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Studies on Brain Monoamine Neurotransmitters in Mice After Prenatal Exposure to Barbiturate

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YANAI, J., P. Y. SZE, C. ISER AND E. MELAMED. *Studies on brain monoamine neurotransmitters in mice after prenatal exposure to barbiturate.* PHARMACOL BIOCHEM BEHAV **23**(2) 215–219, 1985.—Pregnant HS/Ibg mice received 3 g/kg phenobarbital in their milled food; control dams received unadulterated milled food. Their offspring were tested on ages 22 and 50 days for norepinephrine (NE) and dopamine (DA) levels and DA turnover in the hypothalamus and striatum. Additional groups were tested on days 8, 22, and 50 for brain stem tryptophan hydroxylase (TPH) activity. Hypothalamic DA level among offspring with prenatal PhB exposure (B offspring) was 37% below control level on age 22 days and 61% below control on day 50 ($p<0.001$). Hypothalamic NE level of B offspring was reduced on age 50 days (52%, $p<0.05$) and not on day 22. DOPAC level and DA turnover did not differ among B and control groups. Prenatal exposure to PhB did not have a significant effect on TPH activity. The changes in catecholamines may mediate, at least in part, some of the early barbiturate induced neuromorphological and behavioral changes previously found in our laboratory.

Dopamine Hypothalamus Mice Norepinephrine Phenobarbital Prenatal treatment Tryptophan hydroxylase Striatum

SEVERAL neurotransmitter systems are affected by acute or chronic administration of barbiturates to adult animals, possibly in relation to their involvement in CNS adaptation to the drug [8,18].

After prenatal exposure to phenobarbital, mice had at age 21 days alterations in whole brain levels of dopamine and norepinephrine and in uptake of dopamine, norepinephrine and serotonin [15]. These changes were only transient as they all disappeared by age 50 days [16]. On the postsynaptic level, binding studies suggested long lasting alteration in dopamine receptors after prenatal exposure to barbiturate [10]. After early exposure to barbiturate, animals exhibited changes in various behaviors including behaviors related to brain monoamines (see [23] for review). Consequently, in the present experiment we have studied alterations in level and turnover of striatal and hypothalamic catecholamines in mice who were exposed prenatally to barbiturate. Turnover was studied since it serves as an indication for the activity of the respective neurotransmitter system. Discrete brain area, the hypothalamus and the striatum, were chosen because previous studies demonstrated that early exposure to PhB induced behavioral and functional changes which are related to these areas [5, 6, 11, 24, 29]. The metabolic activity of serotonin was evaluated in these offspring by studying the activity of brainstem tryptophan hydroxylase, the rate limiting enzyme in serotonin metabolism.

METHOD

The genetically heterogeneous HS/Ibg mice [14] were employed. The method of drug administration was described before [26]. Briefly adult mice used as parents were housed in mating groups of one male and four females. Their offspring (the subjects of the experiments) received phenobarbital prenatally. Thus, female parent mice were checked daily at 08.00 hr and those that conceived, as evidenced by the existence of a vaginal plug, were separated from the males and housed with other pregnant females. On gestation day 9 (GD 9; the day in which the plug was found is considered GD 1), the females were housed in individual cages. Treated females then received milled mouse food containing 3 g/kg phenobarbital in acid form (their only food source) and water, both available ad lib. Control females received milled food and water. There was no need for pair fed control since barbiturates have no caloric value, and our preliminary studies showed that treated females consumed normal amounts of food. Drug administration continued until GD 18 when the phenobarbital and control diets were replaced with regular mouse pellets. Our previous studies have shown that after prolonged exposure to PhB, pregnant females clear all the PhB within four hours. Yet, special effort was made to control for the possible carryover effect of PhB on maternal milk production or behavior with appropriate fostering [23].

TABLE 1

LONG-TERM EFFECT OF PRENATAL EXPOSURE TO PHENOBARBITAL ON DA AND NE LEVELS IN THE HYPOTHALAMUS AND STRIATUM; RESULTS ARE EXPRESSED IN mm/mg PROTEIN

Prenatal treatment	Hypothalamus		Striatum	
	day 22	50	22	50
DA				
Control	9.23 ± 0.60 (7)	14.64 ± 1.30 (6)	85.52 ± 4.82 (9)	94.08 ± 3.13 (7)
Phenobarbital	5.85 ± 0.46‡ (8)	5.68 ± 0.45‡ (4)	66.63 ± 7.98 (10)	96.53 ± 4.52 (7)
NE				
Control	32.65 ± 3.80 (7)	67.23 ± 11.06 (8)	39.90 ± 4.59 (9)	23.29 ± 3.50 (8)
Phenobarbital	35.53 ± 4.47 (8)	32.53 ± 3.91* (5)	27.81 ± 4.58 (10)	27.38 ± 9.47 (6)

( ) Sample size.
Numbers are mean ± SEM.
*$p<0.05$, †$p<0.01$, ‡$p<0.001$ for the difference from control levels (ANOVA).

