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5. Behavioral assessments of the degree of semantic incongruity were based on subjects' ratings of the last words on a seven-point scale, where 1 was "expected" and 7 was "totally unexpected." The mean rating for congruous words was 1.39 ± 0.13, for moderately incongruous words 6.28 ± 0.16, and for strongly incongruous words 6.96 ± 0.02. The difference between the strong and moderate scores was significant (Mann-Whitney U test, $P < .002$).
6. The small and large letters were 0.89° and 2.64° high.
7. All subjects were normal young adults between 18 and 35 years old who did not know the aims of these studies. The number of subjects run in experiments 1 to 3 were 11, 12, and 9, respectively.
8. The mean peak latencies of N400 at the Pz site were 397 ± 12 msec for the moderate semantic condition and 391 ± 10 msec for the strong semantic condition.
9. As assessed by Mann-Whitney U tests, the N400 area was greater for strong than for moderate semantic deviations at frontal ($P < .01$), central ($P < .025$) and parietal ($P < .05$) electrode sites. Baseline-to-peak measures showed similar levels of significance.
10. Measured as the peak negativity between 300 and 600 msec after the stimulus relative to the baseline voltage averaged over 100 msec before the seventh word, the mean N400 amplitudes were: −6.79 ± 0.74 μV at Fz, −7.14 ± 1.00 μV at Cz, and −7.81 ± 1.49 μV at Pz.
11. The peak N400 amplitudes in the difference waves relative to the baseline before the seventh word were −8.05 ± 0.98 μV at Fz, −9.62 ± 0.72 μV at Cz, and −9.24 ± 1.09 μV at Pz. All statistical evaluations of N400 amplitude to incongruous versus congruous words in experiment 2 were significant beyond the .001 level.
12. The mean latencies of the three peaks at Cz were as follows: P210, 209 ± 4; P350, 351 ± 9; and P560, 558 ± 13 msec. One subject showed a late negative wave instead of these three peaks. The P350 and P560 peaks were greatly attenuated or absent in a control condition where a single, large word was flashed repeatedly [M. Kutas and S. Hillyard, *Brain Lang.*, in press.].
13. The mean N400 areas at Cz were −1213 ± 253 μV-msec for incongruous versus congruous small words and −955 ± 320 μV-msec for incongruous versus congruous large words. The late positive area was 753 ± 492 μV-msec for large versus small congruous words and 794 ± 262 μV-msec for large versus small incongruous words.
14. Late positivity after N400 was assessed in experiment 2 by calculating the difference between the normal and deviant words in area across the 600 to 900 msec range. This difference (for example, 227 ± 270 μV-msec at Cz) was not significant.
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Prenatal Exposure to Diazepam Alters Behavioral Development in Rats

Abstract. *Characteristic potentiation of rat locomotion responses and acoustic startle reflexes that normally appear in the third postnatal week was absent in rats exposed to diazepam during the third week of gestation. Loss of these behaviors suggests a long-term effect that may result from changes in cellular development. Tissue undergoing neuronal differentiation may be especially sensitive to drugs that act on the central nervous system, and the period in which differentiation occurs is perhaps critical for the induction of changes that are later expressed as altered behavior.*

Behavioral alterations in offspring that were exposed prenatally to drugs (*1*) and environmental chemicals (*2*) are noted with increasing frequency. Traditionally, studies of the sequelae of prenatal exposure to drugs and chemicals have concentrated on exposure during organogenesis and on resultant malformation. Exposure during other developmental periods, however, may produce functional disturbances. Given the widespread use of ataractic drugs, including diazepam and chlordiazepoxide (*3*), and the clinical reports (*4*) that prenatal exposure to these agents may yield a variety of regulatory dysfunctions in the neonate, we designed experiments to obtain measures of behavioral activity and reactivity in developing rats exposed to diazepam during the last week of gestation (*5*).

Diazepam was administered subcutaneously once daily between 9:00 and 10:00 a.m. to female rats (Long Evans) on days 13 to 20 of gestation; the females were pregnant for the first time, having been bred at 100 days of age with male rats of the same strain. Day 0 of gestation was the day on which a vaginal smear positive for sperm was first obtained (*6*). On day 13, the pregnant females were weighed and assigned to one of five groups: uninjected controls ($N = 15$); vehicle-injected controls ($N = 3$); and three experimental groups given diazepam (2.5 mg/kg, $N = 2$; 5.0 mg/kg, $N = 3$; and 10.0 mg/kg, $N = 2$) (*7*). On day 20, all females were placed in plastic cages for parturition and, beginning on day 21, were checked every 3 hours from 9:00 a.m. to 10:00 p.m. The day of birth was designated as postnatal day 0.

