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Atypical Location of Cannabinoid Receptors in White Matter Areas during Rat Brain Development

J. ROMERO, E. GARCIA-PALOMERO, F. BERRENDERO, L. GARCIA-GIL, M.L. HERNANDEZ, J.A. RAMOS, AND J.J. FERNANDEZ-RUIZ*
Instituto Complutense de Drogodependencias, Department of Biochemistry, Faculty of Medicine, Complutense University, Madrid, Spain

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ABSTRACT Previous evidence suggests that the endogenous cannabinoid system could emerge and be operative early during brain development. In the present study, we have explored the distribution of specific binding for cannabinoid receptors in rat brain at gestational day 21 (GD21), postnatal days 5 (PND5) and 30 (PND30), and at adult age (>70 days after birth) by using autoradiography with [^{3}H]CP-55,940. Our results indicated that specific binding for cannabinoid receptors can be detected in the brain of rat fetuses at GD21 in the classic areas that contain these receptors in adulthood—in particular, in the cerebellum and the hippocampus and, to a lesser extent, in the basal ganglia, several limbic structures, and cerebral cortex. The density of cannabinoid receptors in all these structures increased progressively at all postnatal ages studied until reaching the classical adult values in 70-day-old animals. Interestingly, cannabinoid receptor binding can also be detected at GD21 in regions, in which they are scarcely distributed or not located in the adult brain and that have the particularity of all being enriched in neuronal fibers. Among these were the corpus callosum, anterior commissure, stria terminalis, fornix, white matter areas of brainstem, and others. This atypical location was quantitatively high at GD21, tended to wane at PND5, and practically disappeared at PND30 and in adulthood, with the only exception being the anterior commissure, which exhibited a moderate density for cannabinoid receptors. Moreover, the binding of [^{3}H]CP-55,940 to cannabinoid receptors in the white matter regions at GD21 seems to be functional and involves a GTP-binding protein-mediated mechanism. Thus, the activation of these receptors with an agonist such as WIN-55,212-2 increased the binding of [^{35}S]-guanylyl-5′-*O*-(γ-thio)-triphosphate, measured by autoradiography, in the corpus callosum and white matter areas of brainstem of fetuses at GD21. This increase was reversed by coincubation of WIN-55,212-2 with SR141716, a cannabinoid receptor antagonist. As this antagonist is specific for the cerebral cannabinoid receptor subtype, called CB_1, we can assert that the signal found for cannabinoid receptor binding in the fetal and early postnatal brain likely corresponds to this receptor subtype. Collectively, all these data suggest the existence of a transient period of the brain development in the rat, around the last days of the fetal period and the first days of postnatal life, in which CB_1 receptors appear located in neuronal fiber-enriched areas. During this period, CB_1 receptors would be already functional acting through a GTP-binding protein-mediated mechanism. After this transient period, they progressively acquire the pattern of adult distribution. All this accounts for a specific role of the endogenous cannabinoid system in brain development. **Synapse 26:317–323, 1997.**

INTRODUCTION

Our laboratory (Rodríguez de Fonseca et al., 1993) and others (Belue et al., 1995, Mailleux and Vanderhaeghen, 1992; McLaughlin and Abood, 1993; McLaughlin

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*Correspondence to: J.J. Fernández-Ruiz, Department of Biochemistry, Faculty of Medicine, Complutense University, 28040-Madrid, Spain.
E-mail: jjfr@eucmax.sim.ucm.es

