Localization of mRNA expression and activation of signal transduction mechanisms for cannabinoid receptor in rat brain during fetal development


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SUMMARY

In the present work, we analyzed cannabinoid receptor mRNA expression, binding and activation of signal transduction mechanisms in the fetal rat brain or in cultures of fetal neuronal or glial cells. Cannabinoid receptor binding and mRNA expression were already measurable at GD14, but they were only located in discrete regions at GD16. Among these, the hippocampus, the cerebellum and the caudate-putamen area, three regions that contain a marked signal for both binding and mRNA in the adult brain. Significant levels of binding and, in particular, of mRNA transcripts were also detected at GD16 in the cerebral cortex, midbrain and brainstem. These structures contain relatively low levels of binding and mRNA in the adult brain, suggesting that cannabinoid receptor gene is transiently expressed in atypical areas during the fetal period. The signal for cannabinoid receptor mRNA in the hippocampus, caudate-putamen and cerebral cortex progressively increased from GD16 up to GD21. At GD18 and GD21, mRNA transcripts could be measured in discrete nuclei, such as septum nuclei, ventromedial hypothalamic nucleus and others. The cerebral cortex exhibited the highest mRNA levels at GD21, although this was not accompanied by a parallel increase in binding. An important aspect is that binding measured at these ages represent binding to functional receptors because their activation by WIN-55,212-2 increased [35S]GTP\textgamma{}S binding in the same areas. This increase was reversed by a specific antagonist, SR141716. The areas where the stimulation was more marked were the midbrain and brainstem. Using cell cultures, we have observed that cannabinoid receptor mRNA is present in cortical and hippocampal neuronal cells, but not in the glial cells. However, WIN-55,212-2 was capable of stimulating [35S]GTP\textgamma{}S binding in membrane fractions obtained from cortical glial cells and this stimulation was reversed by SR141716. This was not seen with hippocampal glial cell cultures, but occurred in hippocampal and cortical neurons. In addition, the activation of these receptors with D\textsuperscript{9}-tetrahydrocannabinol significantly reduced forskolin-stimulated cAMP production in cortical neuronal or glial cell cultures and this effect was reversed by SR141716. In summary, we have detected cannabinoid receptor binding, mRNA expression and activation of signal transduction mechanisms in the fetal rat brain (GD14-GD21), which support the view that the system constituted by these receptors and their putative endogenous ligands might play a role in specific molecular events of the brain development. Of relevance is that binding and mRNA expression appear atypically distributed in the fetal brain as compared with the adult brain, even, that their presence in white-matter-enriched areas might presumably indicate their location in non-neuronal cells. These studies with cell cultures suggest that CB\textsubscript{1} receptor subtype is located in neuronal cells obtained from fetal brain, although preliminary evidence is provided of the existence of another receptor subtype operative in glial cells obtained from the cerebral cortex.

Key words: Cannabinoid receptor, Prenatal ontogeny, Brain development, Autoradiography, Cell culture, PCR amplification, cAMP production, Rat, Signal transduction

INTRODUCTION

Our laboratory (Rodríguez de Fonseca et al., 1993; Fernández-Ruiz et al., 1994) and other groups (Mailleux and Vanderhaeghen, 1992; McLaughlin and Abood, 1993; McLaughlin et al., 1994; Belue et al., 1995) have described the presence of cannabinoid receptor binding and mRNA levels in the developing brain at postnatal ages. However, the presence of these receptors in the brain seems to occur even earlier than the first days of postnatal life, since the prenatal exposure to cannabinoids caused important effects on neurochemical and behavioral development (Walters and Carr, 1986, 1988; Kumar et al., 1990; Rodriguez de Fonseca et al., 1991; Fernández-Ruiz et al., 1992, 1994, 1996; Bonnin et al., 1994, 1996; Vela et al.,
1995, 1998). This supports the proposal that the system constituted by endogenous cannabimimetic ligands and the cannabinoid receptor signaling pathways emerges and is operative early during development, presumably during the last third of gestation, and might play a physiological role in neural development.

Recently, we have presented the first evidence about the presence of these receptors in the brain of fetuses at gestational day (GD) 21 using autoradiography (Romero et al., 1997). We observed that these receptors seem to be transiently distributed in an atypical manner in the brain during fetal and early postnatal ages, compared with their pattern of distribution in adulthood. Thus, cannabinoid receptor binding was detected at GD21 associated with several transverse fibers pathways, such as the corpus callosum, the anterior commissure and the stria terminalis, as well as with white-matter-enriched areas of the brainstem (Romero et al., 1997).

