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Short communication

Prenatal Δ^9-tetrahydrocannabinol exposure modifies proenkephalin gene expression in the fetal rat brain: sex-dependent differences

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Abstract

Perinatal Δ^9-tetrahydrocannabinol (Δ^9-THC) exposure in rats resulted in enhanced morphine self-administration behavior, naloxone-precipitated withdrawal signs or changes in pain sensitivity, which have been related to changes in μ-opioid receptor binding and/or proenkephalin mRNA levels in several brain regions. However, despite exposure of these animals to Δ^9-THC from fetal ages, the effects were studied only when animals matured, whereas there is no study on possible changes caused by this cannabinoid during the prenatal ontogeny of opioidergic neurons. The purpose of the present study was to examine the changes in proenkephalin mRNA levels, measured by using in situ hybridization, in several brain nuclei of rat fetuses that had been daily exposed to Δ^9-THC from the 5th day of gestation. Results were as follows. Prenatal Δ^9-THC exposure altered proenkephalin mRNA levels in most of the brain areas studied at different fetal ages, but the effects were different between sexes. Thus, proenkephalin mRNA levels increased in females, but decreased in males that had been prenatally exposed to Δ^9-THC. This was observed in the caudate–putamen, hypothalamic paraventricular and ventromedial nuclei and cerebral cortex. No changes were observed, however, in the subventricular zones of the caudate–putamen, neocortex and nucleus accumbens. In summary, prenatal Δ^9-THC exposure produced a sex-dependent effect in proenkephalin mRNA levels in several brain structures of rat fetuses. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Cannabinoid; Δ^9-Tetrahydrocannabinol; Prenatal exposure; Proenkephalin gene expression; Opioid peptide

Several studies have demonstrated that perinatal exposure to Δ^9-tetrahydrocannabinol (Δ^9-THC), the prototypical plant-derived cannabinoid, altered the prenatal and/or postnatal ontogeny of several neurotransmitters, which resulted in important long-term effects on several adult behaviors (for review, see Refs. [4–6]). Among other effects observed in rats, perinatal Δ^9-THC exposure altered pain sensitivity [11] and drug-seeking behavior [12], likely indicating an effect of this cannabinoid on the development of opioidergic neurons. Indeed, Kumar et al. [8] reported alterations in the levels of opioid peptides in the hypothalamus of adult rats perinatally exposed to cannabinoids. As regards to the first of these two opioid-related processes, pain sensitivity, we have found that Δ^9-THC, when administered during development, produced, when animals reached the adult age, a certain degree of tolerance to the analgesic effect of morphine, but without modification in basal sensitivity to painful stimuli [11]. As regards to drug-seeking behavior, we have also reported that rats, which had been exposed to Δ^9-THC, exhibited a naloxone-induced withdrawal syndrome at immature ages [11] similar to that observed in morphine-dependent animals. In addition, we have also recently found that these rats, upon reaching adulthood, self-administered more morphine [12], an effect that has been related to a Δ^9-THC-induced increase in μ-opioid receptor binding [12], as well as to a decrease in amounts of proenkephalin mRNA [3], in specific regions related to drug reinforcement, although a marked sexual dimorphism was found for these responses [3,11,12].

Despite the animals used in the above studies [3,11,12] were exposed to Δ^9-THC from fetal ages, the effects were studied only when animals matured and there is no study on possible changes caused by this cannabinoid during the prenatal ontogeny of opioidergic neurons. The purpose of the present study was precisely to examine the changes in opioidergic indices in the brain of rat fetuses that had been

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daily exposed to Δ^9-THC from the 5th day of gestation, according to our previously reported treatment model [5,6]. To this end, we have analyzed proenkephalin mRNA levels, by using in situ hybridization, in selected brain nuclei of rat fetuses, at gestational days (GD) 16, 18 and 21, that had been daily exposed to Δ^9-THC or vehicle.

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 and 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. All experimental procedures and care of the animals were conformed to local rules. Δ^9-THC, kindly 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 5th day of gestation, according to our previously published model in which we have previously reported the absence of any nutritional effects [4–6]. Control rats were fed with vehicle alone. This treatment was daily maintained until the different fetal ages at which pregnant rats were sacrificed: GD16, GD18 and GD21. At each age, fetuses from different litters were used for variation. After the sacrifice of pregnant rats, their fetuses were rapidly removed. At GD21, the sex of fetuses was determined by examination of anogenital distance prior to pup autopsy and further analysis of gonadal location. At earlier gestational ages, the sex could not be determined and the fetuses were used randomly. Whole fetuses (at GD16) or dissected brains (at GD18 and GD21) were rapidly frozen by immersion in 2-methyl-butane previously cold in dry ice. All samples were stored at −70°C until processed. Sagittal (GD16) or coronal (GD18 and GD21) sections 20-μm-thick were cut in a cryostat [1]. 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 analysis of proenkephalin mRNA levels by using the procedure of in situ hybridization previously described by Young et al. [13] with minor modifications [3]. Sections from at least four different animals *per group* were analyzed. For the identification of the different brain structures, adjacent sections to those used for in situ hybridization were stained with Cresyl violet [1]. Data were assessed by Student's *t*-test (GD16–GD18) or by two-way ANOVA (treatment × sex) followed by Student–Newman–Keuls' test (GD21).

