Research report
Arousal from hibernation alters contextual learning and memory
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Abstract
Hibernation is a unique and highly regulated physiological state characterized by profound, albeit periodically reversible, depression in body temperature, metabolism, and consciousness. Hippocampal synapses undergo pronounced remodeling in concert with torpor and arousal. During hibernation, the number of postsynaptic densities, apical dendritic branches, and spine densities decreases substantially in the hippocampus. Upon arousal these parameters increase beyond pre-hibernation levels and peak within 2–3 h. By 24 h after arousal, dendritic parameters remain elevated but have started to subside, consistent with pruning and differentiation. The present study examined the functional consequences of these natural changes in synaptic structure. Wild-caught Arctic ground squirrels (AGS) were trained in a hippocampal-dependent contextual fear conditioning task at 3 h, 24 h, or 4 weeks after arousal (warm-adapted euthermic control group). All groups acquired the fear conditioned response similarly on the training day. During a subsequent retention test session, AGS in the 24 h group exhibited enhanced expression of contextual fear compared to the other two groups. These data suggest that the morphological and biochemical changes occurring at 24 h after arousal from hibernation affect hippocampal-dependent learning and memory. The natural change in synaptic structure during hibernation may provide a unique opportunity to assess the neural substrates underlying cognitive enhancement.

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1. Introduction
Arctic ground squirrels (AGS, Spermophilus parryii) are a hibernating species of rodent indigenous to northern climates. Hibernation is a unique condition characterized by prolonged torpor, defined as a decrease in metabolism and body temperature and a variety of highly regulated physiological adaptations [9,12]. Among these changes, cerebral glucose metabolism decreases to 1–2% of non-hibernating values [13], EEG becomes isoelectric [14], action potentials cease throughout the nervous system [27], and dendritic branching and synaptic profiles (e.g., spine densities) are reduced especially in the hippocampus [20,30]. For example, parameters such as CA3 apical dendritic branching, spine densities, dendritic spine profile area, and the number of postsynaptic densities per spine all decrease during torpor in Spermophilus undulatus, an Arctic species closely related to AGS [16,30,31].

Upon arousal from hibernation, these synaptic changes are rapidly reversed in hippocampus, where the EEG first reappears [29]. Within hours after arousal from hibernation, hippocampal synaptic parameters increase beyond pre-hibernation levels and peak at about 2–3 h. At 24 h after arousal, dendritic parameters are still increased, but re-growth has started to subside [30,31], consistent with active pruning and synaptic differentiation. It is currently unclear if and how the differences in synaptic restructuring at these time points affects hippocampal dependent function following arousal.

In the present study, a learning paradigm that involves the hippocampus was used to assess cognitive function at different time points shortly after arousal from hibernation to examine the effects of hippocampal restructuring. We have shown previously that it is important to select a task with a discrete number of training trials to reduce variability in the performance of wild-caught AGS [39]. Thus we used a contextual fear conditioning task optimized to produce rapid learning that could be evaluated in short, discrete training and testing trials. Training took place at either 3 or 24 h after arousal, times that have been shown to differ with respect to the degree of dendritic branching and changes in...
spine densities, spine area, and other measures of synaptic morphology in hippocampal CA3 apical dendrites [30,31]. Unlike previous reports, this study specifically addresses hippocampal-related function and assesses learning at time points known to be associated with differences in dendritic morphology.

2. Materials and methods

2.1. Subjects

Twenty-six Arctic ground squirrels (AGS, *Spermophilus parryii*) were trapped on the northern slopes of the Brooks Range, Alaska, approximately 11 miles south of the Tolkot Field Station of the University of Alaska Fairbanks (68°38"N, 149°38"W; elevation 899 m) in July 2002 under permit from Alaska Department of Fish and Game. Upon arrival, animals were screened for salmonella and quarantined for 14 days. All animals were housed individually in cages (21 cm high × 46 cm wide × 31 cm deep), fed Purina Rodent Chow 40 (g/6 in an ambient temperature of 18°C, on natural lighting for 64 latitude where light-dark cycle changed from 20:4 to 16:8 until moving to environmental chambers in mid-August. Approximately 2 weeks prior to moving to environmental chambers, AGS were fed 10 sunflower seeds per day to facilitate hibernation. Environmental chambers were maintained at 2-4°C and a 4:20 light-dark cycle. After moving to environmental chambers and throughout the remainder of the experiment, AGS were fed rodent chow ad lib. The Institutional Animal Care and Use Committee of the University of Alaska Fairbanks approved all procedures.

