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BRIEF COMMUNICATION

Dopamine-B-Hydroxylase Activity in Rat Hypothalamus During the Estrus Cycle

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(Received 18 April 1974)

SIMPSON, C. W., C. CUMMINS AND L. V. DICARA. *Dopamine-B-hydroxylase activity in rat hypothalamus during the estrus cycle.* PHARMAC. BIOCHEM. BEHAV. 3(4) 693–696, 1975. – This experiment provides a direct test of our previous suggestion that estradiol regulates dopamine-B-hydroxylase (DBH) activity in hypothalamic loci. Anterior, medial and posterior hypothalamic slices from triplets of rats were taken during estrus and diestrus and assayed for DBH activity using the technique of Molinoff *et al.* [15]. DBH activity was measured in hypothalamic slices on three different occasions from three triplets during the estrous phase of the cycle and also from separate triplets during the diestrous stage of the cycle. Results showed a significant increase in DBH activity during the estrous phase of the cycle. Increased activity did not appear to be anatomically localized within the tissue slices. Explanation of the results has been discussed in terms of possible mechanisms of action.

DBH activity   Estrous cycle   Estrogen inhibition   Uptake mechanisms   De novo synthesis
Hypothalamic slices

RECENTLY we [18] have described behavioral evidence supporting the hypothesis of an interaction effect between estradiol and dopamine B hydroxylase (DBH) activity at hypothalamic loci. Molinoff *et al.* [16] have measured regional levels of DBH in rat brain and report very high concentrations in the hypothalamus. Although DBH activity has not been measured under different conditions of estradiol influence, end product concentrations of norepinephrine (1-NE) have been reported.

Donoso and Stefano [8] first reported significant increases in 1-NE and reciprocal decreases in dopamine in the anterior hypothalamus of ovariectomized female rats. Daily doses of estradiol plus progesterone reversed the 1-NE and dopamine levels in anterior hypothalamus of these animals to those reported in intact rats. This group [19] has also assayed anterior hypothalamic 1-NE during the estrus cycle. Norepinephrine levels are lowest at estrus and increase during diestrus. Donoso and Cukier [6] manipulated endogenous estradiol levels by a number of procedures and reported the subsequent effects on anterior hypothalamic norepinephrine levels. All groups characterized by high endogenous levels of estradiol showed a significant decrease in anterior hypothalamic 1-NE compared to appropriate control groups. Ovariectomy significantly increased 1-NE levels in anterior hypothalamus for all groups. Finally, a number of laboratories [1, 2, 7] have reported significant increases in 1-NE metabolism in anterior hypothalamus following ovariectomy.

This study tested the hypothesis that DBH activity is significantly altered in rat anterior hypothalamus during the estrous as compared to the diestrous stage of the rat estrous cycle.

METHOD

Animals

Eighteen female Sprague-Dawley rats purchased from Spartan Animals served for this experiment. Rats were between 90 and 100 days old when sacrificed. Only rats showing regular 4 or 5 days estrous cycle were used for this experiment. Hormone condition of the animals was determined on the day of the experiment by vaginal smears examined microscopically. The estrous smear was characterized by a large number of nucleated epithelial cells and the absence of leucocytes. Diestrous smears featured an absence of epithelial cells and a large number of leucocytes. Triplets of animals in the same hormone condition were utilized for the assay. Six separate assays on 6 triplets were

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conducted, separated by approximately one week each. Three triplets were assayed in the diestrous condition and three triplets from the estrous stage of the cycle. All animals were overdosed with pentobarbital sodium and brain slices of approximately equal weight were taken from anterior, middle and posterior diencephalon of each animal.

Histology

One rat of similar weight was overdosed with pentobarbital sodium and perfused with formalin through the ascending aorta. Hypothalamic slices of similar weight were taken through anterior, middle and posterior hypothalamus. Sections were cut at 8μ and stained with crysel violet to determine anterior and posterior boundaries for the hypothalamic slices.

Assay Procedures

Methods. Assay of Dopamine-B-Hydroxylase in this paper was based on the method of Molinoff *et al.* [17]. Briefly, this technique utilized bovine adrenal enzyme, Phenylethanolamine-N-methyl transferase, and an artificial substratum phenylethylamine. DBH hydroxylates this compound to phenylethanolamine at the Beta position. PNMT converst phenylethanolamine to its N-methyl derivative, N-methyl-phenylethanolamine. When PNMT is used in excess in this system, DBH becomes rate limiting and the N-methylated product is directly proportional to DBH activity.

Isolation of PNMT. PNMT was isolated from beef adrenal glands according to Molinoff *et al.* [17]. The adrenal glands were obtained fresh from the slaughterhouse. Glands were cleaned of extraneous fat, and homogenized in a Waring Blender in 2 volumes of 1.15 percent KCl. The homogenate was centrifuged at 100,000 X G for 40 min at 4°C., and the red turbid supernatant was frozen at −70 degrees until used in the further purification steps.

