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Activity of Tryptophan Hydroxylase in Brain of Hereditary Predisposed to Catalepsy Rats

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KULIKOV, A. V., E. Y. KOZLACHKOVA AND N. K. POPOVA. *Activity of tryptophan hydroxylase in brain of hereditary predisposed to catalepsy rats.* PHARMACOL BIOCHEM BEHAV **43**(4) 999–1003, 1992.—The activity of the rate-limiting enzyme of serotonin biosynthesis, tryptophan hydroxylase (TPH), was studied in the brain of rats bred for 20 generations for predisposition to catalepsy (an excessive freezing). Increased TPH activity was found in the striatum but not in the hippocampus and midbrain of cataleptic rats compared with Wistar ones. K_m for the enzyme from the striatum of cataleptics was twice as low as that in control rats, although no difference in their V_{max} was found. The increase in TPH activity in the striatum of cataleptics was nonadditive with its activation induced by incubation in vitro of the enzyme under phosphorylating conditions and could be completely reversed with alkaline phosphatase. An administration of *p*-chlorophenylalanine, an irreversible inhibitor of TPH, decreased the duration of freezing in cataleptic rats. These findings indicate that hereditary predisposition to catalepsy is associated with increased TPH activity in the striatum due to local phosphorylation of the enzyme and suggest an essential role of the activation of striatal TPH in genetic predisposition to catalepsy.

Tryptophan hydroxylase Catalepsy Striatum Reversal phosphorylation

SEVERAL lines of evidence indicate that brain serotonin is involved in the regulation of catalepsy (an excessive freezing), a syndrome common to some neurological diseases and schizophrenia (6). Intracerebral serotonin administration induced catalepsy in dogs (21) and an inhibitor of the initial and rate-limiting enzyme of serotonin biosynthesis, tryptophan hydroxylase [(TPH), tryptophan 5-monooxygenase, EC 1.14.16.4], reduced neuroleptic-induced catalepsy in rats. (14).

As was shown in our earlier article (12), two different kinds of catalepsies in rats (catalepsy followed by audiogenic seizures and that induced by chronic administration of methylphenidate) were accompanied by an increase in the activity of TPH in the striatum. Similar increase in TPH activity in the striatum but not in the midbrain, where TPH was synthesized in the midbrain raphe nuclei, has been found in rats selected for 15 generations for predisposition to catalepsy (12,20). The striatum attracts special attention due to numerous data indicating involvement of this region in the regulation of muscular tonus and genesis of catalepsy (10). Thus, the findings of increased TPH activity in hereditary predisposed to catalepsy rats is suggestive although the mechanism of the local increase in TPH activity in the striatum without significant changes in the activity of midbrain TPH is still unknown. It was shown that TPH in the terminals of the serotonergic neurones can be activated in vitro (2) and in vivo (1) by the mechanism involved: Ca^{2+}, calmodulin-dependent phosphorylation.

To elucidate in more detail the role and mechanism of TPH changes in catalepsy, the effect of *p*-chlorophenylalanine (p-CPA), the kinetic properties of TPH, and the influence of phosphorylating conditions and alkaline phosphatase on TPH activity in the striatum of rats bred for predisposition to catalepsy were studied.

METHOD

Animals

Male rats (2 months of age, weighing 200 g) of the strain bred for 20 generations from an outbred Wistar stock for predisposition to catalepsy (11,13) were used. As controls, nonselected Wistar rats of respective age and weight kept under the same conditions were used. Catalepsy was estimated by time during which an animal being lifted with a stick by its forelegs maintained the imposed vertical posture (duration of freezing, seconds). Rats that maintained the posture for no less than 10 s at the first attempt in no less than 3 of 5 tests

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performed on different days are considered cataleptics. One week after the last test, animals were decapitated and their brains were removed and chilled rapidly on ice. The hippocampus, striatum (containing neostriatum and globus pallidus), and midbrain were isolated, rapidly frozen, and stored under liquid nitrogen until use.

