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Prenatal Phenobarbital Treatment and Temperature-Controlling Dopamine Receptors

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KUPRYS, R AND B TABAKOFF *Prenatal phenobarbital treatment and temperature-controlling dopamine receptors* PHARMACOL BIOCHEM BEHAV **18**(3) 401–406, 1983 —Pregnant mice were fed a phenobarbital-containing diet on days nine through 18 of pregnancy Following parturition, the offspring of such animals were allowed to reach adulthood and then were tested for their response to an acute injection of apomorphine Male offspring were less sensitive, while female offspring were more sensitive than matched controls to apomorphine-induced hypothermia The witnessed differences in apomorphine-induced hypothermia could not be attributed to differences in brain apomorphine levels, alterations in the thermoregulation following non-drug challenges to the mouse's thermoregulatory ability, or changes in α-adrenergic receptor function Our results suggest that prenatal phenobarbital administration produces changes in the function of dopamine receptors which regulate body temperature, and that the prenatally-induced changes last well into adulthood

Apomorphine Body temperature Dopamine receptors Hypothalamus Prenatal phenobarbital

ADMINISTRATION of phenobarbital (PB) to pregnant rodents produces in the offspring a number of developmental abnormalities which can be demonstrated, even when the animals reach adulthood As adults, offspring of rodents exposed during pregnancy to PB exhibit changes in brain morphology [28], circulating hormone levels [10, 11, 12, 17], performance in certain behavioral paradigms [1, 13, 15, 18, 19], open field behavior [18, 20, 21, 30], and sexual function [10–12] These long-term effects may be important clinically, since PB is frequently used in humans during pregnancy for the prevention of epileptic seizures in the mother [14], and the prevention and/or treatment of hyperbilirubinemia and kernicterus in the neonate [7, 22, 24 26 27] Despite the use of PB in pregnant women and newborns, the neuropharmacological concomitants of the anatomical and behavioral alterations found with prenatal PB administration have not been investigated

Preliminary evidence, as reported by Tabakoff *et al* [25], suggests that prenatal PB administration alters central dopamine (DA) receptor sensitivity In the present study, apomorphine was employed to investigate further the effects of PB administration to the mother upon the function of postsynaptic DA receptors in her offspring when they are adults Changes in body temperature were studied after injection of apomorphine Apomorphine-induced hypothermia is believed to be mediated via hypothalamic DA receptors [2, 5, 6, 8]

METHOD

Subjects

Subjects were heterogeneous stock (HS) adult male and female mice The HS mice obtained from our breeding colony are an outbred line developed by McClearn *et al* [16] from a cross of eight inbred strains The mice used in the present study were between 46–96 days of age, and were the offspring of dams which had been fed PB on days 9–18 of pregnancy

In Utero Phenobarbital Treatment

Two to three nulliparous or uniparous female mice were housed with one male, and were examined daily for the presence of vaginal plugs Those mice having a vaginal plug were assumed to have been inseminated, and if later found to be pregnant, day one of pregnancy was defined as the day on which a vaginal plug was found Females inseminated on the same day were group-housed and fed Purina Rat Chow pellets and water ad lib

Treatment of pregnant mice with PB was conducted by a modification of the method of Belknap *et al* [3] On day nine of pregnancy, the female mice were housed individually, and were fed a diet of ground Purina lab chow containing 2, 2 5 or 3 mg PB free acid per gram of the ground chow The mice were allowed ad lib access to water On day 18 of pregnancy, the PB diet was removed and replaced with Purina Rat Chow

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pellets Offspring of these dams comprised the PB groups, and these offspring will be referred to as the PB-2, PB-2 5 and PB-3 groups, in reference to the amount of PB that was contained per gram of diet which the mother consumed Two types of control groups were included in our experiments The first of these groups will be referred to as the C group, and these animals were offspring of dams who were fed plain ground chow ad lib The second type of control group included offspring of pregnant mice which were daily fed plain ground chow in the amount of diet consumed by dams of each of the PB groups Offspring of the pair-fed dams comprised the pair-fed control (PFC) groups As an additional control for malnutrition in the PB-3 group, the dietary intake of a group of female mice was restricted to 3 g of plain ground chow daily on days 9–18 of pregnancy Offspring of these dams are designated as the R-3 group Additional details of such feeding regimens are continued in our previous publications [28,29]

