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# Motor Behavior and Nigrostriatal Dopaminergic Activity in Adult Rats Perinatally Exposed to Cannabinoids

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NAVARRO, M., F. RODRÍGUEZ DE FONSECA, M. L. HERNÁNDEZ, J. A. RAMOS AND J. J. FERNÁNDEZ-RUIZ. *Motor behavior and nigrostriatal dopaminergic activity in adult rats perinatally exposed to cannabinoids.* PHARMACOL BIOCHEM BEHAV **47**(1) 47–58, 1994. – We have recently reported several neurochemical alterations, measured at perinatal and peripubertal ages, in the maturation of nigrostriatal dopaminergic neurons following perinatal hashish exposure. In the present work, we tried to undertake whether these neurochemical changes during ontogeny: a) were accompanied by changes of motor behavior, the main neurobiological process regulated by nigrostriatal dopaminergic neurons; and b) persisted in adulthood, leading to disturbances in the expression of an adult motor activity. To this end, two different experiments were performed. In the first, we examined, by using an actimeter, the ontogeny of spontaneous locomotor activity in immature male and female rats born from mothers perinatally exposed to hashish extract. Results showed a complete absence of significant changes in locomotor activity in females, whereas males presented a constant trend to decrease, although never statistically significant, at all ages studied as a consequence of the perinatal cannabinoid exposure. In the second experiment, we evaluated neurochemical indices – dopamine (DA) and L-3,4-dihydroxyphenylacetic acid (DOPAC) contents, tyrosine hydroxylase (TH) activity, and number and affinity of $D_1$ and $D_2$ dopaminergic receptors in the striatum – and behavioral parameters – spontaneous locomotor activity and spontaneous and induced stereotypic behavior – both indicating nigrostriatal dopaminergic activity, in adult female and male rats perinatally exposed to hashish extract. Results were as follows. The spontaneous locomotor activity, measured in the actimeter, was not affected by perinatal hashish exposure in both adult males and females. This was also seen in an open-field test as measured by total number of sector crossings. However, when differentiated between internal and external sectors hashish-exposed males presented a higher number of external crossings than controls, which did not appear in females. Moreover, several induced stereotypic behaviors, such as self-grooming and shaking induced by water spraying, were also altered by hashish treatment in a sexually dimorphic manner, whereas the number of spontaneous rears and self-grooms, measured in the open-field test, was unchanged. Thus, the frequency of water spraying-induced self-grooming was significantly increased in both males and females perinatally exposed to hashish, although the increase was more marked in males (200.4%) than females (121.2%). In addition, the frequency of shaking was also markedly increased in males but remained unchanged in females. These behavioral effects were paralleled by modifications in striatal neurochemical parameters. Thus, there was a significant increase in the DOPAC/DA ratio, indicating increased presynaptic activity, in females perinatally exposed to hashish, but compensated by a lower density of $D_1$ receptors. On the contrary, presynaptic activity in males perinatally exposed to hashish was unchanged, but there was an increase in the density of $D_2$ receptors, which tentatively might explain the increased stereotypy observed in males. Collectively, these results show that the changes in the ontogeny of nigrostriatal dopaminergic neurons originated by perinatal cannabinoid exposure: a) seem to be accompanied by subtle behavioral changes but only in immature males; and b) lead to a set of sexually dimorphic disturbances in the adult functionality of these neurons at the neurochemical and behavioral levels.

Marihuana Cannabinoids $\delta^9$-Tetrahydrocannabinol Nigrostriatal dopaminergic neurons Dopamine
DOPAC Tyrosine hydroxylase $D_1$ and $D_2$ receptors Motor activity Stereotypic behavior

"CANNABIS sativa" preparations (hashish, marihuana) are one of the most widely used psychoactive drugs (30). Their abuse produces multiple physiological effects, mainly at the behavioral and neuroendocrine levels, during both developmental and mature ages [for review, see (18)]. For instance, exposure to marihuana or $\delta^9$-tetrahydrocannabinol (THC), its main psychoactive principle (14,29), has been reported to decrease the release of prolactin (42,50), growth hormone (12,

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42), and gonadotrophins (35,50), increase adrenocorticotrophic hormone secretion (25), alter motor behavior (9,34,39), exacerbate psychotic disorders (2,24), and potentiate brain-stimulation reward (19).

The putamen-caudate area is one of the most important targets for marihuana compounds. This view is based on two facts: the appearance of important extrapyramidal effects associated with their consumption (9,34,39) and the recent description of a higher presence of cannabinoid receptors, likely the most important candidate to mediate marihuana effects at the molecular level, in this brain area (20–22). On this basis, it is well assumed that extrapyramidal effects of cannabinoids should be caused through changes in the activity of neurotransmitter systems located in the putamen-caudate area and involved in the control of motor behavior. Much information exists that suggests that nigrostriatal dopaminergic neurons play a primary facilitatory role in the elaboration of patterns of locomotor as well as stereotyped behavior (7). These neurons, whose cell bodies are located in the substantia nigra of mesencephalic reticular formation, innervate, playing a regulatory action, the forebrain structures composed of executive neurons that carry out the integrative and output processes of motor response (49). A relatively important amount of experimental studies have demonstrated the existence of changes in the activity of these neurons after cannabinoid exposure. This was through an inhibition of neurotransmitter uptake (4,40, 48) and/or a stimulation of its release (51), as well as by modifying the binding of $D_2$ receptors to spiroperidol in striatal membranes (6,45). Moreover, the activity of serotoninergic (4) and GABAergic neurons (34,39) in the striatum was also affected by marihuana or THC exposure.

As mentioned above, one of the highest densities of the recently described cannabinoid binding sites in the brain (13,22) has been found in the nuclei of the basal ganglia (21). These receptors, whose structure and functional expression of the cloned cDNA have been also recently characterized (28), are widely distributed in the brain (21). They are present in a number of extrapyramidal areas (21), although presumably located in GABAergic neurons of the striatonigral pathway (20).

On the other hand, cannabinoids can also affect neurotransmitter development when consumed during prenatal and early postnatal ages because they can be transferred from the mother to the offspring through the placental blood and through the maternal milk [for review, see (18)]. This is especially relevant because of the important "trophic" and "plastic" roles played by several neurotransmitters, such as dopamine (DA) and serotonin, during brain development (18). Thus, we have recently reported that perinatal exposure to cannabinoids markedly affects the maturation of several brain dopaminergic neuronal systems (18,43,44,46). These effects appear early, even before the complete differentiation and maturation of dopaminergic projections into their target areas (46), and might produce important long-term effects in the adult behavior. In this respect, previous studies have shown alterations in adult male copulatory behavior, learning ability, open-field activity, and stress response [(8,10,33); for review, see (11,18)] following perinatal exposure to cannabinoids.