TPH Assay

The samples were removed from liquid nitrogen and homogenized with 5 vol 50 mM Tris-acetate buffer (pH 7.5) containing 1 mM dithiothreitol (Sigma Chemical Co., St. Louis, MO). The homogenates were centrifuged at 18,000 × *g* for 30 min (+4°C). The enzyme activity was determined in supernatant by a fluorescence microassay. This method is a slight modification of one developed earlier (17). The reaction of hydroxylation was carried out in plastic tubes (1.5 ml, Eppendorf) in a total volume of 50 μl that contained: Tris-acetate, pH 7.5, 2.5 μmol; dithiothreitol, 0.05 μmol; 6,7-dimethyltetrahydropteridine (Calbiochem, La Jolla, CA, USA), 0.025 μmol; catalase (Calbiochem) 5 μg and supernatant 30 μl (0.2–0.3 mg protein). The reaction was initiated by addition of 0.04 μmol *l*-tryptophan (Sigma), 10 μl, and carried out at 37°C for 15 min in a shaker. The reaction was stopped by placing the tube into boiling water for 3 min. This procedure was found to be sufficient for complete inactivation of the enzyme. After cooling, 3.5 μg pyridoxal-5-phosphate (Serva) and 1 U decarboxylase extracted from pig kidney (4) in the same buffer (10 μl) were added and the samples were incubated for another 15 min at 37°C for complete conversion of 5-hydroxytryptophan to serotonin. The reaction of decarboxylation was stopped with 0.2 ml 0.5 M borate buffer, pH 10.0. Then, serotonin was extracted by shaking for 3 min with 0.9 ml benzene/butanol (1:1). After centrifugation (2,000 × *g* for 5 min), the organic phase was transferred to other plastic tubes containing 60 μl 0.1% *l*-cystein (Serva Feinbiochemica, Heidelberg, Germany) solution in 0.1 N HCl, shaken, and centrifuged. Then, the organic phase was aspirated and the aqueouse phase containing serotonin was transferred to plastic tubes and 0.25 ml 0.004% *o*-phthalaldehyde (Serva) solution in 10 N HCl was added. The tubes were thoroughly stirred and placed into a water bath at 80°C for 10 min. After cooling the samples, their fluorescence was measured at 470 nm with activation at 360 nm on a Hitachi MF-4 spectrofluorometer. The protein was determined by the method of Lowry et al. (18). The standard and blank tubes containing 200 pmol 5-hydroxytryptophan (Sigma) and the buffer instead of the supernatant were carried out through the entire procedure. It was shown (16) that the fluorescence of the blanks containing the boiled supernatant did not differ from those in which the supernatant was replaced by the buffer. The enzyme activity was expressed in pmol 5-hydroxytryptophan formed per mg of protein per minute.

Kinetic analysis of TPH was carried out at tryptophan concentrations varied from 0.05–1.2 mM in the presence of 0.5 mM 6,7-dimethyltetrahydropteridine. Values of apparent K_m and V_{max} and their SEM were calculated by linear regression (5).

In assay in which alkaline phosphatase was employed, 0.1 U alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) was included in the assay mixture and preincubated with the supernatant for 3 min at 37°C prior to addition of tryptophan.

Phosphorylating conditions that increase TPH activity consisted of 0.5 mM ATP, 5 mM $MgCl_2$, and 0.2 mM $CaCl_2$. These reagents were also included in the reaction mixture to permit preincubation with the enzyme for 3 min at 37°C prior to addition of the substrate (2).

P-CPA methyl ester (Sigma), an irreversible inhibitor of the enzyme, was administered to cataleptics in a dose of 300 mg/kg IP. Control cataleptic rats were treated with saline. Animals were tested for catalepsy 72 h after treatment and then were decapitated.

Statistical Tests

Significance of differences between the enzyme activity or kinetic parameters from cataleptic and noncataleptic rats or between in vitro treatment groups were determined by Student's *t*-test.