Thus, several phenobarbital exposed and control neonates were fostered by control dames within 24 hr after delivery. Because analysis revealed great similarity between the fostered and the nonfostered offspring in all the variables studied, the results of the fostered and the nonfostered offspring were pooled [23]. Following delivery, the barbiturate-exposed offspring (B offspring) and control offspring were maintained with their mothers under standard laboratory conditions and received no further drug treatment. Unless used for biochemical assays in earlier age, the offspring were weaned on day 25 and segregated according to gender.

Tryptophan hydroxylase assays were conducted at ages 8, 22 and 50 days; catecholamines assays were conducted on days 22 and 50.

Tryptophan Hydroxylase Assay

Brainstems were isolated on an ice-cold plate by dissecting out the cerebrum, cerebellum and the olfactory bulbs. Two brainstems were pooled for each sample and were homogenized in 4 vol of 10 mM Tris acetate, pH 7.4. The homogenate was centrifuged at 100,000 g for 30 min. The freshly prepared supernatant was used. The enzyme activity was assayed by the procedure of Ichiyama *et al.* [9] as described by Yanai and Sze [25]. The standard mixture (700 μl) contained 80 mM Tris acetate, pH 7.4; 10 μM L-[1-^{14}C] tryptophan (0.06 μCi; New England Nuclear, Boston, MA); 1.0 mM 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine (Calbiochem, Los Angeles, CA); 2 mM dithiothreitol; 1000 units catalase; 5–10 units aromatic amino acid decarboxylase; 0.7 mM pyridoxal-5′-phosphate; and the 100,000 g supernatant equivalent 5 to 1.5–3.0 mg protein. After incubation at 37°C for 20 min, the $^{14}CO_2$ produced was absorbed in hyamine hydroxide and measured for radioactivity. Protein determination in this assay as well as in catecholamine assays were made using the method of Lowry *et al.* [16]. Tryptophan hydroxylase activity was expressed as cpm $^{14}CO_2$ formed per hr per mg protein.

Catecholamines Assay

Striata and hypothalami were dissected on a ice-cold plate and weighed and frozen at −70°C. Catecholamines were assayed as described by Felice *et al.* [1] and Hefti *et al.* [7] with a few modifications. The tissues were weighed and homogenized by a sonicator in 50 vol (striatum) or 35 vol (hypothalamus) of ice-cold distilled water containing 0.1 M perchloric acid and 0.2 mg/ml sodium metabisulfite. The homogenate was centrifuged (12,000×g for 5 min) and 100 μl of the supernatant fluid were taken for analysis. Catecholamines and DOPAC were isolated using miniature alumina columns as described by Ganchy *et al.* [3]. Small pipet tips were stoppered with glass wool and filled with 20–25 mg alumina (Woelm, activity grade I, activated by boiling in 2 M HCl). Together with adaptors (cut-off larger pipet tips), they were placed in reagent tubes (12×75 mm) and run in a table centrifuge (TJ-6; Beckman Instruments, Palo Alto, CA). The alumina columns were equilibrated with 200 μl 0.05 M Tris HCl buffer (pH 8.6) (100×g, for 2 min). Then 190 μl of a mixture containing the sample (100 μl) and 100 μl of 1 M Tris HCl buffer (pH 8.6, containing 1 mg/ml dithiothreitol) and 10 μl of 0.1 M perchloric acid with 10 ng 3,4-dihydroxybenzylamine (internal standard) were pipetted into the columns, and centrifuged at 100×g for 3 min. The columns were then washed with 200 μl H_2O (100×g for 2 min), transferred into new test tubes and the catecholamines and DOPAC subsequently eluted with 150 μl of 0.1 M phosphoric acid (400×g for 5 min). The eluate was analyzed by reverse-phase high-performance liquid chromatography with electrochemical detection, based on the method described by Felice *et al.* [1]. The column used was C_{18} reverse-phase (3.9×300 mm, μBondapak; Waters) and the electrode was glassy carbon (Bioanalytical Systems). The potential was set at +0.7 V. A