In this earlier report, we hypothesized about the transient expression of cannabinoid receptors during the fetal and early postnatal periods in glial cells, namely the oligodendrocytes or astrocytes, rather than in neuronal elements (Romero et al., 1997). This hypothesis was also based on previous reports from Bouaboula et al. (1995) and Shivachar et al. (1996), who demonstrated that some effects of cannabinoids in glial cell cultures were reversed by the antagonist SR141716, thus pharmacologically proving the presence of these receptors in those cells.

The present report has been aimed to address this hypothesis studying, in a first set of experiments, the distribution of cannabinoid CB1 receptor mRNA transcripts, by using in situ hybridization, in slide-mounted sections obtained from rat fetal brains at different gestational ages (GD14, GD16, GD18 and GD21) and including some representative adult brain sections as a reference. Our objective was to demonstrate that CB1 receptor mRNA signal could be detected in the same white matter (glial) areas where binding levels were detected (Romero et al., 1997). This would indicate the presence of these receptors in glial cells. In contrast, the absence of signal might indicate a preferential location of CB1 receptors in neuronal elements. The analysis of mRNA levels was completed with the mapping of cannabinoid receptor binding, by using autoradiographic analysis of [3H]CP-55,940 binding, in slide-mounted brain sections obtained from different fetal ages (GD14, GD16 and GD18). This completes our earlier report where only autoradiographic data and from GD21 up to adulthood were included (Romero et al., 1997). We also studied the functionality of these receptors by analyzing their capability to activate signal transduction mechanisms. We used autoradiographic analysis of agonist-stimulated [35S]guanylyl-5′-O-(γ-thio)-triphosphate ([35S]GTPγS) binding in slide-mounted brain sections obtained from two representative fetal ages (GD16 and GD18). In a second set of studies, we used cultures of neuronal or glial cells obtained from the cerebral cortex or the hippocampus of fetuses at GD21. These cultures were used for analysis of (i) CB1 receptor mRNA expression, by using polymerase-chain reaction (PCR) amplification; (ii) cannabinoid receptor agonist-inhibition of forskolin-stimulated cyclic adenosine-monophosphate (cAMP) production and (iii) cannabinoid receptor agonist-stimulation of [35S]GTPγS binding using membrane fractions.

**MATERIALS AND METHODS**

**Animals and sampling**

Timed pregnant Wistar rats, sperm-positive on a specific day (GD1), were housed in a room with controlled photoperiod (08:00-20:00 hours light on) and temperature (23±1°C), and free access to standard food and water. Pups from these rats were killed at GD14, GD16, GD18 and GD21. At each fetal age, animals from different litters were used for variation. Whole fetuses (at GD14 and GD16), whole heads (at GD18) or dissected brains (at GD21) were rapidly frozen by immersion in 2-methyl-butane cold in dry ice to avoid morphological damage, which was necessary due to the extreme immaturity of the brain structure at these ages. All samples were stored at –80°C until processed.

**Autoradiographic analyses**

**Tissue preparation**

Frozen serial coronal or sagittal sections (20 μm-thick; obtained from at least three brains per age) were cut in a cryostat, according to several atlases (Altman and Bayer, 1993; Paxinos and Watson, 1986; Paxinos et al., 1991). 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 structures, adjacent sections to those used for in situ hybridization and autoradiographic analysis were stained with cresyl violet.

**Cannabinoid receptor autoradiography**

The protocol used is basically the method described by Herkenham et al. (1991) with slight modifications (Romero et al., 1997). The procedure includes the use of 10 nM [3H]CP-55,940 (DuPont NEN, MA, USA), in the absence or the presence of 10 μM non-labelled CP-55,940 (kindly supplied by Pfizer, Madrid, Spain) to determine the total and the nonspecific binding, respectively. Slides were incubated and washed, and the autoradiograms developed and analyzed using a standard procedure (Romero et al., 1997).

In situ hybridization for CB1 receptor mRNA

In situ hybridization was carried out according to the procedure previously described by Rubino et al. (1994) with slight modifications (Romero et al., 1998). Sections were hybridized with [35S]-labelled oligonucleotide probes (generated by a mixture (1:1:1) of the three 48-mer oligonucleotide probes complementary to bases 4-51, 349-396 and 952-999 of the rat CB1 receptor cDNA (Du Pont, Itisa, Madrid, Spain)), washed and exposed to X-ray film, and the autoradiograms generated and analyzed, using a standard procedure (see Romero et al., 1998). Additional brain sections were co-hybridized with a 100-fold excess of cold probe or with RNase to assert the specificity of the signal. As expected, no hybridization signal was detected in these sections.

