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Involvement of Brain Tryptophan Hydroxylase in the Mechanism of Hibernation

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POPOVA, N. K., I. P. VORONOVA AND A. V. KULIKOV. *Involvement of brain tryptophan hydroxylase in the mechanism of hibernation.* PHARMACOL BIOCHEM BEHAV **46**(1) 9–13, 1993.—Marked changes were revealed in the activity of the key enzyme in serotonin biosynthesis, tryptophan hydroxylase (TPH), during entry into hibernation, hibernation, and arousal in ground squirrels (*Citellus erythrogenys*). An increase in TPH activity was found in the midbrain, hippocampus, and striatum during the prehibernation period in euthermic ground squirrels. A further increase in TPH activity was observed during the entry into hibernation. Significant elevation was found not only in potential TPH activity measured at the incubation temperature of 37°C but also at incubation temperature of 7°C, approximating the body temperature in hibernation. V_{max} in the midbrain of hibernating animals was about 50% higher than in active ones without significant changes in K_m. Thus, brain TPH maintains functionality during torpidity and is activated before the entry into hibernation. The results support the idea that brain serotonin is crucially involved in the transition to and the maintenance of the hibernation state.

Serotonin Tryptophan hydroxylase Hibernation Hibernation trigger

HIBERNATION represents a unique natural model for studies of the mechanisms regulating physiological systems and behavior. The brain mechanisms that are used by hibernators to make the transition from normal, euthermic state to the hibernation characterized by profound alterations in body temperature, state of physiological systems, and behavior are of particular interest. It has been shown (26) that the entry into hibernation is associated with an increase in brain serotonin [5-hydroxytryptamine (5-HT)] levels and a decrease in monoamine oxidase type A (MAO A) activity and 5-hydroxyindoleacetic acid (5-HIAA) levels. The arousal from hibernation is characterized by opposite changes in 5-HT and 5-HIAA levels and in MAO A activity, that is, an increase in MAO A activity, a drop in 5-HT, and a rise in 5-HIAA levels. These data are in agreement with the concept that brain 5-HT is involved as an inhibitory factor in the control of hibernation (12,18,19,30,31,33). However, an alternative explanation cannot be ruled out. The increases in brain 5-HT levels of animals entering hibernation may be due to an inhibitory effect of low temperature on MAO A and may not reflect intensified functional activity of the 5-HTergic system. A marker of the functional state of the brain 5-HTergic system is the activity of the key enzyme in 5-HT biosynthesis, tryptophan hydroxylase (TPH) (20). There are data that suggest an increased TPH activity in hibernating golden hamsters (5) and woodchucks (33). However, there is no information on changes in TPH activity during entry into and arousal from hibernation.

The present study was undertaken to evaluate the activity of TPH in the prehibernation period, during entry into hibernation, during hibernation, and during arousal from hibernation in a typical seasonal hibernators, red-cheeked ground squirrels.

METHOD

Animals

Male red-cheeked ground squirrels (*Citellus erythrogenys* Brandt), weighing 250–300 g, were individually caged. In September, they entered natural hibernation exposed to a special chamber with constant temperature (2–3°C) and housed under conditions similar to natural ones. The body temperature during the entry into hibernation gradually lowered and attained a minimum of 4°C during hibernation. The hibernation period lasts up to 7 mo, during which ground squirrels neither eat nor drink. Experiments were performed in July on active ground squirrels; in September on animals prepared to hibernate (euthermic animals with body temperature of no less than 36°C); in September on animals entering hibernation (body temperature 34–35 and 12°C); in January on hibernating squirrels (body temperature 4°C); and in April on animals arousing from hibernation (body temperature 22 and 35°C).

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Arousal was provoked by transferring animals to a laboratory room with an ambient temperature of 21–22°C. Animals were decapitated when their body temperatures increased to 22 or 35°C. When the body temperature reached 22°C, the orthostatic reflex was restored, animals turned over, paws down, but their eyes usually remained closed. At 37°C, ground squirrels were active, with their eyes opened.

Body temperature was measured in the colon with an electric thermometer.

Biochemical Procedures

After a rapid decapitation, brains were immediately removed in the cold. The hypothalamus, hippocampus, corpus striatum, and midbrain were isolated, immediately frozen in liquid nitrogen, and stored at −190°C until use.