The ontogeny of nigrostriatal dopaminergic neurons was significantly affected by perinatal cannabinoid exposure (44). Thus, we found a marked decrease in the striatal activity of tyrosine hydroxylase (TH) associated with increased densities of both $D_1$ and $D_2$ receptors (45), especially at ages around puberty. However, these effects only were permanent and marked in males, whereas they were transient and small in females. Similar sexual dimorphisms were also observed in the perinatal cannabinoid-induced alterations in the other brain dopaminergic systems (43,44,46). Three important questions have derived from these observations: a) whether these ontogenic effects of perinatal cannabinoids might be accompanied by changes at the behavioral level; b) whether these effects might be maintained until adulthood, indicating a permanent alteration in the functionality of these neurons; and c) whether sexual dimorphisms at behavioral or neurochemical levels might exist in adulthood.

These questions have been studied in the present work in two related experiments. In both, pregnant rats were daily fed with hashish extract from the fifth day of gestation up to and including the lactation period. This period of treatment, similar to that used in our previous reports (43,44,46), was chosen based on a previous work of Mirmiran and Swaab (32) showing that the last week of prenatal and the first 3 weeks of postnatal life in rat are the periods of most vulnerability of the neurotransmitters to the drug action. In the first experiment, we examined, by using an actimeter, the ontogeny of spontaneous locomotor activity in male and female rats of different immature ages—15, 20, 30, and 40 days of life, as in our previous report (44)—born from mothers perinatally exposed to hashish extract. In the second experiment, we evaluated the nigrostriatal dopaminergic activity in adult female and male rats (70 days old) perinatally exposed to hashish extracts by analyzing: a) neurochemical parameters—DA and L-3,4-dihydroxyphenylacetic acid (DOPAC) contents, the ratio between both (DOPAC/DA), which represents an index of presynaptic activity (47), TH activity, and number and affinity of $D_1$ and $D_2$ dopaminergic receptors in the striatum; and b) behavioral parameters—spontaneous locomotor activity (measured in an actimeter and open-field test) and frequencies of spontaneous (self-grooming and rearing, measured in open-field test) and induced (self-grooming and shaking after water spraying) stereotypic behaviors.

## METHOD

### *Animals*

Female virgin rats of the Wistar strain were housed from birth in a room with a controlled photoperiod (light 0800–2000 h and temperature (23 ± 1°C). They had free access to standard food (Panlab, Barcelona, Spain) and water. At adult age (> 8 weeks of life; 150–200 g), daily vaginal smears were taken between 1000–1200 h, and only those animals exhibiting three or more consistent 4-day cycles were used in this study. Females in the proestrous phase were allowed to stay with a male for mating, and a new vaginal smear was taken on the next day. Those animals showing the presence of sperm cells were accepted as probably pregnant and used for the cannabinoid exposure studies. The day on which sperm plugs were found was designated the first day of gestation.

### *Cannabinoid Treatment*

Hashish was obtained from the Spanish Administration (Servicio de Restricción de Estupefacientes y Psicótropos; Dirección General de Farmacia y Productos Sanitarios; Ministerio de Sanidad y Consumo; Madrid, Spain). A crude extract was obtained by maceration with methanol and subsequently dried under a nitrogen flow. The extract contained 14.5% THC and lower percentages of other related cannabinoids (6.9% cannabinol and 11.1% cannabidiol). This was prepared in a sesame oil solution for administration. Pregnant females

received a daily dose of hashish extract (equivalent to 20 mg/kg THC daily) from the fifth day of gestation. This dose is an extrapolation from current estimates of moderate exposure to this compound in humans, correcting for differences in route of administration and body surface area (37). Hashish extract was given orally with the help of a cannula. Control rats were fed with vehicle alone. This treatment was maintained until day 24 after birth, the day on which rats were weaned. Experiments with these animals were always performed between 1000–1300 h. During the whole treatment period, we recorded a set of gestational and lactational parameters, such as mother and neonatal weight gain, mother food and water intake, gestational length, placental and fetal weights, litter size, and maternal plasma THC concentrations, to control the existence of a possible cannabinoid-induced nutritional deficit that could be responsible for part of the brain effects.

*Experiment 1: Effects of Perinatal Hashish Exposure in the Ontogeny of Spontaneous Locomotor Activity in Immature Male and Female Rats*

*Spontaneous locomotor activity.* Spontaneous locomotor activity at different immature ages (15, 20, 30, and 40 days after birth) was measured in actimeter. A standard actimeter was used as previously described (38). Briefly, animals were placed in motility cages (26 × 21 × 9.5 cm each) with photocell motility meters (Actimeter Photoelectrique, Apelab, France). The apparatus was located in a sound-isolated cubicle and the number of crossings was recorded every 10 min. Rats were always placed in the motility cages for a period of 10 min before the onset of the test to become reacclimated. Values are expressed as accumulated scores at 10, 20, and 30 min (only those at 20 min are presented, although the remainder times were considered for a complete statistical analysis).

*Experiment 2: Effects of Perinatal Hashish Exposure on Neurochemical and Behavioral Indices of Nigrostriatal Activity in Adult Male and Female Rats*

*Sampling.* Perinatally hashish-exposed male and female (chosen in the estrous phase of ovarian cycle) rats were used in their adulthood (<70 days after birth) for behavioral and neurochemical studies (performed between 1000–1300 h). Behavioral studies consisted of analysis of spontaneous locomotor (measured in the actimeter and open-field test) activity and frequencies of spontaneous (self-grooming and rearing; measured in an open-field test) and water spraying-induced (self-grooming and shaking) stereotypic behaviors. They were performed in the same animals in subsequent days with resting periods (>7 days). Seven days after the end of behavioral studies, all animals were killed by decapitation. Their brains were quickly removed and both striata dissected and immediately frozen at −70°C until assay for dopaminergic measurements.

