Developmental Alterations in Maturing Rats Caused by Chronic Prenatal and Postnatal Diazepam Treatments

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Abstract—The post treatment effects of early prenatal, late prenatal, early postnatal or combined prenatal and neonatal treatment with diazepam on the development of pain sensitivity, acoustic startle responsiveness, and benzodiazepine receptors in the cerebral cortex were investigated in rats between 14 and 90 days of age. Tail-flick latency was significantly decreased by combined prenatal and neonatal and by early prenatal diazepam treatment, but not by diazepam during the last half of gestation or during the neonatal period alone. Acoustic startle response was decreased by either late prenatal or neonatal diazepam treatment, but not by early prenatal treatment alone. Density of benzodiazepine receptors in the cortex was increased from postnatal day 1 to 21 by either early or late prenatal diazepam treatment. Neonatal diazepam treatment suppressed cortical benzodiazepine receptor or development until postnatal day 21; thereafter, receptor density increased to significantly higher values than in controls at 90 days of age. The results demonstrate that diazepam can alter development of pain sensitivity by actions during early gestation, startle responsiveness by actions late in pregnancy, and cortical benzodiazepine receptors by actions throughout gestation and the early postnatal period.

There have been several clinical reports of deleterious effects of prenatal diazepam usage on behavior development in exposed infants (1, 2). Similarly, chronic treatments of pregnant or nursing rat dams with diazepam have been shown to alter several aspects of behavioral development in exposed offspring (3). Although the daily dose level and the duration of treatment of pregnant rats with diazepam differed somewhat between studies, the prenatal treatment studies have found postnatal alterations in maturation of spontaneous motor activity (3–5), open-field motor activity (4), acoustic startle reflexes (6, 7) and avoidance acquisition (4, 6). However, corollative neurochemical studies in brain tissues of the behaviorally affected rats have failed to identify any specific neuronal mechanisms which might be responsible for the diazepam-induced functional alterations which often persist into adulthood.

In offspring of pregnant rats treated daily with diazepam throughout gestation, the postnatal ontogenesis of [3H]-diazepam binding sites of the whole brain, cerebral cortex, cerebellum, or corpus striatum was not affected by the chronic, prenatal drug administration (8, 9). Similar results were reported by Kellogg et al. (10) in offspring of pregnant rats treated daily with diazepam during the last half of gestation. The most distinctive influence of prenatal diazepam
exposure on brain neurochemical maturation in exposed offspring appears to be limited to a specific and persistent suppression of noradrenergic innervation of the hypothalamus (10). Two-month old offspring of prenatal diazepam-treated rats exhibited substantial deficiencies in hypothalamic norepinephrine concentration, but were not significantly different from offspring of vehicle-treated pregnant rats with respect to norepinephrine content in the cerebral cortex and hippocampus or dopamine levels in any of these brain regions. However, as previously emphasized (10), the mechanistic importance of this diazepam-induced "hypothalamic noradrenergic deficiency" to the alterations in behavioral development caused by prenatal diazepam is tenuous at best and may very well be irrelevant since several different classes of psychoactive drugs, when administered prenatally, induce a similar limited reduction in midbrain norepinephrine content but dissimilar influence on behavioral development (11–13).

Recently, we established that pre and postnatal diazepam exposure reduced the number of opioid receptors in the cortex and striatum of 14 day old rats (14). This evidence supports the existence of a functional link between central benzodiazepine/GABAergic and opioid systems (15–17), and may suggest that the prenatal diazepam exposure alters the behavioral development that relates to the changes in opioid receptor characteristics. Moreover, both opioid and benzodiazepine receptors appear in the central nervous system at almost the same time during gestation (18, 19).

For the above reasons, we examined the development of pain sensitivity, and the acoustic startle response in the offspring of rats exposed to diazepam during the two different stages of gestation and to full gestational in addition to a point 10 days after birth. The development benzodiazepine binding characteristics in rat brain cortex in offspring have also been studied and compared to the results of the behavioral experiments.

Materials and Methods

We have previously described the method of pre and postnatal administration of diazepam to mother rats and their offspring (14).

