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Prenatal Stress: Effect on Development of Rat Brain Serotonergic Neurons

DAVID A. V. PETERS[1]

Department of Pharmacology, University of Ottawa

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PETERS, D. A. V. *Prenatal stress: Effect on development of rat brain serotonergic neurons.* PHARMACOL BIOCHEM BEHAV **24**(5) 1377–1382, 1986.—Maternal low-level stress was found to produce persistent changes in serotonin (5-HT) receptor binding in several brain regions of the offspring. [^{3}H]5-HT binding was increased in cerebral cortex, decreased in hippocampus and unchanged in pons medulla while [^{3}H]spiperone binding was increased in all three regions at 60 days of age. The binding changes appeared to be due to altered numbers of binding sites with no change in dissociation constants. Regional differences were also found when the ability of nerve terminals to synthesize [^{14}C]5-HT from L-[^{14}C]tryptophan was studied. Prenatal stress reduced the rate of [^{14}C]5-HT synthesis in hippocampus but not in cortex or pons medulla. When younger offspring were studied, binding of [^{3}H]5-HT to cerebrocortical membranes was found to be reduced at 16 days of age and increased at 40 days while [^{3}H]spiperone showed only an increased binding at 40 days. In contrast, prenatal stress resulted in increased nighttime locomotor activity whether measured at 23, 40 or 60 days of age. The present study provides additional evidence that prenatal stress affects the development of serotonergic neurons and it is possible that such changes may underlie the reported behavioural deficits in offspring of stressed female rats.

Stress Serotonin Receptor Prenatal Rat Brain Tryptophan hydroxylation Locomotor activity

MANY investigators have reported that if pregnant rats are subjected to repeated stress treatments, the behaviour of the offspring is adversely affected. For example, prenatal stress has been shown to alter some but not all sexual behaviours in male offspring [14, 15, 28, 29]. Several authors have also provided evidence that "emotionality" may be affected by prenatal stress [1, 2, 14, 27] although this conclusion has been challenged [4]. Other investigators have looked for evidence of underlying biochemical abnormalities and there are reports of changes in brain biogenic amine levels following prenatal stress [16, 19, 22].

We have recently reported [19] that low level prenatal stress alters the brain levels of serotonin (5-hydroxytryptamine, 5-HT) and its major metabolite 5-hydroxyindoleacetic acid (5-HIAA). The 5-HT and/or 5-HIAA levels in cerebral cortex and pons medulla were significantly increased at 16 days but unchanged at 23 and 60 days of age. In contrast, the hypothalamus showed a reduced 5-HT level at 16 days and an increased 5-HT level at 60 days of age which suggested that there may have been a long-lasting change in functioning of central 5-HT neurons.

The increased 5-hydroxyindole levels in cerebral cortex and pons medulla at 16 days are consistent with an increased synthesis and release of 5-HT. It was therefore of interest to determine whether the return of 5-hydroxyindole levels towards control values in older animals was due to a loss of this apparent serotonergic hyperactivity or whether an increased activity was maintained by a different mechanism such as by a change in receptor sensitivity. We therefore examined the effect of prenatal stress on two populations of serotonergic binding sites in rats of several ages.

The ability of brain sucrose homogenates to synthesize 5-HT from L-tryptophan has been used as a measure of the biosynthetic capacity of serotonergic nerve terminals [12]. The conversion rate in a synaptosome-containing fraction appears to relate to the enzyme activity in nerve endings rather than to the total tryptophan hydroxylase content although the rate may also be influenced by other factors such as a change in the high affinity uptake of tryptophan [12]. We used this assay to determine whether the postsynaptic receptor changes were associated with changes in the presynaptic neurotransmitter synthetic system.

A further study investigated the effects of prenatal stress on the diurnal pattern of locomotor activity. Alterations in the functional activity of central 5-HT neurons affect a wide range of behavioural processes including gross locomotor activity and we attempted to use this measure as a behavioural correlate of altered brain function.

METHOD

Animals

Forty-eight female Sprague-Dawley rats (150–170 g) were housed in groups of 3 during a 1 week acclimatization period at the end of which an adult male rat (280–300 g) was placed in each cage. After 4 days, the males were removed and the cages randomly assigned to either control or stress groups. Rats assigned to the stress group were given once daily

[1]Requests for reprints should be addressed to Dr. David A. V. Peters, Department of Pharmacology, Faculty of Health Sciences, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, K1H 8M5.