All animals were checked daily for behavioural activity. Hibernation was indicated if respiratory rate was less than six breaths per minute and wood shavings placed on an animal’s back 24 h previously remained undisturbed. AGS were allowed to hibernate for at least 40% of their seventh to ninth bout of torpor and were then aroused by transfer to a warm (18°C) room. All groups hibernated similarly as shown in Table 1. The warm room was maintained on a 12:12 light-dark cycle. Warm room temperature and light cycle inhibited hibernation, but did not induce gonadal genesis. Three groups of animals underwent the fear conditioning procedures described below. One group, the warm-adapted euthermic AGS, was trained 4 weeks after moving to the warm room (n = 7). Two other groups of aroused AGS were trained 3 h (n = 10) or 24 h (n = 9) after the first and typically most intense period of shivering that marks a commitment to fully arouse from torpor. This period, termed peak shivering, occurs approximately 1.5 h after initiating arousal and is characterized by rapid breaths and shivering. AGS in the 3 and 24 h groups were moved to the warm room in pairs, one or two pairs at a time. During arousal respiratory rate was monitored every 30 min and animals were observed for signs of shivering. To ensure that body temperature was consistent among groups without stressing animals prior to behavioural testing, rectal temperatures were measured at 3 and 24 h after peak shivering in other groups of aroused AGS were trained 3 h (n = 9) after the first training trial (baseline freezing) and then during each 6 h interval that separated the training trials (post-shock freezing). For the test day, the 6 min, 24 h session was divided into 64 intervals and freezing behaviour was again scored as present or absent every 8 s. The occurrence of freezing behaviour was then expressed as a percentage of the eight observations per 64 s interval. A score of 100% indicates that freezing behaviour was observed during all eight of the eight observations.

2.3. Behavioural procedures

Animals were trained in a hippocampal dependent contextual fear conditioning paradigm [8,22]. On the training day, AGS were transported to the conditioning chamber in a clean shuttle box and placed in the conditioning chamber. AGS then received three training trials commencing 3 min after placement in the chamber. Each training trial consisted of an unsignalled foot-shock (1 mA, 1 s) delivered though the grid floor. Trials were separated by a 64 s intertrial interval. Sixty-four seconds after the final training trial, the animal was returned to its home cage. Twenty-four hours later, animals were returned to the chamber for a 6 min, 24 h test session during which no shocks were delivered.

2.4. Behavioural observations

Conditioned freezing behaviour served as the measure of learning as described previously [8]. Freezing behaviour is a common measure of fear characterised by a crouching position with no movement except for respiration [6,13]. As subzero ambient temperatures typical of the Arctic environment these animals inhabit, core body temperature drops to as low as −3°C during torpor [3]. In the present study, ‘freezing’ behaviour refers to the motionless behaviour of euthermic animals in the contextual fear conditioning task and is not to be confused with super-cooling, a physiological phenomenon associated with subzero body temperature during torpor.

Freezing behaviour was video recorded and analyzed by an observer blind to experimental condition. On the training day, behaviour was monitored and recorded as present or absent every 8 s for the 64 s period just prior to the first training trial (baseline freezing) and then during each 64 s interval that separated the training trials (post-shock freezing). For the test day, the 6 min, 24 h session divided into 64 intervals and freezing behaviour was again scored as present or absent every 8 s. The occurrence of freezing behaviour was then expressed as a percentage of the eight observations per 64 s interval. A score of 100% indicates that freezing behaviour was observed during all eight of the eight observations.

2.5. Data analysis

Group differences in freezing behaviour were analyzed using a repeated measures analysis of variance (rANOVA) and Fisher’s PLSD for post hoc comparisons. For data gathered on the training day, group (warm-adapted euthermic, 3 and 24 h) was used as the between-subjects variable and trial (baseline, trials 1, 2 and 3) was used as the within-subjects variable. For test data, group and
freezing (< 0.0001]. There were no group differences in post-shock freezing as shown in Fig. 1. Indeed, animals in all groups exhibited increased the conditioned fear response to the training context similarly, as In addition, there were no significant differences in core body temperature between the 3 and 24 h groups (p > 0.2).

During the training session, AGS in all three groups acquired the conditioned fear response to the training context similarly, as shown in Fig. 1. Indeed, animals in all groups exhibited increased freezing behaviour over the conditioning trials [F(3,66) = 17.1, p < 0.0001]. There were no group differences in post-shock freezing (p > 0.4) and no significant group × trial interaction (p > 0.9). During the test session, all groups exhibited a high frequency of freezing behaviour that extinguished over the course of the session as indicated by a significant main effect of Block [F(5,110) = 17.3, p < 0.0001], as illustrated in Fig. 2. However, animals in the 24 h group exhibited greater amounts of freezing compared to either the warm-adapted euthermic group or the 3 h group. A repeated measures ANOVA revealed a main effect of group [F(2,22) = 6, p < 0.01] and a significant group × block interaction [F(10,110) = 2.3, p < 0.02]. Indeed, post hoc analysis indicated that freezing behaviour was significantly increased in the 24 h group versus the warm-adapted euthermic group on the second (p < 0.05), third (p < 0.05), fourth (p < 0.02), and sixth (p < 0.002) 64 s blocks. Compared to the 3 h group, the 24 h group exhibited greater freezing during the third (p < 0.002) and sixth (p < 0.0004) 64 s block. The warm-adapted euthermic group and 3 h group did not differ significantly from each other at any time during the test session.

