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Effects of Maternal Stress During Different Gestational Periods on the Serotonergic System in Adult Rat Offspring

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PETERS, D. A. V. *Effects of maternal stress during different gestational periods on the serotonergic system in adult rat offspring*. PHARMACOL BIOCHEM BEHAV **31**(4) 839–843, 1988.—Pregnant Sprague-Dawley rats were exposed to mild stress treatments during different gestational periods and the offspring were investigated at 60 days of age. In the first study, stress from embryonic day (ED) 11 to ED 20 produced effects similar to those reported following stress throughout pregnancy; increased numbers of 5-HT_2 binding sites in cerebral cortex and a reduced intensity of the behavioral syndrome produced by injections of the 5-HT agonist 5-methoxy-N,N-dimethyltryptamine (5-MeODMT). In the second study, stress from ED 3 to ED 14 had no significant effect on the intensity of the 5-MeODMT-elicited 5-HT syndrome while stress from ED 15 to ED 20 had a similar effect as stress throughout pregnancy. These data provide evidence that the critical period for prenatal stress-induced changes in brain 5-HT neurons is between ED 15 and birth. This suggests that the mechanism involves an interaction with developmental events occurring within this time span such as the growth of nerve axons and the formation of synaptic contacts. Our findings also provide further evidence that stress during the final trimester of pregnancy may have serious adverse effects on fetal brain development.

Prenatal Stress Brain development 5-Hydroxytryptamine 5-HT syndrome 5-HT receptors 5-Methoxy-N,N-dimethyltryptamine

SEVERAL studies have shown that adult behavior in rodents can be significantly influenced by prenatal events such as maternal stress (1, 2, 4, 8). However, there is little evidence at present to suggest a possible mechanism by which rodent behavior may be modified by prenatal stress and even the stage of development during which the effects originate is not known.

We have previously reported that maternal stress affects several biochemical measures associated with central 5-HT neurons in the adult offspring, suggesting the possibility of permanent structural and functional changes. For example, prenatal stress increased 5-HT_2 receptor binding in several brain regions at 60 days of age while 5-HT_1 receptor binding was increased in cortex, decreased in hippocampus and unchanged in hindbrain (15). The increased 5-HT_2 receptor binding was present as early as 21 days of age (15) and persisted until at least 100 days, the latest age studied (unpublished data). Prenatal stress also reduced the intensity of the behavioral syndrome produced by injection of the 5-HT agonist 5-methoxy-N,N-dimethyltryptamine (5-MeODMT) (17) suggesting that the biochemical changes may be functionally significant.

The effect of maternal stress on the intensity of the 5-HT syndrome produced by 5-MeODMT injections appears to be drastically affected by the postnatal environment. For example, when the litters were left with the previously stressed mother until weaning, the intensity of the 5-HT syndrome was consistently increased instead of decreased (16). Moreover, a similar increase could be demonstrated in control offspring that had been reared by a previously stressed mother (16). Maternal stress can therefore affect the intensity of the 5-HT behavioral syndrome both prenatally and during early postnatal life, suggesting that the critical period for stress-induced changes in the development of central serotonergic neurons spans the prenatal and neonatal periods.

In an attempt to define the prenatal critical period more precisely we administered stress treatments at different gestational periods. We now report that both the increased 5-HT_2 receptor binding and the reduced 5-HT syndrome could be demonstrated in adult offspring following stress exposure during the final 10 days of pregnancy alone. A further study showed that the critical period could be narrowed to a stage starting after the peak of differentiation for 5-HT neurons of the raphe nuclei but including the formation of synaptic contacts. These data suggest that the mechanism associated with the stress-induced changes operates in the final few days of gestation. We suggest that a factor released during the stress response interferes with the process of for-

mation and/or verification of synaptic contacts in the serotonergic system, resulting in permanent changes in the functioning of central serotonergic neurons.

METHOD

Pairs of adult male and female Sprague-Dawley rats were placed together overnight between 17:00 hr and 07:00 hr. The morning on which sperm-positive smears were obtained was noted as embryonic day (ED) 0. The females were kept in pairs until the appropriate day of gestation when they were randomly assigned to either the control or stress groups. Stress treatments consisted of crowding (5 females in a 20×40 cm breeding cage) combined with once daily saline injections (0.01 ml saline, intramuscular) as previously described (16). Control females were kept in pairs in identical cages and were undisturbed except for routine cage changing.

For the first study the stress treatment was given from ED 11 to ED 20. At the end of this period the rats were transferred to individual breeding cages and no further treatments were given. Within 6 hours of birth all litters were reduced to 10 pups. At the same time the prenatal stress litters were transferred to control mothers that had given birth within the identical period and the control litters were discarded. A control group of offspring was obtained by cross-fostering litters between control mothers.