*Behavioral studies.* Motor behavior was evaluated by measuring the spontaneous locomotor activity in both the actimeter and open-field test. This last test also allowed observations of stereotypic behavior: rearing and self-grooming. Water spraying-induced self-grooming and shaking behaviors were also evaluated. All the behavioral tests were carried out by one investigator who had no knowledge of the treatment of each rat. Actimeter has been described in Experiment 1. The open-field test allowed the simultaneous measurement of spontaneous locomotor and stereotypic activities. It was carried out simultaneously with the measurement of sexual motivation in a sociosexual approach behavior test (data not shown). It is based on the method described by Meyerson (31). The structure consisted of a circular arena composed of hardboard (diameter: 90 cm) with a surrounding (height: 30 cm), both made of transparent polyvinyl chloride. The floor was divided into five inner and eight outer parts using circles and radial segments. For 2 consecutive days before the test day, each experimental animal was adapted to the apparatus by placing it on the arena for 15 min. On the day of the test, the experimental animal was placed in the center of the arena and its spontaneous activity was recorded in a TV-video system. The duration of the test was 15 min. The apparatus was washed out with an odoriferous solution after each rat had been tested. The following parameters were analyzed in three periods of 5 min: a) spontaneous locomotor activity—number of sector crossings (a single line crossing was defined as the rat placing two front paws into an adjacent quadrant), presented as external, internal, and total crossings; and b) stereotypic behavior—frequencies of spontaneous rearing and self-grooming. Water spraying-induced self-grooming and shaking were evaluated according to Berridge and Fentress (5). Rats were placed on a circular cage (diameter: 50 cm), lightly sprayed with a water mist (10 consecutive sprays at 20 cm of the animal) and the frequency of both stereotypic behaviors videotaped during a period of 5 min to be further evaluated.

*Dopamine and DOPAC determinations.* DA and DOPAC contents were analyzed using high-performance liquid chromatography (HPLC) with electrochemical detection. Tissues were homogenized in 0.4 ml 50 mM Tris buffer (pH 7.4) (used for simultaneous measurement of DA and DOPAC content, TH activity, and $D_1$ and $D_2$ binding site parameters) and diluted until an appropriate volume with ice-cold 0.2 N perchloric acid containing 0.5 mM sodium bisulfite and 0.45 mM EDTA (final ratio: 100–200 vol). Dihydroxybenzylamine was added as an internal standard. The homogenates were then centrifuged and the supernatants injected into the HPLC system. Details of this system have been previously published (15). Values are expressed as ng/mg of tissue weight.

*TH determination.* Aliquots of tissue homogenates in 50 mM Tris buffer (pH 7.4) were processed for measurement of TH activity according to the method described by Nagatsu et al. (36). The amounts of L-dopa formed were evaluated by HPLC according to our previously reported method (17). Values are expressed as ng/mg of tissue weight/h of incubation.

*$D_1$ and $D_2$ dopamine binding site analysis.* Measurements of $D_1$ and $D_2$ binding sites were performed according to the procedures described by Reader et al. (41) and Leysen et al. (26), respectively, with slight modifications. Radioactive ligands were [$^3$H]SCH23390 (60.4 Ci/mmol) for $D_1$ and [$^3$H]spiroperidol (27.5 Ci/mmol) for $D_2$, both purchased from New England Nuclear (Boston, MA). The range of concentrations was 0.125–3.0 and 0.05–0.80 nM, respectively. Protein concentration, measured by the Lowry method (27) in the incubated membrane fractions, was 0.2–0.3 mg/ml of incubation for $D_1$ and 0.15–0.20 mg/ml for $D_2$. 30 μM (±)-SK&F38393 and 1 μM (+)-butaclamol, both purchased from Research Biochemical, Inc. (Natick, MA), were respectively used for measurement of nonspecific binding. The final volume of incubation media was 0.5 ml. Details of the methods have been previously reported (16,43). A Scatchard analysis of the data, using linear regression, was performed to evaluate the dissociation constant ($K_d$), expressed as nM units, and the number of binding sites ($B_{max}$), expressed as fmol/mg of protein.

*Statistics*

For data analysis, normality of distribution was tested and, when appropriate, parametric [Student's *t*-test and two-way or multifactorial analysis of variance (ANOVA)] or nonparametric (Kruskal–Wallis test) were used.

## RESULTS

*Effects of Maternal Hashish Exposure on Several Gestational and Lactational Parameters*

As mentioned in the Method section, during the whole treatment period we recorded a set of gestational and lactational parameters to control the existence of a possible cannabinoid-induced nutritional deficit that could be responsible for part of the neurochemical and behavioral effects. According to our results, this possibility seems unlikely. Thus, we detected high amounts of THC in plasma from hashish-exposed pregnant mothers during the last third of gestation as a consequence of hashish treatment (Table 1), but this did not produce any significant differences in mother weight gain during pregnancy, mother food and water intake during either gestation or lactation, gestational length, placental and fetal weights, neonatal weight gain, and litter size (Table 1). These results were mostly similar to previously published data from our group (46). In that report, we did not find any differences in mother intake but the mother weight gain was smaller in hashish-exposed mothers than controls, likely due to a reduced litter size by a presumably higher prenatal mortality (46), which did not occur in the present experiment.

TABLE 1

MATERNAL FOOD AND WATER INTAKE, MOTHER WEIGHT GAIN, LITTER SIZE, GESTATIONAL LENGTH, PLACENTAL AND FETAL WEIGHTS, MALE AND FEMALE OFFSPRING WEIGHT GAIN, AND MATERNAL PLASMA THC LEVELS MEASURED AFTER GIVING TO PREGNANT AND LACTATING MOTHERS A DAILY DOSE OF HASHISH EXTRACT FROM DAY 5 OF GESTATION UNTIL DAY 24 OF LACTATION

| Parameters | + Oil | + Hashish |
|---|---|---|
| Mother food intake (g)* | | |
| Gestation | 23.4 ± 0.9 | 24.4 ± 0.8 |
| Lactation | 36.8 ± 5.0 | 39.7 ± 6.0 |
| Mother water intake (ml)* | | |
| Gestation | 43.1 ± 1.8 | 48.0 ± 2.2 |
| Lactation | 60.4 ± 6.5 | 70.8 ± 6.9 |
| Mother weight gain (g)† | 155.1 ± 4.2 | 149.5 ± 8.3 |
| Litter size | 14.1 ± 0.4 | 13.4 ± 0.7 |
| Gestational length (days) | 22.5 ± 0.2 | 22.9 ± 0.1 |
| Placental weight (mg)‡ | 742.5 ± 21.8 | 764.0 ± 25.0 |
| Fetal weight (g)‡ | 3.81 ± 0.08 | 3.78 ± 0.06 |
| Male offspring weight gain§ | | |
| Early postnatal (1–20) | 27.2 ± 2.1 | 31.2 ± 0.9 |
| Peripubertal (20–40) | 57.2 ± 3.5 | 56.0 ± 2.5 |
| Adult (40–70) | 233.4 ± 7.5 | 227.1 ± 8.1 |
| Female offspring weight gain§ | | |
| Early postnatal (1–20) | 29.3 ± 2.3 | 30.2 ± 1.1 |
| Peripubertal (20–40) | 44.6 ± 3.2 | 51.8 ± 2.3 |
| Adult (40–70) | 145.8 ± 5.5 | 156.6 ± 5.3 |
| THC concentrations (ng/ml)¶ | ND# | 23.6 ± 9.8** |

Values are means ± SEM of more than 10 determinations per group. Statistical differences were assessed by Student's *t*-test.