TABLE 1

EFFECT OF MATERNAL STRESS ON RADIOLIGAND BINDING IN 60 DAY-OLD MALE OFFSPRING

		(^{3}H)5-HT Binding			(^{3}H)Spiperone Binding		
		Cortex	Pons-medulla	Hippocampus	Cortex	Pons-medulla	Hippocampus
K_D (nM)	Control	3.6 ± 0.5	3.3 ± 0.3	3.4 ± 0.4	0.96 ± 0.11	1.13 ± 0.26	1.20 ± 0.30
	Stress	3.7 ± 0.3 (102)	3.2 ± 0.7 (97)	3.4 ± 0.5 (100)	0.98 ± 0.24 (102)	1.13 ± 0.31 (100)	1.16 ± 0.20 (97)
Bmax (fmoles/mg protein)	Control	182 ± 13	78 ± 6	237 ± 15	440 ± 71	125 ± 12	176 ± 19
	Stress	226 ± 11* (124)	82 ± 12 (104)	186 ± 15* (78)	650 ± 50* (148)	177 ± 15* (142)	200 ± 19 (114)

*Denotes $p<0.05$ when compared to control value by t-test. The figures in parentheses are percent of control. The results are mean ± S.E.M. for 5 estimations by Scatchard plot. Tissues from 5 control rats and 5 prenatally stressed rats from a total of 10 different litters were used for each of the two assays.

stress treatments from day 1 of separation from the males until birth of their litters appeared to be imminent. The stress treatment consisted of once daily removal of the cages to a nearby laboratory where the females received a single saline injection (0.1 ml SC) before being returned to the animal quarters [19]. Control rats were left undisturbed throughout pregnancy except for routine animal care. All females were transferred to individual breeding cages after the final treatment period. From a total of 48 females mated, 20 control litters and 16 stress litters with 10 or more pups were obtained. Within 12 hr of birth, the litters were quickly weighed and culled to 10 pups to a litter. At 16 days of age 1 male and 1 female pup were removed from each litter for the biochemical studies. The remaining animals were weaned at 23 days and separated by sex and treatment group. At 23 days of age 5 control and 5 stress groups of 3 male rats each were selected from all available litters and assigned to the behavioural studies. Further groups of 6 male and 6 female rats selected from the maximum number of different litters were used for the biochemical studies at 16, 23 and 40 days of age and the remaining animals were studied at 60 days.

The rats were killed by decapitation, the brains quickly removed, rinsed with ice-cold saline and dissected on a cooled glass plate. For most assays the cerebral cortex, hippocampus and pons medulla were separated, weighed and frozen in liquid nitrogen prior to storage at −80°C. The remaining brains were divided into cerebellum, corpus striatum, cortex, hippocampus, pons medulla, spinal cord and a remainder and used immediately for the tryptophan hydroxylation assay.

An additional 16 female rats were mated and divided equally into control and stress groups. The stress group was given stress treatments on days 1–19 after removal of the males and rats were killed by decapitation 15 min after the final injection. The pregnant control rats were killed in two groups of 4 within 1 min of removal from the breeding facility on the same day. A blood sample was collected from each rat in heparinized tubes and plasma samples obtained by centrifugation. The plasma was stored at −80°C for later analysis of corticosterone by a fluorometric method [7].

Receptor Binding Assays

The brain tissues were homogenized in 40 volumes of ice-cold tris-HCl buffer (50 mM, pH 7.4) using a Brinkman Polytron. The homogenate was centrifuged at 35,000×g for 20 min and the pellet washed once in the same volume of buffer. The pellet was then resuspended in 40 volumes of fresh buffer and incubated at 37°C for 10 min to remove endogenous 5-HT [17] before a further centrifugation. The final pellet was suspended in 80 volumes of tris-HCl buffer (50 mM, pH 7.4) containing ascorbic acid (5.7 mM) and $CaCl_2$ (4 mM). This preparation was used for both binding assays. The protein concentration of the tissue suspension was determined by the Lowry method [13].