Sprague-Dawley rats (twelve adult males, forty-eight second-pregnancy females and their offspring) were used as the experimental animals. Two female rats and one male rat were housed in the same clear plastic cage for mating. These male rats were coupled at least twice. The gestational zero day was determined when sperm was observed in vagina. The pregnant female rats were kept individually in each cage. These mothers were separated into four groups (twelve mothers/each group). One group of pregnant rats (Group II) received daily subcutaneous injections of diazepam (20 mg/kg) throughout gestation, and their offspring each were treated daily with diazepam (20 mg/kg) from day 1 to day 10 after birth. Other groups were injected with the same dose of diazepam on day 1 through 12 (Group III) or days 13 through 22 (Group IV) of gestation, respectively. The pregnant animals in Group V received only saline injections throughout pregnancy, and their offspring received diazepam injections (20 mg/kg) each day from postnatal day 1 to day 10. The last group (Controls) received no injections during pregnancy, and their offspring remained untreated postnatally, except for daily handling and weighing.

The sex of their offspring was distinguished at birth. Five males and the same number of females were kept in a cage with the mother rat until weaning. Some of these were used for both behavioral tests (i.e., acoustic startle response and tail flick response) on postnatal days 14, 21, 28 and 91, and others were sacrificed for binding assays on postnatal days 4, 7, 14, 21, 28 and 91. Some of the one day old rats, including the surplus rats, were used for the binding assays.

On postnatal days 14, 21, 28 and 91, offspring (equal number of males and females) from each experimental group were studied by behavioral tests. Each result of both behavioral experiments was the averaged data of males and females of the same litter and age. Different groups of offspring were prepared for each behavioral test. Benzodiazepine receptor assays were
performed on the cerebral cortex on postnatal days 1, 4, 7, 14, 21, 28 and 91 for the early and late prenatal treatment group and on postnatal days 14, 21, 28 and 91 for both groups involving neonatal diazepam administration.

**Tail flick response:** At each study point (14, 21, 28 and 91 day old), groups of offspring were tested for sensitivity to pain, using a modification of the tail-flick procedure of D’Amour and Smith (20). The intensity of the beam was set to produce an average control group reaction time of about 5 sec with three trials being run per animal, and each trial was made at least 10 min after the preceding one. Differences between group means were analyzed for significance using Student’s t-test (P<0.05).

**Acoustic startle response:** Offspring of each treatment group were examined on postnatal days 14, 21, 28 and 91 for reactivity to acoustic stimulation by a modification of the method of Davis and Sheard (21). Measurements of acoustic startle reflex were performed by placing individual rats in a stabilimeter cage; each cage (4×6×6 inches) constructed of Plexiglas, with a wire mesh floor, rests on four springs connected to an accelerometer capable of detecting sudden bodily movements. The output voltage of the accelerometer was proportional to the velocity of altered development after perinatal diazepam movement of the rat in the cage so that the resultant peak voltage was taken as the magnitude of the startle response. At testing, offspring were placed in four startle cages housed in a ventilated isolation room. In the center of the room (36 inches from the cages) was a speaker capable of providing white noise or a short acoustic stimulus. Between trials, background noise was continuously provided by a white noise generator at 40–45 dB. The startle stimulus was a 400 Hz, 100 msec tone at 115 and 150 (for 14 and 28 day old rats and both 21 and 91 day old rats, respectively) dB and having a rise-decay time of 5 msec. A test period consisted of 5 min of white noise followed by a series of 10 startle stimuli. The inter-stimulus interval was varied between 20 and 40 sec to minimize anticipatory effects. The results of all trials during the test were combined to determine the mean startle response amplitude for offspring rats of each treatment group. These experiments were always carried out between 9:00 and 14:00 and simultaneously measured the responses of the same aged control (nontreatment) and treatment groups. The mean values obtained for each drug-treated group were compared to the mean value of vehicle-treated group offspring of the same age, and differences between the drug-treated groups and the control group were tested for significance using Student’s t-tests (P<0.05).

**Binding assay:** We have previously described the methods of membrane preparation and receptor assays (14, 22). Rats were decapitated and the brains were dissected over ice. Five whole cortices of 1 or 4 day old rats and three whole cortices of 7 day old rats were pooled in each test tube; the cortices of 14 day rats were each individually prepared. These cortices were stocked at −80°C. The tissue was thawed at room temperature and then homogenized in 10 vol. of ice-cold 0.32 M sucrose and centrifuged at 1,000×g for 10 min. The supernatant was centrifuged at 30,000×g for 30 min. This membrane pellet was resuspended in 10 vol. ice-cold 50 mM Tris HCl buffer (pH 7.4) and centrifuged at 30,000×g for 30 min. This operation was repeated twice; then the membrane suspension was frozen at −80°C for later assay.