Within 24 hours after birth, the litters of both diazepam-injected and control rats were reduced to seven or eight pups and given to uninjected dams for fostering (each litter had one dam). Behavioral testing was begun on postnatal day 12.

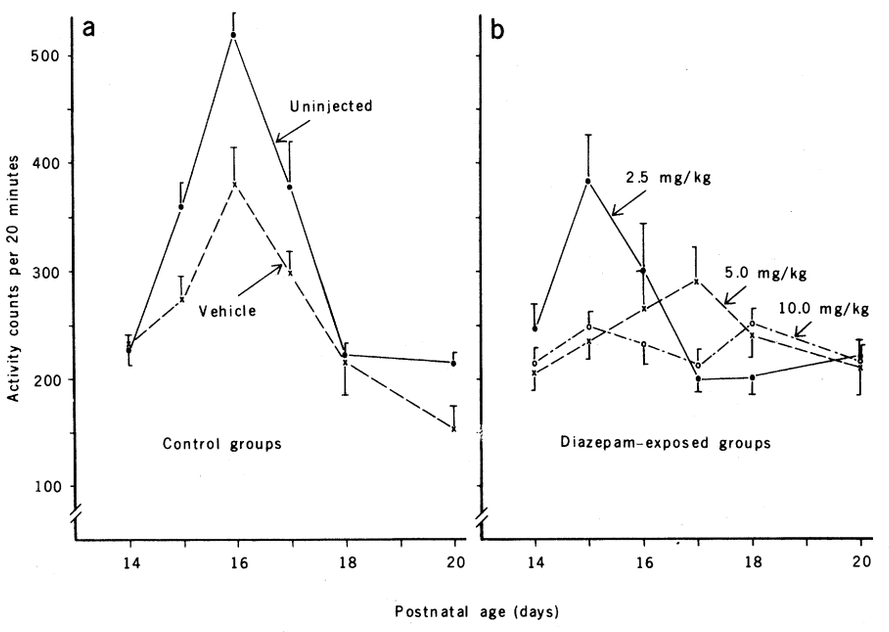

Fig. 1. Development of spontaneous locomotor activity in control and diazepam-exposed rats. Each animal was tested for a 20-minute period on each testing day. Numbers of rats tested were as follows: uninjected, 15; vehicle-exposed, 12; diazepam-exposed, 7, 8, and 7 to 2.5, 5, or 10 mg/kg, respectively. Animals tested represented a minimum of two litters per group. Vertical bars indicate standard error.

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Half of each litter was used to study the development of locomotor activity and half for analysis of the development of the acoustic startle response (*8*). At least seven offspring were tested in each group and (with one exception) represented a minimum of two litters. Separate analyses of the data were performed in which both offspring and litter were used as the experimental unit; the analyses in which litter was the unit compared grouped control litters with grouped drug-exposed litters.

The development of spontaneous locomotor activity follows a characteristic pattern in rodents (*9*), as depicted in Fig. 1a for the controls (*10*). The activity of unexposed animals increased sharply after day 14, peaked on day 16, and then declined abruptly. The activity of the vehicle-exposed controls followed the same pattern but reached a lower magnitude on day 16. Figure 1b shows how this pattern was affected by diazepam. Animals exposed to the lowest dose (2.5 mg/kg) showed an inverted-U pattern of activity, which peaked on day 15 rather than day 16. In the animals exposed to the two higher doses of diazepam, in contrast, the activity was more or less constant. Analyses of variance for repeated measures of the data presented in Fig. 1 (offspring as experimental unit) yielded significant ($P < .01$) effects for treatment and age; age affected locomotor activity in the control groups and the group given the lowest dose of diazepam, but not in the groups exposed to 5 or 10 mg/kg. Analysis of drug-exposed versus control litters yielded reliable effects for treatment, age, and their interaction; trend analysis showed a significant quadratic (increasing, then decreasing) function for age, which interacted significantly with drug treatment. It may be concluded that the transitory period of potentiated locomotor activity is absent in the rats exposed to larger amounts of diazepam prenatally. The relative hypoactivity of such rats cannot be attributed to a generalized motor dysfunction, since at other times there were no reliable differences between the groups. Also, in other tests the treated animals were identical to controls in the onset and performance of body-righting behavior and the geotactic reflex.