[^{3}H]5-Hydroxytryptamine (15–20 Ci/mmol) and [^{3}H]spiperone (20–30 Ci/mmol) were purchased from Amersham Corporation and stored at −20°C. High affinity [^{3}H]5-HT binding was assayed by the method of Nelson *et al.* [17]. For the determination of dissociation constants, 7–9 concentrations of [^{3}H]5-HT (0.4–8 nM) were used while other assays involved a single ligand concentration of 2.0 nM [^{3}H]5-HT. Unlabelled 5-HT (1 μM) was used to define non-specific binding. Aliquots (1 ml) of the tissue suspension were added to glass tubes containing [^{3}H]5-HT (0.05 ml) and either an aqueous solution of unlabelled ligand or water (0.05 ml). The tubes were incubated at 37°C for 10 min in triplicate and rapidly filtered through Whatman GF/B filters under vacuum. The tubes and filters were rinsed twice with ice-cold tris buffer (50 mM, pH 7.4) and radioactivity on the filters was assayed by placing the filters in 10 ml PCS (Amersham Corporation) and counting in a Beckman LS 8100 liquid scintillation spectrometer. Specific binding was determined as the difference in readings from samples incubated in the presence and absence of unlabelled ligand.

[^{3}H]Spiperone binding was assayed using the method of Creese and Snyder [6]. From 8–10 concentrations of the tritiated ligand in the range of 0.02–4 nM were used to determine dissociation constants while a single concentration of 0.5 nM was used for the remaining assays. The binding method used was similar to that described for [^{3}H]5-HT binding except that 0.2 ml of the tissue suspension in a total volume of 1.0 ml was used for the incubation mixture. Cinanserine (1 μM) was used to define non-specific binding.

Values for the number of binding sites (Bmax) and the apparent dissociation constant (K_D) were determined for each individual tissue from Scatchard plots [23].

In Vitro 5-HT Synthesis

Knapp *et al.* [12] have suggested that the regional distribution of soluble and synaptosomal tryptophan hydroxylase

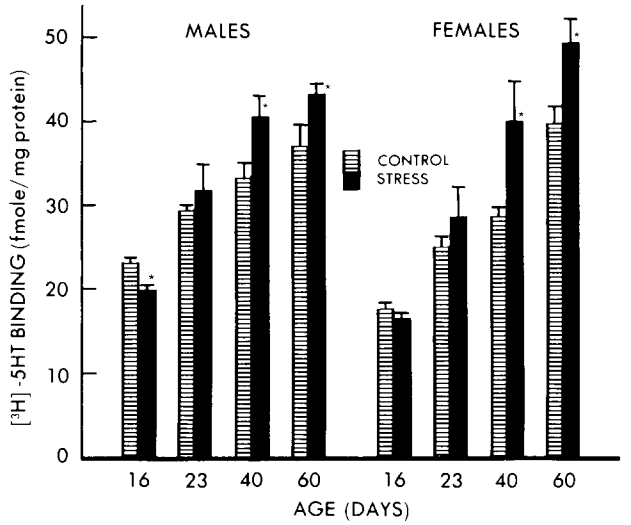

FIG. 1. Effect of prenatal stress on [^{3}H]5-HT binding in cerebral cortex. *Denotes $p<0.05$ when compared to control values by t-test. The binding assay was carried out using a 2 nM concentration of [^{3}H]5-HT. A 1 μM concentration of unlabelled 5-HT was added to a duplicate set of tubes to define non-specific binding. The data presented is the mean±S.E.M. for 6 determinations each done in triplicate. The animals consisted of 12 male-female pairs taken from 6 control and 6 experimental litters.