For the second study the stress treatment was given either from ED 3 to ED 14 (early stress group) or from ED 15 to ED 20 (late stress group). Rats in the early stress group were exposed to the stress treatment up to the end of ED 14 and were then placed under control conditions (2 rats/cage, no further injections). Pregnant females assigned to the late stress group were kept in pairs until ED 15 and were then transferred to crowding/injections stress conditions until the end of ED 20. The litters were cross-fostered within 13 hr of birth as in the first study.

The litters were weaned at 22 days of age and maintained in groups of 4 separated by treatment and sex until testing commenced at day 60. After weaning the animals were kept under a reverse light cycle with lights on at 22:00 hr and off at 10:00 hr. Behavioral testing was carried out between 10:30 hr and 12:00 hr.

5-HT Syndrome

Injection of a 5-HT agonist such as 5-MeODMT produces a group of behaviors usually referred to as the 5-HT syndrome. These include head weaving, forepaw treading, hindlimb abduction, Straub tail, tremor and piloerection (7). We selected three behaviors which we had previously found to occur in almost all 5-MeODMT-treated rats and which could be easily quantified. The types of receptor involved in the different components of the 5-HT syndrome have not all been firmly established. Two of the 5-HT-related behaviors that we examined (forepaw treading and head weaving) are believed to be mediated by 5-HT_{1a} receptors (20) but a different population appears to mediate the hindlimb abduction (20).

Sixty-day-old offspring were given single 4.0 mg/kg intraperitoneal injections of 5-MeODMT in random order and placed individually in 20×40 cm polycarbonate cages. The cages were immediately transferred to a small sound proofed, temperature and humidity controlled cubicle and were viewed through one-way glass. An observer, unaware of the group identity of each rat, rated the animals over a 10 min period for the presence of head weaving, forepaw treading and hindlimb abduction on a scale of 0 (absent), 1 (present), 2 (moderate) and 3 (severe). Four animals were rated simultaneously. The 4 mg/kg dose of 5-MeODMT has been routinely used in our studies because at this dose the different behaviors can be clearly differentiated. At higher dose levels the control animals become completely prostrate and the individual components are not easily distinguished.

[H^3] Spiperone Binding

[H^3] Spiperone was used to label 5-HT_2 receptors in cerebral cortex (6). Sixty day male offspring were killed by decapitation and the brains removed and dissected. The cerebral cortex from each rat was quickly weighed and homogenized in 40 volumes of ice-cold 50 mM pH 7.4 tris-HCl buffer using a Brinkman Polytron. The homogenates were centrifuged at 35000 × g for 20 min and the pellet washed twice more in the same volume of buffer. The final pellet was suspended in 80 volumes of 50 mM pH 7.4 tris-HCl buffer containing ascorbic acid (5.7 mM) and $CaCl_2$ (4 mM). [H^3] Spiperone binding to the suspended membranes was measured by the method of Creese and Snyder (6). Bound ligand was isolated by rapid filtration through Whatman GF/B glass fiber filters followed by two washes of 5 ml ice-cold 50 mM tris-HCl, pH 7.4. The filters were dried, placed in scintillation vials containing PCS (Amersham) and counted in a Beckman LS 8100 liquid scintillation counter. Ten concentrations of the tritiated ligand in the concentration range of 0.02–5 nM were used to determine the number of binding sites (B_{max}) and the apparent dissociation constant (K_d) by the Scatchard method (18). Cinanserine (1 μM) was used to define nonspecific binding. The protein content of the membrane fraction was assayed by the method of Lowry *et al.* (13).

RESULTS

None of the prenatal stress treatments significantly affected the gestation period, the litter size or the birth weight. Similarly, there were no significant weight differences between treatment groups at weaning or at 40 or 60 days of age.

In the first study involving stress on ED 11–20, 8 control and 8 prenatal stress litters were available. This treatment resulted in a significantly reduced intensity of the behavioral syndrome produced by a 4 mg/kg injection of 5-MeODMT (Table 1). A 2-way ANOVA (treatment × sex) showed significant treatment effects for head weaving, $F(1,28)=12.74$, $p<0.005$, forepaw treading, $F(1,28)=14.47$, $p<0.001$, and the hindlimb abduction, $F(1,28)=6.99$, $p<0.025$. There were no significant sex effects or treatment × sex interactions. Scatchard plots of the binding of [H^3] spiperone to cortical membranes showed that stress on ED 11–20 resulted in a significant increase in the B_{max} (Student's t-test; $t=2.55$, $p<0.05$) without affecting the K_d (Table 2).