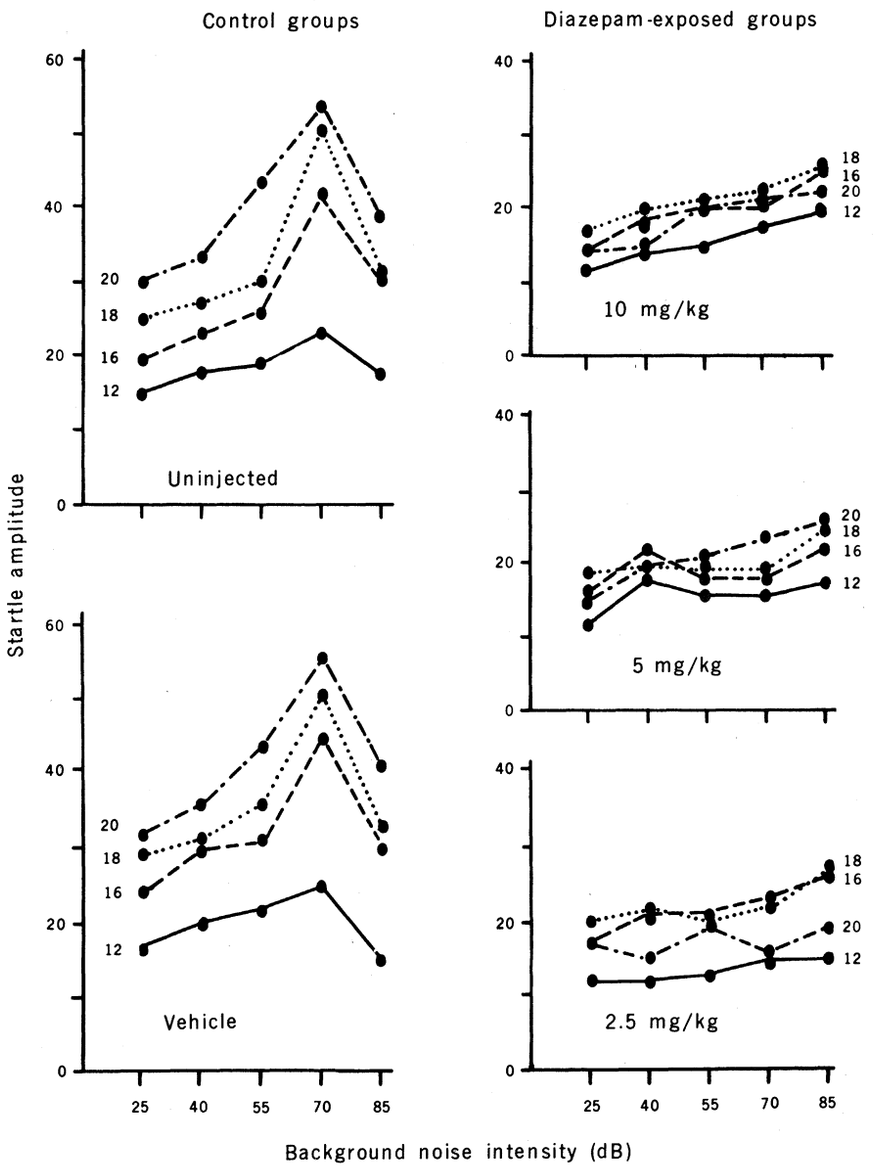

Fig. 2. Development of and effect of background noise intensity on the acoustic startle response of control and diazepam-exposed rats. The stimulus was a 110-dB, 10-kHz, 20-msec tone. Startle amplitude refers to oscilloscope units. Number of rats tested were as follows: uninjected, 11; vehicle-exposed, 8; and diazepam-exposed, 7, 3, and 7 to 2.5, 5, or 10 mg/kg, respectively. Animals tested represented a minimum of two litters except for those exposed to the 5 mg/kg dose of diazepam.

To obtain a measure of reactivity to controlled stimulation, the animals not tested for locomotor activity were exposed to intense auditory tone bursts, which elicited the acoustic startle reflex. This reflex is sensitive to a variety of environmental events in both rats and other species. In the rat, the response is markedly potentiated by stable white noise of moderate intensity. Potentiation is presumed to result from an arousal mechanism activated by the background noise (*11*).

Figure 2 presents the mean startle reactions to noise (*12*). The startle amplitude in vehicle-injected and uninjected controls was markedly potentiated at a background intensity of 70 dB, beyond which amplitude decreased (these are exactly the effects obtained in adult rats). This potentiation was observed at all ages tested but was pronounced after day 16. In contrast, noise did not elicit marked potentiation at the intermediate noise levels in the diazepam-exposed animals at any age. Of 17 animals chosen at random from five litters only one showed the normal inverted-U potentiation function, and even then the potentiation did not enter the range of normal values. Control animals included 11 uninjected and 8 vehicle-exposed animals chosen at random from five litters. All showed the inverted-U function. Analyses of variance on both offspring and litters demonstrated significant main effects for age, background, treatment, and their interactions. Trend analyses revealed reliable linear, quadratic, and cubic functions for background—all of which interacted significantly with drug treatment. The reduction of noise-elicited reflex potentia-

tion in rats prenatally exposed to diazepam is particularly interesting since few manipulations reduce this response in adult rats. However, in adult rats diazepam and flurazepam hydrochloride do reduce the acoustic startle response potentiated by electric shock (*13*).

