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Developmental and behavioral effects of postnatal amitraz exposure in rats

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Abstract

The effects of postnatal amitraz exposure on physical and behavioral parameters were studied in Wistar rats, whose lactating dams received the pesticide (10 mg/kg) orally on days 1, 4, 7, 10, 13, 16 and 19 of lactation; control dams received distilled water (1 ml/kg) on the same days. A total of 18 different litters (9 of them control and 9 experimental) born after a 21-day gestation were used. The results showed that the median effective time (ET_{50}) for fur development, eye opening, testis descent and onset of the startle response were increased in rats postnatally exposed to amitraz (2.7, 15.1, 21.6 and 15.3 days, respectively) compared to those of the control pups (1.8, 14.0, 19.9 and 12.9 days, respectively). The ages of incisor eruption, total unfolding of the external ears, vaginal and ear opening and the time taken to perform the grasping hindlimb reflex were not affected by amitraz exposure. Pups from dams treated with amitraz during lactation took more time (in seconds) to perform the surface righting reflex on postnatal days (PND) 3 (25.0 ± 2.0), 4 (12.3 ± 1.2) and 5 (8.7 ± 0.9) in relation to controls (10.6 ± 1.2; 4.5 ± 0.6 and 3.4 ± 0.4, respectively); the climbing response was not changed by amitraz. Postnatal amitraz exposure increased spontaneous motor activity of male and female pups in the open-field on PND 16 (140 ± 11) and 17 (124 ± 12), and 16 (104 ± 9), 17 (137 ± 9) and 18 (106 ± 8), respectively. Data on spontaneous motor activity of the control male and female pups were 59 ± 11 and 69 ± 10 for days 16 and 17 and 49 ± 9, 48 ± 7 and 56 ± 7 for days 16, 17 and 18, respectively. Some qualitative differences were also observed in spontaneous motor behavior; thus, raising the head, shoulder and pelvis matured one or two days later in the amitraz-treated offspring. Postnatal amitraz exposure did not change locomotion and rearing frequencies or immobility time in the open-field on PND 30, 60 and 90. The present findings indicate that postnatal exposure to amitraz caused transient developmental and behavioral changes in the exposed offspring and suggest that further investigation of the potential health risk of amitraz exposure to developing human and animal offsprings may be warranted.

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- Pesticide neurotoxicology
- Amitraz
- Lactation
- Behavioral teratology

Introduction

Amitraz, a formamidine derivative, is widely used in clinical practice as a pesticide for the treatment of demodicosis (1). The drug has been reported to block monoamine oxidase (MAO) activity both in the liver and brain of rats and is thus classified as a monoamine oxidase inhibitor (MAOI)-like agent (2,3). Exposure to large doses of amitraz causes sedation, loss of the righting reflex, motor incoordination, and coma (1,4,5). According to Flório et al. (6), acute amitraz administration to adult rats induced many behavioral alterations and increased the levels of noradrenaline and dopamine in different brain regions.

During its development, the central nervous system (CNS) can be especially susceptible to the toxic effects of xenobiotics (7-9). Maternal exposure to many xenobiotics during gestation and/or lactation has caused developmental neurotoxicity and/or behavioral abnormalities in the offspring that may persist throughout the lifetime of the animal (10,11). Further evaluation of animal development often includes careful observations of the day of appearance of physical landmarks (12). Both evaluations are often used to identify alterations caused by pre- and postnatal exposure to toxic compounds (13).

Recently, in a cross-fostering experiment, Palermo-Neto et al. (14) observed that prenatal exposure to amitraz caused transient developmental and behavioral changes in the exposed rat offspring. One of the most striking results of that study was the finding that some developmental changes were also observed in the control litters nursed by treated dams, an unexpected finding. Indeed, although a lactational transfer of amitraz is likely to occur (15), with the experimental protocol used the pesticide should be eliminated from the dams before parturition (i.e., the last amitraz administration was performed on day 19 of gestation and the drug has been reported to have a 3-day elimination rate). Since, to our knowledge, no information is available about amitraz neurotoxicology during lactation, we evaluated the effects of amitraz exposure during lactation on some physical landmarks and behavioral parameters in developing rats.

