Neurotoxicology and Teratology, Vol. 21, No. 3, pp. 277–283, 1999

0892-0362/99 $–see front matter

ELSEVIER

PII S0892-0362(98)00058-0

Perinatal Δ^9-Tetrahydrocannabinol Exposure Augmented the Magnitude of Motor Inhibition Caused by $GABA_B$, but not $GABA_A$, Receptor Agonists in Adult Rats

L. GARCIA-GIL, R. DE MIGUEL, J. ROMERO, A. PEREZ, J. A. RAMOS AND J. J. FERNÁNDEZ-RUIZ

Instituto Complutense de Drogodependencias, Departamento de Bioquímica y Biología Molecular III, Facultad de Medicina, Universidad Complutense de Madrid, Ciudad Universitaria, Madrid, Spain

Received 21 July 1998; Accepted 23 October 1998

GARCIA-GIL, L., R. DE MIGUEL, J. ROMERO, A. PEREZ, J. A. RAMOS AND J. J. FERNÁNDEZ-RUIZ. *Perinatal Δ^9-tetrahydrocannabinol exposure augmented the magnitude of motor inhibition caused by $GABA_B$, but not $GABA_A$, receptor agonists in adult rats.* NEUROTOXICOL TERATOL. **21**(3) 277–283, 1999.—We have extensively reported that Δ^9-tetrahydrocannabinol (Δ^9-THC) exposure results in changes in the adult functionality of dopaminergic neurons, in particular, mesotelencephalic pathways, although some changes are evident only after pharmacological challenges. In the present study, we have examined whether similar changes might be observed in γ-aminobutyric acid (GABA) activity, in particular, in those regions where cannabinoid receptors have been reported to be located in GABA-containing neurons. To this end, we first examined GABA content and glutamic acid decarboxylase (GAD) activity in several brain regions of adult male and female rats that had been perinatally exposed to Δ^9-THC or oil. Δ^9-THC exposure did not modify either GAD activity or GABA content in the ventral-tegmental area, nucleus accumbens, substantia nigra, caudate-putamen, and globus pallidus, thus suggesting no changes in the basal presynaptic activity of GABA-containing neurons. Second, we tested the motor response in the open-field test of these animals after a single injection of muscimol, a $GABA_A$ receptor agonist, baclofen, a $GABA_B$ receptor agonist, or vehicle. We observed that the motor inhibition caused by baclofen, in terms of decreased ambulation and stereotypy and increased inactivity, was more marked in magnitude in Δ^9-THC–exposed males and females. This was not observed for the $GABA_A$ receptor agonist, muscimol, indicating a receptor specificity. To extend this observation, we also examined whether the potential differences in the behavioral response found in the above experiment might be due to changes at the level of the efficiency of the activation of these receptors by measuring basal and baclofen-stimulated [^{35}S]-guanylyl-5′-*O*-(γ-thio)-triphosphate ([^{35}S]-GTPγS) binding in adult male and female rats that had been perinatally exposed to Δ^9-THC or oil. However, our results were negative, because perinatal Δ^9-THC exposure did not increase baclofen-stimulated [^{35}S]-GTPγS binding in the areas studied; in particular, in the substantia nigra, an area of interest for the interactions $GABA_B$ receptor/cannabinoid receptor. Collectively, the present results indicate that although perinatal Δ^9-THC did not produce any changes in GABA content and GAD activity in limbic and motor areas in adulthood, it did increase the behavioral response to $GABA_B$ receptor agonists. However, this increase was not due to changes in $GABA_B$ receptor activation of signal transduction mechanisms, as revealed the analysis of the percentage of stimulation by baclofen of [^{35}S]-GTPγS binding in the substantia nigra and other structures of males and females perinatally exposed to Δ^9-THC.

Cannabinoids Δ^9-Tetrahydrocannabinol Perinatal exposure Cannabinoid receptors GABA Glutamic acid decarboxylase $GABA_A$ receptors $GABA_B$ receptors [^{35}S]-GTPγS binding

Requests for reprints should be addressed to J. J. Fernández-Ruiz, Departamento de Bioquímica y Biología Molecular III, Facultad de Medicina, Universidad Complutense, Ciudad Universitaria s/n, 28040-Madrid, Spain. Tel: +34-91-3941450; Fax: +34-91-3941691; E-mail: jjfr@eucmax.sim.ucm.es

CANNABIS sativa preparations (hashish, marihuana) are able to affect the development and differentiation of the central nervous system in rodents when consumed during prenatal and early postnatal ages, because they can be transferred from the mother to the offspring through the placental blood and maternal milk [for review, see (6–9)]. Thus, perinatal exposure to cannabinoids markedly affected the maturation of several neurotransmitter systems, in particular, dopamine (1,2,24,31,32) and serotonin (16). It was also able to alter the development of opioidergic neurons (13), even producing a naloxone-induced withdrawal syndrome at immature ages (29). In the case of dopaminergic neurons, the changes were especially marked and constant in male offspring, whereas the effects in females were usually transient [for review, see (7–9)]. These effects appeared early, even before the complete differentiation and maturation of dopaminergic projections into their target areas (2), in particular, during the last third of gestation and first week after birth, when cannabinoids were able to affect the expression of key genes for dopaminergic neurotransmission (2). All these modifications observed during perinatal and peripubertal periods were followed by important long-term effects on adult behavior, which were also usually more prevalent in male offspring. Thus, adult animals perinatally exposed to cannabinoids exhibited, among others, alterations in a variety of behavioral tests indicating male copulatory behavior (5), open-field activity (10,18), learning ability (6), stress response (15), pain sensitivity (29), social interaction and sexual motivation (19), drug seeking behavior (30), and others [for review, see (6–9)]. In addition, long-term neuroendocrine disturbances have been also reported (4,17).