Maternal body weight was recorded on the first and last day of PB treatment, days nine and 18 of pregnancy, respectively Maternal food consumption was monitored daily during the period of treatment of animals with PB After delivery, the body weight of the pups was recorded

Following parturition, the pups were housed with their natural mothers, or were cross-fostered with dams other than those of their own treatment group until they were 22 days old, at which time they were weaned and separated according to sex The weanling pups were group-housed and fed Purina Rat Chow pellets and water ad lib In the experiments described below, pups from an average of three different litters were used to comprise each control or experimental group

TABLE 1

FOOD INTAKE OF PREGNANT MICE

Group	n	Food Intake (g)*
C	5	8 9 ± 1 36
PB-2	17	5 9 ± 0 95
PB-2 5	10	5 6 ± 0 93
PB-3	7	4 0 ± 1 04

*Values represent mean±SD of the average daily amounts of chow consumed on days 9–18 of pregnancy

During this same period, the body weight of C dams increased 62% (n=5) while PB-2 dams (n=17) showed a 49% increase, PB-2 5 dams (n=11) a 38% increase, and PB-3 dams (n=9) a 23% increase in body weight gain The body weight gain of PFC dams closely paralleled that of their respective PB groups 40% increase for PFC-2 dams (n=26), 33% increase for PFC-2 5 dams (n=10) and a 19% increase for PFC-3 dams (n=6)

Measurement of Body Temperature

During the test period, mice were individually housed at an ambient temperature of 22–24°C The time-course of drug-induced hypothermia in control and experimental mice was monitored with a Telethermometer attached to a lubricated termistor probe (Yellow Springs, Model YSI-402), inserted 27 mm into the rectum Temperature readings were taken before and at various time intervals after drug injection Apomorphine hydrochloride (Sigma) was prepared in a 0 9% saline-0 1% ascorbic acid vehicle and was administered intraperitoneally IP in a volume of 0 1 ml/10 g of body weight Clonidine hydrochloride (Boeringer Ingelheim, Ltd) was dissolved in saline and administered at a dose of 0 5 mg/kg in volumes equivalent to those used for injections of apomorphine The injection of the appropriate vehicle served as a means of determining the effects of the injection per se The ability of PB-3 offspring, as compared to C offspring, to maintain body temperature in the cold was measured at 15, 40 and 65 minutes after placing the mice in a cold (5°C) environment

Measurement of Apomorphine Levels

Whole brain levels of apomorphine were determined in male and female mice, using a modification of the spectrofluorimetric assay of Butterworth and Barbeau [4] Mice were injected, IP, with 3 mg/kg of apomorphine HCl and decapitated at 15 or 30 minutes after the injection These time points were chosen because the apomorphine-induced behavioral changes are maximal between these times Brain tissue was obtained and stored frozen at −70°C until the time of assay In preliminary experiments, it was found that freezing did not affect brain apomorphine levels

Pooled whole brains from two mice were homogenized in 2 ml of 0 01 N hydrochloric acid containing 0 1% ascorbic acid and 0 6 ml of ethyl acetate, using a Brinkmann Polytron Two ml of 0 2 N hydrochloric acid containing 0 1% ascorbic acid, followed by 0 3 g of sodium chloride, were then added to each tube The contents of the tubes were mixed and then centrifuged in a clinical centrifuge The aqueous phase was washed three times with 4 ml of ethyl acetate The organic phase was discarded following each wash After the final wash, the pH of the aqueous phase was adjusted in the range of 6 5–7 5 with solid sodium bicarbonate, and the apomorphine was extracted into 3 ml of ethyl acetate Tissue blanks obtained from untreated mice wre prepared similarly