Material and Methods

Animals

Genetically similar male and female Wistar rats from our own colony, weighing 250-300 g and about 90 days of age, were used. The animals were housed in temperature-controlled (21-23ºC) and artificially lighted rooms on a 12-h light/12-h dark cycle (lights on at 6:00 a.m.) with free access to food and water. The experiments were performed in a different room with the same temperature as the animal colony. The animals were transferred in their home cages 1 h before the experiments. Studies were performed between 8:00 and 12:00 a.m.

Pesticide dose and form of exposure

Amitraz (Triatox®, Coopers do Brasil S/A, São Paulo) diluted in distilled water was administered by gavage to the lactating experimental female rats at doses of 10 mg/kg (1/80 of the LD_{50}). In a previous pilot study, it was observed that a pesticide dose of 20 mg/kg was the highest dose used that did not reduce food or water intake or weight gain by the animal during lactation. Furthermore, this dose induced no hematologic modifications or other clinical or histopathological signs of overt toxicity. These factors were minimized to avoid the confounding effects of maternal toxicity (16). Since the dams were treated with amitraz the exposure of their offspring to the pesticide was limited to that which could be transferred in the milk.

Twenty nulliparous rats were randomly and equally divided into an experimental group and a control group; amitraz was ad-

ministered by gavage to the experimental rats on lactational days 1, 4, 7, 10, 13, 16 and 19 (postnatal day 0 was the day of birth). Control females were treated identically but with distilled water (1 ml/kg). The present treatment schedule was similar to that used in a previous study during gestation (14). The data were collected by experimenters blind to the treatment condition of the offspring. Dams were weighed every other day during pregnancy and lactation. Only those litters born after a 21-day gestation were used. Day 0 of pregnancy was the day when spermatozoa were found in vaginal smears collected daily. Within each group, all litters were randomly culled on the day of birth to six pups: three males and three females. Within each litter, male and female pups were marked on one paw with China ink for individual identification. Weaning was performed on lactation day 21.

Reproductive parameters and maternal behavior

Although amitraz was administered to the experimental dams during the lactation period, the following reproductive parameters were evaluated in both control and experimental animals: pregnancy duration, number of pups alive or dead at birth, and pup weight immediately after birth. Maternal behavior was studied on days 1, 5, 10, 15 and after delivery in dams of all groups, according to a scoring system proposed by Söndersten and Eneroth (17). The grading system was as follows: 0, absence of nest; 1, presence of nest; 2, all rat pups inside their nests; 3, both dam and rat pups inside their nests; 4, all rat pups in their nest, being nursed by their respective dams. A high correlation was found between scores of two independent observers (Pearson's correlation, $r = 0.95$). The body weights of the pups were taken on postnatal days (PND) 5, 10, 15 and 21 (weaning).

Landmark and reflex development

Within each group, 9 male and 9 female pups from different litters were observed daily for total pinna unfolding of both external ears, fur development, incisor teeth eruption, ear, eye and vaginal openings, and testis descent. The presence of a startle response (a whole-body startle in response to the clicking of a spring-loaded metal trap held 2 to 3 cm above and behind the animal) and the grasping hindlimb reflex (the time in seconds spent by an animal in grasping a stick and 70-cm long wire extended horizontally between two 50-cm high poles with one or both of the hindlimbs) were also evaluated. The method for rapid graphic solutions of time-percentage effect curves was employed to calculate the median effective times (ET_{50}) and their confidence limits for each developmental landmark assessed in control and treated pups (18). These curves were constructed using the percentage of animals showing each of the above parameters and their time of appearance.

The following reflexes were also studied in control and experimental pups (9 male and 9 female pups within each group): the surface righting reflex (the time in seconds spent by an animal to assume a normal ventral position after being placed on its back) on PND 2, 3, 4, and 5 and the climbing response (the time in seconds spent by an animal to climb a 45^{o} inclined and 30-cm long rough surface) was also measured on PND 6 to 11.

