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Early maternal deprivation induces gender-dependent changes on the expression of hippocampal CB_1 and CB_2 cannabinoid receptors of neonatal rats

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ABSTRACT

Early maternal deprivation (MD) in rats [24 h, postnatal day 9-10] is a model for neurodevelopmental stress. There are some data proving that MD affects the endocannabinoid system in a gender-dependent manner, and that these changes may account for the proposed schizophrenia-like phenotype of MD rats. The impact of MD on cannabinoid receptor distribution in the hippocampus is unknown. The aim of this study is to evaluate the expression of CB_1 and CB_2 receptors in diverse relevant subregions (DG, CA1 and CA3) of the hippocampus in 13-day-old rats by immunohistochemistry and densitometry. MD induced a significant decrease in CB_1 immunoreactivity (more marked in males than in females), which was mainly associated with fibers in the *strata pyramidale* and *radiatum* of CA1 and in the *strata oriens*, *pyramidale* and *radiatum* of CA3. In contrast, MD males and females showed a significant increase in CB_2 immunoreactivity in the three hippocampal areas analyzed that was detected in neuropil and puncta in the stratum oriens of CA1 and CA3, and in the polymorphic cell layer of the dentate gyrus. A marked sex dimorphism was observed in CA3, with females exhibiting higher CB_1 immunoreactivity than males, and in dentate gyrus, with females exhibiting lower CB_2 immunoreactivity than males. These results point to a clear association between developmental stress and dysregulation of the endocannabinoid system. The present MD procedure may provide an interesting experimental model to further address the role of the endocannabinoid system in neurodevelopmental mental illnesses such as schizophrenia.

INTRODUCTION

Exposing neonatal rats [postnatal day (PND) 9] to a single prolonged 24-h episode of maternal deprivation (MD) has been shown to induce long-term behavioral alterations that resemble certain symptoms observed in schizophrenic patients. Therefore, MD rats showed disturbances in pre-pulse inhibition, latent inhibition, and auditory sensory gating and startle habituation in adulthood (Ellenbroek and Riva, 2003; Ellenbroek et al., 2004). Based on these findings, MD was proposed as a potential animal model for certain aspects of schizophrenia (Ellenbroek and Cools, 2002; Marco et al., 2008).

Several lines of evidence support an association between an altered endocannabinoid system (ECS) and the pathogenesis of schizophrenia. For example, increases in CB_1 cannabinoid receptor expression have been found in the prefrontal cortex (Dean et al., 2001) and cingulate cortex (Zavitsanou et al., 2004) of schizophrenic patients. Also, elevated levels of the endocannabinoid anandamide have been detected in the cerebrospinal fluid of schizophrenics (Leweke et al., 1999; Giuffrida et al., 2004; Leweke et al., 2007). Moreover, frequent cannabis use significantly increases the risk for psychotic symptoms and schizophrenia (see for review, Di Forti et al., 2007; Leweke and Koethe, 2008). Supporting this hypothesis, glutamate transmission is tightly regulated by retrograde endocannabinoid signaling and both, the endocannabinoid system and glutamate are clearly involved in the pathophysiology of major symptoms of schizophrenia (Leweke and Koethe 2008). Following this rationale, it may be possible that MD, as an animal model of schizophrenia, may result in alterations of the endogenous cannabinoid system, as it has been described for the glutamatergic transmission (Pickering et al., 2006; Roceri et al., 2002). Previous behavioral data from our group indirectly supported this hypothesis since MD rats and mice showed altered responses to cannabinoid agonists (see Marco et al., 2008 for review). More recently, we have shown that the level of hippocampal 2-arachidonylglycerol (2-AG), a

ligand for CB_1 and CB_2 cannabinoid receptors, was significantly increased in MD male but not in MD female rats at PND 13. This last finding provided a first direct evidence for a link between MD stress and the ECS that appeared to be expressed in a gender-dependent manner (Llorente et al., 2008).

According to the neurodevelopmental theory of schizophrenia, behavioral deficits observed in MD animals might be mediated by detrimental neurodevelopmental effects of MD-induced increases in corticosterone levels (Ellenbroek and Cools, 2002; Stanton et al., 1988). In order to address this hypothesis, we have recently evaluated possible neurodevelopmental changes induced by MD in the hippocampus, a region that is particularly sensitive to the detrimental effects of glucocorticoids (Gould, 1994; Sapolsky et al., 1988; Sapolsky, 2000). Our results indicated that, at PND 13, MD animals showed significant increases in the number of Fluoro-Jade C (FJ-C) positive (+) cells (suggestive of degenerating neurons) and in GFAP+ cells (suggestive of astrocytosis), being these effects more marked in males than in females (Llorente el al., 2007; Viveros et al., 2008). Interestingly, endogenous glucocorticoid regulates cannabinoid receptors in the brain, providing again a link between MD and the endogenous cannabinoid system (Mailleux and Vanderhaeghen, 1993).