The present study demonstrates for the first time that the presence of Δ^9-THC in the developing brain during the fetal period was able to alter, as previously reported for dopamine, serotonin or GABA (for review, see Refs. [4–6]), the prenatal ontogeny of opioidergic neurons, concretely of enkephalin-containing neurons. Thus, we have observed that GD16 fetuses that had been prenatally exposed to Δ^9-THC exhibited a small, but significant, increase in the levels of proenkephalin mRNA in some structures (see Fig. 1 for a representative autoradiogram) that we have previously found that exhibit significant transcript levels for this opioid precursor in the adult brain [10]. Thus, proenkephalin mRNA levels increased in the

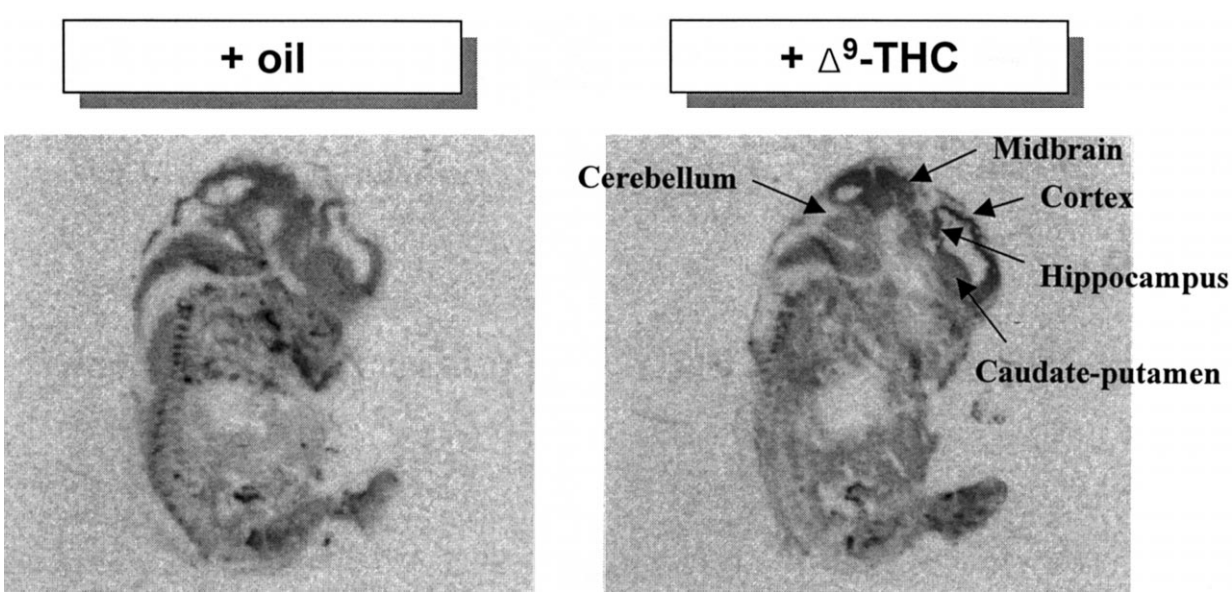

Fig. 1. Representative autoradiogram corresponding to the analysis by in situ hybridization of proenkephalin mRNA levels in a sagittal section of rat fetuses, at GD16, that had been exposed to vehicle or Δ^9-THC since 5th day of gestation. See details in the text.

caudate–putamen (+22.0%; oil: 101.2 ± 2.8, Δ^9-THC: 123.5 ± 2.0; $p < 0.005$), septum nuclei (+17.0%; oil: 101.6 ± 3.1, Δ^9-THC: 118.9 ± 2.0; $p < 0.005$), thalamus (+25.3%; oil: 96.3 ± 3.3, Δ^9-THC: 120.7 ± 5.0; $p < 0.005$), hypothalamus (+29.5%; oil: 94.0 ± 3.0, Δ^9-THC: 121.7 ± 1.6; $p < 0.005$) and midbrain (+11.9%; oil: 106.3 ± 4.9, Δ^9-THC: 119.0 ± 2.9; $p < 0.05$), whereas they exhibited a certain trend to increase in the cerebellum (oil: 99.5 ± 3.5, Δ^9-THC: 112.5 ± 7.6; $p = 0.105$) and no changes in the hippocampus (oil: 103.6 ± 3.0, Δ^9-THC: 110.2 ± 5.7) and the cerebral cortex (oil: 105.5 ± 4.0, Δ^9-THC: 112.5 ± 4.6).