Ammonium sulfate was added to a thawed batch of high speed supernatant to a concentration of 30 percent (176 g/1). The pellet was collected by centrifugation at 10,000 X G for 10 min. The pellet was discarded, and the supernatant was retained. A further 126 g/l of ammonium sulfate was added to the supernatant, bringing the final concentration of ammonium sulfate to 50 percent.

The pellet was collected as before, and the supernatant discarded. The pellet was suspended in a minimal volume of buffer, consisting of 0.001 M Tris, pH 7.4 and was dialyzed overnight against 500 volumes of 0.01 M Tris pH 7.4. Forty volumes of this dialysate were passed over a Sephadex G-200 column (3 X 100 cm.). The samples were eluted with 0.05 M Tris pH 7.4, and 10 ml aliquots were collected.

PNMT activity was assayed according to Molinoff *et al.* [17]. 100 μl aliquots of each of the 10 ml eluates collected from the column were mixed with 25 μl of 0.0146 M Phenylethanolamine (2 mg/ml) 1 μl of SAMe (NEN, specific activity 58 m Ci mmole of Tris buffer, pH 8.6, a total volume of 200 μl. The reaction was terminated by the addition of 0.5 ml of 0.5 M Na Borate buffer, pH 10.0. The N-methylated phenylethanolamine was extracted into 6 ml toluene-3% isoamyl alcohol, and 3 ml of the latter was counted in a Beckman LS-250 liquid scintillation counter.

DBH Assay. For each assay, female rats, 200–250 g, were smeared to determine estrous state. Rats were lightly etherized, decapitated, and the brains removed and placed in ice cold saline until used (usually within 3 hr).

Tissue blocks were removed and homogenized in 40 volumes of 0.005 M Tris, pH 7.5, containing 0.1% Triton X 100. The samples were spun at 10,000 X G for 10 min, and the supernatant was used for the assay.

The solution for the assay of DBH consisted of 25 volumes of 0.5 M Na fumarate, pH 6.0, 5 volumes of 0.006 M pargyline, 5 volumes of catalase (@ 5.5×10^4 units/ml), 5 volumes of 1.0 M Tris buffer, pH 6.0, 5 volumes of 0.03 M phenylethylamine, and 5 volumes of $CuSO_4$, 150 mM.

The reaction was begun by the addition of 100 μl of the DBH assay medium to 200 μl of rat brain supernatant. The initial phase of the assay was allowed to proceed for 80 minutes, and at the end of this time 200 μl of a mixture containing 5 μl of SAMe, 25 μl of PNMT, and 175 μl of Tris buffer at pH 8.6 was added. According to Molinoff *et al.*, the change in pH of the reaction mixture, from pH 6.0 to pH 8.6 inhibits DBH activity by 95% [17]. The PNMT portion of the assay was allowed to proceed for 20 minutes. At the end of this time, the reaction was stopped by the addition of 0.5 ml of 0.5 M Na Borate buffer, at pH 10.0. The entire enzymatic assay was conducted at 37°C.

The sample tubes were layered with 6 ml of toluene−3 percent isoamyl alcohol, and vortexed virogously. The phases were allowed to separate, and 3 ml of the upper phase were removed, placed in scintillation vials containing 10 ml of 1 g dimethyl POPOP, 4.0 g PPO per liter of toluene.

Blanks employed in this assay were tubes containing tissue heated to 100°C for 5 min. Internal standards were also run with each series of experimental tubes. Internal standards consisted of experimental tubes to which were added 10 μl of 0.02 mg/ml phenylethanolamine just prior to the addition of SAMe and PNMT. Percent of PNMT inhibition could be expressed as the ratio of the observed counts per minute to the expected counts per minute. The use of internal standards permitted the calculation of the absolute activity of DBH, and allowed correction for inhibition of PNMT by experimental samples (Molinoff *et al.* [17]).

RESULTS

Table 1 shows the results of the DBH assay. Activity within each of the hypothalamic brain slices is given for both estrous and diestrous hormone conditions for each of the 6 triplets used. It is important to recall that in this assay the formation of product is directly proportional to the amount of enzyme present when phenylethylamine is used as substrate. DBH activity differences were compared by a 2 X 3 split plot analysis of variance. The Hormone condition main effect was significant, $F(1,4) = 12.09$, $p<0.05$. The location variable was not significantly different, $F(2,8) = 2.16$, $p>0.05$, nor was the location X hormone interaction, $F(2,8) = 2.80$, $p>0.05$.