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et al., 1994) have described the presence of cannabinoid receptor binding and mRNA levels in the developing brain, although all these studies were always performed at postnatal ages. However, the presence of these receptors in the brain seems to be even earlier than the first days of postnatal life. This would be based on the important effects caused by prenatal exposure to cannabinoids on neurobehavioral development (for review, see Fernández-Ruiz et al., 1992, 1994). Thus, it seems to support the notion that the system constituted by endogenous cannabimimetic ligands and the cannabinoid receptor signaling pathway would emerge and be operative early during development, presumably during the last third of gestation and that it might probably play a physiologic role in neural development. Recently, we have presented the first evidence about the presence of these receptors in the brain of fetuses at gestational days (GD) 18 and 21 (Fernández-Ruiz et al., 1994). However, these studies were performed by using membrane binding techniques, which do not allow the precise depiction of the anatomic location of these receptors. Hence, a prioritary objective would be to establish, by using autoradiographic analysis, the distribution of cannabinoid receptors in the fetal and early postnatal brain. Our group is presently addressing this objective wholely, and in preliminary studies, we have observed that these receptors seem to be atypically distributed in the brain during fetal and early postnatal ages, comparing with their classical pattern of distribution in adulthood. The present report has been aimed to prove the atypical location of cannabinoid receptors in the fetal and early postnatal brain. We include here the autoradiographic study of cannabinoid receptor binding, using [^{3}H]CP-55,940, in slide-mounted brain sections obtained at ages selected according to the atypical distribution found in the preliminary studies: GD21, 5 (PND5) and 30 (PND30) days after birth, and adulthood (>70 days after birth). In addition, we also include the demonstration that receptor binding found in the fetal brain (at GD21) is not an artifact and corresponds to the CB_1 receptor subtype, which is already functional at this age acting through a GTP-binding protein-mediated mechanism. This has been demonstrated by using an autoradiographic technique to measure agonist-stimulated [^{35}S]-guanylyl-5′-O-(γ-thio)-triphosphate ([^{35}S]-GTPγS) binding in brain sections, according to a recently published procedure (Sim et al., 1996).

MATERIALS AND METHODS

Animals

Timed pregnant Wistar rats, sperm-positive on a specific day (GD1), were housed in a room with controlled photoperiod (08:00–20:00 h light on) and temperature (23 ± 1°C) and free access to standard food and water. Male pups from these rats were sacrificed at GD21, PND5, PND30, and in adulthood (>70 days after birth). At each age, animals from at least three different litters were grouped. After sacrifice, brains were carefully removed and rapidly frozen by immersion in 2-methyl-butane cold in dry ice. All samples were stored at −80°C. Frozen serial coronal sections (20-μm-thick; obtained from at least three brains per age) were cut in a cryostat, according to the Paxinos atlas (Paxinos and Watson, 1986; Paxinos et al., 1991). Sections were thaw-mounted onto gelatin-coated slides and dried briefly at 30°C and stored at −80°C until used. For the identification of the different brain structures, adjacent sections to those used for autoradiographic analysis were stained with cresyl-violet.

Cannabinoid Receptor Autoradiography

The protocol used is basically the method described by Herkenham et al. (1991). Briefly, slide-mounted brain sections were incubated for 2.5 h, at 37°C, in a buffer containing 50 mM TRIS with 5% bovine serum albumin (fatty acid-free), pH 7.4, and 10 nM [^{3}H]CP-55,940 (DuPont NEN, Boston, MA, U.S.A.), in the absence or the presence of 10 μM nonlabeled CP-55,940 (kindly supplied by Pfizer, Madrid, Spain) to determine the total and the nonspecific binding, respectively. After this incubation, slides were washed in 50 mM TRIS buffer with 1% bovine serum albumin (fatty acid-free), pH 7.4, for 4 h (2 × 2 h) at 0°C, dipped in ice-cold distilled water and then dried under a stream of cool dry air. Autoradiograms were generated by apposing the labeled tissues, together with autoradiographic standards ([^{3}H] microscales, Amersham), to tritium-sensitive film ([^{3}H]-Hyperfilm, Amersham) for a period of 2–3 weeks, 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 Dinamics) using the standard curve generated from [^{3}H]-standards.

[^{35}S]-GTPγS Autoradiography

The protocol used is basically the method recently described by Sim et al. (1996). 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, Mannheim, Germany) in assay buffer. Afterward, 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 1 μM WIN-55,212-2 (RBI, Natick, MA, U.S.A.). Basal activity was assessed in the absence of agonist, whereas nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS (Boehringer Mannheim). Moreover, to ensure that WIN-55,212-2-stimulated [^{35}S]-GTPγS binding increase was caused through activation of cannabinoid receptors, additional brain sections were incubated in

TABLE I. Specific binding for cannabinoid receptors in several rat brain regions at fetal (GD21), early postnatal (PND5), immature (PND30), and adult (>70 days after birth) ages[1]