*Average value per day.

†Obtained as the difference between the weight in the day before delivery and the weight in the day of mating.

‡Corresponds to day before delivery.

§Obtained as the difference between the weights at both interval limits.

¶Average value per day measured in plasma of pregnant mothers during the last third of gestation.

#<2.5 ng/ml.

**$p < 0.005$.

*Behavioral Effects (Experiments 1 and 2)*

In the first experiment, we examined, by using an actimeter, the ontogeny of spontaneous locomotor activity in male and female rats of different immature ages born from mothers perinatally exposed to hashish extract. Moreover, adult rats perinatally exposed to hashish (second experiment) were also used for analysis of spontaneous locomotor activity in the actimeter. Both results are presented collectively in Fig. 1. Thus, we found age-dependent differences along the developmental period analyzed, $F(4, 274) = 14.64$, $p < 0.0001$. Concretely, the spontaneous locomotor activity was significantly lesser in 20-day-old rats and higher in 70-day-old rats as compared with the remainder ages. No statistically significant differences were found between sexes, $F(1, 274) = 2.34$, n.s., whereas a small, although collectively significant, decrease was found after cannabinoid treatment, $F(1, 274) = 4.07$, $p < 0.05$. This statistically significant decrease by treatment seemed to correspond exclusively to hashish-exposed males because their spontaneous locomotor activity exhibited a constant trend to be lesser at all ages studied, except at adult age, although the decrease was never statistically significant [treatment–sex interaction, $F(1, 274) = 3.55$, $p = 0.06$; the remainder of the interactions were always clearly nonsignificant].

In the second experiment, we also examined the spontaneous locomotor activity of adult female and male rats perinatally exposed to hashish extract by analyzing the number of sector crossings in an open-field test. Results revealed an absence of statistically significant changes between animals perinatally exposed to hashish and nonexposed in both sexes (Table 2). However, when differentiated between internal and external sectors hashish exposed-males presented a higher number of external crossings than controls, which did not appear in females (Table 2). Moreover, males always presented a lesser number of internal and total crossings than females independently of treatment (Table 2).

Several induced or spontaneous stereotypic behaviors were also altered after hashish treatment, sometimes in a sexually dimorphic manner. Thus, the frequency of water spraying-induced self-grooming was significantly increased in animals perinatally exposed to hashish, $F(1, 60) = 20.64$, $p < 0.001$ (Fig. 2). This increase was not sex-dependent [sex, $F(1, 60) = 0.746$, n.s.; treatment–sex interaction, $F(1, 60) = 0.069$, n.s.], appearing in both sexes although it was more marked in males (200.4%) than females (121.2%). The perinatal exposure to hashish also increased the frequency of water spraying-induced shaking, $F(1, 60) = 8.279$, $p < 0.01$, but this in-

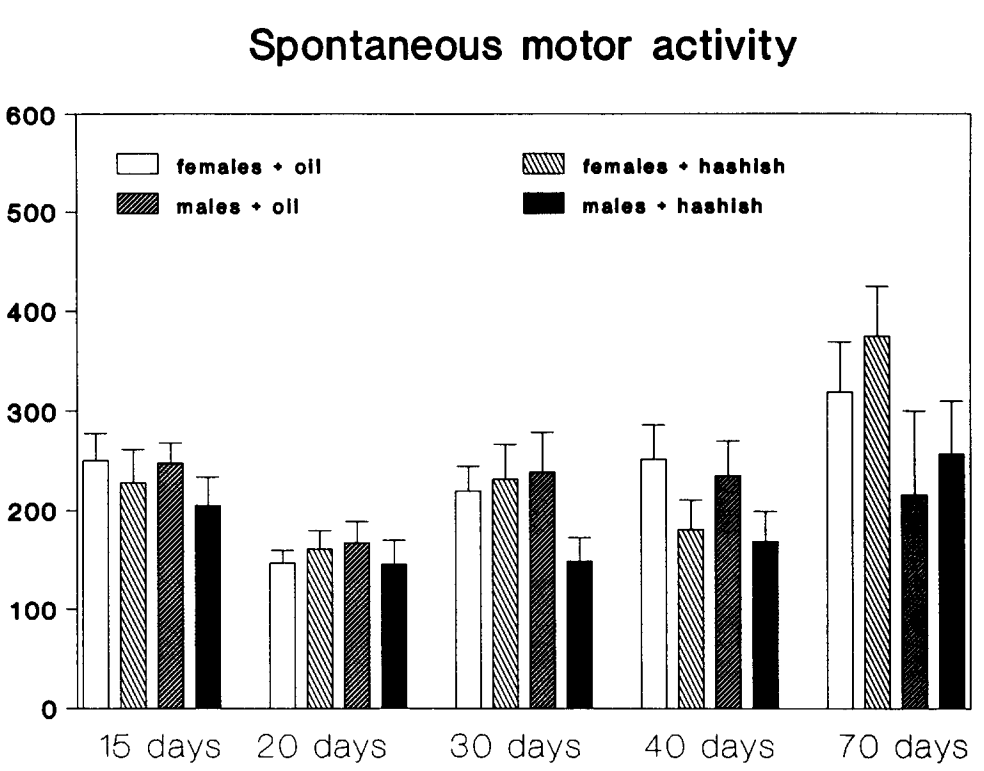


FIG. 1. Spontaneous locomotor activity, measured by an actimeter, in adult males and females perinatally exposed to hashish extract or vehicle (oil). Values corresponded to the data measured at 20 min of the onset of the test. Mostly similar values were obtained at 15 and 30 min (data not shown). Details in the text. Values are means ± SEM of 10–16 determinations per group. Statistical differences were obtained by multifactorial analysis of variance.

crease was sex dependent, appearing only in males [sex, $F(1, 60) = 13.413, p < 0.001$; treatment-sex interaction, $F(1, 60) = 8.598, p < 0.01$] (Fig. 2). The number of spontaneous self-grooms, measured in the open-field test, was unchanged after perinatal hashish treatment, $F(1, 60) = 3.041$, n.s. (Fig. 2). However, this parameter was higher in males than females, $F(1, 60) = 5.761, p < 0.05$, although this effect was independent of treatment [treatment-sex interaction, $F(1, 60) = 0.642$, n.s.] (Fig. 2). The frequency of spontaneous rearing behavior, also measured in the open-field test, was higher in females than males, $F(1, 60) = 4.452, p < 0.05$. This parameter also increased by perinatal drug treatment, $F(1, 60) = 5.005, p < 0.05$, although the effect of treatment was independent of sex [treatment-sex interaction, $F(1, 60) = 0.0073$, n.s.] and did not produce any statistical differences when each hashish-exposed sex was compared with their respective control group (Fig. 2).