Standards of apomorphine HCl (0–5 μg) were prepared on the day of the assay in 0 01 N hydrochloric acid containing 0 1% ascorbic acid Following the addition of 0 3 g of sodium chloride to each standard-containing tube and adjustment of the pH of the aqueous solution to 6 5–7 5 with solid sodium bicarbonate, the apomorphine HCl was extracted into 3 ml of ethyl acetate The amount of apomorphine HCl in the organic phase was measured in a Perkin-Elmer spectofluorimeter (activation wavelength of 285λ and emission wavelength of 360λ) Apomorphine values presented in Table 5 have been corrected for recovery

Drug-induced changes in body temperature of C, PFC and PB mice were compared using an analysis of variance with repeated measures BMD computer program Analysis of other data was done using a two-tailed Student's t-test All results are expressed as mean±S D

RESULTS

In Utero Phenobarbital Treatment

The PB treatment produced a dose-dependent decrease in maternal food intake (Table 1) Maternal body weight gain was also decreased in a dose-dependent manner by the PB treatment (see Table 1) The body weight gain of PFC dams closely paralleled that of their respective PB groups

Although the dams were not monitored directly for signs of withdrawal from the PB treatment, the signs for the pres-

TABLE 2

NEONATAL BODY WEIGHTS

Group	n*	Number of Pups/Litter	Body Weight (g)†
C	10	8 ± 1 6	1 5 ± 0 17
PFC-2	15	9 ± 1 3	1 5 ± 0 11
PB-2	17	7 ± 2 7	1 3 ± 0 19§
PFC-2 5	5	8 ± 2 6	1 4 ± 0 13
PB-2 5	7	6 ± 2 4	1 3 ± 0 15‡
PFC-3	5	6 ± 2 9	1 3 ± 0 33
PB-3	4	4 ± 1 8¶	1 1 ± 0 10#

*Values represent the number of litters
†Values represent mean±S D of the average weight of pups of each litter
‡$p<0$ 05, when compared to the C group
§$p<0$ 02, when compared to the C group
¶$p<0$ 01, when compared to the C group
#$p<0$ 001 when compared to the C group

ence of a PB withdrawal syndrome in the pregnant mice appeared to be minimal During the course of these and other experiments, the incidence of maternal mortality after day 18 of pregnancy was 8% for 465 PB-3 dams and 4% for 56 PB-2 dams, and the length of gestation, which was 20–21 days, did not differ among the C, PFC and PB groups

The body weight of newborn pups in each of the PB groups was significantly less than that of newborn C pups (Table 2) This was the case, despite the absence of a significant difference in the number of pups per litter between the experimental and C groups, with the exception of the PB-3 group, which had significantly fewer pups per litter than the C group As a result of this finding, in each of the behavioral and biochemical experiments described below, the groups were matched for body weight of the experimental subjects

Body Temperature Following Injection of Apomorphine

The time-course of the hypothermic effect produced by apomorphine treatment in adult C and PB-3 male mice is presented in Fig 1 (A-D) The pre-injection baseline body temperature did not differ between these mice A dose-dependent decrease in body temperature was present in both groups, but the extent of this hypothermia following apomorphine treatment differed between C and PB-3 mice

The lowest dose of apomorphine (0 3 mg/kg) was not sufficient to distinguish differences between the C and PB-3 groups (Fig 1A) However, following injection of higher doses of apomorphine (0 4 mg/kg, $F(1,10)=7$ 85, $p<0$ 025, or 0 5 mg/kg, $F(1,21)=6$ 17, $p<0$ 025), PB-3 mice were significantly less sensitive to the hypothermic effects of apomorphine when compared to C mice (Fig 1B and 1C) The hypothermia (both degree and time-course) measured after the highest dose of apomorphine (1 mg/kg) did not differ between C and PB-3 mice (Fig 1D)