The studies of vulnerability to drugs of abuse in adult individuals perinatally exposed to Δ^9-tetrahydrocannabinol (Δ^9-THC) have been of special interest due to their potential social relevance. Thus, we have recently found that adult female, but not male, rats self-administered more morphine when they had been perinatally exposed to Δ^9-THC (30). This effect seems to be related to a Δ^9-THC-induced increase in μ-opioid receptor binding (30) and decrease in proenkephalin gene expression (3) in specific regions directly or indirectly related to drug reinforcement. In addition, we have also found that adult male, but not female, rats that had been perinatally exposed to Δ^9-THC were tolerant to a new cannabinoid exposure with regard to the effects of this compound on dopaminergic activity (8). However, this tolerance was not apparently related to changes at the level of cannabinoid receptor binding and mRNA expression in specific brain regions (García-Gil, Romero, Ramos, and Fernández-Ruiz, unpublished results).

There are no data, however, on the effects of perinatal Δ^9-THC exposure on the development of other neurotransmitters such as γ-aminobutyric acid (GABA), in particular on those GABA-containing neurons related to midbrain dopaminergic neurons. These neurons contain cannabinoid receptors in adult individuals (11,12). The activation of these receptors markedly altered GABAergic activity (14,23,25,26). Thus, the present study was designed to state whether the adult functionality of GABA-containing neurons in motor (caudate-putamen, globus pallidus, and substantia nigra) and limbic (nucleus accumbens and ventral-tegmental area) areas might be altered as a consequence of the contact with Δ^9-THC during early development. To this end, we have performed three consecutive experiments. We first examined GABA content and glutamic acid decarboxylase (GAD) activity in several brain regions of adult male and female rats that had been perinatally exposed to Δ^9-THC or oil. Second, we tested the motor response in the open-field test of these animals after a single injection of muscimol, a $GABA_A$ receptor agonist, baclofen, a $GABA_B$ receptor agonist, or vehicle. We finally examined whether the potential differences in the behavioral response found in the above experiment might be due to changes at the level of the efficiency of $GABA_B$ receptor activation. We used the method recently developed by Sim et al. (27) to measure basal and baclofen-stimulated [^{35}S]-guanylyl-5′-*O*-(γ-thio)-triphosphate ([^{35}S]-GTPγS) binding in the substantia nigra and other structures of adult male and female rats that had been perinatally exposed to Δ^9-THC or oil.

METHOD

Animals and Perinatal Δ^9-THC Exposure

Female virgin rats of the Wistar strain were housed from birth in a room with a controlled photoperiod (0800–2000 h light) and temperature (23 ± 1°C). They had free access to standard food (Panlab, Barcelona, Spain) and water. At adult age (>8 weeks of life; 200–250 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. Δ^9-THC, generously supplied by the National Institute on Drug Abuse (USA), was prepared in a sesame oil solution for administration. Pregnant females received a daily oral dose of Δ^9-THC (5 mg/kg weight) from the fifth day of gestation, according to our previously published model (1,2,18). Control rats were dosed with vehicle alone. This treatment was maintained until day 24 after birth, the day on which rats were weaned. Then, male and female offspring were housed separately (four animals per cage) and left to reach adult age (>10 weeks) to be used for $GABA_A$ and $GABA_B$ receptor agonist studies or analysis of GABA content and GAD activity and baclofen-stimulated [^{35}S]-GTPγS binding in several brain structures. During the whole treatment period, we recorded a set of gestational and lactational parameters, such as maternal and neonatal weight gain, maternal food and water intake, gestational length, placental and fetal weights, litter size, and maternal plasma Δ^9-THC concentrations, to support that potential adult changes were not due to a possible cannabinoid-induced nutritional deficit during the perinatal life. These results have been previously and extensively published (1,18), and are not included here.

Experiments, Sampling, and Tissue Preparation

At adult age (>70 days after birth), both Δ^9-THC– and vehicle-exposed male and female rats were used for three different experiments. In each experiment, six animals per group, taken from at least three different litters, were used to ensure the required experimental variation. In Experiment I, animals were sacrificed by rapid decapitation and their brains were quickly and carefully removed and rapidly frozen by immersion in 2-methyl-butane chilled in dry ice. All samples were stored at −70°C until processed. The day of analysis, brains were thawed and used for manually obtaining coronal slices (around 500-μm thick) at three different levels: (a) substantia nigra/ventral tegmental area; (b) globus pallidus; and (c) caudate-putamen/nucleus accumbens, according to Palkovits and Brownstein (21). Afterwards, the five structures were dis-

sected and processed for the analysis of GABA content and GAD activity, as will be described below. In Experiment II, animals were subjected to a single IP injection of muscimol (1 mg/kg weight), baclofen (2 mg/kg weight) or vehicle (saline). Ten minutes later, animals were subjected to the open-field test. In Experiment III, animals were sacrificed by rapid decapitation, and their brains were quickly and carefully removed and rapidly frozen by immersion in 2-methyl-butane chilled in dry ice. Coronal sections 20 μm-thick were cut in a cryostat, according to the Paxinos and Watson atlas (22). Sections were thaw mounted onto RNAse-free gelatin/chrome alum-coated slides and dried briefly at 30°C and stored at −80°C until used. For the identification of the different brain nuclei, sections adjacent to those used for autoradiographic analysis were stained with cresyl violet and analyzed according to the Paxinos and Watson atlas (22). Sections were stored at −70°C until processed for the autoradiographic analysis of [^{35}S]-GTPγS binding as will be described below.