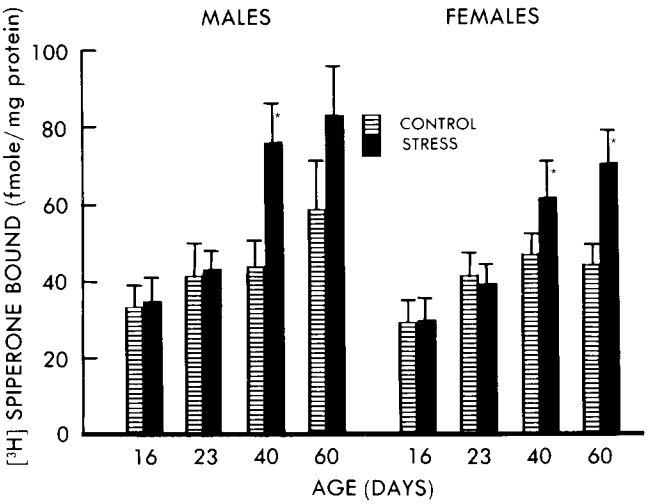

FIG. 2. Effect of prenatal stress on [^{3}H]spiperone binding in cerebral cortex. *Denotes $p<0.05$ when compared to control values by t-test. The binding assay was carried out using a 0.5 μM concentration of [^{3}H]spiperone. A 1 μM concentration of cinanserine was added to a duplicate set of tubes to define non-specific binding. The data presented is the mean±S.E.M. for 6 determinations each done in triplicate. The animals consisted of 12 male-female pairs taken from 6 control and 6 experimental litters.

in rat brain corresponds to regions containing serotonergic cell bodies and nerve endings respectively. Thus, the conversion of L-[^{14}C]tryptophan to [^{14}C]5-HT in a preparation containing intact synaptosomes in the absence of added cofactors or exogenous decarboxylase may be a useful measure of the ability of serotonergic nerve endings to synthesize 5-HT. In our study fresh brain tissue was homogenized in 25 volumes of ice-cold 0.32 M sucrose, a 1000×g precipitate was discarded and the supernatant centrifuged at 40,000×g for 20 min. The pellet was resuspended in the original volume of fresh 0.32 M sucrose. For the measurement of the synaptosomal conversion of L-tryptophan to 5-HT the incubation mixture consisted of 40 μmol tris-acetate buffer pH 8.1, 7 nmol L-tryptophan containing 0.1 μCi L-[methylene-^{14}C] tryptophan (Amersham, 56 mCi/mmol) and 200 μl tissue preparation in a final volume of 700 μl. Blanks contained heat-inactivated tissue homogenate in place of the enzyme-containing preparation. After incubation at 37°C for 30 min the [^{14}C]5-HT formed was separated on a CG-50 ion-exchange column as previously described [20] and counted in a Beckman LS 8100 ligand scintillation system after addition of ACS (Amersham).

Locomotor Activity

Locomotor activity was monitored continuously over 23 hr periods using an equipment which detected movement of the rats by measuring changes in capacitance between metal plates placed above and below the rat cages [21]. The activity was recorded as the number of seconds in each consecutive hour during which movement above a threshold level was detected. In this experiment the threshold level was set so that only large movements such as rearing and locomotion were recorded as activity.

RESULTS

Pregnant rats killed 15 min after the final stress treatment on or about day 19 of gestation showed a significantly higher plasma corticosterone level than uninjected pregnant control rats killed at the same time (stress group 126±13 μg/100 ml, n=7; control group 29±4 μg/100 ml, N=6; $p<0.001$). Values for 1 stress and 2 control females which were found not to be pregnant were omitted from the calculation.

Maternal stress had no significant effect on litter size, birth weight or body and brain weights at 16, 23, 40 and 60 days of age.

The binding of [^{3}H]5-HT and [^{3}H]spiperone to brain membrane preparations from 60 day old male offspring of control and stressed rats was analyzed using Scatchard plots (Table 1). Prenatal stress did not significantly alter the K_D for either [^{3}H]5-HT or [^{3}H]spiperone binding in any of the brain regions studied. However, an analysis of variance on the [^{3}H]spiperone binding data showed a significant main treatment effect for the Bmax values, $F(1,24)=9.48$, $p<0.01$. In contrast, the [^{3}H]5-HT binding data showed no main treatment effect, but a significant treatment × region interaction, $F(2,24)=7.42$, $p<0.005$. Further analysis of the data using t-tests revealed significantly increased binding in cerebral cortex and decreased binding in hippocampus.