The time-course of the hypothermic effect produced by apomorphine treatment in adult C and PB-3 female mice is presented in Fig 2 (AD) The pre-injection baseline body temperature did not differ between these mice The PB3 female mice showed a greater decrease in body temperature after injection of 0 3 or 0 5 mg/kg of apomorphine than their

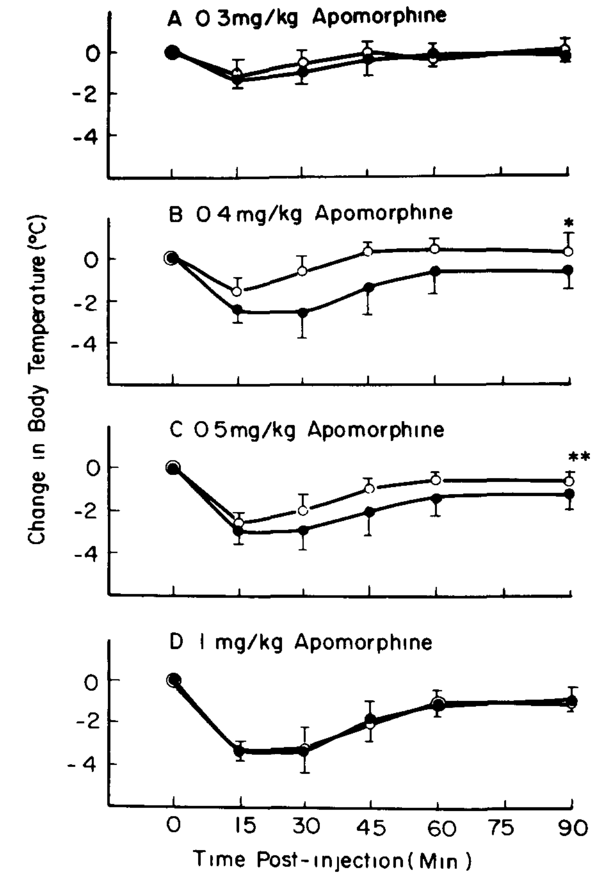

FIG 1 Hypothermic effect of various doses of apomorphine in C (●–●) and PB-3 (○–○) male mice Both are presented as mean±S D of change in body temperature The respective body temperatures (°C) for C and PB-3 mice at zero time were Panel A—36 4±0 64, n=7, 36 6±0 51, n=7, Panel B—36 7±0 66, n=6, 36 6±0 67, n=6, Panel C—37 2±0 34, n=12, 36 9±0 30, n=11, Panel D—38 0±0 40, n=11, 38 0±0 32, n=11 The range for mean body weights for the C and PB-3 groups was 23–26 g *$p<0$ 025, $F(1,10)=7$ 85, when compared to the C group **$p<0$ 025, $F(1,21)=6$ 17, when compared to the C group

weight-matched controls (Fig 2A and 2B) Although these differences were not statistically significant, they were in a direction opposite to those seen between male C and male PB-3 mice Treatment with higher doses of apomorphine (1 or 3 mg/kg) did not demonstrate differences in hypothermia between female C and PB-3 mice (Fig 2C and 2D)

When male and female mice of the PB-2 5 and PB-2 groups were tested with the various doses of apomorphine, no differences in the hypothermic response were found between male C, PFC-2 5, PFC-2 and PB-2 5 and PB-2 groups However, female mice of the PB-2 5 group exhibited a greater sensitivity to hypothermia induced by apomorphine (0 5 mg/kg), compared to the female C and PFC-2 5 controls (Fig 3A) These differences were also in the same direction and statistically significant when female mice of the C, PFC-2 and PB-2 groups were compared (Fig 3B)