There is evidence for a crucial implication of the ECS in brain developmental processes such as neural progenitor proliferation, lineage segregation, migration and phenotypic specification of immature neurons, axonal elongation and synaptogenesis (see for reviews Fernández-Ruíz et al., 2000; Berghuis et al., 2007; Harkany et al., 2008). Retrograde signaling involving endocannabinoids appears to be responsible for the homeostatic control of synaptic transmission and the resulting network patterns in the immature hippocampus (Bernard et al., 2005). Studies on the role of the ECS in the control of neurodevelopmental processes have mainly focused in CB_1 receptors. However, the role

of brain CB_2 receptors in neonatal differentiated neuronal cells has been scarcely investigated. Until recently, it was held that CB_2 receptors were not present in the brain under physiological conditions. In fact, cannabinoid CB_2 receptors appear to be mostly peripherally located on immunological tissues. However, recent data may change the classical view of these cannabinoid receptors. Immunohistochemical analyses have revealed immunostaining for CB_2 receptors in apparent neuronal and glial processes in several adult rodent brain areas, including the hippocampus, under normal-physiological conditions (Van Sickle et al., 2005; Onaivi et al., 2006; Suárez et al., 2008). These results suggest broader functional roles for these receptors.

Based on the above evidence, we have evaluated, by immunohistochemistry and densitometry, the impact of MD on the expression of cannabinoid CB_1 and CB_2 receptors in the hippocampus of 13-day old rats, the same age at which we have previously reported cellular and biochemical (endocannabinoid levels) changes in the hippocampus of MD animals (Llorente el al., 2007; Viveros et al., 2008). Considering the abundant gender differences that we found in our previous studies using this model (Llorente et al., 2007; Marco et al., 2008; Llorente et al., 2008; Viveros et al., 2008), we investigated the possible existence of sexual dimorphisms throughout this study.

MATERIAL AND METHODS

Animals

Subjects were the offspring of Wistar albino rats of both sexes purchased from Harlan Interfauna Ibérica S.A. (Barcelona, Spain) which were mated (one male x two females) in our laboratory approximately 2 weeks after their arrival. All animals were maintained at a constant temperature (22 ± 1 ºC) and humidity (50 ± 1%) in a reverse 12-h dark-light cycle (lights on at 20:00h), with free access to food (commercial diet for rodents

A04/A03; Panlab, Barcelona, Spain) and water. On the day of birth (PND 0), litters were sex-balanced and culled to 8 pups per dam (4 males and 4 females). In all experimental groups, rats of at least four different litters were used in order to reduce litter effect.

The experiments performed in this study are in compliance with the Royal Decree 1201/2005, October 21, 2005 (BOE nº 252), as well as with the European Communities Council Directive of 24 November 1986 (86/609/EEC) about protection of experimental animals.

Maternal Deprivation

Maternal deprivation (MD) took place on PND 9 according to Ellenbroek procedure (Ellenbroek et al., 1998). In summary, mothers from the deprived group were removed early in the morning (beginning at 09:00) and pups were weighed and kept undisturbed in their home cages (in the same room) for 24 hours. On PND 10, pups were weighed again and mothers returned to their corresponding cages. In the control group the mothers were briefly removed on PND 9 and 10 to weigh the pups.

Immunohistochemistry

Animals (n=28; seven per group) were sacrificed by quick decapitation during the dark phase of the cycle (9:00 to 14:00h) at PND 13. Brains were rapidly removed and fixed with 4% paraformaldehyde (Merck) in 0.1 M phosphate buffer (PB), pH 7.2, during 48h at 4ºC. Later, the brains were washed three times, 10 minutes for each of them, in PB 0.1M, pH 7.2, and stored at 4º C in PB with 0.002 % (w/v) of sodium azide. The brains were equilibrated in PBS containing 30% sucrose and 0.01 % (w/v) of sodium azide during 24h. Brains were then cut into 30-µm-thick-transverse sections using a sliding microtome.

Sections were collected from -4.16 to -4.80 Bregma levels to process each antibody and Nissl staining (Paxinos and Watson, 1998).

Several batches of staining were performed for each antibody, each including slices from all the animals studied in order to avoid changes in staining intensity not related to the treatment (see next section).

For each staining batch, free-floating sections were first incubated into H_2Od containing 50 mM sodium citrate (pH 9) for 30 minutes at 80ºC, followed by several washes in PBS, and then with 3% hydrogen peroxide in PBS for 20 minutes at room temperature to inhibit endogenous peroxidase. Then, sections were incubated in blocking solution containing 10% donkey serum, 0,3% triton X-100 and 0,1% sodium azide in PBS for 1 hour.

For this study we used the cannabinoid receptor type 1 (CB_1) and the cannabinoid receptor type 2 (CB_2) antibodies. Both antibodies were produced in the laboratory of Dr. Ken Mackie: the anti-CB_1 was developed in rabbits by using a fusion protein as immunogen containing 73 amino acid residues (401-473) of the rat CB_1 receptor (Wager-Miller et al., 2002); and the anti-CB_2 was produced in rabbits by using a fusion protein containing 14 amino acid residues (328-342) from rat CB_2 receptor. Sections were incubated in the diluted primary antibody (anti-CB_1, diluted 1:500; and anti-CB_2, diluted 1:500) overnight at room temperature. After three washes in PBS, the sections were incubated in a biotinylated donkey anti-rabbit immunoglobulin (Amersham, Little Chalfont, England) diluted 1:500 for 1 hour, washed again in PBS, and incubated in ExtrAvidin peroxidase (Sigma, St. Louis, MO, USA) diluted 1:2000 for 1 hour. We revealed immunolabeling with 0.05% diaminobenzidine (DAB; Sigma), 0.05% nickel ammonium sulphate, and 0.03% H_2O_2 in PBS. All steps were carried out by gentle agitation at room temperature. After sections were washed in PBS, they were mounted on gelatinized slides, air dried, dehydrated in ethanol,

cleared in xylene and coverslipped with Eukitt mounting medium (Kindler GmBH & Co, Freiburg, Germany).