RESULTS

It was shown that the activity of TPH is higher in the striatum of cataleptics compared with Wistar rats, $t(18)$ =

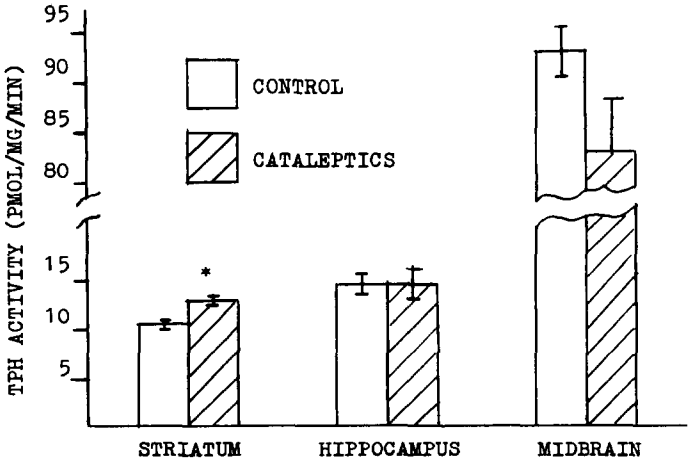

FIG. 1. Activity of TPH in the brain of genetically predisposed to catalepsy (n = 10) and control (n = 10) rats. The activity of the enzyme was assayed in the presence of 0.8 mM tryptophan and 0.5 mM 6,7-dimethyltetrahydropteridine. *$t(18) = 2.3, p < 0.05$, vs. control.

TABLE 1

KINETIC PROPERTIES OF TPH FROM THE STRIATUM AND MIDBRAIN OF CATALEPTIC AND CONTROL RATS

Groups of Animals	Striatum		Midbrain	
	V_{max}	K_m(mM)	V_{max}	K_m(mM)
Control	18.5 ± 1.4	0.18 ± 0.03 (13)	146.7 ± 7.0	0.28 ± 0.03 (16)
Cataleptics	15.7 ± 0.7	0.10 ± 0.01* (14)	137.5 ± 7.4	0.36 ± 0.03 (16)

Seven rats bred for predisposition to catalepsy and seven nonselected Wistar rats were used. Concentration of tryptophan was varied from 0.1–1.2 mM (striatum) and from 0.05–1.2 mM (midbrain) in the presence of 0.5 mM 6,7-dimethyltetrahydropteridine. V_{max} expressed as a mean ± SEM in pmol 5-hydroxytryptophan per mg of protein per minute. Numbers in brackets represent the number of determinations.

*$p < 0.05$ vs. control.

2.3, $p < 0.05$. No differences in the enzyme activity in the hippocampus and midbrain between cataleptics and control animals were found (Fig. 1).

Comparison of the kinetic properties of the striatal enzyme of cataleptics and Wistar rats revealed a decrease in apparent K_m of 60%, $t(23) = 2.12, p < 0.05$ (Table 1), but no significant change in the V_{max} of TPH for the substrate in rats genetically predisposed to catalepsy. At the same time, no differences between cataleptics and noncataleptics in apparent K_m and V_{max} of TPH from the midbrain were found (Table 1).

Preincubation in vitro of the enzyme from the striatum of cataleptics with alkaline phosphatase resulted in the decrease in its activity from 8.5 ± 1.0 to 4.9 ± 0.5 pmol/mg/min, $t(9) = 4.5, p < 0.01$ (Fig. 2A). The activity of TPH in the striatum of cataleptics under dephosphorylating conditions became similar to those in untreated noncataleptic rats [5.8 ± 0.5 pmol/mg/min, $t(18) = 1.8, p > 0.05$]. At the same time, alkaline phosphatase failed to influence the TPH activity in the striatum of control rats, $t(9) = 1.3, p > 0.05$ (Fig. 2A). There was a significant difference found between the effect of alkaline phosphatase on the enzyme activity in cataleptic and control rats, $t(18) = 3.0, p < 0.01$ (Fig. 2A).

The activity of TPH from the striatum in both cataleptic and control rats increased under phosphorylating conditions up to the same value (14.6 ± 1.4 pmol/mg/min in cataleptics and 14.6 ± 1.0 pmol/mg/min in control), but the increase was more pronounced in control than in cataleptic rats, $t(17) = 2.12, p < 0.05$ (Fig. 2A).