TABLE 2

LONG-TERM EFFECT OF PRENATAL EXPOSURE TO PHENOBARBITAL ON DOPAC LEVELS (ng/mg PROTEIN) AND DA TURNOVER (DOPAC/DA) IN THE HYPOTHALAMUS AND STRIATUM

Prenatal treatment	Hypothalamus		Striatum	
	day 22	50	22	50
DOPAC Level				
Control	6.92 ± 1.09 (7)	4.14 ± 0.72 (5)	22.9 ± 2.82 (9)	13.93 ± 2.86 (8)
Phenobarbital	7.23 ± 0.84 (8)	2.79 ± 0.89 (5)	23.90 ± 3.23 (11)	10.91 ± 1.03 (7)
DA Turnover				
Control	0.82 ± 0.09 (6)	0.28 ± 0.03 (5)	0.28 ± 0.03 (9)	0.17 ± 0.03 (9)
Phenobarbital	1.09 ± 0.10 (7)	0.48 ± 0.19 (5)	0.34 ± 0.03 (11)	0.13 ± 0.02 (7)

( ) Sample size.
Numbers are mean ± SEM.
There were no significant differences from control levels (ANOVA).

0.1 M sodium phosphate buffer (pH 3.0), containing 0.6 mM octyl sodium sulfate as ion-pair reagent, served as mobile phase.

All data were analyzed with two way Analysis of Variance (ANOVA, treatment by age) [22].

RESULTS

Since the daily consumption of food by mice is about 20% of body weight, the pregnant females consumed about 600 mg/kg PhB daily. Blood phenobarbital levels in the barbiturate consuming pregnant mice and their fetuses was monitored [30]. Briefly, blood phenobarbital level in the pregnant mice averaged 113±12 μg/ml during most of the phenobarbital feeding period. The phenobarbital levels of the fetuses were similar to that of the mothers.

DA Levels (Table 1)

Hypothalamic DA level among B offspring was 37% below control level ($p<0.001$) on 22 days and 61% below control on day 50 ($F(1,20)=44.1$, $p<0.001$). There was no reduction from control level in striatal DA among B offspring.

NE Levels (Table 1)

Hypothalamic NE level among B offspring was below control level only on age 50 days (52%, $F(1,24)=4.4$, $p<0.05$) and not on day 22. Striatal NE levels did not differ between treatment groups.

DOPAC Levels (Table 2)

There were no statistically significant differences in DOPAC level between B and control offspring in any of the two brain parts or ages studied.

TABLE 3

BRAINSTEM TRYPTOPHAN HYDROXYLASE ACTIVITY (cpm/hr/mg PROTEIN) AFTER PRENATAL EXPOSURE TO PHENOBARBITAL

Prenatal treatment	Age (days)		
	14	22	50
Control	1638 ± 252 (12)	2152 ± 124 (26)	2666 ± 42 (15)
Phenobarbital	1608 ± 214 (12)	2036 ± 134 (24)	2524 ± 206 (15)

( ) Number of samples; every sample was prepared from two brains.
There were no significant differences from control levels (ANOVA).

DOPAC/DA Ratio (Table 2)

This ratio did not differ significantly between B and control in both brain regions and ages studied.

Activity of TPH (Table 3)

There were no significant differences in the activity of TPH between control and B offspring in all ages studied.

Hypothalamic and Striatal Weights (Table 4) and Protein

Although the weight of the striata and hypothalami were somewhat smaller in B groups than in controls, the differences never reached statistical significance. The values of supernatant protein among control groups were: hypothalamus age 22, 67.32 μg/mg tissue, age 50, 97.62 μg/mg. The respective scores of the striatum were 88.87 and 159.08. The respective values of B groups were very similar to controls.

TABLE 4

LONG-TERM EFFECT OF PRENATAL EXPOSURE TO PHENOBARBITAL ON THE WEIGHT OF THE HYPOTHALAMUS AND THE STRIATUM; RESULTS ARE EXPRESSED IN mg ± SEM

Prenatal treatment	Hypothalamus		Striatum	
	day 22	50	22	50
Control	7.72 ± 0.23 (7)	8.92 ± 0.84 (7)	10.28 ± 0.59 (9)	11.95 ± 1.35 (8)
Phenobarbital	7.09 ± 0.87 (8)	8.95 ± 0.81 (4)	9.51 ± 0.47 (10)	10.05 ± 1.10 (7)

( ) Sample size.
There were no significant differences from control levels (ANOVA).