3. Results

One animal from the warm-adapted euthermic group was eliminated from the study because it showed no evidence of freezing either during training or testing. The characteristics of the AGS that remained in the study are presented in Table 1. There were no significant differences in body weight, duration of torpor bout lengths, days in torpor, or total number of torpor bouts between the warm-adapted euthermic, 3 and 24 h groups. In addition, there were no significant differences in core body temperature between the 3 and 24 h groups (p > 0.2).

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4. Discussion

The present study examined the effects of hippocampal synaptic restructuring during arousal from hibernation on learning and memory in AGS trained at different times after arousal. Evidence of cognitive enhancement was found in animals trained 24 h after arousal but not at 3 h after arousal. These results have several important implications. First, these are the first data to shed light on the functional consequences of hippocampal restructuring at different time points after arousal from hibernation. Secondly, the observed increase in freezing in the 24 h group is coincident with pruned dendritic branches, spine density, and spine area occurring after the peak in synaptic regrowth. This suggests that the burst in synaptic growth that peaks within 2–3 h after arousal may not result in functional synapses until there is subsequent pruning and differentiation. This is akin to synaptic alterations and function that occur during neurodevelopment. Lastly, these data indicate that hibernation may represent a useful model to study the functional effects of naturally occurring adult synaptic plasticity and cognitive enhancement in mammals.

Despite the potential utility of hibernation as a natural model of plasticity, there have only been a few published studies examining the effects of hibernation on learning. In a study by Mihailovic et al. [26], hibernation was shown to enhance learning that took place after arousal, consistent with the present findings. However, the behavioural paradigm used in that study (a Hebb-Williams maze) required a prolonged 27-day training period. Thus, it was not possible to determine how the effects on learning were related to the time course of synaptic restructuring associated with arousal. Other studies have assessed how hibernation influences learning that was acquired prior to hibernation [25,28]. These studies have reported mixed effects of hibernation on previously learned information. Moreover, these studies address synaptic stability during periods of prolonged synapse silence while the present study addresses the effects of synaptic restructuring on learning that takes place after arousal. Thus the present findings provide new insight into the functional consequences of hippocampal synaptic restructuring associated

![Image](https://via.placeholder.com/150)

Fig. 1. Freezing behaviour observed during training. Baseline (BL) represents the 64-s interval that preceded the first training trial. Trial 1, 2 and 3 refer to post-shock freezing during the 64-s interval that followed each of three training trials. Freezing behaviour during training did not differ between groups. Data are mean ± S.E.M.

![Image](https://via.placeholder.com/150)

Fig. 2. Freezing behaviour observed during the retention test, 24 h after training. Freezing behaviour was increased in the group trained 24 h after arousal and differed significantly from the warm-euthermic group on blocks 2, 3, 4, and 6. The 24 h group also differed from the 3 h group during blocks 3 and 6. There were no significant differences between the warm-euthermic group and 3 h group. Data are mean ± S.E.M.
with arousal from hibernation. These data also indicate that the contextual fear conditioning procedure is a particularly useful behavioural paradigm to examine the effects of hibernation on learning and memory.

The observation of increased expression of contextual fear in the group trained 24 h after arousal could reflect an enhancement in the retention of contextual fear memory. This could be the result of a more efficient memory consolidation process occurring after the training experience due to the increase in available functional synapses in the 24 h group. As a result, contextual fear memory may be more robust and extinction of the conditioned fear response would therefore be expected to take longer to occur. This explanation is consistent with the results shown in Fig. 2 and previous studies [26]. Indeed, previous studies have reported a similar pattern of enhanced retention of contextual fear due to facilitated memory consolidation after certain pharmacological treatments [11].