In order to demonstrate antibody specificity, hippocampal sections from CB_1 receptor knock-out mice (Ledent et al., 1999), CB_2 receptor knock-out mice (Buckley et al., 2000) and wild-type controls (n = 2 pairs) were also analyzed (Fig. 1). The immunohistochemical protocol was carried out as described above (anti-CB_1, diluted 1:500; anti-rat CB_2, 1:500). We observed that immunostaining was almost completely absent in CB_1 knockout mouse brain, but weak staining could be found in the cerebral peduncle at hippocampal levels. With the exception of this feature, all of the staining in wild-type hippocampus is specifically attributable to CB_1 expression with a similar labeling pattern in rat and mouse hippocampus (Fig. 1A-C). We did not observed labeling in the CB_2 receptor knockout mouse, whereas the wild-type mouse showed similar labeling to that of the rat brain (Fig. 1D-F).

Digital high-resolution microphotographs were taken under the same conditions of light and brightness/contrast by an Olympus BX41 microscope equipped with an Olympus DP70 digital camera. Digital images were mounted and labeled using Adobe PageMaker (San Jose, CA, USA) and analyzed for quantitative measurement of immunostaining (see next section).

Quantification of CB_1 and CB_2 Immunostaining on Hippocampal Slices

There were a total of 4 experimental groups, a male group and a female group for maternal deprivation, as well as male and female control groups. Each experimental group was made up of 7 animals. One immunostaining batch contained 28 slides, (four groups, seven animals each), and the slices corresponding to the four experimental groups were stained simultaneously. 2-3 different batches were run for each primary antibody and for each animal. On each tissue section we focused on CA1 and CA3 areas of Ammon´s horn

and dentate gyrus (DG). For both CA subfields, we carried out separate densitometrical analysis corresponding to: 1) *alveus*/*stratum oriens* (alv; SO), and 2) the rest of the strata, i.e., *pyramidale* (SP) together with *radiatum* (SR), since soma and dendrites of the pyramidale neurons dispose in each layer respectively, and/or *lacunosum-moleculare* (SL-M). For dentate gyrus, we carried out separate densitometrical analysis corresponding to 1) the molecular layer (ml) together with the granular cell layer (gcl), and 2) the polymorphic cell layer (pl).

Quantification of CB_1 and CB_2 immunostaining was carried out on high resolution digital microphotographs by measuring densitometry of the selected areas (see above) (these images were taken with the 10x objective under the same conditions of light and brightness/contrast) using the analysis software ImageJ 1,38x (NIH, USA).

Statistical Analysis

Immunohistochemical data were analyzed by two-way analysis of variance (ANOVA), with the two factors being sex (males and females) and MD (non deprived and deprived animals). The Bonferroni test with a level of significance set at $P<0.05$ was used for post hoc comparisons.

RESULTS

CB_1 Receptors

Figure 2 shows representative microphotographs of CB_1 immunoreactivity from DG, CA3 and CA1 of control and MD of males and females groups respectively. The intense CB_1 immunoreactivity in the hippocampus was associated with a dense network of fibers in the granular cell layer and molecular layer, which are surrounding unstained cell bodies and dendrites of the DG granular cells, respectively (Fig. 2A-D), and in the *stratum pyramidale*

and *stratum radiatum*, surrounding unstained cell bodies and dendrites of the of pyramidal cells in CA1 and CA3 respectively (Fig. 2E-L). We observed that the staining was more intense in CA3 and DG than in CA1. In CA1 and CA3, CB_1 immunoreactivity was associated with fibers in the *stratum radiatum*, with the most intense staining occurring adjacent to the pyramidal cells and to the *stratum lacunosum-moleculare* (Fig. 2E-H). A fine meshwork of stained fibers was evident in the *stratum oriens*, but the most intense staining also occurred adjacent the pyramidal cells. The *stratum lacunosum-moleculare* of CA1 showed a lower density of CB_1 positive (CB_1+) fibers (Fig. 2I-L).

Results corresponding to the quantification of the CB_1 receptor immunoreactivity are shown in Figure 4A. In general, the MD procedure induced a decrease in CB_1+ fibers in the three hippocampal areas analysed (DG, CA1 and CA3), being this effect more marked in males than in females: [SP+SR CA1, MD: $F(1,23)=4.50$, $P<0.05$; SO CA3, MD: $F(1,23)=5.24$, $P<0.05$; SP+SR CA3, MD: $F(1,23)=4.31$, $P<0.05$]. Post hoc comparisons also showed a marked sexual dimorphism in CA3 with females exhibiting higher CB_1 immunoreactivity than males [SO CA3, sex: $F(1,23)=4.95$, $P<0.05$; SP+SR CA3, sex: $F(1,23)=12.96$, $P<0.01$]. Significant interaction between the two factors was found when analyzing CB_1 immunoreactivity in DG and CA3 [pl DG, MD x sex: $F(1,23)=4.88$, $P=0.0373$; SO CA3, MD x sex: $F(1,23)=4.15$, $P=0.05$; SP+SR CA3, MD x sex: $F(1,23)=4.15$, $P<0.05$].