Open-Field Test

Muscimol, baclofen, or vehicle-injected adult male and female rats, that had been perinatally exposed to Δ^9-THC or vehicle, were subjected 10 min after administration to the open-field test. This consisted of a circular floor (diameter: 90 cm) with a surrounding wall (height: 30 cm), both made of transparent polyvinylchloride. The floor was divided into five inner and eight outer parts using circles and radial segments. Animals were placed in the center of the arena, and they were left 5 min for acclimation (this was done to reduce the effects of stress derived of a novel environment on the motor response). After this period, the spontaneous activity of each animal was recorded on a TV video system for a period of 5 min. The apparatus was washed out with an odoriferous solution after each rat had been tested. The following parameters were analyzed: (1) ambulation: number of sector crossings (a single line crossing was defined as the rat placing the four paws into an adjacent quadrant); (2) frequency of stereotypic behaviors; and (3) time spent in inactivity. The scoring of the different behaviors was carried out by investigators who had no knowledge of the treatment of each rat.

Analysis of GABA Content by HPLC with Electrochemical Detection

Analysis of GABA contents in several brain regions were carried out by HPLC with electrochemical detection according to the procedure described by Smith and Sharp (28). Briefly, the dissected brain regions were homogenized in 20 vol of cold 150 mM potassium phosphate buffer, pH 6.8. Each homogenate was divided into two parts: one-half for the analysis of GABA content, and the other half for the analysis of GAD activity. The homogenate part used for the direct measurement of GABA content was diluted (1/2) with 0.4 N perchloric acid containing 0.4 mM sodium disulfite, 0.90 mM EDTA, and 10 μg/ml 5-aminopentanoic acid (5-APA) as internal standard. Afterwards, samples were centrifuged for 3 min (15000 × *g*) and 50 μl of each supernatant removed and neutralized with 100 μl of 0.1 N NaOH. Samples were stored at 4°C until analysis. This was performed by derivatization of GABA and 5-APA through the addition of 15 μl of *o*-phthaldehide (OPA)-sulfite solution (14.9 mM OPA, 45.4 mM sodium sulfite and 4.5% ethanol in 327 mM borate buffer, pH 10.4). Samples were allowed to react at room temperature for a period of 10 min. After this time, 20 μl of each reaction mixture (including derivatizated calibration standards composed of known concentrations of L-glutamate, GABA and 5-APA) were injected into the HPLC system. This consisted in a Spectra-Physics 8810 isocratic pump. The column was a RP-18 (Spherisorb ODS-2; 200 mm, 4.6 mm, 5 μm particle size; Teknokroma, Barcelona, Spain). The mobile phase, previously filtered and degassed, consisted of 0.06 M sodium dihydrogen phosphate, 0.06 mM EDTA and 20–30% methanol (pH 4.4), and the flow rate was 0.8–1.4 ml/min. The effluent was monitored with a Metrohm bioanalytical system amperometric detector using a glassy carbon electrode. The potential was 0.85 V relative to an Ag/AgCl reference electrode with a sensitivity of 50 nA (approx. 2 ng per sample). The signal was recorded on a Spectra-Physics 4290 integrator. The approximate retention times for GABA and 5-APA were 8 and 16 min, respectively. The results were given as area under the peaks and calculated by comparison with the area under the corresponding internal standard peak. Values were expressed as μg/mg of protein.

Assay for GAD Activity

The homogenate part used for the analysis of GAD activity was also diluted (1/2) with 150 mM potassium phosphate buffer, pH 6.8, and processed according to Nicoletti et al. (20). Briefly, 50 μl of each diluted homogenate were incubated for 30 min at 37°C with 50 μl of a mixture of 32 mM L-glutamate and 1.5 mM pyridoxal phosphate prepared in phosphate buffer. After incubation, the reaction was stopped by addition of 50 μl of 0.4 N perchloric acid containing 0.4 mM sodium disulfite, 0.90 mM EDTA and 15 μg/ml 5-APA. Blank tubes for each diluted homogenate were constructed by adding 50 μl of 0.4 N perchloric acid containing 0.4 mM sodium disulfite, 0.90 mM EDTA and 15 μg/ml 5-APA before incubation. Afterwards, both test and blank tubes were centrifuged for 3 min (15000 × *g*), and the supernatants removed. The analysis of GABA formed was done as previously described for the direct analysis of GABA content in the same samples. Values for GAD activity are expressed as μg of GABA formed/mg of protein/h.

Autoradiography of Basal and Baclofen-Stimulated [^{35}S]-GTPγS Binding

The protocol used is basically the method recently described by Sim et al. (27). Briefly, slide-mounted brain sections were rinsed in assay buffer [50 mM TRIS, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, and 0.5% bovine serum albumin (fatty acid-free), pH 7.4] at 25°C for 10 min, then pretreated for 15 min with an excess concentration (2 mM) of GDP (Boehringer Mannheim, Barcelona, Spain) in assay buffer. Afterwards, sections were incubated at 25°C for 2 h in assay buffer containing 0.04 nM [^{35}S]-GTPγS (Amersham Ibérica, Madrid, Spain), 2 mM GDP, and 600 μM baclofen (RBI, Natick, MA). Basal activity was assessed in the absence of agonist, whereas nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS (Boehringer Mannheim). In pilot experiments, additional brain sections were incubated in the presence of the $GABA_B$ receptor antagonist CGP 35348 (1 mM; generously supplied by Ciba-Geigy, Barcelona, Spain) in addition to 0.04 nM [^{35}S]-GTPγS, 2 mM GDP and 600 μM baclofen. CGP 35348 significantly antagonized the increase in baclofen-stimulated [^{35}S]-GTPγS binding, thus supporting that this increase was caused through activation of $GABA_B$ receptors (data not shown). Slices were rinsed twice in 50 mM TRIS buffer, pH 7.4, and once in deionized water, then dried under a stream of cool dry air. Autoradiograms

were generated by apposing the labeled tissues to film (Hyperfilm-βmax, Amersham Ibérica, Madrid, Spain) for a period of 2 days, and developed (D-19, Kodak) for 4 min at 20°C. Developed films were analyzed and quantitated in a computer-assisted videodensitometer (Image Quant 3.3, Molecular Dynamics).