DISCUSSION

Mice which were exposed to phenobarbital during prenatal development had a long term reduction in the level of hypothalamic NE and DA while DA turnover remained unchanged. The effect did not occur in all areas of the brain as striatal NE and DA remained unchanged.

We can not offer an explanation of the fact that the changes in the neurotransmitter levels occurred in the hypothalamus and not in the striatum. This phenomenon is more expected for NE which is not a major striatal neurotransmitter but not for DA which prevails in the striatum. Nevertheless, other DA changes were shown in the striatum as demonstrated by the increase in the number (Bmax) of the postsynaptic DA receptors [10].

The changes in hypothalamic NE after early exposure to PhB were demonstrated on age 50 but not 22 days. This delayed effect shows that the expression of the neurochemical changes is dependent on the stage of development. The present finding is consistent with previous reports on the significance of age at time of testing in neurobehavioral teratology research [20].

The changes in the neurotransmitter level were not accompanied by changes in turnover. However, it should be remembered that the neurochemical changes induced by early PhB administration may be unique as they could be related to neuronal deficits in the respective area [29].

Tryptophan hydroxylase is the rate limiting enzyme in serotonin synthesis in brain. Thus, it may serve as an indicator for the activity of the serotonergic system. Prenatal exposure to phenobarbital did not have long-term effect on TPH activity. Apparently, it has no effect on the ontogenesis of serotonergic activity at least in term of the enzyme activity, since differences did not occur even on age 8 days when the serotonergic system was still developing. It appears, therefore, that CNS alterations induced by prenatal barbiturate administration are specific to both the neurotransmitter system (catecholamines) and the CNS location (hypothalamus) studied.

Previous studies on the state of monoamine neurotransmitters after prenatal exposure to phenobarbital employed whole mouse brains [15,16]. Prenatally treated mice had reductions in whole brain levels of NE and DA but not serotonin and increases in whole brain uptake of all the three neurotransmitters [15]. It is important to note that these changes were only short-term as they could be demonstrated on age 21 days [15], but were abolished by age 50 days [16]. The present results on the hypothalamus are similar to the findings of the previous studies in that the levels of NE and DA were reduced after prenatal phenobarbital exposure. However, there was a basic difference between the two studies: while the previous study showed only transient barbiturate induced changes in catecholamines level, the reductions in hypothalamic NE and DA levels found in the present study were *long-term*. Previous studies demonstrated a transient reduction in uptake of serotonin, NE and DA [15]. The present study did not reveal differences between treated and control offspring for the turnover of hypothalamic DA and the activity of TPH at several ages. Although the measures differ from uptake, all these measures may serve as indicators for synaptic activity. Our study differs from the previous study in several basic experimental conditions such as dose and method and period of drug administration. Moreover, we studied specific brain areas rather than the whole brain. Our data support the notion that the NE and DA systems in the hypothalamus are most sensitive to long-term alteration by prenatal barbiturate exposure.

The present presynaptic changes in neurotransmitters induced by prenatal exposure to phenobarbital together with the postsynaptic DA changes found previously in the striatum [10], may be related to concomitant neuromorphological and behavioral changes published previously. It has been established that at early developmental stages, before activation of neurotransmission, neurotransmitters regulate neuromorphological development in their respective regions [12]. Some of the behaviors that were affected by prenatal exposure are known to be regulated, at least in part, by the neurotransmitter systems that were affected in the present and the previous [10] experiments by prenatal exposure to phenobarbital. For example, learning which is impaired by prenatal phenobarbital administration [17] may be regulated by both the NE [4] and DA [19] systems. Similarly, prenatal phenobarbital exposure induced in mice a long-term resistance to pentobarbital and ethanol narcosis and an accelerated acquisition of barbiturate tolerance [26,27]. It was suggested that the NE [21] and DA [25] systems are involved in these responses. Another example is motor activity which was affected by prenatal phenobarbital exposure [17] and is

regulated at least in part by NE and DA [7]. Similar arguments can be made for the relationship between the hypothalamic neurotransmitters (mainly DA) and the prenatal PhB induced changes in reproductive physiology [5,6] and temperature control [11,24]. Thus, further studies which will correlate early barbiturate induced neurochemical changes with behavioral and neuromorphological alterations may explain the mechanism by which early barbiturate preturb behavioral and neuromorphological development

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