Spontaneous motor activity and open field studies

The spontaneous motor activity of the male and female pups was observed in an open-field on PND 15 to 21. The device used, similar to that proposed by Broadhurst (19), was a round wooden arena (a 50-cm round surface surrounded by a 20-cm high

Table 1 - Effects of amitraz exposure (10 mg/kg) during lactation on indicative landmarks of physical development of suckling rats.

In each group, 9 male and 9 female pups (from 9 different litters) were used. Treatment was given on days 1, 4, 7, 10, 13, 16 and 19 of lactation; control dams were treated with 1.0 ml/kg of distilled water. Data are the median effective days (ET_{50}) plus upper and lower limits; significance (*P<0.05) compared to controls was calculated according to Litchfield (18).

Parameters	Groups	
	Control	Amitraz-treated
Pinna unfolding	6.1 (6.5-6.8)	9.1 (7.7-10.2)
Fur development	1.8 (1.6-2.0)	2.7* (2.4-2.9)
Incisor eruption	5.7 (5.3-5.9)	6.2 (5.7-6.7)
Ear opening	13.6 (12.5-18.1)	19.0 (17.0-22.3)
Eye opening	14.0 (13.6-14.4)	15.1* (14.5-15.7)
Vaginal opening	37.5 (35.0-40.1)	39.0 (36.0-42.2)
Testis descent	19.9 (19.7-21.1)	21.6* (21.0-22.2)
Startle response	12.9 (10.2-13.8)	15.3* (14.3-17.4)
Grasping hindlimb	4.9 (4.3-5.6)	5.2 (4.5-6.0)

enclosure) painted white and subdivided into 19 parts painted black. During the experiments a 40-W white bulb located 74 cm from the floor provided continuous illumination of the arena. A hand-operated counter was employed to score the number of divisions crossed in the open-field; 16 naive pups (8 males and 8 females from different litters within each group) were used. For observation each pup was individually placed in the center of the arena and the number of divisions crossed was counted for 6 min.

A similar device (90 cm in diameter) and identical illumination were employed to study open-field behavior on PND 30, 60 and 90. Hand-operated counters and stopwatches were employed to score ambulation frequency (number of floor units entered with both front feet), rearing frequency (number of times the animals stood on their hind legs), and immobility time (total seconds of no movement); within each group, 9 male and 9 female pups from different litters and already used to study landmark and reflex development were employed. For open-field observations, each rat was individually placed in the center of the arena and its behavior recorded for 6 min.

To minimize the influence of possible circadian changes in pup spontaneous motor activity and open-field behavior, control and experimental animals were alternated, the rats being observed at the same time of day in each session. The open-fields were washed with an alcohol-water solution (5%) before placing the animals to obviate possible biasing effects due to odor clues left by previous rats.

Statistical analysis

Maternal behavior scores were analyzed statistically by Kruskal-Wallis analysis of variance for nonparametric data. The ET_{50} values determined for each landmark were compared between groups using Litchfield (18) procedures. Bartlett's test (20) showed that data on dam and pup weight, spontaneous motor activity, surface righting reflex, climbing response and open-field behavior were parametric (P<0.05). Thus, two-way analysis of variance (treatment x days) followed by the *t*-test for comparison of cell means (StatPac Statistics Analysis Package) was used to analyze these data. An alpha level of P<0.05 was taken to indicate significant differences for all comparisons made.