Body Temperature Following Cold Exposure

The time-course of the hypothermic effect produced by

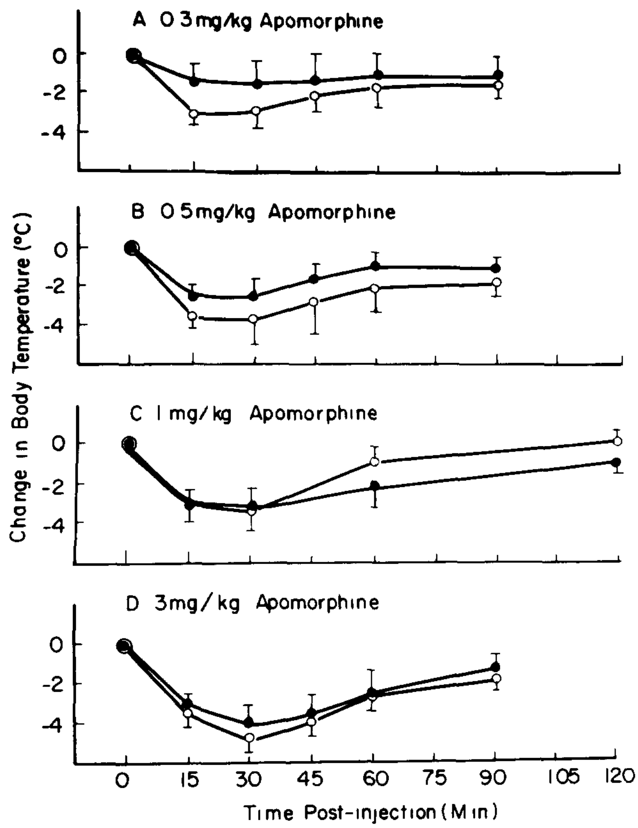

FIG 2 Hypothermic effect of various doses of apomorphine in C (●–●) and PB-3 (○–○) female mice Data are presented as mean±S D of change in body temperature The respective body temperatures (°C) for C and PB-3 mice at zero time were Panel A—36 2±0 86, n=7, 36 8±0 59 n=4, Panel B—36 5±0 50 n=6, 36 4±0 36, n=6 Panel C—37 1±0 52, n=7, 37 0±0 45, n=4, Panel D—35 9±0 62, n=6 36 4±0 32, n=6 The range for mean body weights for the C groups was 20–24 g and 21–23 g for the PB-3 groups

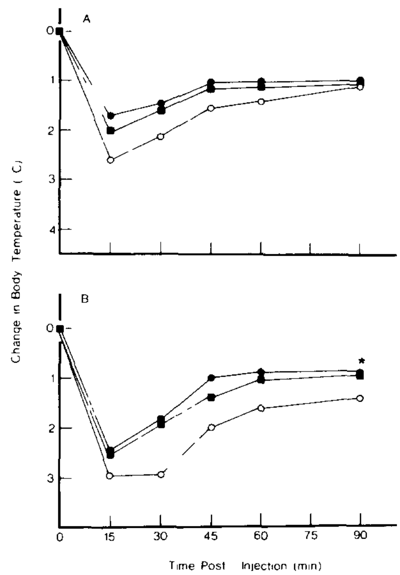

FIG 3 Hypothermic effect of 0 5 mg/kg of apomorphine in C (●–), PFC-2 5 (■–), PB-2 5 (○–○) (Panel A) and C (●–●), PFC-2 (■–■), and PB-2 (○–○) (Panel B) female mice Data are presented as mean±S D of change in body temperature The respective body temperatures (°C) for C, PFC and PB mice at zero time were Panel A—38 1±0 2, n=6, 38 3±0 2, n=8, 38 3±0 3, n=8, Panel B—37 8±0 3, n=9, 37 5±1 1, n=9, 37 4±0 8, n=9 The range for mean body weights for C, PFC and PB groups was 20–24 g *$p<0\ 089$, $F(2,24)=2\ 68$ among groups in Panel B

exposure to the cold in adult C and PB-3 mice is presented in Table 3 Following exposure to the cold, the body temperature of both groups of mice was significantly decreased throughout the test period No significant differences in cold-induced hypothermia, however, could be demonstrated between the two groups of mice