In the second study, stress treatments were given either on ED 3–14 (early stress) or ED15–20 (late stress). Twelve litters in each of the three groups were available. Because the previous study showed no significant sex differences or treatment × sex interactions only male rats were used in this experiment. The early stress appeared to slightly reduce the intensity of the behavioral syndrome elicited by a 4 mg/kg injection of 5-MeODMT (Table 3) but the group means proved not to be statistically different when the data were analysed by Student's t-tests (t values: 0.96, 1.56 and 1.53 for the head weaving, forepaw treading and hindlimb abduction

TABLE 1

EFFECT OF PRENATAL STRESS (GESTATION DAYS 11–20) ON THE 5-HT SYNDROME ELICITED BY 5-METHOXY-N,N-DIMETHYLTRYPTAMINE IN 60–70-DAY-OLD RATS

	Control		Prenatal Stress	
	Male	Female	Male	Female
Head weaving	0.50 ± 0.15	0.70 ± 0.13	0.36 ± 0.8 (72)	0.10 ± 0.05* (14)
Forepaw treading	1.11 ± 0.17	1.22 ± 0.19	0.54 ± 0.14* (49)	0.68 ± 0.07* (56)
Hindlimb abduction	1.07 ± 0.20	1.11 ± 0.18	0.57 ± 0.15* (53)	0.75 ± 0.11 (68)

All rats were given an intraperitoneal injection of 5-MeODMT (4.0 mg/kg) and observed during the first 10 min following the injection. The presence of each of the behaviors was rated on a scale of 0 (not present) to 3 (severe). Results are mean ± s.e.m. for groups of 8 rats. The percentages of control values are given in parentheses.

Student's t-test: *$p<0.05$.

TABLE 2

EFFECT OF PRENATAL STRESS ON EMBRYONIC DAYS 11–20 ON 5-HT_2 RECEPTOR BINDING IN CEREBRAL CORTEX OF 60–70-DAY-OLD RATS

	K_d (nM)	B_{max} (fmole/mg protein)
Control	3.66 ± 0.10	425 ± 8
Stress	3.65 ± 0.05	534 ± 42*
% Control	100	126

[H^3] Spiperone binding to 5-HT_2 receptors was defined as the difference in binding in the presence and absence of 1 μM cinanserine. Values for K_d and B_{max} were obtained from Scatchard plots using 10 ligand concentrations in the range 0.02–5 nM. Results are mean ± s.e.m. for groups of 6 male rats.

Student's t-test: *$p<0.05$.

TABLE 3

EFFECT OF EARLY AND LATE PRENATAL STRESS ON THE 5-HT SYNDROME ELICITED BY 5-METHOXY-N,N-DIMETHYL TRYPTAMINE

	Control	Early Stress	Late Stress
Head weaving	0.56 ± 0.08	0.43 ± 0.11 (77)	0.22 ± 0.06* (40)
Forepaw treading	1.07 ± 0.10	0.87 ± 0.08 (81)	0.59 ± 0.08*† (55)
Hindlimb abduction	1.16 ± 0.11	0.96 ± 0.07 (83)	0.68 ± 0.10*† (59)

All rats were given an intraperitoneal injection of 5-MeODMT (4.0 mg/kg) and observed during the first 10 min following the injection. The presence of each of the behaviors was rated on a scale of 0 (not present) to 3 (severe). Results are mean ± s.e.m. for groups of 12 male rats. The percentages of control values are given in parentheses. Student's t-test *$p<0.01$ (late stress × control), †$p<0.05$ (late stress × early stress).

respectively). In contrast, when the data for the late stress group were examined all three behaviors proved to be significantly reduced (head weaving, $t=3.40$, $p<0.01$; forepaw treading, $t=3.75$, $p<0.01$ and hindlimb abduction, $t=3.23$, $p<0.01$). Moreover, for the forepaw treading and hindlimb abduction the values for the late stress group were also significantly different from the early stress values (head weaving, $t=1.68$, $p>0.05$; forepaw treading, $t=2.48$, $p<0.05$ and hindlimb abduction, $t=2.29$, $p<0.05$).

DISCUSSION

In previous studies we have used several approaches in examining the hypothesis that prenatal stress modifies the development of central 5-HT neurons in altered adult 5-HT-mediated behaviors. One approach has involved measurements of [H^3] spiperone binding in brain homogenates. This ligand appears to label a functional 5-HT receptor population since there is evidence of a strong correlation between 5-HT antagonist potencies at sites labelled by ligands such as [H^3] spiperone and the ability of the antagonists to block a variety of effects mediated by 5-HT agonists (5). The second approach has involved direct measurements of the group of behavioral responses, usually known as the 5-HT syndrome, resulting from administration of 5-HT agonists. Data from early studies in several laboratories suggested that this syndrome was probably also mediated by 5-HT_2 receptors but recent evidence suggests that 5-HT_{1a} receptors are involved in at least some components of the syndrome (20).