CB_2 Receptors

Figure 3 shows representative microphotographs of CB_2 immunoreactivity from DG, CA3 and CA1 of control and MD of males and females groups respectively. The distribution of the CB_2 immunoreactivity in the hippocampus was characterized by a weak to moderate network of CB_2-immunopositive (CB_2+) neuropil and puncta, being more pronounced in the

polymorphic cell layer of the DG (Fig. 2A-D), and in the *stratum oriens* of CA1 and CA3 (Fig. 2E-L). This neuropil showed a mossy aspect, which define numerous unstained cell profiles. Most of these mossy CB_2+ fibers may represent dendritic terminals that are disposed in the *strata radiatum* and *oriens* of the DG to CA3. We also observed an intense immunoreactivity in the *alveus* that may correspond to CA1 interneuronal dendrites (Fig. 2I-L). According to this distribution, we did not observe CB_2 immunoreactivity in the *stratum pyramidale* of CA1 and CA3, or the granular cell layer of DG.

Results corresponding to the quantification of the CB_2 receptor immunoreactivity are shown in Figure 4B. In contrast to the findings in relation to CB_1 receptors, the MD procedure induced significant increases in CB_2 immunoreactivity in the three hippocampal areas analysed (DG, CA1 and CA3), effect that was evident in both, males and females. Statistical analyses rendered significant effects of the MD for all the areas analysed: gcl+ml DG, [$F(1,24)=7.23$, $P=0.01$]; pl DG, [$F(1,24)=30.42$; $P<0.0001$; alveus CA1, [$F(1,24)=7.32$, $P=0.01$]; SO CA1 [$F(1,24)=9.30$, $P<0.01$]; SP+SR CA1 [$F(1,24)=10.39$, $P<0.01$]; SL-M CA1, [$F(1,24)=10.15$, $P= 0.01$]; SO CA3, [$F(1,24)=5.73$, $P<0.05$]; SP+SR CA3, [$F(1,24)=9.62$, $P<0.01$]. We also observed a significant sexual dimorphism in the polymorphic cell layer of DG [pl DG, sex: $F(1,24)=12.36$, $P<0.01$], with females exhibiting lower CB_2 immunoreactivity than males, and a significant interaction between the two factors [pl DG, MD x Sex: $F(1,24)=4.95$, $P=0.0358$].

DISCUSSION

The psychotic-like behavioral alterations displayed by adult rats deprived from their mothers during a 24h- period, at PND 9, have led to propose this experimental procedure as a potential animal model for certain aspects of schizophrenia. Moreover, it was also hypothesized that these behavioral alterations might have a basis in neurodevelopmental

detrimental effects of MD-induced elevated corticosterone on certain brain regions particularly vulnerable to the effects of glucocorticoids, such as the hippocampus (Ellenbroek and Cools, 2002; Ellenbroek and Riva, 2003; Ellenbroek et al., 2004). In support of this hypothesis we previously found that, at PND 13, MD rats showed hippocampal neuronal and glial alterations showing more marked effects in males than in females (Llorente et al., 2008; Viveros et al., 2008). Considering that the endocannabinoid system appears to be critically involved in brain development (Fernández-Ruíz et al., 2000; Berghuis et al., 2007; Harkany et al., 2008), and that several lines of evidences point to a clear association between schizophrenia and disregulation of the ECS system (Leweke et al., 1999; Dean et al., 2001; Giuffrida et al., 2004; Zavitsanou et al., 2004; Leweke et al., 2007), we expected to find an altered ECS in MD animals. The first direct evidence came when we found that the endocannabinoid 2-AG was significantly increased in the hippocampus of MD 13-day old male rats (Llorente et al., 2008). To further investigate possible effects of MD on cannabinoid receptors, we evaluated the expression of CB_1 and CB_2 receptors in diverse relevant subregions of the hippocampus in neonatal rats of the same age by quantitative immunohistochemical techniques. The fact that glutamate transmission and glucocorticoids regulate cannabinoid receptor expression (Mailleux and Vanderhaeghen, 1993 and 1994), MD modifies hippocampal glutamatergic transmission, and data from disrupted glucocorticoid genes, gives support to the present study (Pickering et al., 2006). Moreover, a continuous homeostatic interplay between glutamate, glucocorticoids and endocannabinoids in the hypothalamus, the extended amygdala and the hippocampus, suggests a functional link between these three systems that is essential for homeostatic behaviors (Cota et al., 2006; Rodriguez de Fonseca et al., 1997). The present results show that MD produced a decrease in the expression of hippocampal CB_1 receptors suggesting that these changes could be related to the psychotic-like behavioral alterations found in this

model. Indeed, the CB_1 expression changes were more marked in males than in females. This sexual dimorphism seen here is in agreement with our previous results showing that 13-day old male rats were also more affected than corresponding females regarding hippocampal neuronal and glial alterations (Llorente et al., 2008; Viveros et al., 2008). Since the rats used in this study are obviously pre-pubertal animals, sexual dimorphisms might be attributable to organizational effects of gonadal steroids acting during the perinatal critical period for the sexual differentiation of the brain (McEwen, 1992; Schwarz and McCarthy, 2008). Although there have been very few studies that have analyzed the endocannabinoid system in both sexes, there are data showing gender-dependent differences in both, developmental (Rodriguez De Fonseca et al., 1993) and adult (Rodriguez de Fonseca et al., 1994; Craft, 2005; Fattore et al., 2007; Craft and Leitl, 2008) animals that are attributable to organizational and/or activational effects of gonadal steroids. In fact, existing literature suggest that the endocannabinoid system is markedly affected by sexual dimorphisms.

