PHYSICAL AND BEHAVIORAL DEVELOPMENT IN RATS AFTER LATE PRENATAL EXPOSURE TO DIAZEPAM

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Abstract: The effect of late prenatal exposure to diazepam (DZP) on physical and behavioral development of rat pups was investigated. Prenatal exposure to DZP (20 mg/kg, sc, in last week of pregnancy) did not alter litter size and no gross malformations were noted at birth. Body weight at birth and subsequent weight gain was significantly less in these animals. The development of reflexes and neuromuscular maturation was normal. Open field locomotor activity and rearing scores were significantly decreased. Test of social play in juvenile rats revealed normal pattern of sexual dimorphism with increased masculinized behavior. Acquisition and retention of passive avoidance task was not affected by DZP exposure, however, retention of brightness discrimination task was significantly decreased. The hypnotic effect of a challenge dose of DZP and convulsive effect of pentylene tetrazol remained unaltered. Open field activity test in adult animals revealed increased ambulation. Probe dose of amphetamine in these animals caused paradoxical decrease in activity.

It is concluded that exposure to high dose of DZP during late prenatal period may not manifest in physical or neuromuscular impairment during early development period, except for weight loss, however, it may have long term effects on behavior becoming manifest in adolescence and at maturity.

Key words: diazepam prenatal rat acquisition retention locomotor activity

INTRODUCTION

Diazepam (DZP) given during late pregnancy may lead to ‘floppy infant syndrome’ (1) or ‘withdrawl symptoms’ (2) of short duration. No attempt, however, has been made to follow up these children to monitor late effects of prenatal use of DZP on behavior and performance, probably because of inconsistent animal data in the area. The findings varied depending on the dosage schedule used and duration of prenatal exposure and also the age of the offspring at the time of testing (3). However, learning and memory deficits (4,5), absence of acoustic startle reflex in third postnatal week (3) and impairment of conditioned avoidance response in prenatally exposed rats have consistently been reported (4,6).

In rats, rapid brain receptor development takes place in the third week of gestation and during first three weeks after birth (7). Exposure to chemicals or toxins during this period may result in long lasting subtle behavioral deficits. Most studies conducted so far on the behavioral effects of prenatal exposure to DZP use few preselected tests of behavior and doses upto 10 mg/kg/day (4,8,9,10). Present investigation was aimed at conducting large number of test procedures at different stages of growth and development following a higher prenatal dose (20 mg/kg/day). In addition, the behavioral response to a probe dose of drugs acting at different neurotransmitter mechanisms was also tested in adult offsprings.

METHODS

Albino rats of either sex were used. Nulliparous female rats (100-150g and 2 months of age) were selected as dams and were kept individually in cages at a temperature of 30±2°C with light cycle between 6 AM and 7 PM. Free access to commercial food and tap water was allowed. After one week of acclimatization each animal was exposed to two male rats of proven fertility. The vaginal smears were examined daily for

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the presence of sperms. The sperm positive day was labelled as day one of pregnancy (G1). On G13, the pregnant females were weighed and assigned to one of the three groups viz., (1) Diazepam treated (n = 6); (2) Vehicle injected controls (n = 6) and (3) Uninjected controls (n = 12).

Diazepam (Calmpose, Ranbaxy) was injected between 9.30-10.30 A.M. daily for 7 days from G14 to G20. The dose (20 mg/kg, sc) was calculated on the basis of weight on G13 and was kept constant later. Control animals received equal volumes of vehicle (40% propylene glycol in 10% ethyl alcohol, sc) for the same period. The animals were checked every 4 hr near term (G21) and pups were counted soon after birth (litter size). They were examined for any gross malformations and weighed individually. The litter size was then reduced to eight, and pups were fostered with uninjected dams (delivered within 24-48 hr of that of experimental dams) till weaning (day 21; P21). Body weight of pups was measured on P1, 21, 30, 60.

A total of 92 pups were exposed to vehicle and 106 to DZP in two batches.

Behavioral and performance tests

Preweaning period: Table I summarizes the days on which the appearance of physical signs was noted and reflexes were tested. The tests during preweaning period were performed using 15-20 pups selected randomly from both groups.

<table>
<thead>
<tr>
<th>Postnatal day (P)</th>
<th>Physical signs/reflexes</th>
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<tbody>
<tr>
<td>0</td>
<td>Day of birth</td>
</tr>
<tr>
<td>2</td>
<td>Surface righting</td>
</tr>
<tr>
<td>4</td>
<td>Pinna detachment</td>
</tr>
<tr>
<td>8</td>
<td>Cliff avoidance</td>
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<tr>
<td>9</td>
<td>Negative geotaxis</td>
</tr>
<tr>
<td>11</td>
<td>Forelimb grasp</td>
</tr>
<tr>
<td>15</td>
<td>Barholding</td>
</tr>
<tr>
<td>16</td>
<td>Eye opening</td>
</tr>
<tr>
<td>16</td>
<td>Forelimb hanging</td>
</tr>
<tr>
<td>16</td>
<td>Air righting</td>
</tr>
<tr>
<td>17</td>
<td>Development of fur</td>
</tr>
<tr>
<td>19</td>
<td>Ear unfolding</td>
</tr>
<tr>
<td>21</td>
<td>Rotadrum test</td>
</tr>
<tr>
<td>25-30</td>
<td>Descent of testis and opening of vagina.</td>
</tr>
</tbody>
</table>

To test the motor ability (holding capacity for 180 sec) the individual animal was placed on a rotating drum (15 cm D, 2 rpm) 3 times on 2 consecutive days.