For the second experiment, cerebral cortices from 16, 23, 40 and 60 day old offspring were examined for [^{3}H]ligand binding (Figs. 1 and 2). A 3-way analysis of variance on the [^{3}H]5-HT binding data showed significant treatment, $F(1,80)=1.25$, $p<0.005$, and age, $F(3,80)=20.8$, $p<0.005$, effects. Further analysis indicated a reduction in binding at 16 days of age, $F(1,20)=4.45$, $p<0.05$, and significant increases in binding at 40 days, $F(1,20)=7.27$, $p<0.05$, and 60 days, $F(1,20)=12.4$, $p<0.005$. The effect of prenatal stress on [^{3}H]spiperone binding in cerebral cortex was similar with significant increases in binding at 40 days, $F(1,20)=8.54$, $p<0.05$, and 60 days, $F(1,20)=5.81$, $p<0.05$, but no treatment effects at either 16 or 23 days of age.

Table 2 shows the effect of prenatal stress on tryptophan

TABLE 2

EFFECT OF PRENATAL STRESS ON THE CONVERSION OF L-(^{14}C)TRYPTOPHAN TO (^{14}C)5-HT IN TISSUE HOMOGENATES

	Tryptophan hydroxylation (nmol/g/hr)		
	Control	Prenatal Stress	Difference (%)
Cerebellum	0.249 ± 0.014	0.216 ± 0.023	−13
Cortex	0.68 ± 0.08	0.74 ± 0.03	+9
Hippocampus	1.31 ± 0.07	1.05 ± 0.07*	−20
Pons-medulla	1.14 ± 0.06	1.12 ± 0.04	−2
Spinal cord	0.48 ± 0.05	0.52 ± 0.10	+8
Striatum	1.68 ± 0.09	1.65 ± 0.13	−2

Results are mean ± S.E.M. for groups of 6 male rats, 60 days of age.

*Denotes $p<0.05$ when compared to the control value by t-test.

TABLE 3

EFFECT OF PRENATAL STRESS ON LOCOMOTOR ACTIVITY IN MALE RATS

		Age (days)		
		23	40	60
Night (1800–0600 hr)	Control	147 ± 9	276 ± 14	412 ± 38
	Stress	205 ± 22*	316 ± 20	526 ± 39*
	Difference (%)	+39	+14	+28
Day (0600–1800 hr)	Control	71 ± 13	138 ± 9	154 ± 12
	Stress	88 ± 13	117 ± 14	158 ± 16
	Difference (%)	+24	−15	+2

Results are given as the mean ± S.E.M. for the average number of seconds in each hour during which activity was recorded. Each cage contained 3 rats from the same litter and the experiment was repeated 5 times using rats from different litters.

*Denotes $p<0.05$ when compared to control rats by t-test.

hydroxylation in several brain regions of 60 day old rats. Prenatal stress had little effect on the rate of synthesis of [^{14}C]5-HT in sucrose homogenates and only the hippocampus showed evidence of an impaired ability of nerve terminals to synthesize 5-HT.

The effect of maternal stress on locomotor activity in male rats is summarized in Table 3. When tested by ANOVA, nighttime activity showed a significant main treatment effect ($p<0.01$) whereas daytime activity showed neither a main treatment effect nor a treatment × age interaction. The apparent effect of age on locomotor activity was, at least in part, an artifact due to the increasing body weight with age; in older animals more movements register on the activity counters.

DISCUSSION

Blood levels of hormones such as corticosterone, prolactin and growth hormone are highly responsive to stress. For example, merely handling rats for 6 sec has been shown to drastically elevate blood corticosterone and prolactin and decrease growth hormone levels within 15 min [25]. However, in a pilot study we found that handling alone was insufficient to consistently elevate blood corticosterone level after 14 days of daily treatments whereas handling combined with a saline injection elevated blood corticosterone levels at least 3-fold even after 20 once daily treatments (Peters and Tang, unpublished). We now report a similar elevation in pregnant female rats after 19 days confirming that the selected treatment was sufficient to repeatedly elicit a typical stress response. As expected [3], the plasma corticosterone levels in control pregnant females were somewhat higher than usually reported for non-pregnant rats.