It is important to note that both, hipoglutamatergic state and glucorticoid administration reduces cannabinoid mRNA expression in the rat brain, although those early studies were only performed in adult male animals (Mailleux and Vanderhaeghen, 1993 and 1994). Additional data obtained from male adult rats also suggest that hippocampal cannabinoid CB_1 receptor signaling could be reduced under certain conditions associated with hypersecretion of glucocorticoids, such as chronic stress (Hill et al., 2008). MD induces significant increases in corticosterone levels that can still be detected at PND 13 (Llorente et al., 2008; Viveros et al., 2008). It is possible that, at this early postnatal age, hippocampal CB_1 receptors are under the negative regulation of glucocorticoids which may explain, at least in part, the reduced CB_1 receptors in MD animals. Another possible mechanism leading to the reduced expression of hippocampal CB_1 receptors is the receptor down-regulation in response to an increase in endocannabinoid levels. In fact, as it has been indicated above, we

have observed that, at PND 13, MD males showed significantly increased levels of hippocampal 2-AG (Llorente et al., 2008). However, the induction of CB_2 receptors after MD indicates that endocannabinoid-driven down regulation may not underlie the decrease in the hippocampal CB_1 receptors.

Rearing rats in isolation for 6-8 weeks following weaning has long term effects that may arise from the lack of sensory information and social contact important for the normal structural and functional neurodevelopment. Similar to the MD model, isolation rearing produces a number of post-pubertal behavioral and neurochemical changes, some of which are similar to those observed in psychoses, in particular, a disruption in prepulse inhibition which is indicative of deficits in sensorimotor gating (Geyer et al., 1993; Cilia et al., 2005). Isolation rearing in rats has also been shown to alter hippocampal function, including the serotonergic system (Muchimapura et al., 2002; Muchimapura et al., 2003). Moreover, raising rats in isolation led to a significant decrease in CB_1 receptor expression in the caudate putamen and the amygdale, as well as a significant increase in FAAH expression in the caudate putamen and the nucleus accumbens core and shell (Malone et al., 2008). Taken as a whole, the present results together with those reported by Malone et al. (2008) support the view that the endocannabinoid system is altered in animal models related to different aspects of psychosis. These environmentally based non invasive experimental models may provide new insights into the role of the endocannabinoid system in the development of this mental illness and other related neuropsychiatric disorders.

This study also provides an original (non-previously reported) description of CB_1 distribution within the hippocampus of neonatal rats (PND-13). The CB_1 staining pattern, showing CB_1+ axons in the DG and hippocampal CA1 and CA3 areas, is in agreement with previous descriptions in adult animals (Egertova and Elphick, 2000) suggesting that, at this post-natal age, the CB1 is already in its final adult distribution.

Whereas CB_1 receptors are among the most abundant and widely distributed G-protein coupled receptors in the brain (highly expressed in brain regions such as cerebellum, basal ganglia and hippocampus), CB_2 receptors have been traditionally considered as peripherally located receptors, mainly expressed in immunological tissues. This receptor has also been found within the central nervous system on neurons and glial cells but their expression was mainly related to conditions of inflammation. However, recent findings of brain CB_2 receptors under normal conditions suggest broader functional roles for these receptors in the central nervous system (see for review Viveros et al., 2007; Mackie, 2008; Svíženská et al., 2008). Using reverse transcription polymerase chain reaction (RT-PCR), pharmacological, and behavioral evidence, Van Sickle et al. (2005) showed that functional cannabinoid CB_2 receptors are present in brainstem neurons. More recently, Hill et al. (2007) investigated the expression of CB_1 and CB_2 in identified neurons of rat neocortical slices using single-cell RT-PCR. They found that as much as 49% of pyramidal neurons expressed CB_1, whereas CB_2 was only observed in a small proportion of neocortical neurons. In a recent paper, we have provided a detailed analysis of the distribution of relevant proteins of the endogenous cannabinoid system (including CB_1 and CB_2 receptors) in the rat cerebellum (Suárez et al., 2008). Gong et al. (2006) described multifocal expression of CB_2 immunoreactivity in apparent neuronal and glial processes in a number of rat brain areas, including the hippocampus, cerebral cortex, striatum, thalamic nuclei, amygdala, substantia nigra and periaqueductal gray, among others. In vitro, CB_2 immunoreactivity was also present in hippocampal cell cultures. Although the physiological significance of brain CB_2 receptors is still unclear, recent data provided evidence that CB_2 receptors in the thalamus may play a functional role in the modulation of responses of neurons in the ventral posterior nucleus of the thalamus in neuropathic rats (Jhaveri et al.,