Incubation samples contained 0.2–0.5 mg protein, 50 μl of 6 different concentrations of [3H]-diazepam (final concentration 1–15 nM, 86.6 Ci/mmol. New England Nuclear), with or without 50 μl of cold ligand diazepam (final concentration of 3 μM) and 50 mM Tris-HCl buffer (pH 7.4 at 0°C). Finally, following 30 min of incubation on ice (0–4°C), samples were rapidly filtered under reduced pressure through Whatman GF/B filters. Each filter was washed 3 times with 5 ml of ice cold buffer, then counted for radioactivity in 10 ml of scintillation mixture. Protein was determined by the method described by Lowry et al. (23). Values of binding parameters (Bmax and Kd) were obtained from Scatchard plots of specific [3H]-diazepam binding saturation curves by
linear regression.

**Results**

**Tail-flick response:** Diazepam treatment throughout pregnancy or during either half of gestation had no significant influence on the body weight of the pregnant rats, length of gestation, litter size or postnatal offspring mortality. Tail-flick latency (pain sensitivity): Combined prenatal and postnatal diazepam treatment significantly increased pain sensitivity in 14 day old offspring; but on postnatal days 21, 28 and 91, the pups did not differ from controls of the same age in tail-flick latency (Table 1). There were no significant differences in tail-flick latency between offspring exposed only postnatally to diazepam and control pups at either test age, indicating that increased responsiveness in

| Group | n | 14 day old | 21 day old | 28 day old | 90 day old |
|-------|---|------------|------------|------------|------------|
| Control | 9 | 4.27±0.33 | 4.52±0.24 | 4.43±0.18 | 4.73±0.24 |
| II | 9 | 3.05±0.10* | 4.08±0.18 | 4.02±0.15 | 4.48±0.33 |
| III | 8 | 3.00±0.12* | 3.99±0.26 | 4.61±0.21 | 4.54±0.18 |
| IV | 8 | 4.35±0.34 | 4.26±0.30 | 4.74±0.25 | 4.68±0.11 |
| V | 9 | 4.10±0.41 | 3.91±0.31 | 4.55±0.11 | 4.58±0.21 |

The sensitivity of analgesia was measured by a modification of the tail-flick procedure of D'Amour and Smith (20). The intensity of the beam was set to produce an average control group reaction time of about 5 sec with three trials being run per animal, and each trial was run at least 10 min after the preceding one. Values are the group mean±S.E. for response latency (sec) of the averaged data of male and female pups of the same litter at each age as one experiment. Significant differences between control and drug treatment groups are denoted by an asterisk (*P<0.05).

**Acoustic startle response:** As shown in Table 2, magnitude of the response to acoustic startle were significantly lower than control values in the 14 and 21 day old offspring of the prenatal plus postnatal, only postnatal, and late gestational treatment groups (decreases of 55, 40 and 50%, respectively). At 28 days after birth, these reductions of the pre plus postnatal and only postnatal treatment group disappeared, and the only late gestational treatment group

| Group | n | 14 day old | 21 day old | 28 day old | 91 day old |
|-------|---|------------|------------|------------|------------|
| Control | 8 | 12.34±0.90 | 22.47±1.94 | 12.16±1.47 | 25.97±2.42 |
| II | 6 | 5.27±0.50* | 13.79±1.36* | 13.06±1.62 | 21.86±1.84 |
| III | 8 | 18.24±1.98* | 21.68±2.21 | 14.10±2.06 | 22.80±2.39 |
| IV | 8 | 8.04±0.69* | 13.35±1.06* | 17.78±1.64* | 22.03±2.03 |
| V | 8 | 6.79±0.97* | 10.86±1.26* | 13.74±1.27 | 25.68±2.18 |

Measurements of the acoustic startle response were performed by placing individual rats in a stabilimeter cage; each cage (4×6×6 inches, constructed of plexiglass, with a wire mesh floor) rests on four springs connected to an accelerometer capable of detecting sudden bodily movements. The startle stimulus was a 400 Hz. 100 msec tone at 115 and 150 (for 14 and 28 day old and 21 and 91 day old rats, respectively) dB and having a rise-decay time of 5 msec. A test period consisted of placing a rat in each stabilimeter, closing the chamber, and after 5 min of white noise, presenting a series of 10 stimulus tones. The inter-stimulus interval varied between 20 and 40 sec to minimize anticipatory effects. Values are the group mean±S.E. for the magnitude of initial bodily responses to acoustic stimulus (magnitude, in arbitrary units, proportional to velocity of bodily movement). Significant differences between the control and drug treatment group are denoted by an asterisk (*P<0.05).
showed a significant enhancement of this response. The 14 day old offspring of the early gestational treatment group responded more vigorously than controls to acoustic startle; but at 21 days of age, they were not different from control offspring. None of the groups had different responses from the control group on postnatal day 91. The results of the 14 and 21 (weaning) day old rats and the 28 and 91 day old rats are data from the same offspring. In this experiment, we used two differential stimuli to exclude the possibility of acclimation. One was 115 dB, which was treated to the 14 and 28 day old rats, and the other was 150 dB, which was treated to the 21 and 91 day old rats.