Several different tritiated ligands have been used to identify 5-HT binding sites. Two different populations are widely recognized, the 5-HT$_1$ sites labelled by [^{3}H]5-HT and the 5-HT$_2$ sites labelled by the neuroleptic [^{3}H]spiperone. Evidence that the high affinity [^{3}H]5-HT and [^{3}H]spiperone binding can be increased or decreased by appropriate treatments [18,26] suggests that these sites probably represent functional receptors associated with 5-HT neurons. Available evidence suggests that the [^{3}H]5-HT binding sites have a postsynaptic location. For example, both electrolytic raphe lesions [24] and intracerebral injection of 5,7-dihydroxytryptamine [17] increase the density of [^{3}H]5-HT binding sites while kainic acid lesions of postsynaptic neurons have the opposite effect [5]. The sites labelled by [^{3}H]spiperone are also presumably located postsynaptically since destruction of 5-HT containing neurons does not decrease the [^{3}H]spiperone binding [9,24].

We previously observed that prenatal stress reduced cortical 5-hydroxyindole levels in 16 day old rats but had no apparent effect at later ages [19]. The present studies show that in the same tissue prenatal stress reduced [^{3}H]5-HT binding at 16 days and increased both [^{3}H]5-HT and [^{3}H]spiperone binding at 40 and 60 days of age. However, the finding of increased nighttime locomotor activity at 23, 40 and 60 days of age suggests that at least some behavioural changes resulting from prenatal stress exposure are independent of the age of testing in the post weaning period. A possible interpretation of these data is that prenatal stress may result in a functional hyperactivity of serotonergic neurons commencing at about 16 days of age. Initially, increased synthesis and release of 5-HT may be involved while in older animals an increased number of 5-HT receptors may mediate the hyperactivity.

Some postsynaptic receptors appear to develop independently of the presynaptic neurons [11]. Our finding of a decreased [^{3}H]5-HT binding in cerebral cortex at 16 days of age therefore suggests an alternative explanation for our biochemical data; that the initial effect is a reduction in the number of target cells for 5-HT neurons. It is possible that when the 5-HT synapses became functional in the neonatal period there is an increased synthesis and release of neurotransmitter as a short-term compensation for reduced receptor numbers. This might then be followed by an increased formation of postsynaptic receptors as the longer term compensatory process. The failure to demonstrate any apparent change in the ability of brain homogenates to synthesize [^{14}C]5-HT from L-[^{14}C]tryptophan is consistent with the view that in cerebral cortex any functional change in adult animals is more likely to be mediated by altered receptor sensitivity than by changes in presynaptic neurotransmitter synthesis and release.

The effect of prenatal stress on hippocampal 5-HT

neurons differed in that (a) there was no evidence of a transient increase in 5-hydroxyindole levels [19], (b) the number of [^{3}H]5-HT binding sites was decreased rather than increased at 60 days of age and (c) the hippocampus was the only one of 3 regions studied in which [^{3}H]spiperone binding was not significantly increased. Furthermore, this tissue was the only one of 6 brain regions in which the ability of homogenates to synthesize [^{14}C]5-HT from L-[^{14}C]tryptophan was significantly reduced. The reason for this regional difference is not known, but a possible explanation is that prenatal stress may have altered the distribution of serotonergic terminals in the rat brain resulting in a decreased number of serotonergic synpatic contacts in hippocampus.

The present study provides evidence that prenatal stress results in marked changes in the distribution of serotonergic receptors in the adult central nervous system. Whether the changes are brought about as a compensatory response to an impaired development of non-serotonergic neurons or whether there are more direct effects on the development of serotonergic neurons is unknown but, in either case, it is possible that an altered functioning of central serotonergic neurons may underlie the behavioural changes initiated by prenatal stress exposure. It is of interest that we find evidence of a consistently elevated nighttime locomotor activity following prenatal stress since treatments that would be expected to produce 5-HT receptor "supersensitivity" have been associated with enhanced locomotor stimulation by serotonergic agonists [8]. However, at present we have no direct evidence that the increased locomotor activity that we observed is directly linked to the serotonergic system.

It has been observed that 5-HT may have a modulatory role in the CNS since alterations in its functional activity appear to affect virtually all behavioural and physiological processes [10]. Our finding that prenatal stress appears to affect the development of central 5-HT neurons resulting in persistent changes in the distribution of 5-HT receptors suggests that exposure to stress during pregnancy may cause wide-ranging behavioural changes in the offspring.

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