2008). To the best of our knowledge, here, we provide the first evidence for the presence of CB_2 receptor in the hippocampus of neonatal rats for both genders. The staining pattern highly suggests that immunostained structures correspond to dendritic terminals (Brusco et al., 2008), and not to microglial cell bodies as could be initially thought based on old reports confining CB_2 receptors to immune system-related cells. These results are partially in contrast with recent reports on CB_2 expression within the hippocampus of adult animals (Gong et al 2006), and the discrepancies could be related to differential expression of CB_2 receptor during postnatal development suggesting that, at PND-13, CB_2 expression has not still reached its adult final distribution. Therefore, the differential expression pattern between CB_1 and CB_2 receptors must reflect the different physiological roles of both types of receptors. According to the literature, the localization of both receptors seems compatible with a cannabinoid-dependent synaptic modulation of neuronal transmission, as proved by CB_1 immunolabeling suggesting a presynaptic localization from external hippocampal input, i.e. perforant fibers from entorhinal cortex, and from internal (excitatory and inhibitory) hippocampal circuits, (Hájos et al., 2001; Kawamura et al., 2006; Takahashi and Castillo, 2006; Nyíri et al., 2005); and CB_2 immunolabeling seem to be restricted to a postsynaptic localization in dendritic terminals according to a previous study (Brusco et al., 2008). These anatomical features suggests a predominant role of CB_1 in modulating external hippocampal input and a role for CB_2 in modulating internal hippocampal flux of information. However, CB_1 and CB_2 receptors may also serve to promote fiber growth, stabilization and plasticity of CB_1/CB_2-containig neurons during the pre-pubertal stages. Moreover, our data demonstrate that CB_2 receptor was also clearly affected by the MD procedure and therefore this alteration could, together with CB_1 expression changes, account for the psychotic-like behavioral alterations found. Interestingly, the alterations of CB_1 and CB_2 receptors were in opposite directions, i.e. MD animals showed a clear increase in CB_2 receptor but a decrease

in CB_1 hippocampal receptor. This striking opposite pattern may suggest some kind of functional compensation and/or interaction between both types of cannabinoid receptors. Another difference regarding both receptors is that the CB_2 type was affected equally in the two genders, whereas CB_1 showed a clear sexual dimorphism with a more predominant effect of MD in males. As mentioned above, up to date few studies have analyzed the gender-related differential expression of cannabinoid receptors after neurodevelopmental stress, and the scarce studies available only refer to CB_1 receptors. The present data further support sexual dimorphisms affecting this receptor subtype, but further work will be needed in order to elucidate the extension of this gender-specific CB1 alteration as well as the relative plasticity of brain CB_2 receptors and their susceptibility to show alterations in response to both hormonal and environmental (external) factors.

In conclusion, we provide the first evidence for significant effects of MD on CB_1 and CB_2 cannabinoid receptors expression in the hippocampus of male and female neonatal rats. The present MD procedure may provide relevant information about the specific role of the endocannabinoid system in neurodevelopmental events that could lead to schizophrenia and other neuropsychiatric diseases. Importantly, the present data provide the first evidence for the expression of CB_1 and CB_2 receptors in the hippocampus of male and female pre-puberal rats, and demonstrates that these receptors are in fact affected by stressful events occurring early in life that could be related to long-term behavioral alterations. In view of recent reports on the presence of CB_2 receptors in diverse brain regions of adult rats and mice and, also considering the present findings, the functional roles of CB_2 receptors in the brain of naïve rodents clearly deserve further investigation. Lastly, but not least, our data indicate that the analysis of both genders in animal models of psychiatric diseases, such as schizophrenia, may shed some light on a possible neurodevelopmental basis for existing gender differences in these pathologies.

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FIGURE LEGENDS

Figure 1. CB_1 and CB_2 immunoreactivity in the hippocampus of Wistar adult rats (**A** and **D**), wild type mice (**B** and **E**) and CB_1 or CB_2 receptor knockout mice (**C** and **F**), respectively. We observed an identical distribution of CB_1 and CB_2 immunoreactivity between rat and mouse hippocampus, but the immunostaining was completely absent in knockout mice. Abbreviations: Cx, cortex; Hc, hippocampus. Scale bars are included in each image.

Figure 2. Representative microphotographs of CB_1 immunoreactivity in relevant subregions (DG, CA1 and CA3) of 13-day-old rat hippocampus. Differences are illustrated between control (Co) and maternal deprivation (MD) procedures in male and female animals. The intense CB_1 immunoreactivity in the hippocampus was associated with a dense network of fibers in the granular cell layer and molecular layer surrounding the unstained cell bodies and dendrites (respectively) of the granular cells of the dentate gyrus (**A**-**D**), and in the *stratum pyramidale* and *stratum radiatum* surrounding the unstained cell bodies and dendrites (respectively) of the pyramidal cells of CA1 (**E**-**H**) and CA3 (**I**-**L**). Abbreviations: alv, *alveus*; CA, *cornu ammonis*; Co, control; DG, dentate gyrus; gcl, granular cell layer; MD, maternal deprivation; ml, molecular layer; pl, polymorphic cell layer; SL-M, *stratum lacunosum-moleculare*; SO, *stratum oriens*; SP, *stratum pyramidale*; SR, *stratum radiatum*. Scale bars are included in images **A**, **E** and **I**.

A, E, I: Male Co; **B, F, J**: Male MD; **C, G, K**: Females Co; **D, H, L**: Female MD;

Figure 3. Representative microphotographs of CB_2 immunoreactivity in relevant subregions (DG, CA1 and CA3) of 13-day-old rat hippocampus. Differences are illustrated between control (Co) and maternal deprivation (MD) procedures in male and female animals. The distribution of the CB_2 immunoreactivity in the hippocampus was characterized by a weak to moderate network of CB_2-immunopositive (CB_2+) neuropil and puncta, being more pronounced in the polymorphic cell layer of the dentate gyrus (**A-D**) and in the *stratum oriens* of CA1 (**E-H**) and CA3 (**I-L**). Of note, we can also observe an intense immunoreactivity in the *alveus*. Abbreviations are listed in the legend of figure 2. Scale bars are included in images **A**, **E** and **I**.