Statistics

The three-way analysis of variance was used to assess the data on open-field responsiveness after muscimol or baclofen challenge (sex × perinatal treatment × challenge) and the data on basal and baclofen-stimulated [^{35}S]-GTPγS binding (sex × perinatal treatment × agonist stimulation). Data on GABA contents and GAD activity were assessed by two-way analysis of variance (sex × perinatal treatment). In the three cases, Student–Newman–Keuls test was used as the post hoc test.

RESULTS

Experiment I: Analysis of GAD Activity and GABA Content

In this experiment, we examined the effects of the perinatal Δ^9-THC exposure on the presynaptic activity of GABA-containing neurons in motor and limbic areas, by measuring GAD activity and GABA content (see Table 1). The two-way analysis of variance revealed that the variable perinatal treatment, analyzed alone, did not reach statistical significance in any of the five regions analyzed, either for GAD activity or for GABA content. The interaction of this variable, perinatal treatment, with the other, sex, did not reach statistical significance either. The variable sex, analyzed alone, exhibited, however, statistical significance in some cases. For instance, GABA content in the caudate-putamen were higher in females than males [Table 1; $F(1, 15) = 4.24$, $p < 0.05$]. However, this was observed independently of the other variable, perinatal treatment, as reflected by the absence of changes in the above-mentioned two-way interactions for each structure. The remaining areas did not reach any statistically significant differences with regard to the variable sex, analyzed alone, either for GAD activity or for GABA content.

Experiment II: Challenges with GABA-A and -B Receptor Agonists

In this experiment, we tested the open-field response of adult males and females that had been perinatally exposed to Δ^9-THC or oil, to a single administration of a $GABA_A$ receptor agonist, muscimol, a $GABA_B$ receptor agonist, baclofen, or vehicle (see Table 2). The administration of baclofen produced the expected motor inhibition, reflected by decreased ambulation and stereotypy and increased inactivity (Table 2), as revealed the three-way analysis of variance [ambulation: $F(1, 36) = 49.76$, $p < 0.00005$; inactivity: $F(1, 36) = 46.84$, $p < 0.00005$; stereotypy: $F(1, 36) = 57.19$, $p < 0.00005$]. The variable perinatal treatment did not reach statistical significance when analyzed alone, thus suggesting that the presence of Δ^9-THC during GABAergic development did not alter by itself the basal motor activity when animals matured (Table 2). The variable sex, analyzed alone, indicated higher frequency of stereotypy in females than males [Table 2; $F(1, 36) = 7.00$, $p < 0.05$), but independently of the other two variables. No changes were observed by sex in inactivity and ambulation.

The analysis of the different two-way and three-way interactions for these parameters revealed that the magnitude of baclofen effects might be affected by the perinatal treatment with Δ^9-THC in some cases. Thus, perinatal treatment × challenge interaction did achieve statistical significance for ambulation, $F(1, 36) = 3.28$, $p < 0.05$. As the three-way interaction for this parameter did not reach statistical significance, this indicated that the effect was seen in both sexes. The transformation of these data to % of change revealed that the decrease in ambulation was effectively more marked in animals exposed to Δ^9-THC (females: −81.1%; males: −83.9%) compared with their respective oil-exposed groups (females: −57.7%; males: −75.4%). No changes were seen for the other two-way interactions, perinatal treatment × sex and sex × challenge. In the case of inactivity and frequency of stereotypy, no changes were seen for the different two-way and three-way interactions, except for the interaction between challenge and sex for stereotypy, $F(1, 36) = 6.30$, $p < 0.05$, thus reflecting higher effects of baclofen in females than males, but independently of the perinatal treatment (Table 2).

Finally, the administration of muscimol also produced the expected motor inhibition, with a main result in this case in

TABLE 1

GLUTAMIC ACID DECARBOXYLASE ACTIVITY AND GABA CONTENT IN SEVERAL BRAIN REGIONS OF ADULT FEMALE AND MALE RATS THAT HAD BEEN PERINATALLY EXPOSED TO Δ^9-TETRAHYDROCANNABINOL (Δ^9-THC) OR VEHICLE (OIL)

Brain Structure	Females + Oil	Females + Δ^9-THC	Males + Oil	Males + Δ^9-THC
GAD Activity (μg/mg of Protein/h)				
Nucleus accumbens	36.6 ± 1.9	38.2 ± 2.9	35.0 ± 1.7	40.2 ± 4.2
Ventral-tegmental area	29.1 ± 3.5	30.4 ± 2.7	34.1 ± 3.5	29.7 ± 3.7
Caudate-putamen	19.2 ± 1.0	17.9 ± 1.6	19.0 ± 1.5	20.4 ± 2.0
Globus pallidus	45.7 ± 8.4	51.9 ± 5.4	52.3 ± 3.3	56.5 ± 4.5
Substantia nigra	54.9 ± 4.1	70.0 ± 6.5	70.9 ± 2.1	71.8 ± 5.8
GABA Content (μg/mg of Protein)				
Nucleus accumbens	3.7 ± 0.5	3.6 ± 0.5	3.1 ± 0.5	3.8 ± 0.5
Ventral-tegmental area	4.6 ± 0.3	4.1 ± 1.0	3.8 ± 0.6	4.4 ± 0.5
Caudate-putamen	2.8 ± 0.3	2.4 ± 0.2	2.1 ± 0.2	2.1 ± 0.1
Globus pallidus	4.5 ± 0.5	5.2 ± 0.6	4.6 ± 0.3	4.5 ± 0.6
Substantia nigra	6.3 ± 0.6	6.9 ± 0.3	7.4 ± 0.2	7.0 ± 0.2

Details in the text. Values are means ± SEM of six determinations per group. Data were assessed by two-way analysis of variance (sex × perinatal treatment) followed by the Student–Newman–Keuls test.