Age	Corpus callosum	Anterior commissure	Fornix	Stria terminalis	Stria medullaris	Fasciculus retroflexum	Brainstem	Hippocampus	Cerebellum	Caudate-putamen	Septum nuclei	Nucleus accumbens	Cerebral cortex
GD21	++++	+++	++++	++++	+++	+++	++	+++	++	+	++	+	+
PND5	++++	+++	++	++++	++	++	++	+++	++	+	++	+	+
PND30	ND	++	ND	+++	+	ND	+	++++	+++++	+++++	++	++	++
Adult	ND	+++	ND	ND	ND	ND	+	+++++	+++++	+++++	+++	+++	++++

[1]Details in the text. Values of specific binding for each region were obtained as the mean of at least three animals per age and the means assigned to the following semiquantitative table: ND, <3 fmol/mg tissue; +, specific binding = 3–30 fmol/mg tissue; ++, specific binding = 30–60 fmol/mg tissue; +++, specific binding = 60–90 fmol/mg tissue; ++++, specific binding = 90–120 fmol/mg tissue; +++++, specific binding >120 fmol/mg tissue.

the presence of 0.3 μM SR141716 (kindly supplied by Sanofi Recherche, Montpellier, France) in addition to 0.04 nM [^{35}S]-GTPγS, 2 mM GDP, and 1 μM WIN-55,212-2. 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) for a period of 10 days and developed (D-19, Kodak) for 4 min at 20°C. Developed films were analyzed and quantitated in a computer-assisted viodeodensitometer (Image Quant 3.3, Molecular Dinamics).