To determine whether the behavioral consequences of prenatal exposure to diazepam could have resulted from diazepam persisting in the brain tissue of the offspring, two procedures were used (*14*). A competitive binding procedure did not reveal diazepam or its active metabolites in brain tissue of 10-day-old rats that had been exposed to diazepam (5 mg/kg) prenatally, whereas some inhibition of the binding was apparent in 2-day-old rats. Additionally, when ^{14}C-labeled diazepam (administered with unlabeled diazepam at 2.5 mg/kg) was given to dams, no radioactivity could be detected in the brains or livers of their offspring at 20 days of age. These results suggest that the behavior observed during the third week of postnatal life was not being influenced by persisting diazepam.

It is evident that prenatal exposure to diazepam in the third (final) week of gestation has consequences for the development of arousal processes in the rat. We have demonstrated that prenatal exposure to diazepam during this period of profound neuronal differentiation induces pronounced alterations in behavioral development that normally takes place in the third postnatal week. The characteristic developmental pattern of locomotor activity (presumed to be a consequence of isolation in the unweaned rat) has been attributed to the asynchronous rates of maturation of arousal structures and arousal-inhibitory structures (*9*). Similarly, the facilitation of acoustic startle behavior by background noise at moderate intensities is part of a generalized arousal process seen in rats in a variety of reflexive and nonreflexive behaviors. The fact that both of these phenomena are lost after prenatal exposure to diazepam suggests that the drug alters neuronal development in such a way that the behavioral consequences of arousal are suppressed. Administration of other drugs during the third week of gestation in the rat has been shown to interfere with neuronal differentiation (*15*). This period may be a time of great sensitivity to drugs that act on the central nervous system, and may be critical for the induction of behavioral teratology.

The practical significance of these results will be revealed only by further testing in which the intended therapeutic usage of these drugs is taken into account. The effects of maternal anxiety and stress may also be deleterious for rat offspring (*16*), and diazepam may counter such effects without increasing the overall risk to the offspring. Future studies should address the effects of the interaction of maternal stress and ataractic drugs administered during pregnancy on the development of progeny.

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5. The gestational period in the rat is 21 to 22 days. Neuronal multiplication occurs during the third week, a period comparable to the second trimester of human pregnancy.
6. Animals were housed individually in supervised quarters with constant temperature and humidity and a 12-hour light-dark cycle (lights on at 6:00 a.m.). A ratio of two to three females per male was used for breeding. The animals were placed together at 5:00 p.m.; vaginal smears were obtained by 9:30 the following morning, at which time the animals were separated.
7. The diazepam was the injectable solution prepared by Hoffmann-La Roche. The doses were based on the weight of the experimental rats on day 13 and were held constant throughout. Volumes administered ranged from 0.16 ml to 0.67 ml. Volume of the vehicle was also varied among the three vehicle-injected animals to correspond to the different doses of diazepam given the experimental rats.
8. One litter exposed to diazepam (5 mg/kg) and one uninjected group were used for analysis of the development of thermoregulatory function; no differences were observed.
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10. Locomotive activity was recorded on postnatal days 14, 15, 16, 17, 18, and 20. Animals were individually placed in an activity chamber for a 20-minute period between 12:00 noon and 4:00 p.m. The chamber, built in our laboratory, records horizontal activity on the basis of changes in resistance measured by drinkometer-type circuitry. The floor of the chamber (18 by 10 inches) receives an input from an oscillator (10,000 cycle/sec) and is divided into 45 1-inch squares, each of which is connected to one of three amplifiers. When the rat's paws cross between the main board and the inside of a square, the output of the oscillator (50 μA) passes through the paws, completing a circuit, and a signal is sent to an amplifier. The counts were recorded every 2.5 minutes on an Anadex printer. The cumulated activity during the 20-minute period was used for analysis.
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12. Measurement of the acoustic startle response was conducted in a double-walled sound-attenuating chamber. Rats were placed individually in a small perforated Plexiglas cage. An accelerometer mounted below the cage detected the startle response. Output from the accelerometer was fed to a Grass polygraph (7P122B), amplified, and exhibited on a cathode-ray tube as a change in voltage. The startle stimulus was a 10-kHz, 110-dB, 20-msec tone that had rise and decay times of 5 msec. Background white noise was fed into the testing chamber by a Grason-Stadler white noise generator. Presentation of the different intensities was determined by Latin squares; there were five trials at each background intensity. All sound measurements were made with 20 μN/m^2 as the reference level. Previous studies demonstrated that there was no difference in the acoustic startle response measured on day 21 between rats tested daily from day 12 and rats tested only on day 21 [T. Parisi and J. R. Ison, *Dev. Psychobiol.* **12**, 219 (1979)].
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