TPH activity was determined by fluorometric assay (14). The samples were homogenized with 5 vol 50 mM Tris acetate (pH 7.5) containing 1 mM dithiothreitol. The homogenate was spun at 18,000×*g* for 30 min at 4°C. The supernatant was incubated for 15 min at 37°C with 0.8 mM l-tryptophan, 0.5 mM 6,7-dimethyl-5,6,7,8- tetrahydropteridin, and 10 μg catalase in 200 μl 50 mM Tris acetate buffer containing 1 mM dithiothreitol. The supernatant from a brain region of hibernating and active animals was divided in half: One part was incubated at 37°C and the other incubated at 7°C in the same incubation solution. The reaction was stopped by placing the samples into boiling water for 3 min. After cooling, 15 μg piridoxal-5- phosphate and 5 U decarboxylase (50 μl) were added and the samples were incubated for another 25 min at 37°C for complete conversion of 5-hydroxytryptophan (5-HTP) into 5-HT. The decarboxylation reaction was stopped with 0.5 M borate buffer, pH 10. 5-HT was extracted with heptane : butanol (2 : 1), then reextracted from the organic phase with 0.1% l-cystein in 0.1 N HCl. After condensation with *o*-phthalaldehyde, the concentration of 5-HT corresponding to the amount of 5-HTP was determined fluorometrically on a Hitachi MF-4 spectrofluorometer. Protein was determined by Lowry. The standard and blank tubes contained 0.5 nmol 5-HTP and buffer. TPH activity was expressed in pmol of 5-HTP formed per mg of protein per minute.

Reagents were purchased from the following sources: 5-HT creatinine sulphate from Reanal (Hungary), 6,7-dimethyl-5,6,7,8-tetrahydropteridin and bovine catalase from Calbiochem, (Switzerland), l-tryptophan and dithiothreitol from Sigma Chemical Co. (St. Louis, MO), and l-cystein and *o*-phthalaldehyde from Serva (Germany).

Statistics

The statistical evaluation of results was made using Student's *t*-test and analysis of variance (ANOVA) followed by multiple comparisons according to Scheffe (16). The kinetic characteristics of TPH was calculated by linear least square regression analysis (4) using 14 initial concentrations of tryptophan from 0.05–1.2 mM.

RESULTS

TPH activity in hibernating ground squirrels was increased in comparison with active animals (Fig. 1). A significant elevation in potential TPH activity, that is, activity measured at an incubation temperature of 37°C, was found in the midbrain, $t(9) = 4.06, p < 0.01$, and striatum, $t(9) = 6.63, p < 0.001$. An increased TPH activity in the midbrain, $t(10) = 4.78$, $p < 0.001$, and hippocampus, $t(10) = 3.14, p < 0.05$, of hibernating ground squirrels was also found at an incubation temperature of 7°C, that is, a temperature close to the body temperature in hibernation. At this low temperature, TPH activity decreased both in hibernating and active animals; however, during hibernation TPH activity was higher than that observed in active ground squirrels.