GD18 fetuses also exhibited some small, but significant, changes in proenkephalin mRNA levels in several brain structures due to prenatal Δ^9-THC exposure (see Table 1). Thus, proenkephalin mRNA levels increased in the cerebral cortex (+14.7%) and caudate–putamen (+11.5%) of fetuses that had been prenatally exposed to Δ^9-THC, but they exhibited no changes in the ventromedial and paraventricular hypothalamic nuclei (Table 1), despite these were altered at GD16. It is possible that this might be related to the fact that, when the sex of the fetuses could be easily determined, as occurred at GD21, we observed a sex-dependent response to Δ^9-THC with increases in female pups and decreases in male pups in most of the brain structures analyzed, such as the caudate–putamen, cerebral cortex and paraventricular and ventromedial hypothalamic nuclei. According to this, the absence of changes at GD18 in some regions might be the result of the sum of opposite effects Δ^9-THC as a function of sex, since the animals were taken randomly due to the extreme difficulty to assess the sex of fetuses. This might be relevant in some regions at GD18, because sexual differentiation of the brain caused by androgens produced in the fetal testes starts at this GD, but it is unlikely that has an impact in the effects observed at days earlier than GD18 and, hence, before the sexual differentiation of the brain.

As observed in GD16 and GD18, proenkephalin mRNA levels were also altered, although to a lesser, but significant extent, in several brain structures of GD21 fetuses that had been perinatally exposed to Δ^9-THC, but, at this age, it could be observed that the changes were sexually dimorphic, with increases in female fetuses and decreases in male fetuses (see Table 1). In this respect, it is important to point out that sexual dimorphism is frequently observed in the responses of developing brain to drugs of abuse, stress or other insults (for review, see Refs. [4–6]). This also occurred in our precedent studies on the effects of perinatally administered cannabinoids on several opioid-related behaviors [3,11,12]. Thus, the above-mentioned enhanced morphine self-administration [12], increased μ-opioid receptor binding [12] and decreased proenkephalin gene expression [3], observed in adult rats that had been perinatally exposed to Δ^9-THC, were only observed in females, but not in males, whereas other responses were found only in males [11]. In our present study, the two-way ANOVA of the data observed in GD21 fetuses supported the existence of a sexually dimorphic response, because

Table 1
Proenkephalin mRNA levels in several brain structures of rat fetuses, at GD18 and GD21, that had been exposed to Δ^9-THC or vehicle since 5th day of gestation

Details in the text. Values are means ± S.E.M. with the number of animals analyzed *per* group in parentheses. Data were assessed by Student's *t*-test (GD18) or two-way ANOVA (sex × treatment) followed by Student–Newman–Keuls' test (GD21).

Age	Brain structure	Sex	+ Vehicle	+ Δ^9-THC
GD18	subventricular zone of the neocortex	not determined	111.3 ± 2.4 (6)	100.6 ± 1.6 (6)**
	subventricular zone of the striatum		113.2 ± 2.5 (6)	102.9 ± 1.9 (6)**
	subventricular zone of the nucleus accumbens		118.9 ± 2.2 (6)	109.6 ± 2.0 (6)*
	cerebral cortex		107.4 ± 4.3 (5)	123.2 ± 4.9 (6)*
	paraventricular hypothalamic nucleus		115.2 ± 6.6 (5)	120.8 ± 3.5 (5)
	ventromedial hypothalamic nucleus		113.2 ± 5.0 (5)	121.2 ± 2.2 (5)
	caudate–putamen		128.7 ± 3.7 (5)	143.5 ± 3.1 (6)**
GD21	subventricular zone of the neocortex	females	114.0 ± 4.4 (6)	107.5 ± 2.3 (5)
		males	114.2 ± 6.6 (5)	113.0 ± 5.6 (6)
	subventricular zone of the striatum	females	111.2 ± 4.4 (6)	107.6 ± 3.5 (5)
		males	114.8 ± 8.0 (5)	112.8 ± 6.3 (5)
	subventricular zone of the nucleus accumbens	females	116.4 ± 4.7 (6)	110.9 ± 2.2 (5)
		males	115.7 ± 5.7 (5)	114.2 ± 5.2 (6)
	cerebral cortex	females	113.0 ± 3.9 (5)	119.8 ± 2.2 (5)
		males	128.9 ± 1.0 (5)	108.1 ± 2.3 (5)**
	paraventricular hypothalamic nucleus	females	121.9 ± 4.9 (5)	139.2 ± 4.8 (6)*
		males	146.1 ± 2.8 (5)	127.4 ± 3.2 (5)**
	ventromedial hypothalamic nucleus	females	102.5 ± 2.8 (6)	119.5 ± 1.3 (5)**
		males	120.3 ± 6.2 (5)	106.9 ± 2.5 (5)*
	caudate–putamen	females	117.9 ± 4.5 (5)	134.6 ± 4.6 (5)*
		males	133.2 ± 5.7 (5)	111.1 ± 3.8 (5)*