Binding assay (ontogenesis of benzodiazepine receptors): Binding affinities ($K_d$) for specific binding of $[^3H]$-diazepam to cortical membranes from offspring of all diazepam treatment groups were similar to values for control offspring at all study ages (data are not shown). However, prenatal diazepam treatment did influence the postnatal development of apparent benzodiazepine receptor density in the cerebral cortex (Fig. 1). Receptor densities in the cerebral cortex ($B_{max}$ values) were significantly higher than control values ($B_{max}$ of postnatal 1, 4, 7, 14, 21, 28 and 91 days: 0.525±0.065, 0.607±0.029, 0.860±0.051, 1.208±0.075, 1.478±0.150, 1.750±0.089 and 1.468±0.026 pmol/mg protein, respectively) for offspring of both the early and the late gestational diazepam treatment groups (III, IV) throughout the neonatal period (birth to postnatal day 7) as well as at the time of weaning.

For offspring of the two groups involving postnatal diazepam treatments (II, V),

![Fig. 1. Developmental alteration of the rat brain cortical benzodiazepine receptors due to chronic prenatal diazepam treatments. The procedures of dissection and membrane preparation of rats of each age group were described in Materials and Methods. The maximum of $[^3H]$-diazepam specific binding sites ($B_{max}$) were obtained from Scatchard analysis. The control group received no injections during pregnancy, and their offspring remained untreated postnatally, except for daily handling and weighing. Group III or IV was treated with 20 mg/kg (s.c.) of diazepam on day 1 through 12 or day 13 through 22 of gestation, respectively. Each of the values are shown as the mean±S.E. of 4–5 experiments, each done in triplicate. Significant difference between control and drug treatment groups at each postnatal day is indicated by an asterisk (*$P<0.05$, **$P<0.01$). The $B_{max}$ values for postnatal days 1, 4, 7, 14, 21, 28 and 91 of the control group 0.525±0.065, 0.607±0.029, 0.860±0.051, 1.208±0.075, 1.408±0.150, 1.275±0.089 and 1.468±0.026 pmol/mg protein, respectively.]}
cortical benzodiazepine receptor densities were unaffected by the drug when results were compared to control values between 14 and 28 days of age (Fig. 2); but, by postnatal day 90, receptor densities were significantly higher in the cortex of offspring from both the postnatal only and the prenatal plus postnatal diazepam groups (52% and 50%, respectively, vs. controls).

**Discussion**

The early gestational treatment of diazepam significantly decreased the tail-flick latency of the offspring rats. In contrast, the acoustic startle response was decreased by the late gestational or neonatal diazepam treatment. Density of benzodiazepine receptors in the cortex was increased from postnatal day 1 to 21 by either early or late prenatal diazepam treatment, but these increases were inhibited by the neonatal treatment of diazepam.

Braestrup et al. (8) and Massotti et al. (9) reported that prenatal exposure to diazepam did not alter the development of $[^3H]$-diazepam binding densities in the offspring rat brain. The behavioral experiments, however, suggested that the duration of treatment of pregnant rats with diazepam changed the development of some behavioral characteristics (e.g., locomotor activities and acoustic startle responses) (4, 6, 7). Recently, Simmons et al. (24) demonstrated that the late gestational treatment of diazepam (gestational day 13 through day 20) decreased the level of norepinephrine and turnover rate of norepinephrine in the hypothalamus of 90 day old rats. Moreover, concurrent administration of the specific benzodiazepine antagonist Ro 15-1788 with diazepam (2.5 mg/kg) to pregnant rats effectively reversed the effects of diazepam in the hypothalamus of the adult offspring. Prenatal exposure to diazepam therefore seems to induce alteration of some behavioral characteristics and to decrease the hypothalamic norepinephrine activity which may be mediated via the benzodiazepine receptor. However, the alteration of development of norepinephrine levels in the hypothalamus of rats, which is induced by the prenatal exposure to diazepam, does not have any
correlation with the changing of behavioral development (10, 24).

