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The Brain Tryptophan Hydroxylase Activity in the Sleep-Like States in Frog

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KULIKOV, A. V., I. G. KARMANOVA, E. Y. KOZLACHKOVA, I. P. VORONOVA AND N. K. POPOVA. *The brain tryptophan hydroxylase activity in the sleep-like states in frog.* PHARMACOL BIOCHEM BEHAV **49**(2) 277–279, 1994. – The activity of the rate-limiting enzyme of serotonin biosynthesis, tryptophan hydroxylase, was determined in the brain stem in active awake frogs, and frogs in three sleep-like states: with plastic muscle tone (SLS-1), with rigid muscle tone (SLS-2), and with relaxed muscle tone (SLS-3). Significant decrease in the enzyme activity has been found in frogs in SLS-1 and SLS-2 compared to awake animals. The development in frogs a cataleptic-like immobility after treating the animals with rhythmic lighting was accompanied with a decrease in the brain tryptophan hydroxylase activity. These results provide strong evidence for the involvement of the brain serotonin in frogs in the control of evolutionary ancient sleep-like states, probably by the regulation of muscle tone.

Frog Tryptophan hydroxylase Sleep-like states Catalepsy

THE brain serotonergic system was shown to be involved in the regulation of sleep-wakefulness cycle in mammals (13). Inhibition of serotonin biosynthesis with *p*-chlorophenylalanine induced a secondary insomnia (3). An alteration in the activity of the rate-limiting enzyme of serotonin biosynthesis, tryptophan hydroxylase, in mouse brain during the sleep–waking cycle was found (10).

In frogs, three forms of sleep-like states were elucidated, regarded as the evolutionary ancient resting forms, a primary sleep in the phylogenesis of vertebrates (4). These sleep-like states are distinct from wakefulness and from each other in some behavioral, somato-vegetative, and neurophysiological characteristics and in the tone of somatic muscles (4). The first form of sleep-like state is characterized by a plastic muscle tone of cataleptic type (SLS-1). The second form is identified by the rigid muscle tone of catatonic type (SLS-2). The third resting state (SLS-3) is characterized by the relaxed tone of skeletal musculature. The involvement of brain serotonin in the regulation of the sleep-like states in frog has been shown earlier in sleep-like states with changed muscle tone (12). Frogs in the sleep-like state with plastic muscle tone were characterized by a decrease of the level of the main serotonin metabolite, 5-hydroxyindole acetic acid, in the brain by 25%. Pronounced decrease both in serotonin and its metabolite levels were found in the brains of frogs in the sleep-like state with rigid muscle tone. The magnitude of the serotonin decrease in SLS-2 in frog kept at the constant ambient temperature (20°C) was similar to that induced by hypobiosis (12). An inhibitor of tryptophan hydroxylase, *p*-chlorophenylalanine, decreased brain serotonin level and elicited the SLS-2. It was suggested that the way of the serotonergic control of the resting forms in frogs is connected with a reduced serotoninergic transmission and, therefore, is different from that in the regulation of sleep in endothermic animals. The neurochemical mechanism of the serotonin and its metabolite decrease remained unclear, although the experiments with *p*-chlorophenylalanine administration inducing sleep-like state suggested an implication of the key enzyme of serotonin biosynthesis, tryptophan hydroxylase in the regulation of the primary sleep in frog.

In this report, the activity of tryptophan hydroxylase in the brain of frogs in the different sleep-like states have been studied.

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METHOD

Animals

Rana ridibunda, weighing 22–25 g, were used. Frogs were collected from the wild in the fall and maintained in the laboratory at 7°C in a special chamber. The experiments were performed in winter on frogs transferred from 7°C to 20°C and acclimated at this ambient temperature for 2 weeks.

Procedures

Frogs were kept in separate glass chambers. Different forms of the sleep-like states were determined as described elsewhere (4,12). Animal hypnosis (immobility reflex) was produced by manually forcing a frog into an inverted position. To induce photogenic catalepsy frogs were treated with an additional rhythmic lighting (10 s lighting and 10 s pause) for 30 min twice a day for 3 successive days. Two 150 W light bulbs placed 50 cm far from the chamber were used for the lighting. The procedure induced cataleptic-like immobility in all frogs treated. Awake, active frogs were used as a control.