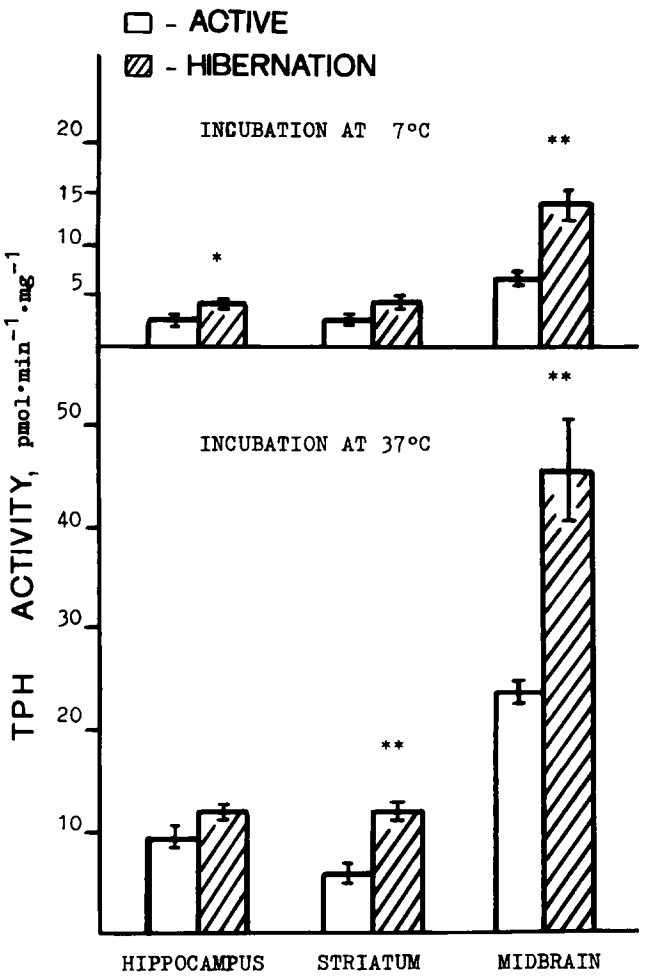

FIG. 1. Tryptophan hydroxylase (TPH) activity in brain regions in hibernating and active ground squirrels. TPH activity was determined after incubation of brain at 37 or 7°C, that is, the temperature corresponding to the body temperature of hibernating ground squirrels. Bars represent mean ± SEM $*p < 0.05$, $**p < 0.01$ vs. active animals.

Kinetic studies of TPH in hibernating animals did not reveal any significant changes in apparent K_m. Concomitantly, V_{max} in hibernating animals was about 50% higher than in active animals, $t(25) = 4.4, p < 0.001$ (Table 1).

To determine whether the activation of TPH precedes hibernation and does not depend upon a depression in the body temperature, the enzyme activity was monitored in active and in normothermic ground squirrels prepared to enter hibernation (body temperature about 36°C). Marked changes in TPH were found in some brain regions of animals in the prehibernation period and in ground squirrels entering hibernation. A pronounced increase in TPH activity in the midbrain, $t(10) = 3.75$, $p < 0.01$, hippocampus, $t(10) = 3.44$, $p < 0.01$,

TABLE 1

KINETIC PROPERTIES OF TRYPTOPHAN HYDROXYLASE FROM THE MIDBRAIN OF ACTIVE AND HIBERNATING GROUND SQUIRRELS

Groups of Animals	V_{max} ± SEM (pmol · mg^{-1} · min^{-1})	K_m ± SEM (μM)
Active	42.7 ± 3.10	0.52 ± 0.07
Hibernation	60.8 ± 2.78*	0.49 ± 0.04

Fourteen concentrations of *l*-tryptophan varied from 0.05–1.2 mM in the presence of 0.5 mM 6,7-dimethyl-5,6,7,8-tetrahydropteridine were used.

*$p < 0.01$ vs. active.

and striatum, $t(9) = 2.74$, $p < 0.05$, was observed in normothermic ground squirrels prepared to enter hibernation. TPH activity was increased further during entry into hibernation, which was characterized by a decrease in body temperature (Fig. 2).

During arousal from hibernation, TPH activity in the midbrain decreased and reached levels equal to that in active animals (Fig. 3). In the striatum, a twofold elevation in TPH activity was found when the body temperature increased to 22°C, $t(8) = 5.43$, $p < 0.001$; further elevations in body temperature resulted in enzyme activity decreasing to levels characteristic of active ground squirrels. No significant changes were found in the hippocampal TPH activity during arousal.

Changes in TPH activity observed in the hypothalamus were different from those observed in the other brain regions studied (Fig. 4). Although there were significant changes in enzyme activity during the hibernation cycle, $F(6, 35) = 5.6$, $p < 0.001$, no significant changes in hypothalamic TPH activity were found during the prehibernation period. In addition, no differences in TPH activity between hibernating and active ground squirrels were observed with incubation temperatures of 37°C [19.5 ± 2.5 pmol/mg/min in hibernating and 19.3 ± 1.4 pmol/mg/min in active animals, $t(10) = 0.07$, $p > 0.05$] or 7°C [6.4 ± 0.7 pmol/mg/min in hibernating and 7.3 ± 0.8 pmol/mg/min in active animals, $t(10) = 0.88$, $p > 0.05$]. However, in animals entering hibernation with body temperatures of 12°C TPH activity in the hypothalamus decreased and TPH activity displayed a significant increase during arousal. By using multiple-comparisons Scheffe (15) at