Results

Reproductive parameters and maternal behavior

No differences in body weight were found ($F_{1,54}$ = 1.15, P>0.05) between control and

experimental dams throughout pregnancy (data not shown). There was no significant difference in average number of pups alive at birth between the control group (10 ± 3) and the experimental group (10 ± 2). Furthermore, no pups were found dead at birth and no differences in pup weight were detected immediately after birth; malformations were also absent. Although the length of gestation did not differ between control and experimental dams, only the 18 litters (9 of them control and 9 experimental) born after a 21-day gestation were included in the experiment. No differences were found in the maternal behavior of control and experimental dams (H_{40} = 5.8, P>0.05). Thus, during all the days of observation and for both groups, the median of the maternal behavior scores was 4. In addition, no differences were found in dam weight during lactation ($F_{1,72}$ = 0.15, P>0.05) or in pup weight ($F_{1,424}$ = 1.01, P>0.05) on PND 5, 10, 15 and 21 (weaning) (data not shown).

Landmark and reflex development

Since the time-percent effect curves constructed to study the effects of amitraz exposure during lactation on pup development were parallel, we compared the data for the two groups. As shown in Table 1, amitraz exposure during lactation increased the ET_{50} for the development of fur, eye opening and testis descent (P<0.05). Table 1 also shows that amitraz exposure during lactation increased the ET_{50} for the appearance of the startle response (P<0.05). The ET_{50} for the other parameters studied, such as incisor eruption, total pinna unfolding of the external ears and ear and vaginal openings were not modified by postnatal amitraz exposure (P>0.05). Finally, no differences in the time spent to perform the grasping hindlimb reflex were detected between control and experimental animals (P>0.05).

Figure 1 shows that significant differences between control and experimental data

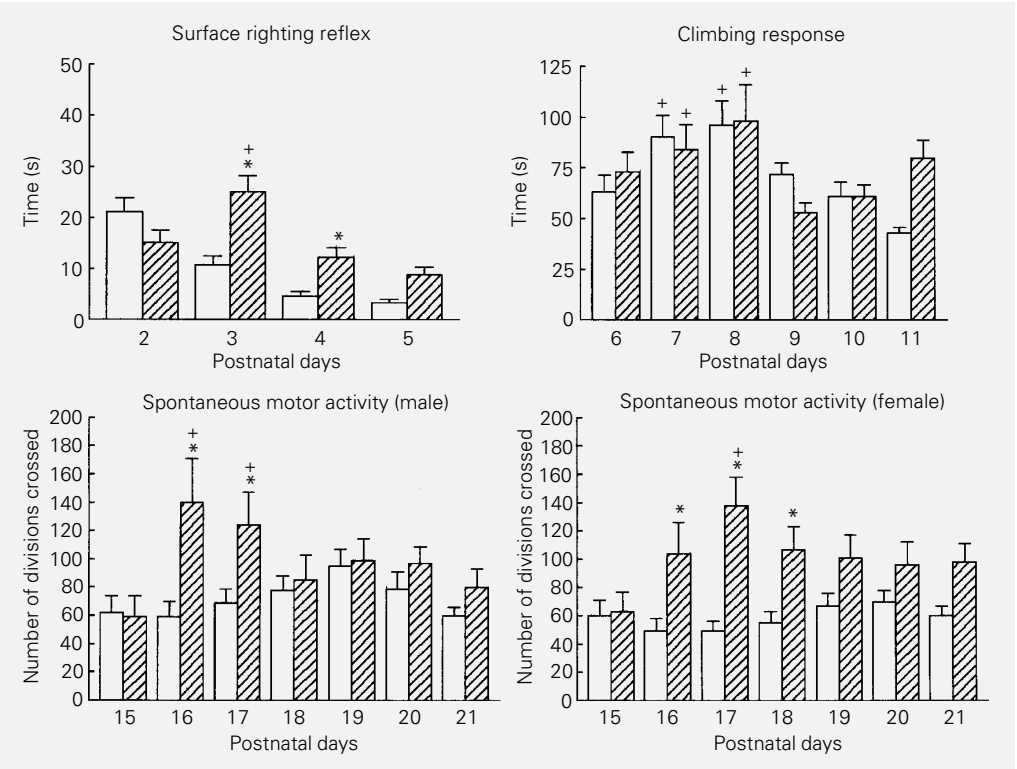

Figure 1 - Effects of postnatal amitraz exposure (10 mg/kg) on spontaneous motor activity, climbing response and surface righting reflex of pups. Treatment was given on postnatal days 1, 4, 7, 10, 13, 16 and 19 of lactation. In each group, 9 male and 9 female pups from different litters were used to observe the surface righting reflex and the climbing response. The spontaneous motor activity was observed in 16 naive animals (8 males and 8 females from different litters for each group). The open and hatched columns correspond to offspring of control and amitraz-treated dams, respectively. Data are reported as the mean ± SEM. *P<0.05 compared to control Groups; +P< 0.05 compared to the other days (two-way ANOVA).