TABLE 2

AMBULATION (NUMBER OF SECTOR CROSSINGS), TIME SPENT IN INACTIVITY, AND FREQUENCY OF STEREOTYPY, MEASURED IN THE OPEN-FIELD TEST, AFTER A CHALLENGE WITH BACLOFEN (2 mg/kg), MUSCIMOL (1 mg/kg) OR VEHICLE TO ADULT FEMALE AND MALE RATS THAT HAD BEEN PERINATALLY EXPOSED TO Δ9-TETRAHYDROCANNABINOL (Δ9-THC) OR OIL

Parameter	Challenge	Females + Oil	Females + Δ9-THC	Males + Oil	Males +Δ9-THC
Ambulation	+vehicle	27.2 ± 4.0	37.4 + 3.5	23.0 + 5.9	30.4 + 4.9
	+baclofen	11.5 + 5.7*	7.1 + 4.5†	5.7 + 2.9*	4.9 + 2.8†
Inactivity	+vehicle	0	0	20.2 + 13.5	2.8 + 2.8
	+baclofen	138.0 + 54.5*	185.5 + 44.5†	135.3 + 42.6*	204.2 + 42.4†
Stereptypy	+vehicle	9. 4 ± 1.1	8.2 ± 0.8	4.3 ± 1.5	5.0 ± 2.3
	+baclofen	0.9 ± 0.9†	0.4 ± 0.3†	1.0 ± 0.7*	0.1 ± 0.1*
	+muscimol	3.0 ± 1.2†	4.2 ± 1.4*	1.6 ± 0.5*	1.7 ± 1.0

Details in the text. Values are means ± SEM of six determinations per group. Data were assessed by three-way analysis of variance (sex × perinatal treatment × challenge) followed by the Student–Newman–Keuls test (*$p < 0.05$, †$p < 0.005$).

the reduction of stereotypic movements [see Table 2; $F(1, 36) = 19.38$, $p < 0.005$] but less marked effects in other parameters, such as ambulation and inactivity (data not shown). The three-way analysis of variance revealed that, as expected, the frequency of stereotypy varied by sex, when analyzed alone, $F(1, 36) = 10.77$, $p < 0.005$, indicating higher frequency in females than males (Table 2). Also as expected, the variable perinatal treatment, when analyzed alone, did not reach statistical significance. An absence of statistically significant variations was also seen in the three different two-way interactions and in the three-way interaction, thus suggesting that the inhibitory effects of muscimol on stereotypy were of similar magnitude in animals perinatally exposed to Δ9-THC than controls in both sexes.

Experiment III: Basal and Baclofen-Stimulated [35S]-GTPγS Binding

In this experiment, we used the procedure recently developed by Sim et al. (27) to measure $GABA_B$ receptor activation of signal transduction mechanisms. Similarly to that previously reported by these authors, we have observed that the incubation of slide-mounted brain sections in the presence of baclofen increased [35S]-GTPγS binding over the basal binding measured in the absence of the agonist in those areas containing $GABA_B$ receptors. The coincubation of baclofen with CGP 35348, a specific antagonist, reversed this increase, supporting the involvement of $GABA_B$ receptor activation.

Using this procedure, we analyzed the magnitude of the stimulation by baclofen of [35S]-GTPγS binding in the substantia nigra, globus pallidus, cerebellum, and other structures (analyzed as controls) of adult males and females, that had been perinatally exposed to Δ9-THC or oil. Baclofen produced the expected increase in [35S]-GTPγS binding in the substantia nigra [see Table 3; $F(1, 33) = 5.38$, $p < 0.05$] and in other structures [i.e., Ammon's horn: $F(1, 36) = 9.35$, $p < 0.005$; dentate gyrus: $F(1, 36) = 10.45$, $p < 0.005$; cerebral cortex: $F(1, 34) = 36.31$, $p < 0.00005$; cerebellum: $F(1, 33) = 21.50$, $p < 0.0005$; see Table 3], but not in the globus pallidus. The stimulation was small in most of the structures analyzed

TABLE 3

BASAL AND BACLOFEN-STIMULATED [35S]-GTPGγS BINDING IN THE SUBSTANTIA NIGRA, CEREBELLUM, GLOBUS PALLIDUS, AMMON'S HORN, DENTATE GYRUS, AND CEREBRAL CORTEX OF ADULT MALES AND FEMALES THAT HAD BEEN PERINATALLY EXPOSED TO Δ9-TETRAHYDROCANNABINOL (Δ9-THC) OR OIL