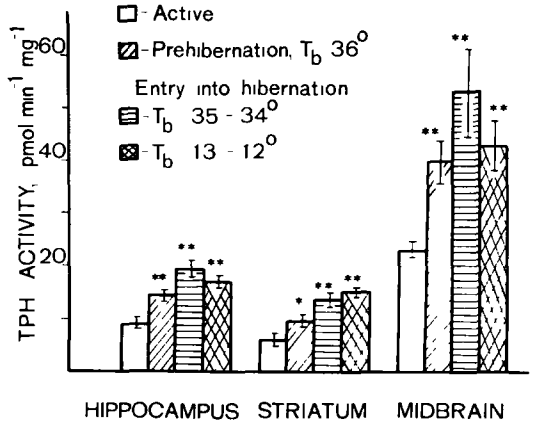

FIG. 2. Tryptophan hydroxylase (TPH) activity in brain regions of ground squirrels during prehibernation and entry into hibernation. *$p < 0.05$, **$p < 0.01$ vs. active animals.

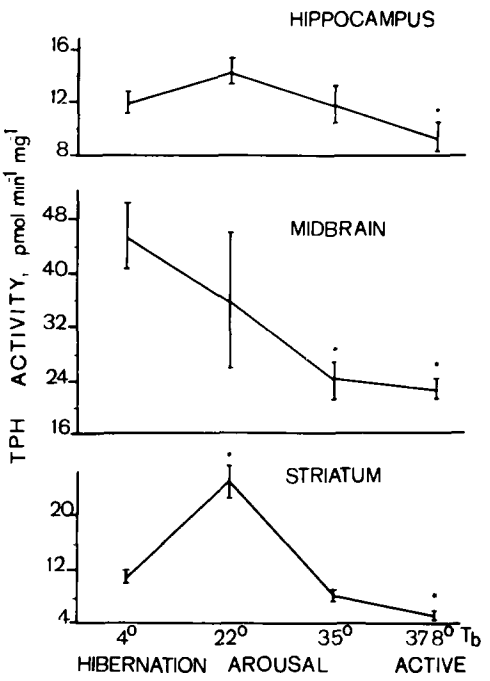

FIG. 3. Changes in brain tryptophan hydroxylase (TPH) activity during arousal from hibernation. Groups of six ground squirrels were decapitated in April during hibernation (body temperature 4°C) and after transferring them to a warm room (t° 21–22°C) when their body temperature had increased to 22 or 35°C. On the horizontal line is the body temperature. *$p < 0.05$ vs. hibernation state.

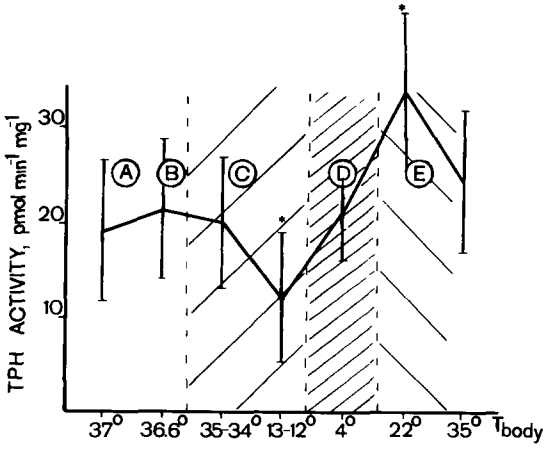

FIG. 4. Changes in tryptophan hydroxylase (TPH) activity in hypothalamus in different physiological state of ground squirrels. A–, active state; B–, prehibernation; C–, entry into hibernation; D–, hibernation; E–, arousal from hibernation. Bars represent mean ± its Scheffe's 95% confidence interval. *The point of the hibernation cycle was significantly different from other points at 95% confidence level.

a 95% confidence level, it was found that these two points of the hibernation cycle were significantly different from other points and from each other.

DISCUSSION

Our results demonstrate that in the brain of hibernators the key enzyme of serotonin biosynthesis, TPH, displays increased activity during hibernation, and this activation of TPH occurs before the onset of the hypothermic state of hibernators. The high level of TPH activity suggests an intense synthesis and release of 5-HT. Increased TPH activity was found in the midbrain, hippocampus, and striatum during the prehibernation period, a period in which animals were prepared to enter hibernation but still maintained euthermic body temperatures. This indicates two important points: First, the changes in brain TPH were not a consequence of body temperature change, nor were they induced by low temperatures; second, alterations in TPH activity preceded the entry into hibernation, strongly supporting the paradigm of participation of the brain serotonergic system in the mechanisms controlling the preparation for and induction of the forthcoming hibernating state.