were also observed for surface righting reflex on PND 3 and 4 ($F_{1,64}$ = 4.45, P<0.05); pups from dams treated with amitraz during lactation spent more time to perform this reflex. Furthermore, the time spent by the experimental animals to perform this reflex on PND 3 was greater ($F_{3,64}$ = 7.33, P<0.05) than that found for both control and experimental animals in the remaining days of observation. No differences were found between control and experimental data for the climbing response on PND 6, 7, 8, 9, 10 and 11 ($F_{1,96}$ = 1.55, P>0.05); nevertheless, differences were detected among the days of observation. Indeed, the values for the climbing response were greater in both control and experimental animals on PND 7 and 8 ($F_{5,96}$ = 5.22, P<0.05).

Spontaneous motor activity and open-field studies

Postnatal amitraz exposure increased the number of divisions crossed by experimental male ($F_{1,98}$ = 5.32, P<0.05) and female ($F_{1,98}$ = 18.84, P<0.05) pups in the open-field, compared to that of controls on PND 16 and 17 and 16, 17 and 18, respectively. Differences were also found among the data recorded for the experimental pups along the observation days; thus, activity recorded on PND 16 and 17 for experimental males ($F_{6,98}$ = 2.16, P<0.05) and on PND 17 for experimental females ($F_{6,98}$ = 3.05, P<0.05) were the highest recorded. No qualitatively gross differences were observed between the various postures displayed by the pups during the 6-min observation period (position of the head and limbs and contact with the surface). Nevertheless, the elevation of the heads, shoulder and pelvis matured one or two days later in the experimental pups.

Finally, no differences were found (P>0.05) between locomotion (LO) and rearing (RE) frequencies or immobility time (IT) of male (LO: $F_{1,48}$ = 1.3; RE: $F_{1,48}$ = 0.23; IT: $F_{1,48}$ = 0.007) and female (LO: $F_{1,48}$ = 2.55; RE: $F_{1,48}$ = 2.96; IT: $F_{1,48}$ = 0.42) pups on PND 30, 60 and 90. Nevertheless, in the male pups LO ($F_{2,48}$ = 10.10, P<0.05) and RE ($F_{2,48}$ = 14.7, P<0.05) frequencies recorded on PND 60 were higher than those recorded on PND 30 and 90. Further analysis showed that female pups had smaller frequencies of LO ($F_{2,48}$ = 16.52, P<0.05) and RE ($F_{2,48}$ = 60.26, P<0.05) and higher IT ($F_{2,48}$ = 47.50, P<0.05) on PND 30. Male pups also had higher IT on PND 30. These data are illustrated in Figure 2.

Discussion

Postnatal amitraz exposure in rats changed the time course of development of some physical landmarks (increased the ET_{50} for the development of fur, eye opening and testis descent) and increased the ET_{50} for the appearance of the startle response. Pups from dams treated with amitraz during lactation also spent more time to perform the surface righting reflex. Further spontaneous motor activity, as detected by the number of crossings in the open-field, was higher in experimental offspring postnatally treated with amitraz. Some qualitative differences in spontaneous motor behavior were also observed, with the elevation of the head, shoulder and pelvis maturing one or two days later in the amitraz-treated offspring. No differences were found in the length of gestation or the body weight of the pregnant dams used in this experiment and the body weights of their offspring were similar at birth. No differences in maternal behavior were observed between control and amitraz-treated rats. Finally, no differences were found in dam or pup weight throughout lactation. These findings are important, since malnutrition during pregnancy and lactation as well as alterations in maternal behavior often result in differences in the median times of maturation of physical features and reflexes (21).

