 ± 0.5°C. The combined data showed that within each of three dose levels of ACh (50, 100, 200 μg/μl), the magnitude of the thermal responses evoked by stimulation of
the MRF was larger in the later portions of the bout than in the earlier portion. Within individual animals, it was clear that thermal response magnitude increased progressively as time in the bout elapsed. This is illustrated by three experiments, shown in Fig. 1, in which the MRF of one animal was activated by ACh (200 µg/µl) during three different bout quarters. The first experiment (top panel) begins with the animal in the final stage of entrance into hibernation. A single microinjection of ACh, given at a Tmb of 6.2°C, produced a small response after a latency of 56 min that had a maximum amplitude of 0.3°C. The rising phase of this response was variable and slow, with a maximum rate of rise of 0.02°C/min, prior to reversing at a Tmb of 6.5°C and subsequently returning to baseline hibernating levels.

Responses with these characteristics have been classified as type I thermogenic responses (3). CNS responsiveness was low during the first quarter, as evidenced by the small magnitude of the evoked thermal response. Testing in a later quarter evoked a larger thermogenic response, demonstrating increased CNS responsiveness over that observed during the final phase of entrance into hibernation. This is shown in the middle panel of Fig. 1. Microinjection of ACh (200 µg/µl) at a Tmb of 5.2°C produced a rise in Tmb of 7.1°C after a latency of 35 min. The rising phase of this response was initially slow, like that seen in the first quarter. After approximately 1.5 h, the rate increased to 0.07°C/min (during the interrupted portion of the record). The rate of increase continued to accelerate, reaching a maximum of 0.12°C/min prior to sharply reversing at a Tmb of 12.9°C and subsequently returning to hibernating levels.

Responses such as these have been classified as type II thermogenic responses (3) and are presumed to involve activation and subsequent inhibition of the arousal triggering process. A continuing increase in CNS responsiveness in the third and fourth quarters is evidenced by the triggering of full arousal following a single microinjection of ACh (200 µg/µl) in the fourth quarter, shown in the bottom panel. Following microinjection at a Tmb of 5.4°C, temperature began to increase after a latency of 38 min, reaching a euthermic level of approximately 36°C in 2 h.

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**TABLE 1. Changes in temperature following chemical stimulation in MRF during hibernation**

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<tr>
<th>Stimulus</th>
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<td>Isotonic saline</td>
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<td>(2)</td>
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<td>ACh, 50 µg</td>
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<td>ACh, 100 µg</td>
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<td>(4)</td>
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<tr>
<td>ACh, 200 µg</td>
<td>0.10</td>
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Temperatures are mean values, °C. Values in parentheses are number of observations. FA, full arousal.

Five animals were tested with ACh at a dose of 200 µg/µl (Table 1). The results show that as time in the bout elapsed, transient increases in Tmb that were confined to the hibernation state increased in magnitude. In the first quarter, the mean increase of three type I responses was 0.10°C. During the second quarter, the mean amplitude of four type I responses was 1.84°C. Trigger responses first appeared in the second quarter, and were evoked exclusively in all subsequent quarters by MRF stimulation at this dose. Thus, in four experiments, one full arousal was triggered in the second quarter, one type II response and one full arousal in the third quarter, and one full arousal in the fourth quarter.

Activation of neurons in the MRF with lower concentrations of ACh evoked progressively larger thermogenic responses over succeeding quarters, but at a lower
overall magnitude than that produced by 200 μg/μl of ACh. This is illustrated by the experiments shown in Fig. 2. The record begins during the final stage of entrance into hibernation (top panel). Microinjection of 100 μg/μl of ACh at a Tmb of 5.6°C produced no change in Tmb, and the animal remained in deep hibernation. A second experiment during the second quarter of this bout yielded the same results (not shown in the figure). During the third quarter of the same bout, 100 μg/μl of ACh, microinjected at a Tmb of 5.6°C, produced an increase in Tmb of 1.3°C after a latency of 58 min. The rising phase of this response was slow and variable, reaching a maximum rate of rise of 0.04°C/min (type I response). After reversing at Tmb 6.9°C, temperature returned smoothly to baseline hibernating levels. During the fourth quarter of this bout, a single microinjection of ACh (100 μg/μl) at a starting temperature of 5.6°C triggered full arousal from hibernation after a latency of 33 min. The animal reached a euthermic temperature of approximately 37°C with a rise time of 2.5 h.

The results of 15 experiments on five animals at a dose of 100 μg/μl of ACh (Table 1) shows that temperature was unchanged in three experiments during the first quarter, increased 0.08°C (mean) in four experiments during the second quarter, and increased 0.35°C (mean) in four experiments during the third quarter. All of these responses were type I responses. In the fourth quarter, stimulation of the MRF at this dose level produced two full arousals as well as two type I responses with a mean amplitude of 0.15°C.