Tryptophan Hydroxylase Assay

The enzyme activity was determined in brainstem of frogs being in the state of wakefulness; SLS-1; SLS-2; SLS-3; animal hypnosis, and in the state of photogenic catalepsy at the third day of the lighting procedure. Frogs were decapitated, their brains were quickly removed, and brain stem containing thalamus, hypothalamus, midbrain, and pons was isolated, frozen, and kept under liquid nitrogen until use. Samples were homogenized in ice-cold 50 mM Tris acetate buffer, pH 7.5, containing 1 mM dithiothreithol and spun at 20,000 × *g* for 30 min (+4°C). Tryptophan hydroxylase activity was assayed in the supernatant by fluorescence microassay (9) using 6,7-dimethyl-5,6,7,8-tetrahydropteridine (0.5 mM, Sigma, MO) as the cofactor and l-tryptophan (0.4 mM, Sigma) as the substrate. In the preliminary study these concentrations of the substrate and cofactor were found to be saturating for the enzyme from the frog brain. The hydroxylase reaction was carried out at 20°C for 15 min and stopped by placing the samples into boiling water for 3 min. 5-Hydroxytryptophan was converted into serotonin by incubation of the samples with 1 U of l-aromatic amino acids decarboxylase from hog kidney for 30 min at 37°C. Serotonin was isolated by extraction with benzene : butanol mixture and its fluorescence was assayed after its condensation with *o*-phthalaldehyde. The enzyme activity was evaluated as pmol of 5-hydroxytryptophan synthesized per mg of protein per min.

Statistics

The comparisons between the tryptophan hydroxylase activity in the brain of active, awake frogs and the enzyme activity in the brain in frogs in the different sleep-like states were made using two-tailed Student's *t*-test.

RESULTS

A significant decrease in the tryptophan hydroxylase activity was found in the brain stem of the frog in the SLS-1, $t(16) = 2.9$, $p < 0.02$, and SLS-2, $t(16) = 5.3$, $p < 0.001$, compared to active awake animals (Fig. 1). The enzyme activity in the frog in sleep-like state of catatonic type (SLS-2) was almost twice as lower as in the active frogs. At the same time, no significant difference in tryptophan hydroxylase activity between the awake frogs and the animals in the sleep-like state with a relaxed muscle tone (SLS-3) has been revealed, $t(16) = 1.4$, $p > 0.05$, although it should be noted some tendency to decrease the enzyme activity (Fig. 1).

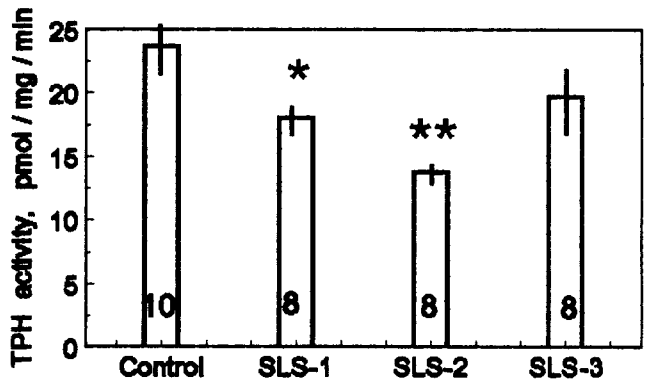

FIG. 1. Tryptophan hydroxylase activity in the brain stem in awake frogs (control) and in frogs in sleep-like state with plastic muscle tone (SLS-1), with rigid muscle tone (SLS-2), and with relaxed muscle tone (SLS-3). Bars represent the mean ± SEM. The numbers in the bars represent numbers of determinations. *$p < 0.02$, **$p < 0.001$ vs. control.

A cataleptic-like state induced by treatment of the frogs with an additional rhythmic lighting (photogenic catalepsy) was found to be accompanied with a significant decrease of tryptophan hydroxylase activity in the brain stem compared to active animals, $t(19) = 2.8$, $p < 0.02$ (Fig. 2). However, manually forcing a frog into an inverted position and holding it for several seconds induced the cataleptic-like immobility, but did not affect the brain tryptophan hydroxylase activity, $t(18) = 0.15$, $p > 0.05$ (Fig. 2).