Postweaning period: Following tests of increasingly complex nature were performed on the day specified. The mean scores of individual groups in each were calculated and compared.

1. Open field activity: P35 During a 2 min observation period, ambulation was scored, in a special arena (circular 60 cm D with 18 floor sectors) by the number of sectors the animal crossed by all four limbs. Rearing and grooming was also scored along with the number of faecal pellets and urine deposits.

2. Social play behavior in juvenile rats: P38 At weaning 6 males and 6 females were separated from each and housed in monosexual groups of three. Three animals were marked as test animals and the others as stimulus on P38. Only the animals designated as test were removed from their social housing unit and placed individually in cages. Play behavior was tested 48 hr after separation. The test animal was in its isolation cage into which a stimulus animal of the same sex and age was introduced. The animals were marked and observed from a distance of 2 meters from the cage. The number of pins made by each test animal and stimulus animal of a pair was recorded for a 10 min-session. A pin was defined as an instance when one rat had its dorsal surface to the floor with another animal hovering on top in a dominant stance.

3. Passive avoidance test: P35 A modified method of File (11) was used. The animal was placed in the illuminated compartment facing away from the sliding door. As soon as the animal entered the dark compartment, the door was closed and a shock of 100 V, 20/min frequency for 3 msec. was given. The animal was retested 24 hr later and its ability to learn to avoid the shock by remaining in the illuminated compartment for more than 3 min was expressed as learned passive avoidance.

4. Brightness discrimination maze test: P60 Acquisition and retention of brightness discrimination task was tested on a four unit simultaneous choice black-white discrimination maze (4). The animal was deprived of water 24 hr before the test and training consisted of
eight trials. The running time was recorded as the time elapsed when the animal was left in the start box till it reached the goal box, containing water. Errors were scored each time the animal entered at least 4 cm into the black incorrect alley. Recall of the task memory was tested by two additional trials in the maze under similar conditions ten days later.

5. Hypnotic and hypothermic effects after a probe dose 20 mg/kg, ip) of diazepam: P<0.01 The time from loss of righting reflex to its recovery was taken as the sleeping time. Rectal temperature was measured by a thermometer before and one hr after the administration of the drug.

6. Amphetamine induced activity in open field: P<0.05 The ambulation score in individual animal was recorded before and half an hour after the administration of amphetamine sulphate (SKF) given at a dose of 1 mg/kg, ip.

7. Pentylene tetrazol induced convulsion: P<0.01 Pentylene tetrazol (40 mg/kg, ip) was administered and each animal was observed for 20 min period. The duration of tonic clonic convulsion was recorded. The percentage incidence of convulsions and mortality in each group was calculated and compared.

RESULTS

Weight gain of dams during the gestation period in two groups was not significantly different. Gestation period was not affected by DZP exposure and the litter size was similar in both groups. Birth weight was significantly less (P<0.001, 't' test) in DZP exposed group (5.82±0.36g, against 6.45±0.31g in vehicle treated group) although the mean litter size in DZP group (6.67±0.67) did not differ from that in vehicle treated group (8±0.73, "CH²" test).

The age of appearance of physical signs was not different in two groups except 7-8 days delay in full development of fur. Weight gain was markedly reduced in these pups (Fig.1) in tests of reflexes, neuromuscular maturation and motor ability (surface righting, cliff avoidance, negative geotaxis, forelimb grasp, bar holding, air righting ) no difference was observed between pups exposed to DZP or vehicle.

Open field activity of pups exposed to DZP and vehicle on P<0.05 is shown in Table II. Ambulation and rearing scores were markedly reduced in treated animals.

TABLE II: Open field activity on P<0.05 of pups exposed to diazepam or vehicle during prenatal period.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ambulation (Score Mean ±SEM)</th>
<th>Rearing (Score Mean ±SEM)</th>
<th>Grooming (Score Mean ±SEM)</th>
<th>Defecation (Score Mean ±SEM)</th>
<th>Urination (Score Mean ±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DZP</td>
<td>4.1±0.16 ±2.63** 11.35±1.31**</td>
<td>4.14±0.35 1.36±0.39 0.42±0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>51.35±2.14 16.92±1.69 3.57±0.38 1.8±0.25 0.77±0.21</td>
<td></td>
<td></td>
<td>**P&lt;0.01 (t-test)</td>
<td></td>
</tr>
</tbody>
</table>

Results of social play behavior test in juvenile rats at P<0.05, indicated normal pattern of play behavior viz male animals made more pins than females and
test animals showed more play behavior; however, DZP exposed group had more masculanized behavior (Table III). Learning and retention of a conditioned passive avoidance response in DZP and vehicle exposed groups was not different.