Nine experiments at a dose of 50 μg/μl of ACh were performed on three animals (Table 1). No changes in body temperature were observed in any of seven experiments performed during the first three quarters of the bouts. During the fourth quarter, stimulation of the MRF produced increases of 0.2°C in two experiments.

Ten control experiments were performed on four animals in which microinjections of isotonic saline (0.9% NaCl) were administered into the MRF during each of the bout quarters. Microinjections produced no change in body temperature in two experiments in each of the first and second quarters, and three experiments in the third quarter. In the fourth quarter, however, three experiments resulted in a mean increase in temperature of 0.07°C (individual values: 0.1°C, 0.1°C, and no change in Tmb), which was similar to that during euthermia.

The data presented in Table 1 were subjected to an analysis of covariance. The statistical tests showed that elapsed time in the bout was a significant variable across all drug treatments (F = 6.47; df = 1.48; P < 0.02). Elapsed time in the bout was a significant treatment factor for experiments with 0.9% NaCl as well, indicating that the progressive return in responsiveness of the CNS reached the point that neurons in the MRF became sensitive to the ancillary effects of microinjection (such as mechanical stimulation or a transient decrease in local pH) during the last quarter of the bout.

Microinjection of ACh into the MRF during the fourth quarter of the bouts resulted in the production of thermogenic responses with all the doses tested. Only the lowest dose (ACh, 50 μg/μl) exclusively produced thermogenic responses that were confined to the hibernation state, whereas the higher doses triggered full arousal.

In order to compare the changes in thermodine response magnitude that occurred during the onset and successive phases of deep hibernation with the magnitude of responses during euthermia, we tested six animals during euthermia with MRF microinjections of ACh at a dose of 50 μg/μl. One of these experiments is shown in Fig. 3. The record begins with Tmb stabilizing after undergoing changes which normally occur as a result of restraining the animal while connecting the thermistor leads and inserting the microinjection cannulas. At the time of microinjection, the animal had been resting quietly. Microinjection of ACh at a Tmb of 38.1°C produced an increase in Tmb of 0.6°C after a latency of 1 min. The rise time to the peak of the response was 8 min. The animal began to show exploratory-type behavior beginning 2 min after the start of the microinjection (1 min after the beginning of the rise in
Tmb) and continued to show this behavior until well after the rise in Tmb reached its peak and began to decline toward baseline levels. In the majority of experiments (n = 9), the animals remained quiet throughout the change in Tmb. The mean increase in Tmb, (±SE) produced by microinjections of ACh (50 μg/μl) during euthermia in 23 experiments was 0.48 ± 0.07°C. This value was statistically greater than the mean increase in Tmb (±SE) following microinjections of 0.9% NaCl (nine experiments) of 0.12 ± 0.05°C (t = 2.57, df = 30, P < 0.05).

Histological examination of the brains of all animals used in this study confirmed that microinjections were made into the MRF. The tips of the cannula guides were located at coordinates A-P: 4.1 to 3.5, L: ±2.5, and H: +5.5 to 6.5 in the atlas of Joseph et al. (21). This placement of the guide tubes positioned the tips of the microinjection cannulas within the substance of the MRF. The guide tube placement of one animal is shown in Fig. 4.

**DISCUSSION**

These experiments demonstrate that thermogenic responses evoked by ACh stimulation of the MRF are markedly diminished in magnitude during entrance and the early phase of the hibernation bout, followed by a progressive return in magnitude as time in the bout elapses. Thus, a given concentration of ACh produced little or no response early in the bout and larger responses later in the bout. The results also show (Table 1) that the minimum concentration of ACh required to evoke a thermogenic response decreased as the hibernating period progressed. These results, and those of previous work (2), suggest that the MRF is an important component of the CNS mechanism that controls onset of and arousal from hibernation.

Our observations bear a strong similarity to the phenomenon of "progressive irritability" previously described by Twente and Twente (40, 41). They showed that the local peripheral stimulation associated with intraperitoneal injections of isotonic saline in hibernating C. lateralis did not evoke full arousal during the first half of the expected bout duration. After that period, the number of full arousals produced by the injection procedure increased as the hibernating period progressed (40). Similarly, experiments with intraperitoneally injected epinephrine showed that the minimum dose of epinephrine required to evoke full arousal decreased as the hibernating period progressed (41). Twente and Twente (41) suggested that the duration of each normal hibernating period is limited by the development of a progressive irritability in the CNS that culminates in the triggering of spontaneous arousal.

We propose that our results and those of Twente and Twente (41) are due to characteristics of the CNS in which inhibitory mechanisms in the limbic system and hypothalamus may act on the MRF to decrease its responsiveness during the onset and early portions of the hibernation bout and that as time in the bout pro-
progresses, the inhibitory influence diminishes. This is a working hypothesis, based upon our work as well as upon studies that have examined the function of different regions of the CNS and the characteristics of peripheral effector mechanisms during hibernation.