TABLE 3

HYPOTHERMIC RESPONSE FOLLOWING EXPOSURE TO A COLD (5°C) ENVIRONMENT

	Change in Body Temperature (°C)		
Group	15 min	40 min	65 min
C	1 5 ± 0 90*	1 8 ± 0 69	1 5 ± 1 04
PB-3	1 3 ± 0 80	1 4 ± 0 98	1 4 ± 1 12

C mice and PB-3 male mice were placed at zero time into a cold room at 5°C

*Values represent mean±SD and are significantly decreased at all time points ($p<0\ 01$) from their respective zero time values, but the decrease does not differ between C and PB-3 groups Body temperatures for C and PB-3 mice at zero time were 37 0±0 47 (n=7) and 36 5±0 64 (n=7), respectively

Body Temperature Following Injection of Clonidine

To assess the effect of non-dopaminergic drugs, the hypothermic response of C and PB-3 mice was also examined following treatment with clonidine (0 5 mg/kg) The time-course of clonidine-induced hypothermia is presented in Table 4 The hypothermia produced by 0 5 mg/kg of clonidine was greater and more prolonged than that produced by 0 5 mg/kg of apomorphine in both C and PB-3 groups, however, there was no significant difference in hypothermic response between the C and PB-3 mice

Brain Apomorphine Levels

The brain weights were found to be lower ($p<0\ 05$) for PB-3 female mice compared to C female mice (PB-3 females=830±42 6 mg, n=15, and, C females=858±39 5 mg, n=24) Although male PB-3 mice also had lower mean brain weights compared to male C mice, these differences were not statistically significant (PB-3 males=808±40 7 mg, n=10, C males=842±52 1 mg, n=10) Whole brain levels of apomorphine in C and PB-3 male and female mice are presented in Table 5 Fifteen minutes after injection, the levels of apomorphine in brains of PB-3 female mice were not statistically different from those of C female mice, and no

TABLE 4

CLONIDINE-INDUCED HYPOTHERMIA

	Change in Body Temperature (°C)			
Group	15 min	30 min	60 min	120 min
C	2.3 ± 0.29*	3.7 ± 0.53	4.4 ± 0.81	3.5 ± 1.07
PB-3	2.5 ± 0.40	4.0 ± 0.71	4.8 ± 0.70	4.0 ± 0.82

C and PB-3 male mice were injected at zero time with clonidine HCl (0.5 mg/kg, IP).

*Values represent mean±S.D. Body temperatures for C and PB-3 mice at zero time were 37.2 ± 0.39 (n=7) and 37.7 ± 0.59 (n=4) respectively.

TABLE 5

WHOLE BRAIN APOMORPHINE LEVELS FOLLOWING INJECTION OF APOMORPHINE

	Apomorphine Concentration (μg/g)*			
Group	15 min	N	30 min	N
Female				
C	0.57 ± 0.30	(15)	0.48 ± 0.41	(9)
PB-3	0.61 ± 0.45	(10)	0.33 ± 0.12	(5)
Male				
C	0.95 ± 0.29	(5)	0.53 ± 0.21	(5)
PB-3	0.94 ± 0.18	(5)	0.46 ± 0.06	(5)

C and PB-3 mice were injected at zero time with apomorphine HCl (3 mg/kg, IP).

*Values represent mean±S.D.

statistically significant differences in brain apomorphine levels were found between PB-3 and C male mice.

By 30 minutes after drug injection, the levels of apomorphine were decreased from levels present at 15 minutes post-injection in PB-3 and C mice. The drug levels at the 30-minute time point were not significantly different between the PB-3 and C mice of either sex.