* $p < 0.05$.
** $p < 0.005$.

the interaction treatment/sex reached statistical significance in the cerebral cortex ($F(1,12) = 24.65$, $p < 0.0005$), caudate–putamen ($F(1,15) = 17.87$, $p < 0.005$), ventromedial hypothalamic nucleus ($F(1,15) = 14.14$, $p < 0.005$) and paraventricular hypothalamic nucleus ($F(1,16) = 17.37$, $p < 0.005$). Thus, proenkephalin mRNA levels increased in the caudate–putamen (+14.2%) and ventromedial (+16.6%) and paraventricular (+14.2%) hypothalamic nuclei, but exhibited only a trend in the cerebral cortex, of female fetuses that had been perinatally exposed to Δ^9-THC (see Table 1). By contrast, proenkephalin mRNA levels decreased in the caudate–putamen (−16.6%), cerebral cortex (−16.1%) and ventromedial (−11.1%) and paraventricular (−12.8%) hypothalamic nuclei of male fetuses that had been perinatally exposed to Δ^9-THC (see Table 1). Other brain regions, such as the paraventricular thalamic nucleus (females: oil: 115.4 ± 2.5, Δ^9-THC: 121.5 ± 2.0; males: oil: 119.1 ± 1.0, Δ^9-THC: 117.0 ± 2.8), did not exhibit, however, this sexually dimorphic response.

Another interesting issue observed in the present study was the localization of proenkephalin gene transcripts in several forebrain regions, such as the subventricular zones of the neocortex, striatum and nucleus accumbens, that have the common feature of being proliferative areas for neurons and glial cells during brain development [7]. Enkephalin-containing neurons are likely to proliferate from these regions, subsequently migrating until to their target fields. Even, it has been suggested that expression of proenkephalin in proliferative regions may be indicative of a role of this opioid peptide in cell proliferation (for review, see Ref. [9]). Interestingly, Δ^9-THC exposure affected the levels of proenkephalin mRNA in these three areas at GD18 (Table 1). However, by contrast with the above-mentioned regions, proenkephalin mRNA levels slightly decreased in these three regions of fetuses that had been perinatally exposed to Δ^9-THC (subventricular zone of the neocortex: −9.6%; subventricular zone of the striatum: −9.1%; subventricular zone of the nucleus accumbens: −7.8%) (see Table 1). However, these decreases disappeared at GD21 (see Table 1). This is concordant with the role suggested for the endocannabinoid system in the process of neuronal or glial cell proliferation (for review, see Ref. [6]), which is based in the localization of high levels of cannabinoid receptor mRNA in these proliferative regions [2]. It could be concluded that Δ^9-THC, by acting through cannabinoid receptors located in these proliferative regions might be altering the process of proliferation and migration of opioid neurons, which could be responsible for the changes produced by Δ^9-THC in the other brain structures observed in the present study and, in particular, for the long-term effects in opioidergic neurotransmission reported previously [3,11,12].

Taken together, the results found in this study strengthen the hypothesis that cannabinoids, given during the ontogeny of opioidergic neurons, produce not only long-lasting alterations in the functionality of endogenous opioid system, as reported in precedent studies [3,11,12], but also during the prenatal maturation of these neurons. Thus, prenatal Δ^9-THC exposure altered proenkephalin mRNA levels in the rat brain during the fetal period. The effects exhibited a marked sexual dimorphism at GD21, with increases in females and decreases in males. It is true that most of the effects observed were small in magnitude (usually not greater than 10–20%), but this does not imply that they have not physiological relevance in terms of producing an alteration in the maturation of opioidergic neurons and, consequently, in opioid-related behaviors. Brain development is a period of maximal vulnerability to epigenetic influences, and whatever insult, even of small magnitude, might produce significant and irreversible phenotypical changes.

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