RESULTS

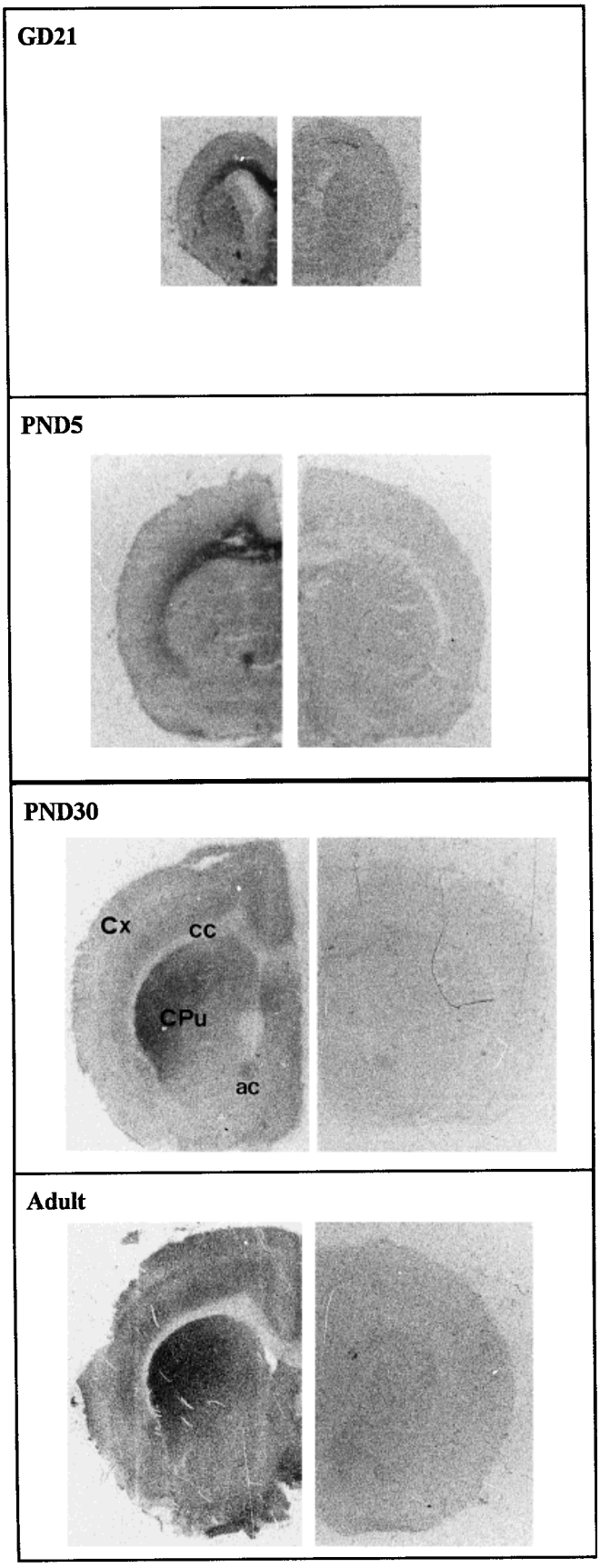
Cannabinoid Receptor Autoradiography

Specific binding for cannabinoid receptors could be detected in the brain of rat fetuses at GD21 in the classic areas that contain these receptors in adulthood—in particular, in the cerebellum and the hippocampus (Table I). Other classic structures such as the basal ganglia (caudate-putamen), several limbic structures (septum nuclei, nucleus accumbens), and cerebral cortex also exhibited specific binding for cannabinoid receptors at GD21 (Table I, Fig. 1) but to a lesser extent than in the cerebellum and hippocampus. The density of cannabinoid receptors in all these regions increased progressively at all postnatal ages studied until reaching the current adult values in 70-day-old animals (Table I).

Specific binding for cannabinoid receptors could also be detected at GD21 in regions in which they are scarcely distributed or not located in the adult brain and that have the particularity of all being characterized by the presence of neuronal fibers. Among these were the corpus callosum, the anterior commissure, the stria terminalis, the stria medullaris, the fornix, the fasciculum retroflexus, and the white matter areas of brainstem (Table I, Fig. 1). Even a possible gradient in the appearance of specific binding for cannabinoid receptors was observed in the corpus callosum at GD21, because, in coronal sections, central parts of the corpus callosum exhibited a higher signal than lateral parts (Fig. 1). This atypical location of cannabinoid receptors in neuronal fiber-enriched areas was high at GD21, tended to wane at PND5, and practically disappeared at PND30 and in adulthood (Table I), with the only exception being the anterior commissure, which exhibited a moderate density for cannabinoid receptor binding in adult brain (Table I), as previously reported (Herkenham et al., 1991; Jansen et al., 1992).

[^{35}S]-GTPγS Autoradiography

It could be considered that the high presence of cannabinoid receptor binding in the white matter areas might be, at least partially, an artifact due to expected quenching when using a tritium labeled ligand before



full maturation of myelinated tracts. However, the second methodologic approach performed in the present study clearly demonstrates that (1) the signal found in white matter regions at GD21 represents binding of [^{3}H]CP-55,940 to cannabinoid receptors and (2) these receptors seem to be functional at this age and act through a GTP-binding protein-mediated mechanism. Thus, the activation of cannabinoid receptors with an agonist such as WIN-55,212-2 increased the binding of [^{35}S]-GTPγS, measured by autoradiography, in the corpus callosum of fetuses at GD21 (Fig. 2), and this increase was reversed by coincubation of WIN-55,212-2 and SR141716, a cannabinoid receptor antagonist (Fig. 2). Incubation of brain slices with WIN-55,212-2 also increased the binding of [^{35}S]-GTPγS in other regions, such as the cerebellum and white matter areas of brainstem (Fig. 2).

DISCUSSION

Our present results demonstrate that fetal and early postnatal brain contain specific binding for cannabinoid receptors in areas that do not contain or have a small density of these receptors in adult brain. The most interesting aspect was that these atypical areas have the common characteristic of being constituted by neuronal fibers rather than by cell bodies. Another characteristic was that the location of these receptors in these atypical areas seems to be transient because, during the course of postnatal development, cannabinoid receptors progressively acquired the classic pattern of distribution observed in the adult brain. Transient expression of other types of receptors in developing brain has been also previously reported (Barg and Simantov, 1991).

Thus, our present study clearly demonstrates that cannabinoid receptors are present in the brain of fetuses at GD21 in very important white matter areas, in which they are scarcely distributed or not located in the adult brain. It is remarkable that cannabinoid receptors appear associated with transverse or commissural pathways—in particular, the corpus callosum, anterior commissure, and stria terminalis. This signal likely represents specific binding to receptors and not artifactual lipophilic association with myelin, because, as also pointed by Jansen et al. (1992), (1) levels of binding not different from background were found in the white matter of cerebellum and (2) the stria termi-

Fig. 1. Representative autoradiograms corresponding to specific binding for cannabinoid receptors in a brain section (5×), selected according to Paxinos atlas (it was chosen for its diversity of anatomic structures) and obtained from male rat brains at fetal (GD21), early postnatal (PND5), immature (PND30), and adult (>70 days after birth) ages. Autoradiograms were processed according to the conditions described in Materials and Methods. Left hemisection is total binding, whereas right hemisection in nonspecific binding, for each age. (Cx, cortex; cc, corpus callosum; CPu, caudate-putamen; ac, anterior commissure.)