DISCUSSION

The results of this study provide evidence for the presence of long-term changes produced by prenatal PB treatment in sensitivity or function of certain central nervous system (CNS) DA receptors. Adult male offspring of mice which had been treated with 3 mg of PB/gm food on days 9–18 of pregnancy were found to be less sensitive than offspring of control mice to the hypothermic effects of an acute injection of apomorphine (Fig. 1B and 1C). In contrast to these results, the female offspring of dams fed PB were, in general, more sensitive than offspring of control mice to the hypothermic effects of apomorphine (0.5 mg/kg) (Figs. 2 and 3). Statistically significant differences were noted between female C-2, PFC-2 and PB-2 groups, and the direction of differences was maintained when the female offspring of dams receiving higher doses of PB were compared to their respective control groups.

These changes in sensitivity did not appear to be mediated by prenatal nutritional factors, since offspring of food-restricted mothers (i.e., R-3 and PFC groups) did not differ significantly from the C groups. A general change in the ability to regulate body temperature in mice prenatally-exposed to PB also could not explain the results obtained after injection of apomorphine (see data in Tables 3 and 4). Alterations in apomorphine metabolism do not appear to play a role in the subsensitivity of male PB-3 mice to apomorphine-induced hypothermia, since brain levels of the drug at 15 or 30 minutes after injection were quite similar in the PB-3 and C groups (Table 5). Differences in brain levels of apomorphine also cannot be used as an explanation for greater sensitivity of the female PB-3 mice to the hypothermic effects of apomorphine. Brain apomorphine levels were comparable in female PB-3 and C mice at both 15 and 30 minutes after drug injection and, yet, differences in apomorphine-induced hypothermia were evident at these time points (Fig. 3).

The subsensitivity of the PB-3 male mice to apomorphine treatment may reflect a change in the affinity of the postsynaptic hypothalamic DA receptor for the receptor agonist. This is suggested by the fact that the decreased response was evident following administration of the 0.4 or 0.5 mg/kg doses of apomorphine, but not following the higher 1 mg/kg dose of apomorphine. At the high dose of apomorphine, DA receptors in both PB-3 and C mice may be fully saturated with the apomorphine, and would demonstrate a similar maximal response of the system (i.e., one can postulate no change in B_{max}). The lowest dose of apomorphine (i.e., 0.3 mg/kg) may not produce changes of sufficient magnitude to differentiate the experimental and control groups of animals. Treatment of the dams with doses of PB lower than 3 mg PB/g food apparently was not sufficient to produce changes in the sensitivity of DA receptors in the brains of male offspring.

The increased sensitivity of female PB mice to apomorphine-induced hypothermia may also be due to an alteration in the affinity of the hypothalamic DA receptor for apomorphine. In this case, one would have to conclude that an increased affinity of the hypothalamic DA receptor for apomorphine exists in these animals. Altered DA receptor sensitivity may arise as a result of factors such as long-term alterations of neurotransmitter release at particular DA synapses.

The presence of long-term alterations in CNS dopaminergic receptors found in the present study in adult HS offspring of mice exposed during pregnancy to PB may be complimentary to reported changes in brain DA levels and synaptosomal uptake of DA in young C57 offspring of mice treated during pregnancy with PB [21].

Although we have concentrated our discussion on possible changes in DA receptors produced by PB, changes in other neuronal systems may well contribute to the witnesses differences. Apomorphine-induced hypothermia, while being initiated through the hypothalamic dopaminergic system [2, 5, 6, 8], is also dependent upon other CNS neuronal components. For an example, acute apomorphine treatment has been shown to increase CNS 5-hydroxyindoleacetic acid levels [9], indicating that apomorphine can stimulate serotonin turnover in serotonergic neurons. Moreover, male rats maintained on a low tryptophan diet showed less of a body temperature decrease following acute apomorphine treatment than did normally fed rats [25]. Further experiments are necessary to fully elucidate the mechanism of the observed effects.

Our results do, however, show that prenatal PB treatment produces changes in central DA receptor function which extend into adulthood, and indicate that drug usage during pregnancy can produce complex and long-lasting physiological changes in the offspring Furthermore, male and female mice respond differently to the effects of prenatal PB treatment The results of our study also suggest that nutritional deficits produced in the dam during treatment with PB have to be noted and controls for such deficiencies instituted

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