The time course of TPH activity in the hibernation cycle was different in various brain regions, probably reflecting dissimilar functional activity of these brain areas and their different roles in the control of hibernation. Increased TPH activity has been shown in the striatum, which is involved in the regulation of skeletal muscle tonus (11). An elevation in TPH activity during entry into hibernation and hibernation was found in the midbrain the site of the raphe nuclei, the primary sites for brain 5-HT synthesis. The enhancement of TPH activity in the midbrain during hibernation is probably a result of de novo synthesis because TPH kinetic parameters during hibernation show an increase in V_{max} without any alterations in K_m.

The elevated midbrain TPH activity observed during hibernation suggests that the serotonergic system is functionally active. This idea is supported by the data demonstrating an association of TPH activity with the functional activity of brain serotonergic neurons (3).

Attention is drawn to the increase in TPH activity during the prehibernation, entry into hibernation, and hibernation states in hippocampus. These results are in good agreement with our earlier data showing that the most marked change in 5-HT levels were seen in the hippocampus, where the 5-HT/5-HIAA ratio increased sixfold (26) and an elevation of 5-HT level preceded the entry into hibernation (12). Recently, it has been shown in hamsters (6) that the 5-HT-induced inhibition and rebound excitation patterns in hippocampal pyramidal cells are unchanged over a temperature range of 35 to 15°C, without any decrease in the modulatory effect of 5-HT. At the same time, several lines of electrophysiological evidence indicate that the hippocampus represents a brain structure whose neuronal activity is maintained even in deep hibernation. This suggests that the hippocampus could be a pacemaker for the induction and maintenance of the hibernation state (28,29). Together, these observations strongly suggest a functional significance for 5-HT in the hippocampus during the transition to the hibernation state.

The significance and mode of action of maintained 5-HT activity has yet to be determined. Notwithstanding a lot of evidence implicating the brain 5-HT in the thermoregulation of mammals (2,15,17,21,27), there are some data that suggest a minor thermoregulatory role in hibernation: a) In contrast to TPH in the hippocampus, striatum, and midbrain, no TPH activation was found in the hypothalamus during prehibernation or hibernation; b) 5-HT administration into the preoptic area of the hypothalamus failed to affect the temperature curve of ground squirrels arousing from hibernation (1); c) no significant effects on thermoregulation during arousal were found after ICV administration of 5-HT or its precursor 5-HTP (22), whereas IP injection of 5-HT or 5-HTP produced a drastic inhibitory effect on thermoregulation of ground squirrels arousing from hibernation (18,22). It was hypothesized (19,24) that the peripheral rather than the central 5-HT system could participate in the control of thermoregulation in hibernation by a decrease of thermogenesis and a decrease of heat loss.

It can be suggested that the maintenance of the functional activity of the brain serotonergic system in hibernation produces a sleep-like state, on the one hand, and the changes in postural tonus, on the other. Some support for this hypothesis comes from the following observations: a) Numerous data implicate brain 5-HT in the control of sleep in mammals (7–10); b) the involvement of brain 5-HT in the control of sleep-like states with rigid and with plastic muscle tonus has been shown in frogs (23); c) an increased TPH activity in the striatum was revealed in the hibernation cycle in ground squirrels as well as in rats with hereditary predisposition to catatonia (13). Therefore, 5-HT could be a neurotransmitter or neuromodulator involved in the induction of sleep-like state with changed muscular tonus in the entry into hibernation and in hibernation.

In contrast, arousal from hibernation is characterized by an inactive serotonergic system, evidenced by increased brain 5-HT metabolism (26) and decreased 5-HT synthesis. TPH activity in arousing animals decreased in all brain regions studied. Only a short-term activation of the enzyme was found in the hypothalamus and striatum at the body temperature of 22°C. The changes in TPH activity in the striatum may be associated with changes in postural muscle tonus in arousing animals. As for the hypothalamus, the increase in TPH activity in this structure may be linked to the involvement of hypothalamic 5-HT in cardiovascular control (32) or in restoration of the stress response (25). However, an understanding of the significance of this temporal activation of the 5-HT system in the hypothalamus and striatum will depend upon more information about the central mechanisms that control the arousal from hibernation.

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