As indicated in the Method section, the measurement of spontaneous locomotor and stereotypic behaviors was carried out simultaneously with the measurement of sexual motivation in a sociosexual approach behavior test. A priori, this could presumably represent that motor parameters might be influenced by the presence of incentive animals. However, there was not a particular correlation between the number of sector crossings or the frequency of a specific stereotypic behavior and the presence of the experimental animal in the vicinity of a particular goal (data not shown), excepting the expected correlation due to the different times spent by animals in each incentive area.

*Neurochemical Effects (Experiment 2)*

Behavioral effects observed in Experiment 2 were paralleled by several modifications in the activity of nigrostriatal neurons at the neurochemical level. Thus, there was a significant increase in the DOPAC/DA ratio, indicative of increased presynaptic activity, in females perinatally exposed to hashish (Fig. 3). This effect was exclusive of a treatment-sex interaction [treatment, $F(1, 28) = 0.342$, n.s.; sex, $F(1, 28) = 0.406$, n.s.; treatment-sex interaction, $F(1, 28) = 7.009, p < 0.05$]. The density of $D_1$ receptors was lesser in females than males, $F(1, 32) = 13.681, p < 0.001$. This parameter was affected by treatment only in a sex-dependent manner [treatment, $F(1, 32) = 3.903$, n.s.; treatment-sex interaction, $F(1, 32) = 5.545, p < 0.05$]. Thus, hashish-exposed females exhibited a lower density of $D_1$ receptors as compared with the remainder groups (Fig. 4). Males perinatally exposed to hashish exhibited a small, although significant, increase in the density of $D_2$ receptors (Fig. 4). This effect also depended on the treatment-sex interaction, $F(1, 32) = 12.368, p < 0.01$, because we did not observe any significant modifications by treatment, $F(1, 32) = 0.327$, n.s., or sex, $F(1, 32) = 1.703$, n.s., analyzed

TABLE 2

SPONTANEOUS LOCOMOTOR ACTIVITY, MEASURED IN THE OPEN-FIELD TEST AS THE NUMBER OF EXTERNAL, INTERNAL, AND TOTAL SECTOR CROSSINGS, IN ADULT MALES AND FEMALES PERINATALLY EXPOSED TO HASHISH EXTRACT OR VEHICLE (OIL)

| Parameters | Females | | Males | |
|---|---|---|---|---|
| | + Oil | + Hashish | + Oil | + Hashish |
| External crossings | 47.8 ± 6.6 | 43.5 ± 4.3 | 34.0 ± 5.2 | 44.9 ± 3.5* |
| Internal crossings | 41.5 ± 5.5 | 58.6 ± 10.4 | 21.8 ± 3.5† | 25.0 ± 5.4† |
| Total crossings | 88.4 ± 10.2 | 100.8 ± 11.7 | 56.1 ± 6.5† | 68.5 ± 7.3† |

Values corresponded to the sum of data measured in periods of 5 min over a total duration test of 15 min. They are summed because no significant differences were seen in the different periods. Details in the text. Values are means ± SEM of 10–16 determinations per group. Statistical differences were obtained by Kruskal–Wallis test.

*$p < 0.05$ vs. oil-exposed for the same sex.

†$p < 0.01$ vs. the other sex and similar treatment.