The notion that active inhibitory mechanisms are a prominent feature of the hibernating CNS is suggested by the presence of regional differences in the amplitude and regularity of EEG activity during both onset and deep hibernation (32, 35, 39). Because the limbic system remains almost continuously active in deep hibernation (32, 35, 39), it is reasonable to assume that some neurons within this region are responsible for inhibiting the activity of neurons in other areas of the CNS. The inhibitory influence might be expected to be particularly effective in the MRF, because extensive pathways from the limbic system terminate in this region (7).

We have postulated that the responses obtained in the present experiments reflect changes in the activity of CNS mechanisms. We have found no evidence in the literature to support the possibility that a decrease in responsiveness of thermogenic effector mechanisms, such as skeletal muscle or brown fat, could also account for our findings. Studies on skeletal muscle demonstrated a hyperresponsiveness to ACh (25, 27). The enhanced responsiveness was, moreover, observed on the first day of the bout and was comparable to that observed several days later, which indicates that such peripheral changes are rapid in onset and are not progressive. Studies on brown fat (10, 12, 34) also give no indication that the ability of this important thermogenic organ to increase its metabolic heat production during hibernation is diminished. Measurements of norepinephrine turnover and content in brown fat of hibernating animals have been interpreted as indicating that brown fat is active during hibernation (10, 12) and, in fact, might be primed for rapid activation (12) by the network of sympathetic fibers that innervate it (6).

The mechanism responsible for the progressive return in the magnitude of thermogenic responses evoked by MRF stimulation, or the “progressive irritability” of Twente and Twente (40, 41), has not been determined. There are at least three possibilities that should be considered. First, the low level of neural (EEG) activity present in the CNS during hibernation (32, 35, 39) might induce an effect analogous to disuse supersensitivity (31). If the progressive increase in CNS responsiveness were due to such a mechanism, then the initial activation of neurons by microinjected ACh and the contiguous sustained activation during the period of transient (type I or type II) thermogenic responses would be expected to diminish the changes that disuse might have produced (31). Yet, in our experiments, microinjections of ACh into the MRF at successive portions of a bout did not disrupt the development of progressive increases in evoked thermogenic response magnitude or the expected time of arousal. This does not discount the possibility that supersensitivity-like phenomena in the CNS might contribute to the increases in response magnitude we observed, but does indicate that such changes cannot completely account for our results.

A second possibility is that an inactivation of acetylcholinesterase, due to the low hibernating body temperatures, might produce a progressive increase in MRF responsiveness by increasing the duration of action of ACh. This is unlikely, though, because experiments have shown that acetylcholinesterase is only slightly affected by the low temperatures that are common during hibernation (25, 27).

A remaining alternative is that the inhibitory mechanism, that we and others (16, 32, 37) have assumed to play a major role in producing onset and maintenance of hibernation, in some way reduces its level of activity in a progressive fashion once the state of deep hibernation has been attained. According to this view, we would expect the threshold for MRF-activated thermogenic responses to decrease as time in the bout elapses, as our data indicate.

This threshold effect is demonstrated in Table 1 by the diagonally oriented row of values, extending from the lower left to the upper right corner that separates combinations of stimulus strength and elapsed time in the bout that result in no thermal response from those that produce thermogenic responses. Other studies on marmots (26, 36) and ground squirrels (17, 19) have demonstrated a threshold for the activation of thermogenic responses by local cooling of the PO/AH, and this has been reported to shift during the bout (17, 36). Our results suggest that the level of this threshold might be determined by the amount of ascending input reaching the PO/AH from the MRF.

Hammel and co-workers (15, 16, 19) have postulated that neurons in the brainstem reticular formation, including the MRF, sustain the activity of neurons in the PO/AH that drive thermogenic responses. These investigators speculated (16) that the insensitive state of the PO/AH thermoregulator during hibernation (19, 20) was due to an inactivation of neurons in the reticular formation. Our observation in the present study of a reduction in the magnitude of MRF-evoked thermogenic responses during hibernation is consistent with this view. Extending this hypothesis, we note that in view of the convergence of peripheral input upon neurons of the MRF (4, 22) and of the influence of the MRF upon hypothalamic (11, 28) and hippocampal (29, 30) neurons, it is reasonable to assume that an initial decrease, followed by a progressive return in the responsiveness of the MRF during hibernation might serve as a gating mechanism for controlling the flow of ascending neural impulses that influence neurons in the hypothalamus (5, 9) and hippocampus (13, 14) which, in turn, might function in the process of triggering arousal from hibernation (9, 32). Input ascending through the MRF reaching rostral brainstem and limbic areas in progressively increasing levels would eventually result in attaining the threshold for activating the trigger process for arousal from hibernation, resulting in spontaneous arousal.

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