Furthermore, it is interesting to consider these data in light of recent studies on the structural basis of long-term potentiation (LTP) in single dendritic spines. Matsuzaki et al. [23] examined single proximal apical dendritic spines of pyramidal neurons in the CA1 region of rat hippocampal slices. Smaller spines were preferred sites for LTP induction, suggesting that small spines play a leading role in initial learning. Larger spines appear to be resistant to LTP and possibly represent physical traces of long-term memory. These data are consistent with our present findings and support the notion that smaller, pruned spines facilitate new learning. The increase in the expression of conditioned fear when AGS were trained 24 h after arousal suggests that synchronized spine restructuring and pruning may prime the hippocampus for learning. Indeed, during hibernation and arousal, cyclical changes in mossy fiber innervation of CA3 pyramidal cells are associated with changes in expression of the intercellular neuronal adhesion molecule PSA-NCAM. This molecule has been shown to be associated with a high neuropsychiatric potential in adult mammals [4]. Other aspects of hippocampal anatomy such as neurogenesis or changes in glutamate receptor subunit expression [19,32,33,36] may also contribute to synaptic plasticity. Because contextual fear conditioning is known to depend largely on the hippocampus [1,2,10,17] and changes in synaptic morphology during hibernation are especially noticeable in hippocampus, the present results likely reflect a difference in hippocampal function at different times after arousal. Dendritic spines disappear upon cooling of mature mouse hippocampus in vitro, and proliferate upon rewarming in part due to loss of ion homeostasis [18,34]. It is unclear if similar mechanisms are involved in hibernating mammals during cooling and rewarming since these animals are uniquely adapted to cold tissue temperatures and protected from disruptions in ion homeostasis [12]. Nonetheless, warming of mouse hippocampus in vitro can also induce spinogenesis without loss of ion homeostasis [18] and may therefore have mechanisms or functional consequences in common with rewarming in hibernating animals.

There are several possible alternative explanations and interpretations for the results of this study. For instance, it is possible that performance differences between the groups could account for the increase in freezing behaviour observed in the 24 h group. This is unlikely, however, since all characteristics including sex, body weight, duration of torpor bout lengths, number of days in torpor prior to arousal and total number of torpor bouts were similar for all groups of AGS. In addition, core body temperature was not different at 3 h versus 24 h after arousal, consistent with previous studies [37]. Furthermore, no significant group differences were noted in freezing before the first training trial (no main effect of group or a group × trial interaction), suggesting that activity levels were comparable in all three groups at the start of behavioural training. This indicates that differences in freezing during the test session cannot be explained by temperature-related changes in mobility and are more likely related to differences in hippocampal synaptic morphology at the time of training. It is also important to note that although previous studies indicate that hippocampal damage prior to training does not have a dramatic effect on the subsequent expression of contextual freezing, it is largely accepted that the hippocampus is a central component of the preferred pathway for processing contextual information in intact animals (e.g., see review by Maren, 2001 [21]). It has been argued that absent the hippocampus, other mechanisms and brain regions can contribute to contextual information processing. However, it is unclear whether or not those mechanisms are available in an animal arouses from hibernation. In addition, it is important to keep in mind that a hippocampal lesion is quite different, neurobiologically and probably behaviourally, from alterations in hippocampal plasticity associated with hibernation. The hippocampus is very much intact during and after hibernation compared to a lesion, so it is possible that the hippocampus is still the primary area processing contextual information in a recently hibernating animal. Indeed, the effect observed in the present study is an enhancement in freezing, presumably associated with alterations in hippocampal plasticity, not impairment as occurs following lesions. Nonetheless, the potential involvement of other areas awaits further biochemical investigation of the effects of hibernation on other brain areas such as the amygdala.

The present results are interpreted as an enhanced retention of contextual fear in animals trained at 24 h after arousal rather than impaired retention in animals trained at 3 h after arousal because freezing behaviour in the 3 h group was similar to that exhibited by the warm-adapted euthermic control animals. Warm-adapted euthermics were chosen as the control group rather than cold-adapted euthermics to avoid potential effects of cold stress on learning and memory and to prevent these animals from hibernating during the prolonged euthermic period. The period of prolonged euthermia occurred before natural arousal in the spring and was timed to avoid gonadalgenesis and circannually entrained increases in gonadal steroids that could influence cognition. The warm-adapted euthermic group hibernated normally before they were moved to a warm room 4 weeks prior to training. Compared to the other groups, the warm-adapted animals did not differ in characteristics such as body weight, number of torpor bouts or torpor bout duration. At the time of training and testing, the groups also had similar activity levels and vocalizations when handling.

The present study indicates contextual learning and memory is altered at specific times after arousal. Hibernating animals
may provide a useful natural model of adult mammalian synaptic plasticity. Other studies reverse these deficits. In birds, seasonal changes in song provide disease and sleep deprivation. Indeed, these are but a few of the many conditions known to impair cognitive function [3,24]. Hippocampal dependent processes and plasticity are among the first to decline in many of these conditions and not enough is known about mechanisms of adult synaptic plasticity to possibly reverse these deficits. In birds, seasonal changes in song provide an avian model of adult synaptic plasticity [7]. Other studies are beginning to provide evidence of genetic and pharmacological enhancement of cognitive function in adult mammals [19,36,38]. To our knowledge, hibernation is the only known natural model of synaptic changes in the adult mammal, and may thus contribute significantly to our understanding of synaptic plasticity.

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