At the same time, the enzyme from the midbrain of cataleptic and control rats increased to the same level after in vitro

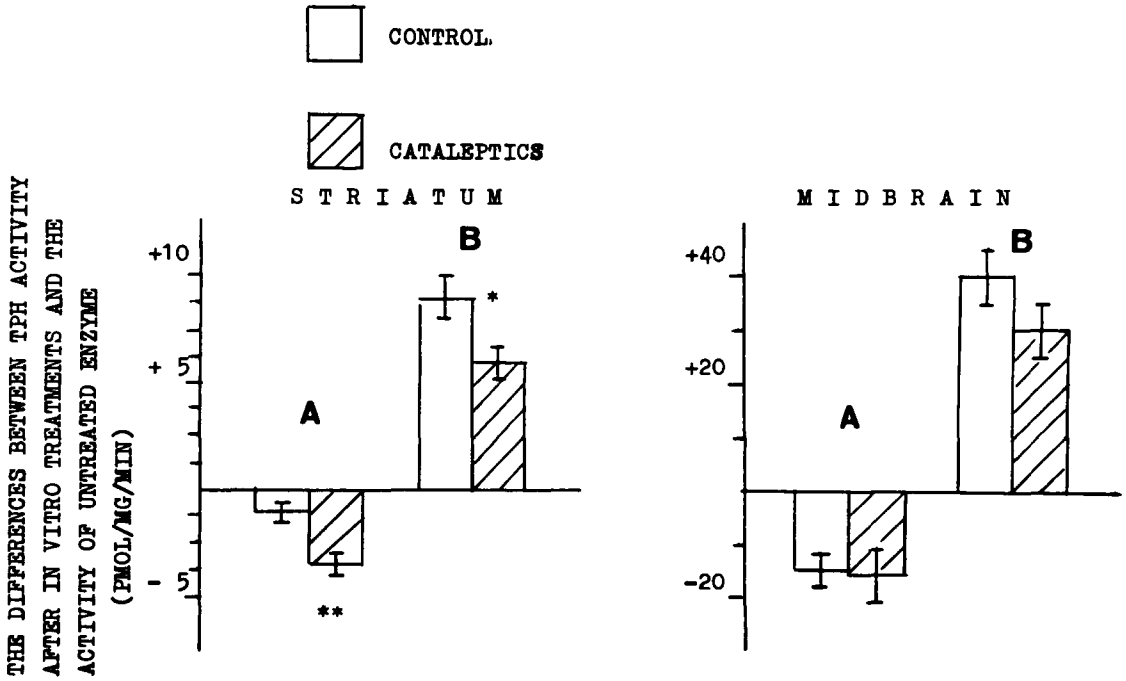

FIG. 2. Effects of alkaline phosphatase (A) and phosphorylating conditions (B) on in vitro activity of TPH in the striatum and midbrain of rats bred for predisposition to catalepsy and control rats. The enzyme activity was determined in the presence of 0.2 mM *l*-tryptophan and 0.5 mM 6,7-dimethyltetrahydropteridine. Bars represent the means ± SEM of the differences (Δ) between the initial and posttreated enzyme activities. There are 10 differences in the striatum and 6 in the midbrain. *$t(17) = 2.12, p < 0.05$, vs. control. **$t(18) = 3.0, p < 0.01$, vs. control.

TABLE 2

EFFECT OF p-CPA ON TPH ACTIVITY IN THE BRAIN OF GENETICALLY PREDISPOSED TO CATALEPSY RATS AND DURATION OF THEIR FREEZING

Treatment	n	Duration of Freezing (seconds)	TPH Activity (pmol/mg/min)	
			Striatum	Midbrain
Saline	6	88.3 ± 7.5	12.0 ± 1.0	81.2 ± 10.9
p-CPA	17	14.7 ± 1.8*	ND	11.3 ± 1.5*

The duration of freezing was determined 72 h after pCPA methyl ester administration (300 mg/kg). Then, animals were decapitated. TPH activity was assayed in the presence of 0.8 mM tryptophan and 0.5 mM 6,7-dimethyltetrahydropteridine. Comparisons were made between pCPA- and saline-treated animals. ND, the activity was too low to be determined.

