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Effect of Hibernation on the Enteric Nervous System of Thirteen-lined Ground Squirrel Colon

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Abstract

The enteric nervous system is the intrinsic nervous system within the gut that controls gastrointestinal motility, ion secretion, and local blood flow. During hibernation, gastrointestinal motility is slowed down. The study aims to investigate the changes in the enteric nervous system that may contribute to the lowered level of gastrointestinal motility. Summer active, winter torpor, and interbout arousal thirteen-lined ground squirrels were used. Immunofluorescence staining was used to examine the changes in neurochemical codes in the enteric nervous system of the colon. Cell counts were carried out to obtain the number of cell bodies immunoreactive to choline acetyltranferase (ChAT), a marker for cholinergic neurons, nitric oxide synthase (NOS), an enzyme which catalyzes the synthesis of the inhibitory neurotransmitter nitric oxide, and substance P (SP), a marker for a subset of excitatory motor neurons. There was a significant decrease in the number of ChAT-immunoreactive (IR) neurons in the myenteric plexus of winter torpor ground squirrels. In the summer active group, there were 14.17 ± 1.45 ChAT-IR neurons/myenteric ganglion. In the winter torpor group, there were 8.48 ± 1.14 ChAT-IR neurons/myenteric ganglion ($P < 0.05$, $n = 5$). In the interbout arousal group, there were 11.38 ± 1.44 ChAT-IR neurons/myenteric ganglion ($P > 0.05$, $n = 5$). There was no significant difference in expression of NOS or of SP between summer active, winter torpor, and interbout arousal ground squirrels. Selective downregulation of ChAT in the myenteric plexus of the ground squirrel colon may contribute to gut hypomotility during winter torpor.

Keywords: Hibernation, gastrointestinal motility, enteric nervous system, thirteen-lined ground squirrel

1. Introduction

Hibernation is a state of inactivity and metabolic depression that certain endothermic animals undergo during winter. During hibernation, these animals experience dramatic changes in their morphology, physiology, and behavior. For example, the animals' body temperature drops, breathing and heart rate slow, and renal function stagnates. These changes help the hibernators reduce the cost of maintaining a constant high body temperature and increase the chance of survival during long periods of cold and food shortage. While some aspects of hibernation on animals' physiological function have been well documented, the effects of hibernation on the digestive system have received less attention.

The digestive tract is one of the most metabolically active organs of the animal body and consumes high amount of energy, thus it is expensive to maintain. A few early studies found that hibernators selectively reduce the tissue mass of the digestive tract when it is not in use during hibernation. For example, Carey (1) and Carey et al. (2) reported decreases in mucosal wet mass and protein content per centimeter of the small intestine in hibernating thirteen-lined ground squirrels. The villus height, villus density, and mucosal surface area were also reduced in the small intestine of hibernating ground squirrels (1, 2). Interestingly, the enzymes responsible for digestion of food were only slightly

decreased during hibernation and not by a statistically significant amount (3, 4). Although absorption of ions and nutrients across the hibernator intestine was low when tested at 7 °C, warming up the hibernator intestinal tissue to 37 °C brought the absorptive function back to the normal level as seen in summer active squirrels (1). Surprisingly, the absorption rate was even higher in hibernator intestine than the summer active squirrel intestine when normalized to mucosal surface area (1), suggesting that the plasma membrane proteins involving in nutrient absorption are well preserved during hibernation.

Motility in the gut functions to mix digestive enzymes and food, move food through the digestive tract, and clear out the gut lumen between times of digestion (6). Gut motility is generated by smooth muscles in the wall of the digestive tract and is controlled by the enteric nervous system, a "little-brain-in-the-gut" (5). A recent study conducted in our laboratory found that there was a statistically significant decrease in gastrointestinal motility during hibernation (7), however, the underlying mechanisms contributing to the hypomotility is unknown. The current study aimed to investigate the control of gut motility during hibernation by the enteric nervous system, using the thirteen-lined ground squirrel as an animal model. The enteric nervous system is made up of two neuronal plexuses, the myenteric plexus that mainly controls gut motility, and the submucosal plexus that mainly controls epithelial secretion and local blood flow (5, 6). The enteric neurons release specific neurotransmitters to control specific gut function. This study tested four neurotransmitters in the myenteric plexus that control gut motility. These neurotransmitters included two excitatory (acetylcholine and substance P) and two inhibitory (nitric oxide and vasoactive intestinal peptide) neurotransmitters (5, 6). Understanding the effects of hibernation on gut function and alteration in the control systems of the gut function may stimulate new approaches to improve success rates of gut transplantation, which requires low-temperature storage of the donor gut.