Experimental offspring treated with amitraz during lactation did not show changes

in activity in the open-field. Locomotion frequency measured in the open-field has been used as an index of both arousal (22,23) and "emotionality" (24); the decrease or absence of movement within the apparatus normally indicates a reduction in arousal or an increase in the level of emotionality (25). In light of the present findings, it seems reasonable to suggest that amitraz exposure during lactation did not change arousal and/or emotionality of the offspring. This hypothesis is not in accordance with that proposed for

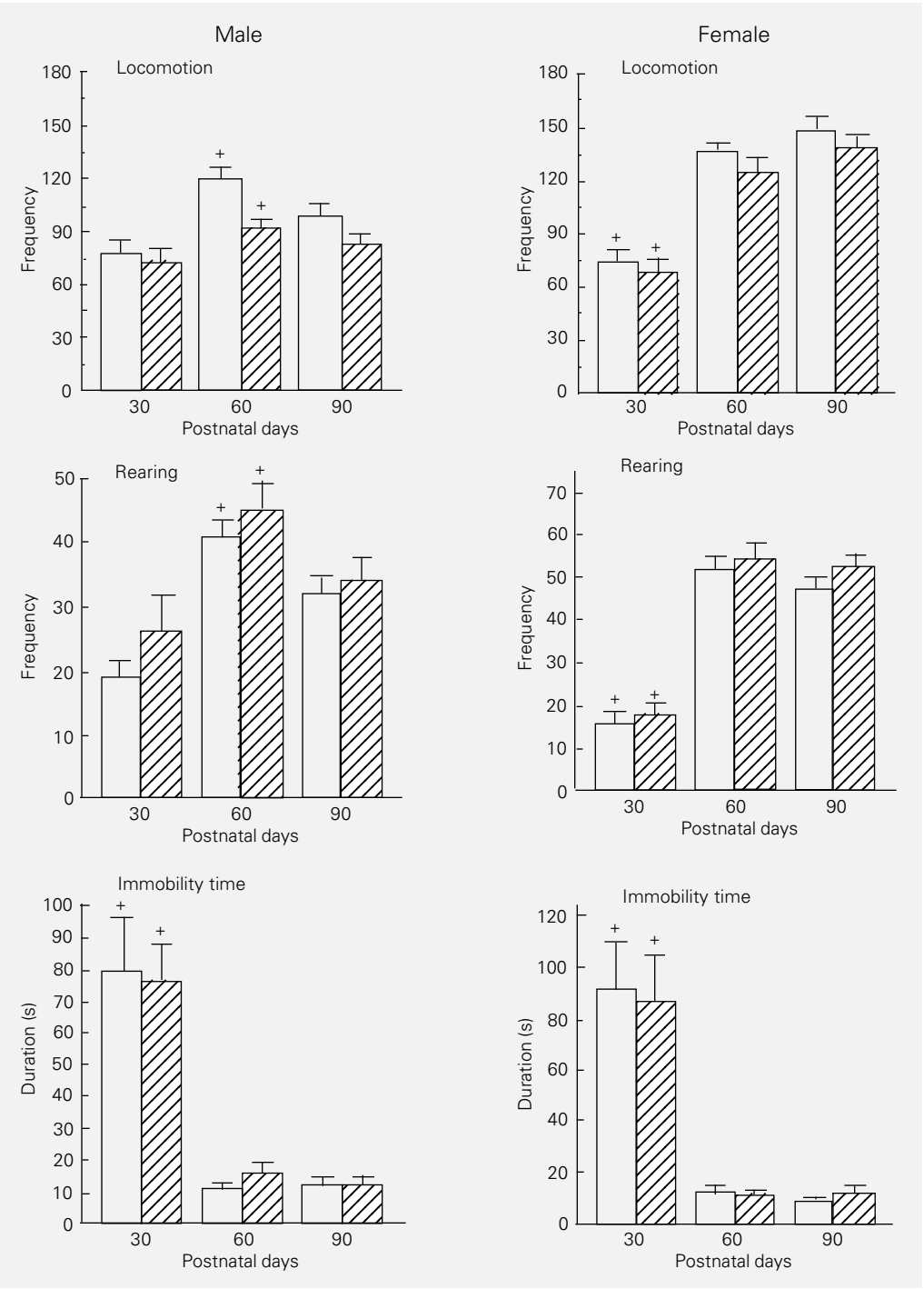

Figure 2 - Effects of postnatal amitraz exposure (10 mg/kg) on locomotion and rearing frequencies, as well as on immobility time of male and female rats observed in an open-field at 30, 60 and 90 days of age. Treatment was given on days 1, 4, 7, 10, 13, 16 and 19 of lactation. In each group, 9 male and 9 female pups from different litters were used. The open and hatched columns correspond to offspring of control and amitraz-treated dams, respectively. Data are reported as the mean ± SEM. $^{+}P<0.05$ compared to the other days (two-way ANOVA).

amitraz given during gestation (14). In that experiment, a lactational transfer of amitraz was reported to be responsible for the observed increase in activity of the experimental offspring on PND 30. In light of the present protocol, it seems possible to suggest that the stress of being previously manipulated to observe landmark and reflex development might have equalized the reaction of both control and experimental pups to the apparatus. Nevertheless, it seems relevant to point out that this procedure did not induce a uniform open-field behavior of the rats previously studied in the cross fostering experiment (14). In this respect, a modification of mother-offspring interaction in our previous study may have also occurred after the exchange of treated progeny with control mothers, thus being responsible for these significant data and for the discrepancies observed in the present study. Indeed, this criticism was already made for fostering procedures (26).

Several relevant postnatal developmental modifications occur in the hypothalamic-pituitary-testicular axis of rats, including testis descent (27). Therefore, the decrease in ET_{50} for testis descent detected here might suggest changes in pup sexual development and probably in their sexual activity when adults. Nevertheless, as usually observed (13,28), female rats had higher activity scores in the open-field than males on PND 90, i.e., amitraz given during lactation did not change the sexual dimorphism of rats observed in the open-field.