*$p < 0.001$.

treatment with phosphorylating conditions and revealed a similar decrease after its preincubation with alkaline phosphatase (Fig. 2B).

To investigate whether inherited catalepsy is related to the TPH activity, the effect of pCPA on the enzyme activity and duration of freezing has been studied (Table 2). Profound decrease in the activity of TPH in the striatum, $t(21) = 11.6$, $p < 0.001$, and midbrain, $t(21) = 6.4$, $p < 0.001$, 72 h after pCPA methyl ester administration (300 mg/kg, IP) to cataleptics was found. In the striatum of pCPA-treated rats, TPH activity was too low to be determined. At the same time, there was six-fold decrease in the duration of freezing found in pCPA-treated cataleptic rats, $t(21) = 9.5$, $p < 0.001$.

DISCUSSION

We were able to confirm our previous observations that selection of rats for predisposition to catalepsy was accompanied by activation of TPH in the striatum while no changes in the enzyme activity were revealed in the hippocampus and midbrain. The study of the enzyme kinetic properties showed that TPH in the striatum but not in the midbrain of predisposed to catalepsy rats had a lower value of K_m and therefore higher affinity to the substrate than the enzyme from the same region of noncataleptics. These data are in good agreement with the idea of selective changes in TPH in the striatum in catalepsy (20). They strongly suggest that the increased TPH activity is rather a result of a modification of the enzyme than an increase of its synthesis de novo, so V_{max}, reflecting the amount of the enzyme, was similar in predisposed to catalepsy and control rats.

TPH activity in the striatum in cataleptics was decreased to the level of noncataleptics after dephosphorylation of the enzyme whereas the activity of TPH from the striatum of control rats remained unchanged. At the same time, under phosphorylating conditions higher activation of TPH from the striatum in control than in predisposed to catalepsy rats was shown. These data are sufficient to conclude that, in contrast to control animals, TPH in the striatum of cataleptics is phosphorylated. This phosphorylation may explain the increased affinity of the enzyme from the striatum of cataleptics in that it has been shown (7) that phosphorylation of TPH resulted in decreasing K_m for the substrate and cofactor.

Although some authors demonstrated the phosphorylation of the enzyme in vitro in brain slices after its depolarization (3,7,15) and in vivo after exposing rats to auditory stimuli (1), the present data give the first evidence that the breeding of rats for predisposition to catalepsy is associated with increased TPH activity in the striatum due to phosphorylation of this enzyme. This fact, taken together with the finding that TPH inhibition by pCPA suppressed this catalepsy response, supports the essential role of TPH phosphorylation in the striatum in the mechanism of hereditary predisposition to catalepsy.

Taking into consideration that autoregulation of serotonin neuronal activity and the release of the neurotransmitter are controlled by serotonin$_{1A}$ and serotonin$_{1B}$ autoreceptors (8,19), it can be supposed that the activation of TPH in the striatum of genetically predisposed to catalepsy rats may be related to hereditary deficiency of serotonin$_{1A}$ or serotonin$_{1B}$ receptors in their striatum.

At present, it is commonly accepted that a deficiency in striatal dopaminergic receptors is a major factor contributing to catalepsy (9,22). The present data point to the essential role of the functional activity of the striatal serotonergic system in the inherited predisposition to catalepsy.