Brain Structure	[35S]-GTPγS Binding	Females + Oil	Females +Δ9-THC	Males + Oil	Males +Δ9-THC
Substantia nigra	Basal	100 ± 5.7	114.7 ± 7.1	119.7 ± 5.0	108.4 ± 8.7
	Baclofen	132.4 ± 16.5*	120.3 ± 9.4	139.0 ± 9.1*	115.7 ± 9.6
Cerebellum	Basal	100 ± 5.7	104.5 ± 14.1	104.6 ± 9.5	125.4 ± 11.0
	Baclofen	161.1 ± 18.9†	144.0 ± 18.9*	153.4 ± 16.3*	170.7 ± 15.2*
Globus pallidus	Basal	100 ± 15.4	91.4 ± 8.5	99.6 ± 13.1	103.4 ± 21.5
	Baclofen	107.3 ± 18.8	108.0 ± 8.5	114.1 ± 9.1	118.0 ± 18.6
Ammon's horn	Basal	100 ± 6.1	113.3 ± 3.6	108.4 ± 7.4	105.0 ± 7.8
	Baclofen	117.1 ± 6.0*	133.8 ± 10.0*	135.6 ± 14.8*	122.5 ± 7.9*
Dentate gyrus	Basal	100 ± 5.4	103.5 ± 2.9	101.3 ± 9.4	99.4 ± 8.4
	Baclofen	127.4 ± 6.5†	119.7 ± 5.7*	126.5 ± 13.8*	110.6 ± 6.3
Cerebral cortex	Basal	100 ± 4.6	108.4 ± 7.8	111.1 ± 6.1	102.0 ± 7.2
	Baclofen	141.2 ± 4.2‡	137.7 ± 5.6†	144.5 ± 14.1*	137.1 ± 6.2†

Details in the text. Values are means ± SEM of data transformed to % over the basal binding in oil-exposed females. The number of determinations per group was 6. Data were assessed by three-way analysis of variance (sex × perinatal treatment × agonist stimulation) followed by the Student–Newman–Keuls test (*$p < 0.05$, †$p < 0.01$, ‡$p < 0.005$).

(with the only exception of the cerebellum) (Table 3), in concordance with the moderate presence of $GABA_B$ receptor binding. The other two variables, perinatal Δ^9-THC treatment and sex, analyzed alone did not exhibit any statistically significant differences for [^{35}S]-GTPγS binding in the substantia nigra, and the same occurred in the other structures (Table 3). The different two- and three-way interactions did not in any case reach statistical significance. A priori, this represents that the magnitude of baclofen-stimulated [^{35}S]-GTPγS binding was similar in the animals of both sexes that had been perinatally exposed to Δ^9-THC. However, it is important to point out that the post hoc analysis revealed that the differences between baclofen-stimulated and basal [^{35}S]-GTPγS binding only reached statistical significance in males (% of stimulation: 16%) and females (32.4%) perinatally exposed to oil, but not in those exposed to Δ^9-THC (males: 6.7%; females: 4.9%) (see Table 3). This did not occur in the other brain structures analyzed (Table 3), indicating certain specificity.

DISCUSSION

The present study again demonstrates that the exposure to Δ^9-THC during critical periods of brain development may cause long-term changes in the functionality of certain neurotransmitters in the adulthood. Most of our studies have been focused to dopamine (1,2,7–9,24,31,32) and, more recently, to opioid peptides (3,13,29,30). The present study extends these observations to GABA, because we have seen that the motor inhibition caused by a $GABA_B$ receptor agonist, baclofen, in the open-field test was more marked in magnitude in the adult animals that had been perinatally exposed to Δ^9-THC. There was a receptor specificity in this observation, because no differences were seen after challenging with muscimol, a $GABA_A$ receptor agonist. This is in concordance with the predominant role proposed for $GABA_B$ over $GABA_A$ receptors in the relationships between GABA and cannabinoids in the basal ganglia of adult individuals (25).

These changes in the motor responsiveness to baclofen mimicked those observed previously with dopaminergic antagonists (10), which were also different in animals perinatally exposed to Δ^9-THC. Moreover, as occurred in that earlier study for dopamine (10), we have seen here that the differences in the baclofen-induced motor inhibition occurred in parallel, with no changes in the basal activity of GABA-containing neurons in the areas related to motor activity. Thus, perinatal Δ^9-THC exposure did not produce any changes in GAD activity and GABA content in motor (caudate-putamen, globus pallidus, and substantia nigra) and limbic (nucleus accumbens and ventral-tegmental area) structures in adulthood. In addition, we could not demonstrate that the differences in baclofen response might be due to changes in the magnitude of the activation by this agonist of signal transduction mechanisms, using the recently developed methodology for the autoradiographic analysis of agonist-stimulated [^{35}S]-GTPγS binding (27). A priori, it would appear reasonable that baclofen, as was able to produce a higher degree of motor inhibition in Δ^9-THC–exposed animals, should also increase the efficiency of $GABA_B$ receptor activation in an area, such as the substantia nigra, which is a key area for the motor interactions between $GABA_B$ and cannabinoid receptors (25,26). However, our results did not confirm this initial hypothesis. In fact, it appeared that the magnitude of baclofen stimulation of [^{35}S]-GTPγS binding was reduced in animals perinatally exposed to Δ^9-THC, although this effect did not reach statistical significance.