DISCUSSION

The decrease of the activity of the rate-limiting enzyme of serotonin biosynthesis in the brain of frogs in SLS-2 is in good

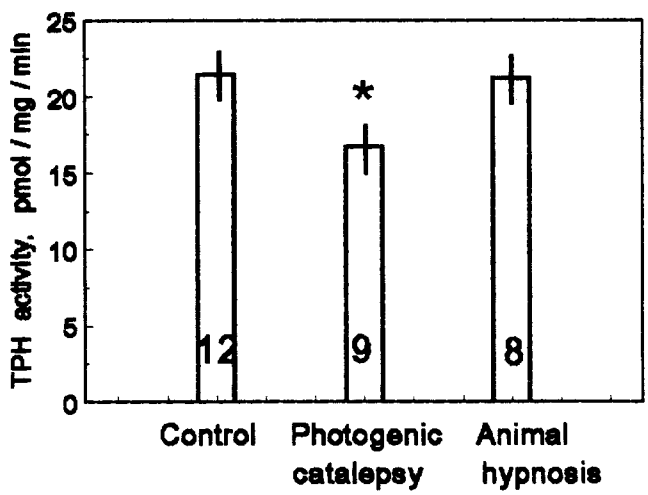

FIG. 2. Tryptophan hydroxylase activity in the brain stem in awake frogs (control) and in frogs in the cataleptic-like immobility induced by rhythmic lighting (photogenic catalepsy) and by forcing a frog into an inverted position (animal hypnosis). Bars represent the mean ± SEM. The numbers in the bars represent numbers of determinations. *$p < 0.02$ vs. control.

agreement with the earlier results showed a decrease in this state of rest in the brain concentration of serotonin and its main metabolite, 5-hydroxyindole acetic acid (12). A significant lowering of tryptophan hydroxylase activity in the brain of frogs in SLS-1 also agrees with the decrease of 5-hydroxyindole acetic acid content (12). Therefore, the decrease in the brain tryptophan hydroxylase activity may be considered as a cause of the alterations in the brain serotonin and 5-hydroxyindole acetic acid level. Thus, the development of SLS-1 and SLS-2 states is associated with a decrease in the activity of the rate-limiting enzyme of serotonin metabolism.

It should be noted that the decrease in the tryptophan hydroxylase activity in this study as well as a decrease in the level of serotonin and its metabolite (12) are found only in the states with a plastic or rigid muscle tone, but not in the sleep-like state with relaxed muscle tone. The alteration in the activity of the rate-limiting enzyme of serotonin biosynthesis in a frog with plastic and rigid muscle tone gives another strong evidence for the suggestion (12) that the serotoninergic system is involved in the brain mechanism of the regulation of muscle tone in a frog.

This suggestion was confirmed in experiments with photogenic catalepsy. This kind of catalepsy was found to be also associated with the decrease in the brain enzyme activity. However, another type of cataleptic-like immobility (animal hypnosis) elicited with forcing a frog into an inverted position was not accompanied with an alteration of the tryptophan hydroxylase activity. It is likely that instantly developing so-called animal hypnosis is regulated via serotonin receptors rather than through tryptophan hydroxylase activity modification.

It is commonly accepted that the temperature is the main environmental factor regulating the activity of the enzymes in poikilotherms. In this study, the pronounced alterations (by 30%) in the tryptophan hydroxylase activity in the brain of frog being kept at the same ambient temperature, but in different functional states (activity/sleep-like states of rest) have been demonstrated. The rhythmic lighting without changing ambient temperature also induces a deep change in the brain enzyme activity. These results provide strong evidence that not only temperature, but other external or internal factors as well regulate the brain tryptophan hydroxylase activity. The main neurochemical mechanism of rapid alteration in the brain tryptophan hydroxylase activity in mammals is a reversal phosphorylation of the enzyme (1). It may be suggested that the decrease in the enzyme activity observed in the frogs in the states of rest with plastic and rigid muscle tone, and in the photogenic catalepsy results from an inhibition of the mechanism of phosphorylation or activation of dephosphorylation of the tryptophan hydroxylase.

Therefore, the development of evolutionary ancient sleep-like states with cataleptic- or catatonic-like types of immobility in frog is associated with decrease in the tryptophan hydroxylase activity in the brain and, evidently, with attenuation of the activity of the brain serotoninergic system. Numerous data have revealed that immobility reflex in chickens was also associated with an inhibition of the brain serotonergic transmission (2). On the contrary, the brain serotonergic system in mammals seems to facilitate the development of immobility reaction. An increase in the tryptophan hydroxylase activity has been shown in striatum of cataleptic rats (5,7,11) and mice (8), while *p*-chlorophenylalanine administration prevented the development of haloperidol-induced catalepsy in rat (6) and reduced immobility time in hereditary predisposed to catalepsy rats (7). Thus, although the brain serotonergic system is involved in the mechanism of the regulation of cataleptic- and catatonic-like states in frog as well as in mammals, the role of the brain serotonin is opposite in these two classes of animals: serotonin attenuates catalepsy in frogs and plays a cataleptogenic role in mammals.

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