REFERENCES

1. Boadle-Biber, M. C.; Corley, K. C.; Graves, L.; Phan, T.-H.; Rosencrans, J. Increase in the activity of tryptophan hydroxylase from cortex and midbrain of male Fischer 344 rats in response to acute or repeated sound stress. Brain Res. 482:306–316; 1989.
2. Boadle-Biber, M. C.; Johannessen, J. N.; Narasemhachari, N.; Phan, T.-H. Tryptophan hydroxylase: Increase in activity by electrical stimulation of serotonergic neurons. Neurochem. Int. 8:83–92; 1986.
3. Boadle-Biber, M. C.; Phan, T.-H. Involvement of calmodulin-dependent phosphorylation in the activation of brainstem tryptophan hydroxylase induced by depolarization of slices or other treatments that raise intracellular free calcium levels. Biochem. Pharmacol. 36:1174–1176; 1987.
4. Christenson, J. G.; Diarman, W.; Udenfriend, S. Preparation and properties of a homogeneous aromatic 1-amino acid decarboxylase from hog kidney. Arch. Biochem. Biophys. 141:356–367; 1970.
5. Cornish-Bowden, A. Principles of enzyme kinetics. London, UK: Butterworths; 1976.
6. Garver, D. L. Disease of the nervous system: Psychiatric disorders. In: Kaplan, L. A.; Pesce, A. J., eds. Clinical chemistry: Theory analysis and correlations. St. Louis, MO: C. V. Mosby; 1984:864–881.
7. Hamon, M.; Bourgoin, S.; Hery, F.; Simonnet, G. Activation of tryptophan hydroxylase by adenosine triphosphate, magnesium, and calcium. Mol. Pharmacol. 14:99–110; 1979.
8. Hoyer, D. Functional correlates of serotonin 5-HT$_1$ recognition sites. J. Receptor Res. 8:59–81; 1988.
9. Klemm, W. R. Cholinergic-dopaminergic interactions in experimental catalepsy. Psychopharmacology (Berl.) 81:24–27; 1983.
10. Koffer, K. B.; Berney, S.; Hornykiewiez, O. The role of the corpus striatum in narcoleptic- and narcotic-induced catalepsy. Psychopharmacology (Berl.) 47:81–86; 1978.
11. Kolpakov, V. G.; Barykina, N. N.; Chepkasov, I. L.; Alekhina, T. A.; Parvez, H. On animal models of schizophrenia. In: Par-

vez, H.; Nagatsu, T.; Nagatsu, X.; Parvez, U., eds. Methods in biogenic amines research. Amsterdam: Elsevier Press; 1983:997–1020.
12. Kolpakov, V. G.; Kulikov, A. V.; Barykina, N. N.; Alekhina, T. A.; Popova, N. K. Catalepsy and increased tryptophan hydroxylase activity in rat striatum. Biogenic Amines 2:131–136; 1985.
13. Kolpakov, V. G.; Parvez, H. Experimental models of endogenous psychoses. Biogenic Amines 3:135–152; 1985.
14. Kostowski, W.; Gumulka, W.; Czlonowski, A. Reduced cataleptogenic effects of some neuroleptics in the rats with lesioned midbrain raphe and treated with p-chlorophenylalanine. Brain Res. 48:443–446; 1972.
15. Kuhn, D. M.; O'Callaghan, J. P.; Juskevich, J.; Lovenberg, W. Activation of brain tryptophan hydroxylase by ATP-Mg^{2+}: Dependence on calmodulin. Proc. Natl. Acad. Sci. USA 77:4688–4691; 1980.
16. Kulikov, A. V. A rapid nonisotopic method for assay of tryptophan hydroxylase activity in brain. Vopr. Med. Chimii. 1:135–139; 1982 [in Russian].
17. Kulikov, A. V.; Voronova, I. P.; Janaeva, E. Y. The sensitive fluorometric assay of tryptophan hydroxylase activity in brain structures. Vopr. Med. Chimii. 2:120–123; 1988 [in Russian].
18. Lowry, O. H.; Rosenbrough, M. J.; Farr, A.; Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275; 1951.
19. Peroutka, S. J. 5-Hydroxytryptamine receptor subtypes. Annu. Rev. Neurosci. 11:45–60; 1988.
20. Popova, N. K.; Kulikov, A. V.; Kolpakov, V. G.; Barykina, N. N.; Alekhina, T. A. Changes in the brain serotonergic system in rats genetically predisposed to catalepsy. J. Vyssh. Nervn. Deyat. 33:742–746; 1985 [in Russian].
21. Sacchi, V.; Garello, L.; Dolce, G.; Bonanini, F. L'azione cataletizzante della 5-idrossitriptamine introdotta nella cisterna magna del cane. Boll. Soc. Ital. Biol. Sperim. 31:663–665; 1955 [in Italian].
22. Sanberg, P. R.; Bansey, M. D.; Giordano, M.; Norman, A. B. The catalepsy test: Its ups and downs. Behav. Neurosci. 102:748–759; 1988.