2. Materials and Methods

2.1 Animals

Five summer active, 5 winter torpor, and 5 interbout arousal thirteen-lined ground squirrels were used. Ground squirrels were born in captivity and housed at the animal facility of the University of Wisconsin-La Crosse (UWL). All procedures were approved by the institutional IACUC. Non-hibernating animals were housed individually in rooms with a Wisconsin photoperiod (9 h in December gradually increasing to 15.5 h in June and then decreasing again). In October when an animal's body temperature dropped to ambient temperature (25 °C), they were moved into a 4 °C hibernaculum. In January and February, the ground squirrels in deep torpor (body temperature was 9.8 ± 2.1 °C) were euthanized and segments of colon were collected. During hibernation season, the torpor bouts are regularly interrupted by interbout arousal, wherein the ground squirrels rapidly rewarm to 37 °C. Status of the ground squirrels was checked daily. Once a ground squirrel was in interbout arousal, it would be euthanized and colon samples would be collected, until 5 interbout arousal squirrels were reached. Colon samples were also collected from euthanized summer active animals in June–July.

2.2 Immunofluorescence Staining

Segments of the colon were opened along the mesenteric border, stretched tautly, pinned out flat with mucosa side up onto Sylgard-coated Petri dishes, and fixed in Zamboni's fixative (4% formaldehyde plus 1.5% picric acid in 0.1M phosphate buffered saline). After fixation, colonic segments were washed in phosphate buffered saline three times, 10 min each. Whole-mount preparations of myenteric and submucosal plexuses were dissected from these segments. To minimize non-specific binding and to permeabilize the tissues, the preparations were placed in phosphate buffered saline containing 10% normal donkey serum and 0.3% Triton X-100 for 30 min at room temperature and used for immunofluorescence staining. The preparations were incubated in primary antibodies for human neuronal protein C/D (HuC/D; mouse, 1:50, cat# A-21271, ThermoFisher Scientific), choline acetyltransferase (ChAT, goat, 1:100, cat# AB144P, Millipore), nitric oxide synthase (NOS, sheep, 1:500, cat# AB1529, Millipore), or substance P (SP; rat, 1:500, cat# MAB356, Millipore) overnight at 4 °C. After being washed, the tissues were incubated with appropriate fluorescein isothiocyanate-conjugated secondary antibody at room temperature for 1 h. The tissues were washed in phosphate buffered saline and cover slipped with ECTASHIELD mounting medium (Vector labs). Immunofluorescence labeling was examined using a Nikon 80i fluorescence microscope. Numbers of immunostained neurons were counted from 30 ganglia in each whole-mount preparation from each animal, and five animals in each treatment group were studied. The 30 ganglia were selected randomly from the whole-mount preparation at six

different locations, two on the left, two in the middle, and two on the right, to minimize the influence of ganglionic size on neuronal counting. One-way analysis of variance was used to determine whether there were any statistically significant differences between the means of number of specific neurons among summer active, winter torpor, and interbout arousal groups. If the analysis variance yielded a significant F-ratio, Tukey's HSD post hoc test would be performed to determine which group means are significantly different from other group means. $P < 0.05$ was considered statistically significant.

3. Results

First, HuC/D was used as a neuronal marker to examine if there was neuronal loss in the enteric nervous system during hibernation. As shown in figure 1, no statistical significant difference was found between the neuron counts in either the myenteric or submucosal plexus between summer active, winter torpor, and interbout arousal ground squirrels. This suggests that neurons in the enteric nervous system were well preserved during hibernation.

Acetylcholine is a neurotransmitter found in the excitatory motor neurons in the myenteric plexus and the secretory motor neurons in the submucosal plexus (5). ChAT, a key enzyme for the synthesis of acetylcholine, is commonly used as a marker for cholinergic neurons. The number of ChAT-immunoreactive (IR) neurons in the myenteric plexus decreased significantly in the winter torpor ground squirrels compared to the summer active animals (winter torpor: 8.48 ± 1.14 neurons/myenteric ganglion vs. summer active: 14.17 ± 1.45 neurons/myenteric ganglion; $p < 0.05$, $n = 5$; Fig. 2). Surprisingly, the number of ChAT-IR neurons in the interbout arousal animals (11.38 ± 1.44 neurons/myenteric ganglion) rapidly returned to the level found in the summer active state (interbout arousal: 11.38 ± 1.44 neurons/myenteric ganglion vs. summer active: 14.17 ± 1.45 neurons/myenteric ganglion; $p > 0.05$, $n = 5$; Fig. 2). ChAT-IR neurons in the submucosal plexus did not show significant change during hibernation (Fig. 2).