In this report, we chronically treated diazepam in three different groups of mother rats (e.g., the early gestational treatment group, the late gestational treatment group and whole gestational treatment group), in order to hopefully better define the gestational point of the possible effect of diazepam. Richards and Mohler indicated that the benzodiazepine receptors appear on the gestational day 14 (18) and Zhang and Pasternak reported that opioid receptors appear about the same time (25). Given neuronal development during the second week in the rat (24, 26), we divided the animals into experimental groups consisting of an early gestational drug regimen, a separate late gestational drug regimen, and a total gestational regimen so as to better define the point in development where benzodiazepine receptors could induce pharmacologic effects which might later be manifest.

We have shown that group II (pre plus postnatal treatments) and III (only gestational treatment) are much more sensitive to analgesia than the control, group IV (only late gestational treatment) and group V (neonatal treatment). These results support our previous findings (14). Namely, pre plus postnatal exposure to diazepam reduced the densities of [3H]EKCZ binding sites in the offspring cortex and striatum. It is well-known that opiate receptors are related to the mechanism of analgesic function. Pasternak and his co-workers demonstrated that the development of high affinity binding of a variety of opiates and enkephalins have a good correlation with the change in analgesic sensitivity of the offspring, when they were 2 days old and 14 days old (25, 27). According to their results, the density of high affinity binding increased about 3-fold between 2 and 14 days after birth. Additionally, morphine's analgesic ED50 is 40-fold greater in 2 day old rats than in 14 day old rats.

Pre plus postnatal exposure to diazepam not only decreased the densities of the opioid receptor in rat cortex and striatum, but it also increased the pain sensitivities in 14 day old rats (14). In addition, 14 day old rats of the early gestational treatment groups (III) also had higher sensitivity to pain than the control group of the same age. Other treatment groups (e.g., IV and V) did not show this hypersensitivity to pain at any age. These results indicate that early gestational (1 day through 12 days of gestation) treatment of diazepam might directly or indirectly decelerate the development of opioid receptors in the brain and then induce the hypersensitivity for pain. Evidence for this has not been published yet. Additionally, these results show a good correlation between the results of binding assays and the behavioral experiments.

Depressed startle responsiveness was observed in the late gestational (gestational day 13 through 22) and/or neonatal treatment group at 14 and 21 days after birth. Treatment of benzodiazepine or other drugs during the third week of gestation in the rat has been clearly shown to interfere with the development of behavior and receptor densities in the offspring (3, 7, 28). However, only the early gestation (day 1 through 12) treatment group showed an enhanced startle response at 14 days after birth. This enhancement was inhibited by the late gestational and neonatal treatment of diazepam (e.g., the result of group II). In addition, the early gestational exposure to diazepam-induced hyper startle responsiveness has already recovered at 21 days after birth. These results indicated that the late gestational and/or neonatal exposure to diazepam affected the development of auditory temporal acuity or feeling of fear, apprehension and dread as was reported by other workers (7, 29). The early gestational treatment of diazepam also alters the development of the acoustic startle response, but these effects are completely opposite from those obtained for diazepam exposure in the third week of gestation and neonates.

In benzodiazepine receptor maturation, daily diazepam treatment during pregnancy does not appear to alter the number of sites and the affinity characteristics of [3H]-diazepam binding in offspring as the previous reports (8–10). However, prenatal exposure to diazepam (either early or late gestation)
stimulated development of benzodiazepine receptor density between 1 and 21 days after birth. Postnatal treatment of diazepam (either post only or pre plus post) inhibited the further development of benzodiazepine receptor density during 14 days through 21 days of age. However, on postnatal 91 days, postnatal treatment groups (II and V) showed a significant increase of binding density. The previous papers also observed the gestational administration of drug-induced increase of receptor density over the 8-week postnatal period, the so-called “rebound effects” (13, 26). Our results of benzodiazepine receptor maturation after the chronic treatment of diazepam to mother rats are slightly different from the previous reports (8–10). These discrepancies are difficult to explain at this time, but some possible causes are the different strain of experimental animal, administration route, dose of drug and stress in the animals due to handling.

Several conclusions can be made from these results: First is that the early gestational exposure to diazepam appears to alter the pain sensitivity in the juvenile age with changing of opioid receptor densities. This result may be supported by previous papers (15–17). Second is that prenatal (either earlier or later gestation) and/or postnatal exposure to diazepam affects the variational development of startle responsiveness, this being independent on the changing of benzodiazepine receptor maturation.

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