Hence, we must relate the differences in the behavioral response of baclofen to reasons other than pharmacodynamic changes, because we could not obtain the expected changes in receptor activation. Among these plausible reasons might be those related to Δ^9-THC–induced differences in the pharmacokinetic profile of baclofen or to Δ^9-THC–induced changes in processes located subsequently to activation of GTP binding proteins by $GABA_B$ receptor agonists (i.e., kinase proteins, effector systems). However, the complexity of the extrapyramidal circuitry involved in the regulation of motor behavior allows another explanation. The control of the activity of nigrostriatal dopaminergic neurons by GABA is exerted by at least three different series of GABA containing neurons: (1) a direct inhibitory influence exerted by striatonigral GABAergic neurons, which it is known to contain cannabinoid receptors (13); (2) a second direct inhibitory influence coming from the pallidonigral GABAergic system, but which is under the inhibitory control of striatopallidal GABA-containing neurons; cannabinoid receptors are located only in these last neurons (13); and (3) a direct stimulatory influence, exerted by glutamatergic neurons coming from the subthalamic nucleus, which are under the control of pallidosubthalamic GABAergic neurons; cannabinoid receptors are located only in subthalamonigral glutamatergic neurons [for review, see (26)]. Then, it might be possible that the differences in the action of baclofen, producing a higher motor inhibition, but accompanied by an apparently lesser stimulation of [^{35}S]-GTPγS binding in the substantia nigra, both in Δ^9-THC–exposed animals, might be the result of multiple interactions among these different inputs. For instance, if we accept that the activation of $GABA_B$ receptors might be less efficient in the substantia nigra of Δ^9-THC–exposed animals, it would be possible to expect that the same effect might be occurring in the $GABA_B$ receptor synapses in other nuclei, such as the globus pallidus (stimulation was very poor in this nucleus, however) or the subthalamic nucleus. If this were true, the effect of systemic administrations of baclofen on motor activity might result in a more pronounced effect rather than the expected loss of effect. This is because we can suspect both: (a) an activation of pallidonigral GABAergic neurons, resulting in a higher neuronal inhibition in the substantia nigra, and (b) a loss of stimulatory inputs coming from the subthalamic nucleus due to increased activity in pallidosubthalamic GABA-containing neurons. Both facts would be concordant with a more pronounced motor inhibition, as observed in Δ^9-THC–exposed animals. At any rate, further research would be required to validate this hypothesis.

Collectively, the present results indicate that perinatal Δ^9-THC, although it did not produce any changes in GABA content and GAD activity in limbic and motor areas in adulthood, did increase the behavioral response to $GABA_B$ receptor agonists. However, this increase was not due to changes in $GABA_B$ receptor activation of signal transduction mechanisms, as revealed by analysis of the percentage of stimulation by baclofen of [^{35}S]-GTPγS binding in the substantia nigra and other structures of males and females perinatally exposed to Δ^9-THC.

ACKNOWLEDGEMENTS

This work has been supported by grants from CICYT (PM96-0049) and UCM (PR294/95-6189). J. Romero is a postdoctoral fellow of the "Comunidad Autónoma de Madrid". The authors are indebted to the National Institute on Drug Abuse (USA) for kindly supplying Δ^9-THC and to Ciba-Geigy (Barcelona, Spain) for the gift of CGP-35348. The authors wish to thank the technical assistance of Ana Jurado.