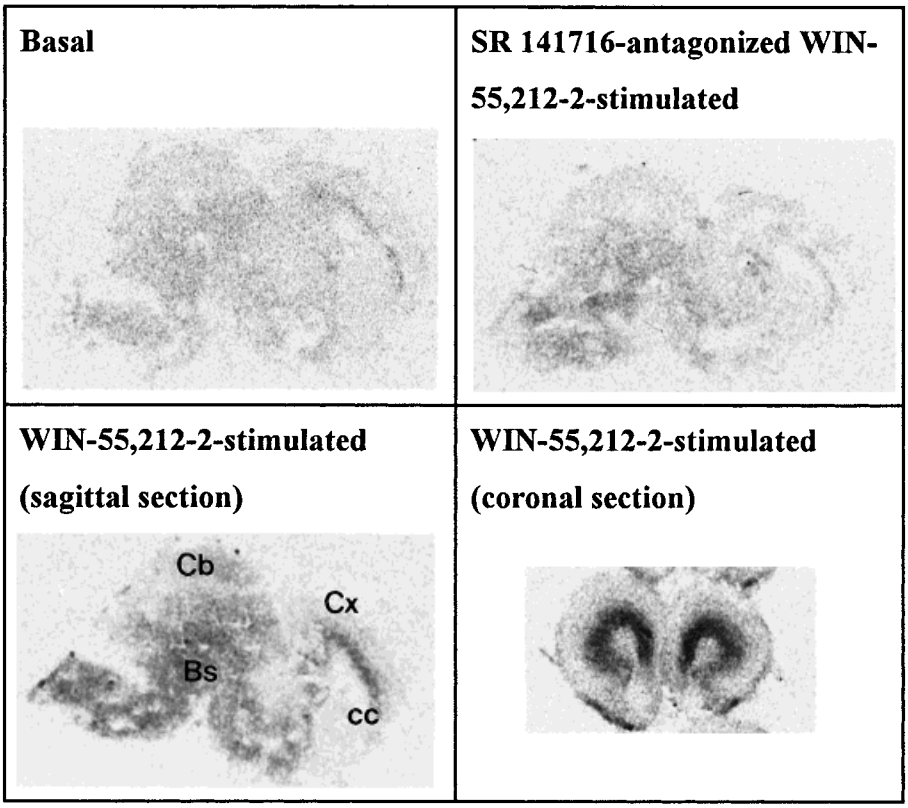


Fig. 2. Representative autoradiograms corresponding to basal (**left top**), WIN-55,212-2-stimulated (**bottom**) and SR141716-antagonized WIN-55,212-2-stimulated (**right top**) [^{35}S]-GTPγS binding in a brain section (5×) obtained from male rat brains at fetal age (GD21). Autoradiograms were processed according to the conditions described in Materials and Methods. (Cx, cortex; cc, corpus callosum; Cb, cerebellum; Bs, brainstem.)

nalis is an unmyelinated pathway but contains a high density of cannabinoid receptors. This can be also concluded from the studies of agonist-stimulated [^{35}S]-GTPγS binding, which clearly support that cannabinoid receptor binding found in the corpus callosum in the fetal brain at GD21 is already functional at this age and acts through a GTP-binding protein-mediated mechanism. This can be asserted from the observation that the activation of cannabinoid receptors with WIN-55,212-2 increased the binding of [^{35}S]-GTPγS in the corpus callosum and, also, in other regions. The specificity of this increase through the activation of cannabinoid receptors could be demonstrated because the increase was reversed by the specific cannabinoid receptor antagonist, SR141716. Moreover, as this antagonist is specific for the cerebral cannabinoid receptor subtype, called CB_1, we can assert that the signal found for cannabinoid receptor binding in the fetal and early postnatal brain likely corresponds to the CB_1 receptor subtype.

This atypical location of cannabinoid receptors in neuronal fiber-enriched areas appeared to be high at GD21, tended to wane at PND5, and practically disappeared at PND30 and in adulthood, except in the anterior commissure, which presents important levels of specific binding, as previously reported by Jansen et al. (1992). These authors also found the signal for cannabinoid receptors in other white matter areas of adult rat brain. They reported very low levels in the brainstem and spinal cord but moderate levels in unmyelinated fiber bundles such as the stria terminalis and in several myelinated ones such as the corpus callosum and the fornix, although always in lesser magnitude than in the anterior commissure. Our data revealed, however, the absence of specific binding in all these areas in adult brain, except the anterior commissure.