The anatomical data are summarized below. Anterior slices begin in the lateral septal area slightly anterior to the optic chiasm and extend through the anterior hypothalamus to the initial development of the hippocampus. The area used for the assay lies between König and Klippel Plates 16 b and 30 b. The anterior boundary of the medial hypothalamic slices was the level of the ventro-medial and dorsomedial hypothalamus. Posteriorly, the medial section terminated at the level of the prerubal fields of Forel, dorsal pre-mammilary nucleus and arcuate area. Medial sections were taken between Plates 32 b to 40 b from the

TABLE 1

THE ABSOLUTE LEVEL OF DBH ACTIVITY EXPRESSED AS nm PRODUCED/HR/gm WEIGHT FOR ANTERIOR, MEDIAL AND POSTERIOR HYPOTHALAMIC TISSUE SLICES TAKEN FROM RATS DURING ESTROUS OR DIESTROUS PHASES OF THE RAT ESTROUS CYCLE

Hormone Condition	Section	Activity (nm produced/hr/gm weight)
Estrous	Anterior	1.40
	Medial	1.12
	Posterior	1.71
Estrous	Anterior	3.13
	Medial	3.37
	Posterior	3.80
Estrous	Anterior	3.18
	Medial	5.22
	Posterior	3.71
Diestrous	Anterior	0.72
	Medial	0.40
	Posterior	0.56
Diestrous	Anterior	2.68
	Medial	1.33
	Posterior	1.84
Diestrous	Anterior	1.70
	Medial	1.31
	Posterior	3.25

rat atlas. The most posterior hypothalamic sections extended from mammillary bodies to the supramammillary decussation. Posterior hypothalamic slices used or the DBH assay were taken from the area described between Plates 42 b and 44 b of the König and Klippel atlas.

DISCUSSION

The significant increase in DBH activity measured in our assay system demonstrates that estradiol effects enzyme levels at different stages of the estrous cycle.

As pointed out in the results section, DBH activity for different hypothalamic tissue slices both within each assay and between assay conditions were very consistent for estrous and diestrous hormone conditions. This lack of anatomical specificity is not too surprising, when it is recalled that the slices were not microdissected from different areas, but were taken as a continuous slice of approximately 2–3 mm. In addition, all slices contained varying proportions of both estrogen sensitive receptors as localized by Stumpf [20], and receptor areas innervated by both dorsal and ventral noradrenergic fiber bundles as discussed by Ungerstedt [21]. While more microdissection experiments are necessary to assess regional differences in activity, our data tend to support a more general effect of estrogen on DBH activity, wherever DBH activity is localized.

Norepinephrine levels in the brain are undoubtedly controlled by a complex array of conditions. DBH plays a significant role in this scheme of controls, in that it catalizes the rate limiting step from dopa to norepinephrine [3].

There are at least two primary ways in which DBH activity can be regulated; (1) allosteric or feedback control, or (2) de novo synthesis.

DBH does not seem to be regulated directly by NE concentrations in an *in vitro* system [11], and this is most probably true also *in vivo*, inasmuch as physical data do not suggest allosteric binding sites on purified preparations of enzyme [10].

Both estrogen and neuronal electric activity can increase enzyme activity and the *de novo* synthesis of proteins [4, 5, 10, 11, 17]. Jensen [13] has shown that the synthesis of a wide variety of proteins can be stimulated by estrogen treatment of uterine tissue. Since estrogen receptor sites have been shown by Stumpf in the hypothalamus [20], it is possible that we are demonstrating the direct synthesis of DBH in response to high levels of estrogen. Tyrosine hydroxylase, an enzyme, is thought to catalize the rate limiting step in the biosynthesis of NE. Beattie and his colleagues [4] have recently measured this enzyme in the hypothalamus of rats after ovariectomy and under conditions of estrogen replacement therapy. Although tyrosine hydroxylase in rat hypothalamus increased after ovariectomy, replacement therapy (1 mg/day estradiol, for 7 days) further increased tyrosine hydroxylase by 30 percent.

The possibility exists, on the other hand, that the increase in DBH activity could be due to *de novo* synthesis in response to an increase in nerve cell activity, induced by estrogen. Zolovic *et al.* [22] reported that a major NE and dopamine catabolizing enzyme monoamine oxidase increased markedly during estrous. These authors suggested that nerve cell activity was the cause for this increase. Molinoff [16] noted that after reserpine depletion of NE in the rat stellate ganglion preparation, DBH increased dramatically. They interpreted this finding to mean that as NE is depleted, there is an increase in DBH activity, due to *de novo* synthesis.

Along these same lines, it is interesting to note that estrogen incubated in a synaptosomal preparation decreased re-uptake of NE [12]. If this system parallels the *in vivo* effect of estrogen, a NE depletion would occur. This, in turn, would increase the activities of the intermediate enzymes, and at the same time tend to deplete the vesicular bound NE. Thus, the mechanisms of *de novo* synthesis and membrane re-uptake inhibition could account for the data presented here and elsewhere [6,16], which shows an increased DBH activity, and a substantially low NE level.

It is impossible to determine from these data the exact mechanism mediating the increase in DBH demonstrated in this experiment. However, both mechanisms are fundamentally consistent with the original hypothesis that estrogen profoundly effects the DBH activity in the rat hypothalamus during the estrous cycle.

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