A, E, I: Male Co; **B, F, J**: Male MD; **C, G, K**: Females Co; **D, H, L**: Female MD;

Figure 4. Quantification of CB_1 (**A**) and CB2 (**B**) receptor immunoreactivity in relevant subregions (DG, CA1 and CA3) of the hippocampus of 13-day-old male and female rats after early maternal deprivation (MD). **A**) MD induced significant decreases in CB_1 immunoreactivity (more marked in males than in females), which was mainly associated with a lower network of fibers in the *strata pyramidale* and *radiatum* of CA1 and the *strata oriens*, *pyramidale* and *radiatum* of CA3. Differences of CB_1 immunoreactivity in the layers of DG were not significant. **B**) MD induced a significant increase in CB_2 immunoreactivity in the three hippocampal areas analyzed and this effect was evident in both, males and females. Marked sex dimorphism was observed in CA3 with females exhibiting higher CB_1 immunoreactivity than males (A), and in DG with females exhibiting lower CB_2 immunoreactivity than males (B). Significant interaction between the two factors was also

found when analyzing CB_1 immunoreactivity in DG and CA3, and CB_2 immunoreactivity in DG. Abbreviations are listed in the legend of figure 2.

Histograms represent the mean ± S.E.M. (7 animals per experimental group). Two-way ANOVA and Bonferroni post hoc test: *P<0.05; **P<0.01; ***P<0.001; ns, no significance.

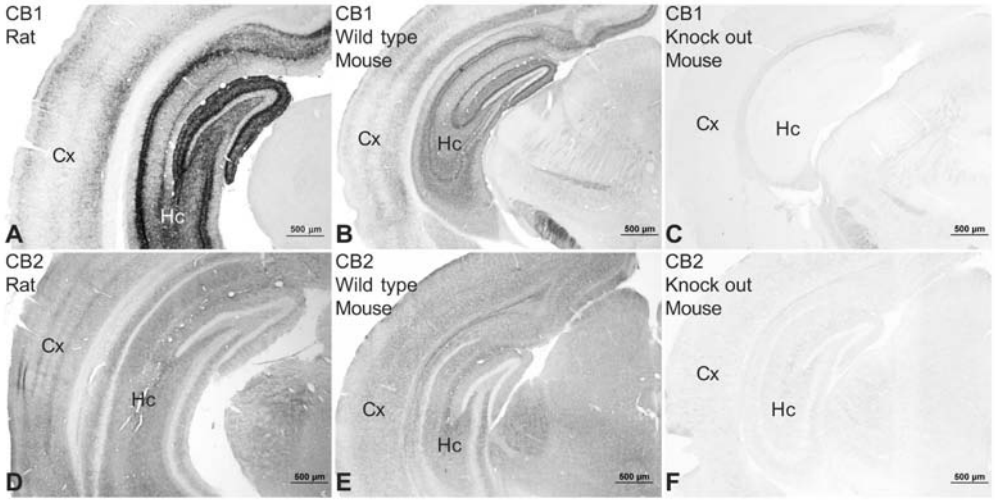

Figure 1. Controls for CB1 and CB2 immunohistochemistry in rat, mouse and CB1 and CB2 knockout mice
178x90mm (600 x 600 DPI)