The present results show that amitraz administration during lactation may change the development of the suckling pups. Accordingly, it seems feasible to think that the developmental effects previously reported in control pups nursed by dams exposed to amitraz (20 mg/kg) during gestation (14) were related to a lactational transfer of the drug and/or its metabolites to the suckling neonates. The mechanism of the effects of postnatal amitraz exposure is unknown and future experiments are needed to determine pesticide toxokinetics both in lactating dams and suckling pups. Indeed, two possibilities arose from the present data: 1) the 3-day elimination rate proposed for amitraz (29) is not valid for pregnant and/or lactating rats and 2) amitraz metabolites might induce behavioral and developmental changes in rat offspring. Alternatively, the role of amitraz and/or its metabolites on amine levels and activity in dam and pup brain and also in their endocrine status seems to warrant further investigation. Indeed, it was recently suggested that the acute effects of amitraz on motor function of rats are the consequence of the inhibitory effects on MAO activity, most probably through the increases in catecholamine levels produced within the CNS (6). Clearly, more information is needed to interpret the present findings. Efforts are currently being undertaken to study the toxokinetics of amitraz in both lactating dams and suckling pups.

The present data further support the view that nervous tissue, especially the brain, is more sensitive to foreign chemicals and that toxic effects can be manifested as subtle disturbances of behavior long before classical symptoms of poisoning become apparent. In this respect, several papers have discussed the qualitative and quantitative comparability of human and animal developmental neurotoxicity (8,9,30). Amitraz is widely used in the treatment of demodicosis and the present data raise concern about the safety of the use of this treatment during lactation. In addition, the present findings support the use of developmental and behavioral evaluations in animals when assessing the potential developmental neurotoxicity of these chemicals in humans.

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