Nitric oxide is an inhibitory gas neurotransmitter in the enteric nervous system. NOS, a key enzyme for the synthesis of nitric oxide, is used as a marker for inhibitory motor neurons in the myenteric plexus (5). NOS is not present in the submucosal plexus (5). There was no significant difference between the neuron counts of NOS expression between summer active, winter torpor, and interbout arousal ground squirrels (Fig. 3).

Substance P is another excitatory neurotransmitter in the enteric nervous system and is found in a subset of excitatory motor neurons in the myenteric plexus (3). There was no significant difference between the neuron counts of SP expression between summer active, winter torpor, and interbout arousal ground squirrels (Fig. 4).

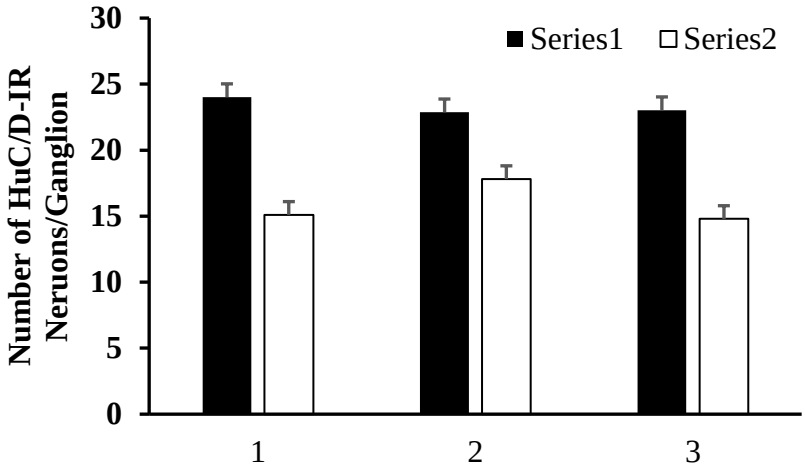

Fig. 1 Effects of hibernation on the total number of neurons in the myenteric and submucosal plexuses of the ground squirrel colon. No significant difference was found between the counts of HuC/D-IR neurons in summer active, winter torpor, and interbout arousal animals.

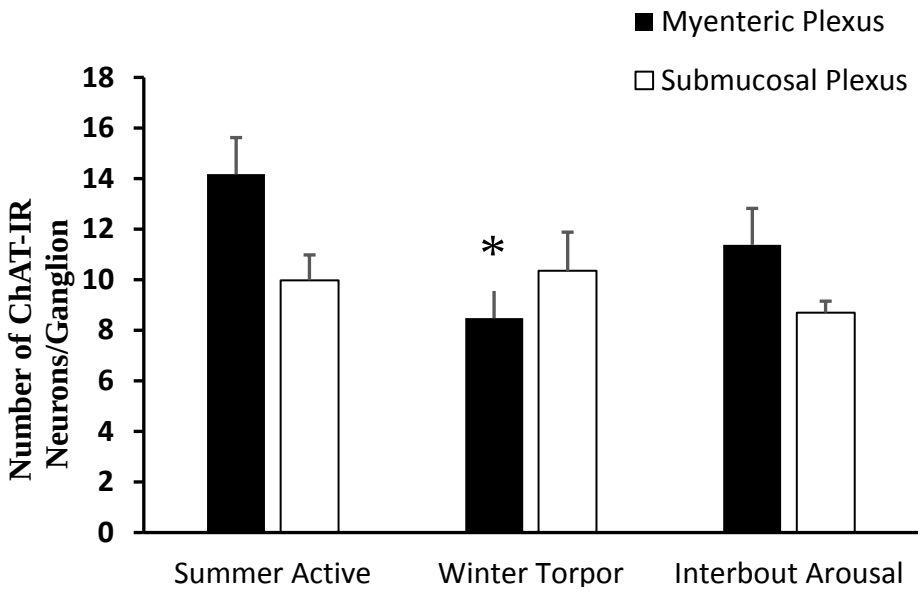

Fig. 2. Effects of hibernation on the number of cholinergic neurons in the myenteric and submucosal plexuses of the ground squirrel colon. Number of ChAT-IR neurons in the myenteric plexus was significantly reduced in winter torpor ground squirrels. * $P < 0.05$ compared to summer active.