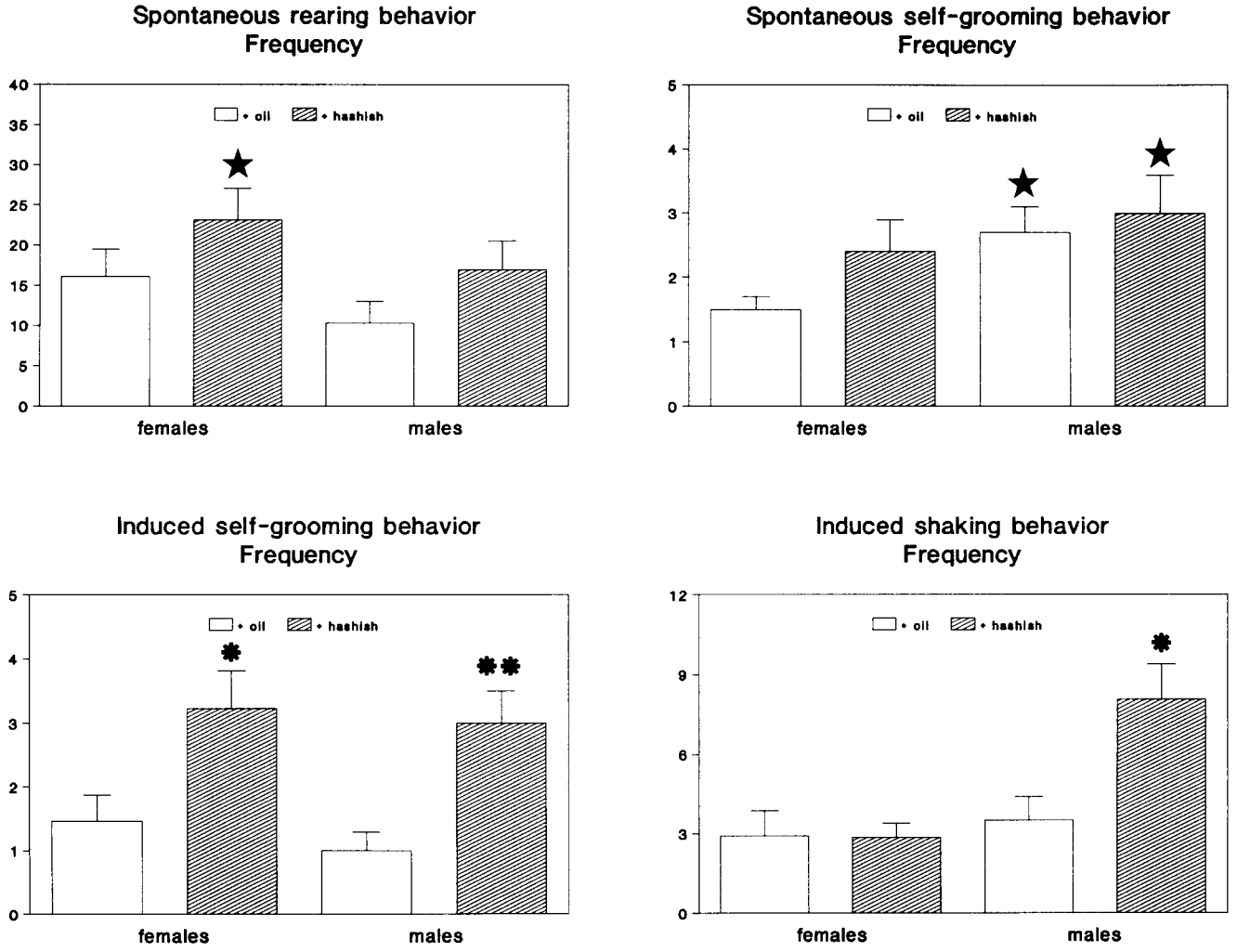


FIG. 2. Frequency of spontaneous rearing and self-grooming (measured in open-field test) and water-induced self-grooming and shaking behaviors in adult males and females perinatally exposed to hashish extract or vehicle (oil). Details in the text. Values are means ± SEM of 10–16 determinations per group. Statistical differences were obtained by two-way analysis of variance (*$p < 0.05$, **$p < 0.01$ vs. the control for same sex; ★$p < 0.05$ vs. the control of different sex).

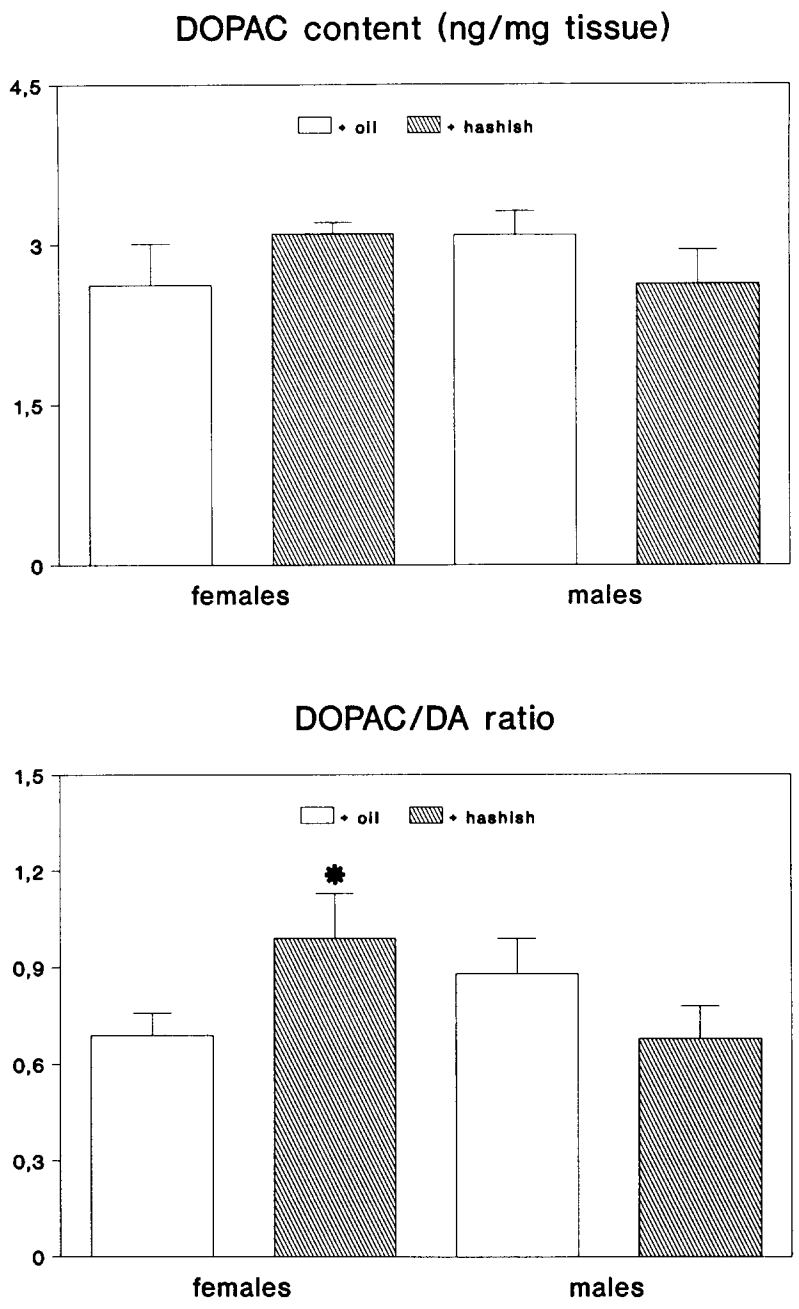


FIG. 3. L-3,4-Dihydroxyphenylacetic acid (DOPAC) content and its ratio with dopamine (DOPAC/DA) in the striatum of adult males and females perinatally exposed to hashish extract or vehicle (oil). Details in the text. Values are means ± SEM of six to eight determinations per group. Statistical differences were obtained by two-way analysis of variance (*$p < 0.05$ vs. the control of same sex).

individually. This male-specific effect tentatively might explain the increased behavioral expression observed in hashish-exposed males. No changes were observed in the remainder parameters as a consequence of perinatal cannabinoid exposure [TH, $F(1, 28) = 1.410$, n.s.; DA, $F(1, 28) = 1.669$, n.s.], although some sex-dependent differences could be observed [TH, $F(1, 28) = 5.349$, $p < 0.05$; DA, $F(1, 28) = 4.566$, $p < 0.05$] without affecting treatment [TH, $F(1, 28) = 1.054$, n.s.; DA, $F(1, 28) = 0.952$, n.s.] (Table 3).