The pattern developed by cannabinoid receptor binding in classic areas that contain the highest densities of this receptor in the adult brain is practically opposite to that observed in white matter regions. Thus, moderate levels of specific binding were found in the cerebellum and the hippocampus of fetuses at GD21, whereas low levels were observed in the caudate-putamen area, limbic structures, and cerebral cortex. The specific binding in all these areas progressively increased from GD21 up to adulthood, when it practically exhibits the classic pattern.

The transient location of specific binding for cannabinoid receptors in white matter areas during fetal and early postnatal periods might indicate that these receptors could be located presynaptically onto axonal endings that would be elongating during late pregnancy to get to their final target sites in the process of synapse formation. However, most of the growing axons might have reached these target sites at PND5, although important amounts of specific binding still remain at this age in the corpus callosum, stria terminalis, anterior commissure, and others. Moreover, specific binding for cannabinoid receptors is present in the anterior commissure of adult brain, when axonal elongation does not occur. An alternative possibility would be that specific binding in these white matter areas would indicate the axonal transport of newly synthesized receptor protein coming from the cell bodies to the axonal terminals, but this process would also occur in adult ages and only the anterior commissure presents a signal for these receptors at this age.

Another possibility would be that, during fetal and early postnatal periods, cannabinoid receptors might be expressed transiently in non-neuronal cells. In the adult brain, these receptors are mainly located in neuronal elements (Howlett et al., 1990), but during certain periods of development, they might be also expressed in astrocytes and/or oligodendrocytes, which play important roles in neural development (metabolic and trophic support, guidance of neuronal migration and axonal elongation, formation of myelin). In this respect, recent studies have demonstrated that the cannabinoid receptor agonist-induced arachidonic acid mobilization (Shivachar et al., 1996) and kros-24 expression (Bouaboula et al., 1995) in cultures of astrocytes were both blocked by SR141716, a specific antagonist for cannabinoid receptors, thus indicating the presence of these receptors in astrocytes. This is concordant with the recent observation that astrocytes can bind [^{3}H]anandamide (Di Marzo et al., 1994). Concerning the possible presence of cannabinoid receptors in oligodendrocytes and, hence, in the myelin, it is interesting to recover the studies from Nye et al. (1985, 1988), performed during the past decade. These authors described in 1985 the first high-affinity binding site for cannabinoids using a radioactive synthetic analog, although this binding site did not appear to be involved in psychotrophic effects of cannabinoids. The most intriguing was that myelin basic protein, which is responsible for the compaction of myelin, was reported to be an endogenous inhibitor of the binding of cannabinoids to this receptor, which raised the possibility of an effect of cannabinoids altering the structure of myelin and, in turn, affecting signal conduction in myelinated axons. Moreover, Nye et al. (1988) specifically mentioned as unpublished data that high-affinity cannabinoid binding sites exist in purified myelin fractions, although we have been unable to find any further publication of these results.

Hence, according to the findings from all these reports, the endogenous cannabinoid system might have a remarkable functional role in the molecular events underlying the functions of glial cells during development. The high level of binding found in some white matter regions, along with the absence or low signal in others, predicts that cannabinoid receptors could be differentially expressed in some subpopulations of glial cells that remain to be identified. In support of this hypothesis, it can be argued that cannabinoid compounds were able to inhibit adenylate cyclase activity in several astrocytome cells (Bouaboula et al., 1995) but not in others (Howlett et al., 1990). In any case, further research would be required to elucidate this hypothesis.

In summary, all these data suggest the existence of a transient period of the brain development in the rat, around the last days of the fetal period and the first days of postnatal life, in which CB_1 receptors appear to be located in neuronal fiber-enriched areas. This might indicate either (1) the presynaptic location of these receptors in terminals that would be elongating to their final target sites or (2) their transient expression in non-neuronal cells such as astrocytes or oligodendrocytes, which play a role in axonal development. We have also observed that during this period, CB_1 receptors would be already functional acting through a GTP-binding protein-mediated mechanism. After this transient period, they progressively acquire the pattern of adult distribution. All this accounts for a specific role of the endogenous cannabinoid system in brain development.

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