We have previously reported that administration of stress treatments throughout pregnancy resulted in increased brain 5-HT_2 receptor binding in the offspring (15,17). Provided that

the offspring were reared by control females from birth to eliminate postnatal effects were were also able to demonstrate decreased behavioral responses to 5-MeODMT. It was of interest to determine the critical period of these prenatal stress-induced changes to provide evidence that would be useful in formulating hypotheses on possible mechanisms.

Serotonergic neurons make their appearance early in brain development (14). Recent immunohistochemical studies (11, 12, 21) have generally confirmed the timetable for 5-HT neuron development established earlier from fluorescence histochemical observations (14,19). Thus, the earliest appearance of 5-HT perikarya occurs on ED 12–13 in the mesencephalon and 1–2 days later in the medulla. Growth of axons proceeds rapidly after the first detection of 5-HT immunoreactivity and by ED 17 5-HT axons reach the basal forebrain. [H^3] Spiperone binding to 5-HT_2 sites becomes measurable after ED 15 (3). By ED 19 the 5-HT pathways to all major regions of the forebrain have been established (11) but innervation of the cerebral cortex is not complete until the end of the third postnatal week (11). From ED 19 until the end of the first postnatal week there is a rapid growth of 5-HT dendrites (12).

For our first study we chose ED 11–20 as the stress period. This period extends from immediately before the first appearance of 5-HT-containing cells until shortly before birth, at which time pathways to all major areas of the forebrain have been established and the extended period of development of terminal fields just begun (11). Stress exposure during this time produced similar changes to those reported for stress treatments throughout gestation, namely, increased 5-HT_2 receptor binding and a reduced 5-HT syndrome (17), confirming that the critical period was between ED 11 and birth.

The second study attempted to discriminate between effects on early events such as 5-HT cell proliferation and growth of axons, and later stages such as the period of development of terminal fields and synapse formation. Three groups of pregnant rats were studied, unstressed controls, an early stress group treated on ED 3 to ED 14, and a late stress group treated on ED 15 to ED 20. There is considerable overlap between the stages of 5-HT neuron development and it is impossible to completely separate the stages. We selected the beginning of ED 15 as the break between the early and late stress period because both 5-HT cell subdivision and the period of initial axon elongation are nearly complete at this time, while formation of the terminal fields has only just begun. ED 15 is also about the time that forebrain 5-HT_2 receptors become detectable (3) suggesting that some synapse formation may have occurred.

Delaying the start of stress treatments until ED 15 again resulted in a reduction in the intensity of the 5-HT syndrome. In contrast, stress treatments starting on ED 3 and ending on ED 14, a period which included the peak period of raphé cell differentiation, did not significantly affect the intensity of the 5-HT syndrome. Thus, the critical period for changes in the central serotonergic system appears to start after the peak of 5-HT raphé cell differentiation and corresponds to a period of rapid formation of synaptic contacts. Since the process of synapse formation in the rat is incomplete at birth, these data are consistent with our previous finding that the same biochemical and behavioral parameters can also be influenced during the early postnatal period.

Several authors have concluded that monoamines may play an important trophic role in the early development of brain cells before synaptic transmission is established [see discussion in (10)]. There is evidence that 5-HT may influence the development of cells which will later receive 5-HT synaptic contacts. For example, the 5-HT-depleting drug p-chlorophenylalanine (PCPA) was found to retard the onset of neuronal differentiation in regions known to contain 5-HT terminals or to have a high 5-HT content in the adult while the stress of daily vehicle injections had the opposite effect (9). It is of interest that there is evidence that prenatal stress elevates fetal brain levels of tryptophan and 5-HT (D.A.V.P., unpublished observation). At least some of the effects of maternal stress on fetal development may therefore be mediated by a stress-induced increase in fetal brain 5-HT synthesis. If such an increase occurred during the period starting about ED 15, when the peak of differentiation of cells in 5-HT terminal fields takes place (10), then the development of neurons which receive a serotonergic input may be affected.

In summary, we report evidence that the critical period of prenatal stress-induced changes in the intensity of at least some 5-HT mediated behaviors is during the final 7 days of pregnancy. Since this period commences toward the end of the period of differentiation of 5-HT raphé neurons and after the time that these cells synthesise 5-HT, it is suggested that prenatal stress affects the later stage of neuron development such as the formation of synaptic contacts. Moreover, if 5-HT acts as a differentiation signal then stress-induced increases in fetal brain 5-HT may also affect the development of nonserotonergic neurons.

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