## DISCUSSION

As mentioned in the introductory section, we have recently reported that perinatal exposure to hashish extracts modifies

the maturation of nigrostriatal dopaminergic neurons at the neurochemical level (43,44,46). Concretely, we observed a significant decrease in the activity of TH in the striatum, which was constantly maintained during the immature period (15–40 days after birth). This effect was sex dependent because it appeared only in males. Moreover, it was accompanied by a significant increase in the postsynaptic sensitivity of these neurons during the same age period, as revealed by the increases in the striatal density of $D_1$ and $D_2$ receptors. Three important questions remained to be addressed after these pre-

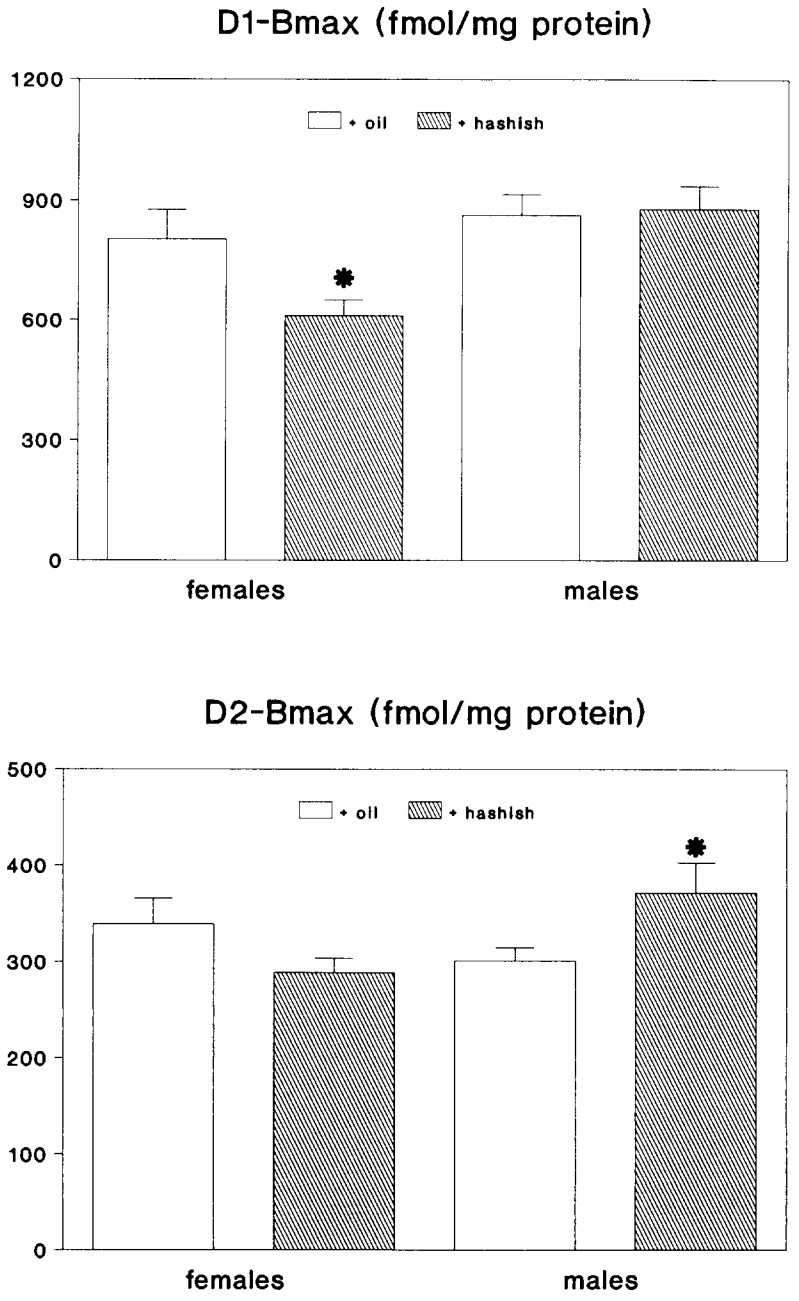


FIG. 4. Number ($B_{max}$) of $D_1$ and $D_2$ dopaminergic receptors in the striatum of adult males and females perinatally exposed to hashish extract or vehicle (oil). Details in the text. Values are means ± SEM of six to eight determinations per group. Statistical differences were obtained by two-way analysis of variance (*$p < 0.05$ vs. the control of same sex).

TABLE 3

DA CONTENT, TH ACTIVITY, AND $D_1$ AND $D_2$ BINDING SITE AFFINITY ($K_d$) IN THE STRIATUM OF ADULT MALES AND FEMALES PERINATALLY EXPOSED TO HASHISH EXTRACT OR VEHICLE (OIL)

| | Females | | Males | |
|---|---|---|---|---|
| Parameters | + Oil | + Hashish | + Oil | + Hashish |
| DA (ng/mg) | 3.34 ± 0.39 | 3.45 ± 0.46 | 3.76 ± 0.38 | 4.57 ± 0.42* |
| TH (ng/mg/h) | 99.8 ± 11.3 | 102.2 ± 11.5 | 119.2 ± 18.7 | 152.6 ± 22.9* |
| $D_1$ $K_d$ (nM) | 0.53 ± 0.08 | 0.52 ± 0.08 | 0.54 ± 0.09 | 0.53 ± 0.06 |
| $D_2$ $K_d$ (nM) | 0.13 ± 0.02 | 0.13 ± 0.02 | 0.09 ± 0.02 | 0.12 ± 0.02 |

Details in the text. Values are means ± SEM of six to eight determinations per group. Statistical differences were obtained by two-way analysis of variance.

*$p < 0.05$ vs. females + oil.

vious results: first, whether these neurochemical effects might modify the ontogeny of motor behavior that is controlled by these neurons; second, whether these neurochemical effects and their potential behavioral modifications might be maintained until adulthood; and, third, whether sexual dimorphisms in behavior might appear in adulthood. The present experiments attempted to answer these questions.

Regarding the first question, the present results showed a constant trend of males perinatally exposed to hashish to exhibit a lesser spontaneous locomotor activity than male controls during the immature period that did not exist in females, where fluctuations were randomly distributed. It is true that the effects in males were really small and always nonsignificant, but, in support of these results, it may be argued that this undervalued behavioral modification could be the result of compensatory mechanisms. This is supported by the fact that our original observation of decreased striatal TH activity was accompanied by increases in receptor densities (44), presumably leading to normalized functional alterations.