**TABLE III**: Number of pins (Mean±SEM) made during a 10 min. test session by test and stimulus animals exposed to DZP or vehicle prenatally.

<table>
<thead>
<tr>
<th></th>
<th>DZP</th>
<th>Vehicle</th>
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<tbody>
<tr>
<td>Male</td>
<td>10.5±2.97</td>
<td>7.2±2.18</td>
</tr>
<tr>
<td>Female</td>
<td>5.2±2.69</td>
<td>6.0±3.03</td>
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</tbody>
</table>

* P<0.05 (Mann Whitney U test)

In the brightness discrimination task, DZP as well as vehicle treated animals were able to learn the task as indicated by downward trend in the error scores in eight trial session. The difference in running time in the two groups was not statistically significant (Table IV). However, after 10 days, when retested, the DZP exposed animals made more errors and the running time to the goal box was also significantly prolonged (P<0.05).

**TABLE IV**: Acquisition and retention of brightness discrimination in terms of running time and error (Mean±SEM) in animals exposed to diazepam or vehicle during prenatal period.

<table>
<thead>
<tr>
<th></th>
<th>Acquisition (Mean±SEM of 8 trials)</th>
<th>Retention (Mean—SEM of 2 trials)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Running time</td>
<td>Error score</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DZP</td>
<td>1.92±0.22</td>
<td>1.32±0.66</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1.72±0.22</td>
<td>1.13±0.14</td>
</tr>
</tbody>
</table>

* P < 0.05 ('t' test and Mann Whitney U test)

The hypnotic and hypothermic effect of a probe dose of DZP was not reduced in exposed group.

At P< 0.75 the ambulation score in open field was significantly more than control rats which decreased paradoxically after amphetamine in the former while increased significantly in the vehicle controls (Fig.2).

The response to pentylene tetroxol measured in terms of duration and percentage incidence of convulsions and mortality was similar in both groups.

**DISCUSSION**

Prenatal exposure to diazepam did not affect the litter size, and no gross malformations in pups were noticed at birth, the finding being in agreement with other studies (4,9,12). Significant decrease in birth weight observed here, has not been reported by others, probably because they used low doses (1-5 mg/kg). Markedly less weight gain in these pups from P 1-60 could be attributed to impaired suckling as has been reported after prenatal exposure to alcohol (13). Maternal neglect or decreased milk production due to diazepam may not be responsible for this effect, as pups were fostered with uninjected dams. Exposure to low doses of DZP (5 and 10 mg/kg), on the other hand, is shown to cause weight loss in some studies while in others, insignificant effect on weight gain has been reported (4,9,12,14).

The physical development, appearance of reflexes and neuromuscular maturation in exposed pups remained unaffected except for slight delay in fur development.
which could be due to nutritional deficiency as indicated by weight loss.

Decreased open field activity at P3 has also been reported after low doses of DZP (4). In another study normal ontogenic potentiation of locomotor activity at P15-20 was not seen in exposed pups (3). This hypoactivity is unlikely to be due to persistence of DZP and its active metabolite in brain tissue of pups or due to motor disability, however, reduced curiosity and fearfulness has been suggested (3,4). Though evidence for neurotransmitter mechanisms involved in hypoactivity is lacking, decreased hypothalamic norepinephrine concentration reported at this age in DZP exposed rats (10) may possibly be correlated with this effect.

Social play in juvenile rats is an important test to detect sexual dimorphism (15). DZP exposed animals showed more masculanized normal pattern of behavior. Some hormonal change in intrauterine environment of developing fetus could be responsible for this effect (15). Acquisition and retention of conditioned passive avoidance task remained unaffected, the findings being in accordance with others (9). However retention of brightness discrimination task was poor in these animals. In a similar study with low doses of DZP, both learning and retention deficits have been reported (4,12).

The tolerance to hypnotic and hypothermic response to the probe of DZP was not observed as has been described for alcohol and barbiturates (16). In addition the response to pentylenetetrazol remained unchanged, both findings together support the existence of unaltered state of DZP receptors in offspring (8).

Increased ambulation at P7 was an interesting finding when considered in relation to hypoactivity observed in the same animal at P3. Asynchronous rates of maturation of inhibitory and excitatory neurotransmitter mechanisms in the brain may possibly be responsible for this observation. The ontogenic increase in locomotor activity during preweaning period (17) might be delayed with normal maturation of inhibitory mechanisms (18) in these animals. Another possible contributory factor for hyperactivity at P7 may be decreased striatal dopamine turnover (19). The paradoxical locomotor response to amphetamine observed here further supports the above suggestion (20).

Presence of hyperactivity with memory deficit in these animals could be compared to minimal brain damage syndrome in children; indeed such rats have been recommended as useful models for learning disabilities in children (12).

In conclusion, chronic DZP treatment of pregnant rats during last week of gestation led to significant changes in behavioral development of the offspring. Some of these changes were specific to age as hypoactivity and masculanized social behavior were predominant in young juveniles, whereas hyperactivity, memory deficit and paradoxical locomotor response to amphetamine were observed in mature animals.

REFERENCES