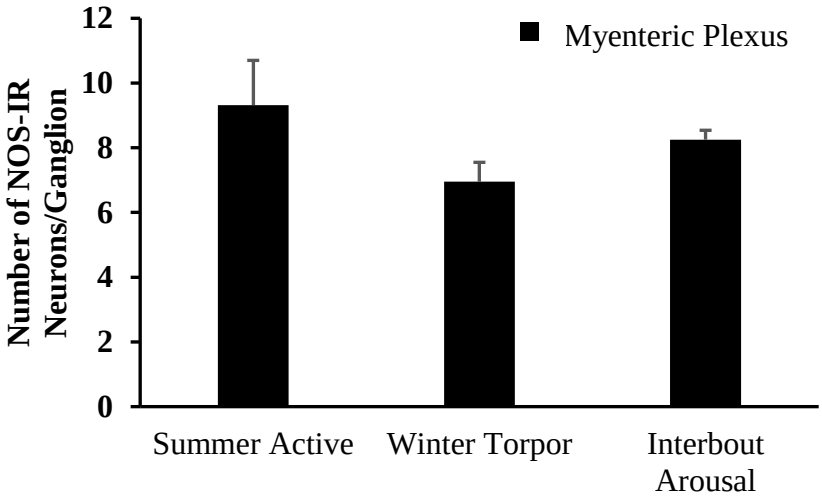

Fig. 3. Effects of hibernation on the number of NOS-IR neurons in the myenteric plexus of the ground squirrel colon. No significant difference was found between the counts of NOS-IR neurons in summer active, winter torpor, and interbout arousal animals.

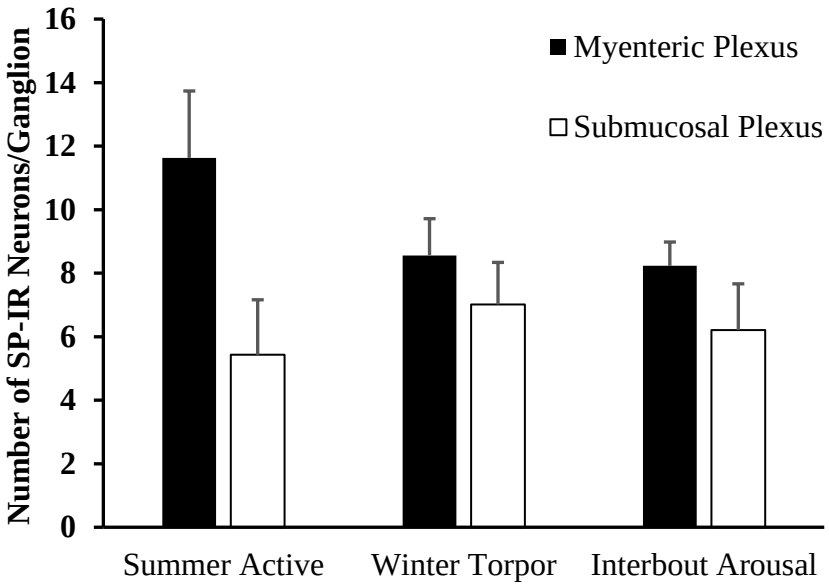

Fig. 4. Effects of hibernation on the number of SP-IR neurons in the myenteric and submucosal plexuses of the ground squirrel colon. No significant difference was found between the counts of SP-IR neurons in summer active, winter torpor, and interbout arousal animals.

4. Discussion

Four neurochemical markers in the enteric nervous system were observed in the study. The first neurochemical marker, HuC/D, was used to mark all the neurons to determine if there was neuronal loss in the enteric nervous system during hibernation. Although there were dramatic decreases of core body temperature, shortages of nutrients in the gut lumen, and decreases of blood flow to the gut, the total number of neurons stayed the same during hibernation, indicating that the enteric nervous system is well protected during hibernation through unknown mechanisms.

ChAT is an enzyme that synthesizes the neurotransmitter acetylcholine. Acetylcholine is an excitatory neurotransmitter found in the excitatory motor neurons in the myenteric plexus (5). There was a significant decrease in ChAT-IR neurons counted in the myenteric plexus during hibernation. A downregulation of the levels of ChAT in the myenteric plexus during hibernation may underline the reduction in gastrointestinal motility found in the hibernating ground squirrels (7). The number of neurons immunoreactive to SP, another excitatory neurotransmitter in the myenteric plexus, did not change significantly during hibernation.

NOS is an enzyme used in the synthesis of nitric oxide, which is an inhibitory neurotransmitter found in the inhibitory motor neurons of the myenteric plexus (5). An upregulation of NOS during hibernation would also cause hypomotility in the gastrointestinal tract. However, there was no significant change in NOS-IR neurons counted during hibernation, suggesting that NOS may not play a role in tuning down gastrointestinal motility during hibernation.

In summary, the study found that the total number of neurons in the enteric nervous system stays unchanged during hibernation, but the number of cholinergic neurons decreased significantly in the myenteric plexus. Selective downregulation of ChAT in the myenteric plexus of the ground squirrel colon may contribute to gut hypomotility during winter torpor.

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