An additional point to this first question should be also considered. In our previous report (44), we hypothesized about the sequence of neurochemical effects originated by perinatal hashish exposure in nigrostriatal dopaminergic neurons of developing males. Two possibilities could be potentially considered. The primary effect might be presynaptic—a decrease in the ability to synthesize DA—which could secondarily produce increases in postsynaptic sensitivity. The other possibility could be that cannabinoids primarily increase the number of dopaminergic receptors including the presynaptic receptors, which have an inhibitory effect on presynaptic functionality, leading secondarily to an inhibition of DA synthesis. Our present results seem to support the first possibility because of the inhibitory trend observed at the behavioral level.

Regarding the second question, persistence of ontogenic neurochemical and behavioral effects of perinatal hashish exposure in adulthood, a variety of aspects should be considered. First, both the previously published neurochemical changes in the ontogeny of nigrostriatal dopaminergic neurons (43,44,46) and the alterations in their related behaviors observed in the present study were only partially maintained in adult animals. This observation might be consistent with the fact that dopaminergic systems undergo a significant amount of maturation during the postnatal period and, hence, a "normalization" due to compensatory mechanisms during the course of development is not an unreasonable assumption. For instance, as mentioned above, the perinatal cannabinoid-induced decrease in the ontogeny of striatal TH activity was paralleled by an increase in $D_1$ and $D_2$ receptor densities (44). In this context, basal nigrostriatal activity could appear as unchanged in adulthood due to compensatory mechanisms, but the response of these neurons to excitatory or depressor situations could be different in animals perinatally exposed to cannabinoids and controls. This might be made evident by examining their behavioral response to a variety of challenges. Thus, although spontaneous locomotor activity and rearing and self-grooming behaviors were not altered by perinatal hashish treatment in adult males, this enhanced the frequency of shaking and self-grooming induced by water spraying. At the neurochemical level, the decrease in TH activity and the increase in $D_1$ receptor density in the striatum observed in males perinatally exposed to hashish (44) did not appear in 70-day-old rats. However, the increase in the density of striatal $D_2$ receptors in males observed during ontogeny (44) still appeared in adulthood. Whether these altered behaviors might be related to modifications in $D_2$ receptors is still unknown, but previous studies have reported that interactions between both $D_1$ and $D_2$ receptors seem to be in the origin of eliciting stereotypic behaviors [for review, see (3)]. Further studies by using pharmacological challenges with $D_1$ and $D_2$ agonists and antagonists will be needed to demonstrate in depth this relationship and establish the degree of perinatal cannabinoid affectation.

On the other hand, no effects in spontaneous locomotor activity, measured in the actimeter and open-field test, were found in both adult males and females perinatally exposed to hashish, which agrees with the results obtained from other open-field studies in rats prenatally exposed to cannabis extracts when tested at 75 days of age (1). However, hashish-exposed males presented a higher number of external crossings than controls, indicating possible parallel changes in motivation and/or emotional reactivity.

Hence, the existence of behavioral and neurochemical changes in adult rats perinatally exposed to hashish supports the view of a "permanent" alteration in the activity of nigrostriatal dopaminergic neurons as a consequence of cannabinoid exposure during critical periods of development of these

neurons. It is unlikely that these changes depend on the presence of the drug in adulthood. Previous studies have indicated that THC and its metabolites disappear from the whole rat with a half-life of approximately 17 h (23). It is therefore unlikely that significant amounts of cannabinoids would have been present in the brain of offspring 70 days after birth. In this respect, our results contrast with the observations of Walter and Carr (52), who observed a disappearance of marihuana-induced neurochemical effects after weaning, although these authors did not analyze males and females separately.

The existence of perinatal cannabinoid-induced subtle changes in the adult functionality of nigrostriatal dopaminergic neurons would suggest possible modifications in key proteins for dopaminergic neurotransmission at the genomic level. In support of this hypothesis, we have recently found a decrease in the amounts of TH mRNA in adult males perinatally exposed to hashish (Bonnin, Fernández-Ruiz, Ramos, and Santos, unpublished results), although, as observed in the present study, this does not represent a loss of the activity of this enzyme but might influence the response to several challenges. Studies using cDNA for $D_1$ and $D_2$ receptors are actually in progress. These might be important considering the results of our present work and the recent studies from Herkenham and coworkers (20), who reported that dopaminergic receptors, mainly $D_1$ receptors, are present in the same striatal neurons where cannabinoid receptors seem to be located.

Regarding the third question, the present results proved that sexual dimorphisms originated by perinatal cannabinoid exposure at the neurochemical level also appear at the behavioral level during adulthood. Thus, shaking behavior induced by water spraying was not affected by perinatal hashish treatment in adult females, contrarily to that observed in males. In addition, induced self-grooming behavior was increased by hashish treatment in adult females as in males, but the increase was less marked. No changes were seen in the spontaneous locomotor activity of females in both the actimeter and open-field test. Moreover, the higher number of external crossings measured in the open-field test in males perinatally exposed to hashish did not appear in females. When we examined neurochemical activity of nigrostriatal neurons in females, we found an increase of the striatal DOPAC/DA ratio, indicative of increased presynaptic activity, after perinatal hashish exposure. However, this effect was probably compensated by a parallel decrease in the density of $D_1$ receptors, suggesting downregulation of this receptor.

Finally, we could also observe some additional sexual differences in control animals. Thus, males always presented a lesser number of internal and total crossings than females independently of treatment. The number of spontaneous rears and self-grooms, measured in the open-field test, were unchanged in both sexes after perinatal hashish exposure, but the frequency of rearing behavior decreased in males as compared with females, whereas self-grooming frequency was higher in males. These differences would be likely related to previous sexual differences in the functionality of nigrostriatal dopaminergic neurons (18).

In summary, the results of this study tend to support the three proposed hypotheses. Thus, our previously reported observations that perinatal hashish exposure decreases the presynaptic activity of nigrostriatal dopaminergic neurons in male rats of different immature ages can be associated with the slight decrease in the spontaneous locomotor activity observed in immature males perinatally exposed to hashish, whereas this did not appear in females. Moreover, although the spontaneous locomotor activity was not affected by perinatal hashish exposure in adult males they exhibited increases in several induced stereotypic behaviors (self-grooming and shaking) in parallel with increases in striatal $D_2$ receptors. Adult females also exhibited a decrease, less marked than males, in induced self-grooming behavior. This was accompanied by an increase in the DOPAC/DA ratio although compensated for by a decrease in striatal $D_1$ receptors. These findings do not discard the possibility that perinatal cannabinoids might also affect other nondopaminergic neurons, even located in nonstriatal areas, which have been also involved in the control of motor behavior (7,49).

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