REFERENCES

1. Bonnin, A.; de Miguel, R.; Hernández, M. L.; Ramos, J. A.; Fernández-Ruiz, J. J.: The prenatal exposure to Δ^9-tetrahydrocannabinol affects the gene expression and the activity of tyrosine hydroxylase during early brain development. Life Sci. 56:2177–2184; 1995.
2. Bonnin, A.; de Miguel, R.; Castro, J. G.; Ramos, J. A.; Fernández-Ruiz, J. J.: Effects of perinatal exposure to Δ^9-tetrahydrocannabinol on the fetal and early postnatal development of tyrosine hydroxylase-containing neurons in rat brain. J. Mol. Neurosci. 7:291–308; 1996.
3. Corchero, J.; García-Gil, L.; Manzanares, J.; Fernández-Ruiz, J. J.; Fuentes, J. A.; Ramos, J. A.: Perinatal Δ^9-tetrahydrocannabinol exposure reduces proenkephalin gene expression in the caudate-putamen of adult female rats. Life Sci. 63:843–850; 1998.
4. Dalterio, S.; Bartke, A.: Perinatal exposure to cannabinoids alter male reproductive function in mice. Science 205:1420–1422; 1979.
5. Dalterio, S. L.: Perinatal or adult exposure to cannabinoids alters male reproductive functions in mice. Pharmacol. Biochem. Behav. 12:143–153; 1980.
6. Dalterio, S. L.: Cannabinoid exposure: Effects on development. Neurobehav. Toxicol. Teratol. 8:345–352; 1986.
7. Fernández-Ruiz, J. J.; Rodríguez de Fonseca, F.; Navarro, M.; Ramos, J. A.: Maternal cannabinoid exposure and brain development: Changes in the ontogeny of dopaminergic neurons. In: Bartke, A.; Murphy, L. L., eds. Neurobiology and neurophysiology of cannabinoids, biochemistry and physiology of substance abuse, vol. IV. Boca Raton, FL: CRC Press; 1992:119–164.
8. Fernández-Ruiz, J. J.; Bonnin, A.; Cebeira, M.; Ramos, J. A.: Ontogenic and adult changes in the activity of hypothalamic and extrahypothalamic dopaminergic neurons after perinatal cannabinoid exposure. In: Palomo, T.; Archer, T., eds. Strategies for studying brain disorders, vol. 1. England: Farrand Press; 1994;357–390.
9. Fernández-Ruiz, J. J.; Romero, J.; García-Gil, L.; García-Palomero, E.; Ramos, J. A.: Dopaminergic neurons as neurochemical substrates of neurobehavioral effects of marihuana: Developmental and adult studies. In: Beninger, R. J.; Archer, T.; Palomo, T., eds. Dopamine disease states. Madrid, Spain: CYM Press; 1996;359–387.
10. García-Gil, L.; de Miguel, R.; Ramos, J. A.; Fernández-Ruiz, J. J.: Perinatal Δ^9-tetrahydrocannabinol exposure in rats modifies the responsiveness of midbrain dopaminergic neurons in adulthood to a variety of challenges with dopaminergic drugs. Drug. Alcohol Depend. 42:155–166; 1996.
11. Herkenham, M.; Lynn, A. B.; Johnson, M. R.; Melvin, L. S.; de Costa, B. R.; Rice, K. C.: Characterization and localization of cannabinoid receptors in rat brain: A quantitative in vitro autoradiographic study. J. Neurosci. 11:563–583; 1991.
12. Herkenham, M.; Lynn, A. B.; de Costa, B. R.; Richfield, E. K.: Neuronal localization of cannabinoid receptors in the basal ganglia of the rat. Brain Res. 547:267–274; 1991.
13. Kumar, A. M.; Haney, M.; Becker, T.; Thompson, M. L.; Kream, R. M.; Miczek, K.: Effect of early exposure to Δ^9-tetrahydrocannabinol on the levels of opioid peptides, gonadotropin-releasing hormone and substance P in the adult male rat brain. Brain Res. 525:78–83; 1990.
14. Maneuf, Y. P.; Crossman, A. R.; Brotchie, J. M.: Modulation of GABAergic transmission in the globus pallidus by the synthetic cannabinoid WIN 55,212-2. Synapse 22:382–385; 1996.
15. Mokler, D. A.; Robinson, S. E.; Johnson, J. H.; Hong, J. S.; Rosecrans, J. A.: Neonatal administration of δ^9-tetrahydrocannabinol alters the neurochemical response to stress in the adult Fischer-344 rat. Neurotoxicol. Teratol. 9:321–326; 1987.
16. Molina-Holgado, F.; Amaro, A.; González, M. I.; Alvarez, F. J.; Leret, M. L.: Effect of maternal Δ^9-tetrahydrocannabinol on developing serotonergic system. Eur. J. Pharmacol. 316:39–42; 1996.
17. Murphy, L. L.; Steger, R. W.; Bartke, A.: Psychoactive and non-psychoactive cannabinoids and their effects on reproductive neuroendocrine parameters. In: Watson, R. R., ed. Biochemistry and physiology of substance abuse, vol. 2. Boca Ratón, FL: CRC Press; 1990;73–94.
18. Navarro, M.; Rodríguez de Fonseca, F.; Hernández, M. L.; Ramos, J. A.; Fernández-Ruiz, J. J.: Motor behavior and nigrostriatal dopaminergic activity in adult rats perinatally exposed to cannabinoids. Pharmacol. Biochem. Behav. 47:47–58; 1994.
19. Navarro, M.; de Miguel, R.; Rodríguez de Fonseca, F.; Ramos, J. A.; Fernández-Ruiz, J. J.: Perinatal cannabinoid exposure modifies the sociosexual approach behavior and the mesolimbic dopaminergic activity of adult male rats. Behav. Brain Res. 75:91–98; 1996.
20. Nicoletti, F.; Grandison, L.; Meek, J. L.: Effects of repeated administration of estradiol benzoate on tubero-infundibular GABAergic activity in male rats. J. Neurochem. 44:1217–1220; 1985.
21. Palkovits, M.; Brownstein, J.: Maps and guide to microdissection of the rat brain. New York: Elsevier Science; 1988.
22. Paxinos, G.; Watson, C.: The rat brain in stereotaxic coordinates. San Diego: Academic Press; 1986.
23. Pertwee, R. G.; Greentree, S. G.; Swift, P. A.: Drugs which stimulate or facilitate central GABAergic transmission interact synergistically with Δ^9-tetrahydrocannabinol to produce marked catalepsy in mice. Neuropharmacology 27:1256–1270; 1988.
24. Rodríguez de Fonseca, F.; Cebeira, M.; Fernández-Ruiz, J. J.; Navarro, M.; Ramos, J. A.: Effects of pre- and perinatal exposure to hashish extracts on the ontogeny of brain dopaminergic neurons. Neuroscience 43:713–723; 1991.
25. Romero, J.; García-Palomero, E.; Fernández-Ruiz, J. J.; Ramos, J. A.: Involvement of GABA-B receptors in the motor inhibition produced by agonists of brain cannabinoid receptors. Behav. Pharmacol. 7:299–302; 1996.
26. Romero, J.; de Miguel, R.; Ramos, J. A.; Fernández-Ruiz, J. J.: The activation of cannabinoid receptors in striatonigral GABAergic neurons inhibited GABA uptake. Life Sci. 62:351–363; 1998.
27. Sim, L. J.; Selley, D. E.; Childers, S. R.: *In vitro* autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl-5′-[γ-[^{35}S]thio]-triphosphate binding. PNAS (USA) 92:7242–7246; 1995.
28. Smith, S.; Sharp, T.: Measurement of GABA in rat brain microdialysates using *O*-phthdialdehyde-sulfite derivatization and high-performance liquid chromatography with electrochemical detection. J. Chromat. B 652:228–233; 1994.
29. Vela, G.; Fuentes, J. A.; Bonnin, A.; Fernández-Ruiz, J. J.; Ruiz-Gayo, M.: Perinatal exposure to Δ^9-tetrahydrocannabinol (Δ^9-THC) leads to changes in opioid-related behavioral patterns in rats. Brain Res. 680:142–147; 1995.
30. Vela, G.; Martin, S.; García-Gil, L.; Crespo, J. A.; Ruiz-Gayo, M.; Fernández-Ruiz, J. J.; García Lecumberri, C.; Pelaprat, D.; Fuentes, J. A.; Ramos, J. A.; Ambrosio, E.: Maternal exposure to Δ^9-tetrahydrocannabinol facilitates morphine self-administration behavior and changes regional binding to central μ opioid receptors in adult offspring female rats. Brain Res. 807:101–109; 1998.
31. Walters, D. E.; Carr, L. A.: Changes in brain catecholamine mechanisms following perinatal exposure to marihuana. Pharmacol. Biochem. Behav. 25:763–778; 1986.
32. Walters, D. E.; Carr, L. A.: Perinatal exposure to cannabinoids alters neurochemical development in the rat brain. Pharmacol. Biochem. Behav. 29:213–216; 1988.