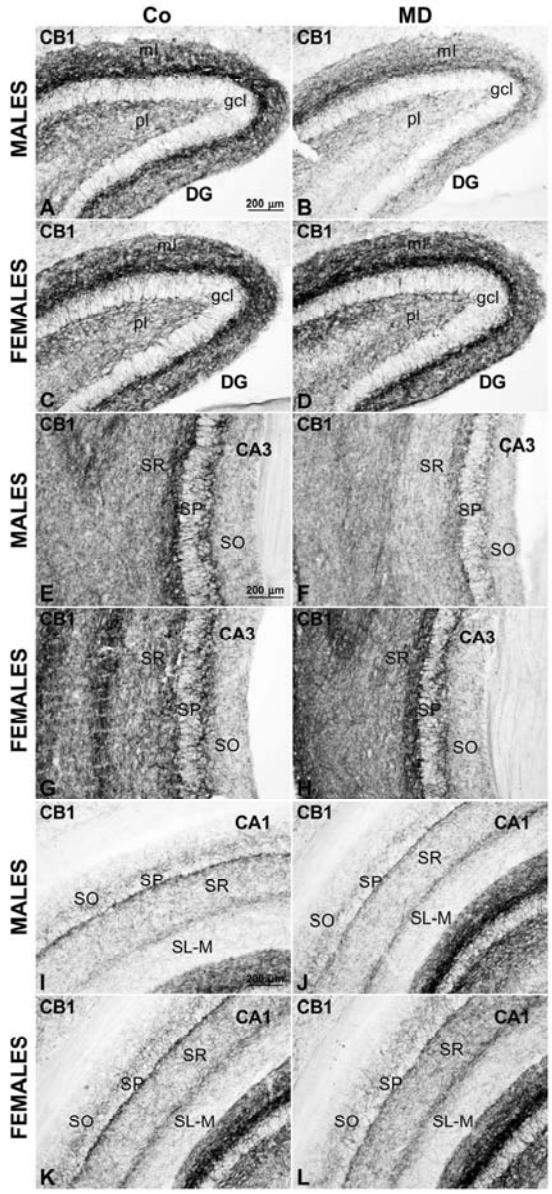

Figure 2. CB1 immunohistochemistry in hippocampus of control and maternal deprivation of male and female 13-day old rats.
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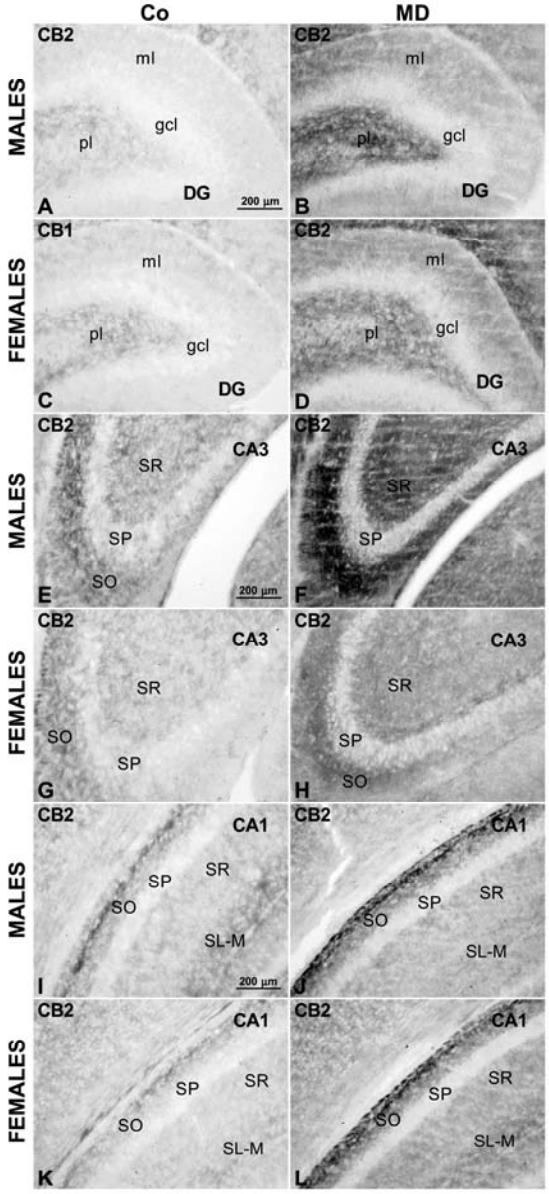

Figure 3. CB2 immunohistochemistry in hippocampus of control and maternal deprivation of male and female 13-day old rats.
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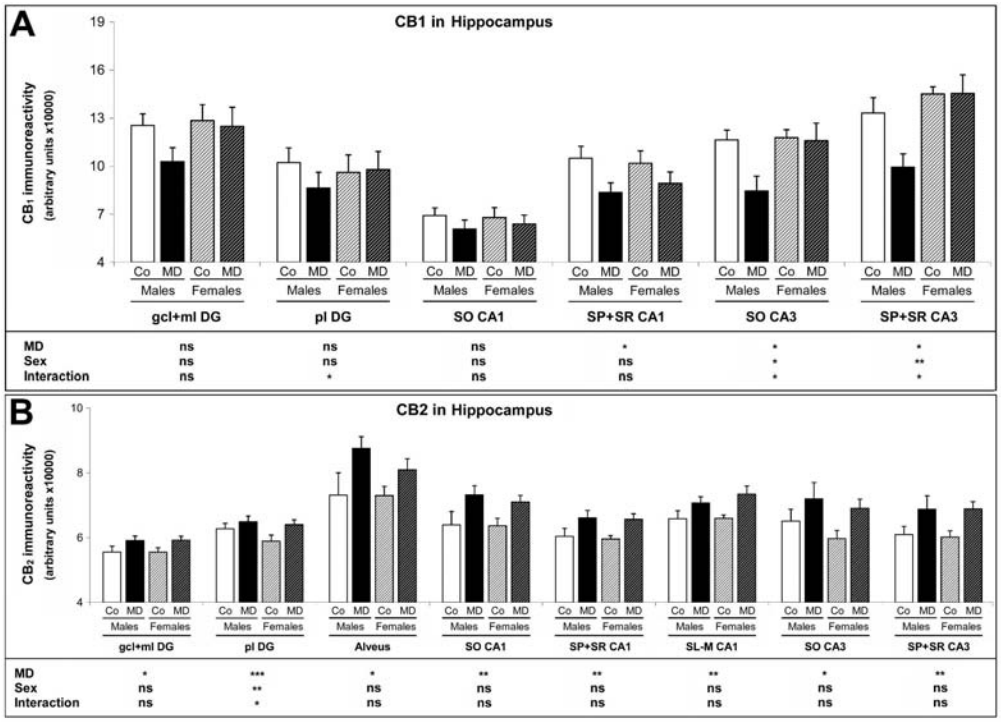

Figure 4. Quantification of CB1 and CB2 immunostaining in 13-day-old rat hippocampus
177x128mm (600 x 600 DPI)