**[35S]GTPγS autoradiography**

The protocol used is basically the method recently described by Sim et al. (1996). The procedure includes the use of 0.04 nM [35S]GTPγS (Amersham Iberica, Madrid, Spain) and 2 mM GDP in the absence or presence of 10 μM WIN-55,212-2 (RBI, Natick, MA, USA) and/or 3 μM SR141716 (kindly supplied by Sanofi Recherche, Montpellier, France). Nonspecific binding was measured in the presence of 10 μM unlabeled GTP S (Boehringer Mannheim, Germany). Slices were incubated and rinsed, and the autoradiograms generated and analyzed using a standard procedure (see Romero et al., 1998).

**Experiments with cultures of rat fetal neuronal or glial cells**

**Cell dissociation**

Primary neuronal and glial cell cultures were prepared from either the cerebral cortex or the hippocampus of rat fetuses by a combination of...
enzymatic and mechanical dispersion using the method described by Galve-Roperh et al. (1997). This procedure includes the use of 0.1% trypsin followed by 0.1% deoxyribonuclease type I (DNase I) and mild trituration. Dissociated cells were then centrifuged, resuspended in fresh complete medium containing 15% fetal calf serum, counted and plated.

Cell plating

Cells for neuronal cell-enriched cultures were plated at a density of 10^5 cells/cm² onto 35 mm tissue culture plates, previously coated with poly-D-lysine (50 μg/ml, Sigma Chem., Madrid, Spain), in a total volume of complete medium of 2 ml/plate. Cells were cultured at 37°C in an atmosphere of CO₂/O₂ (5%/95%) saturated with water. After 24 hours, the complete medium was changed to a chemically defined medium (CDM). CDM is composed of a 1:1 mixture of Minimum Essential Medium and Nutrient Mixture Ham’s F12 (Gibco, Life Technologies, Barcelona, Spain), supplemented with glucose (6 mg/ml), insulin (25 μg/ml), human transferrin (100 μg/ml), putrescin (20 μM), sodium selenite (30 nM) and progesterone (20 nM). This medium without serum limits the glial cell growth to 1-2% of the total cells (for review, see Dreyfus and Black, 1990). After plating, cells were cultured for a period of 7 days and then used for RNA extraction for PCR amplification of CB₁ receptor-mRNA, preparation of membrane fractions for analysis of WIN-55,212-2-stimulated [35S]GTPγS binding and quantification of Δ²-tetrahydrocannabinol (Δ²-THC) inhibition of forskolin-stimulated cAMP production. Cells for glial cultures were plated at a density of 30,000/cm² into tissue culture flasks (25 cm²), previously coated with poly-L-ornithine (5 μg/ml) in water, in a total volume of 5 ml of complete medium containing 10% fetal calf serum. Cells were cultured at 37°C in an atmosphere of CO₂/O₂ (5%/95%) saturated with water. The complete medium containing 10% fetal calf serum was changed every 3-4 days. In this medium, neuronal cells do not survive after 1 or 2 weeks, whereas glial cells continue to undergo mitosis (for review, see Dreyfus and Black, 1990). Cells were cultured for a minimum period of 15 days and then used for the same experiments done with neuronal cell-enriched cultures.

Detection of CB₁ receptor-mRNA by reverse transcriptase-PCR

The presence of mRNA for the cannabinoid receptor subtype CB₁ was evaluated by reverse transcriptase-PCR, according to the procedure described by Bouaboula et al. (1995) with slight modifications. RNA was isolated, treated with DNase I (Pharmacia Biotech, Madrid, Spain), purified, precipitated and subjected to reverse transcriptase reactions using a standard procedure (see Bouaboula et al., 1995). The primers used for PCR amplification correspond to bases 1041 to 1065 and 1286 to 1310 of the rat CB1 receptor mRNA sequence (Matsuda et al., 1990), yielding an expected amplification product of 270 bp. PCR reactions were carried out using the sequence reported by Bouaboula et al. (1995). At the end, 1/3 of each PCR reaction was electrophoresed on a ethidium bromide-stained 2% agarose gel. In order to confirm that amplification products were derived from cDNA and not from possible genomic DNA contamination, a control reaction without reverse transcriptase was carried out for each sample. Samples of total RNA from rat cerebellum and from rat liver were run in parallel as positive and negative controls, respectively, of the PCR amplification.

WIN-55,212-2-stimulated [35S]GTPγS-binding assays

We used the method described by Traynor and Nahorski (1995) to determine [35S]GTPγS binding. Briefly, membrane fractions (200-300 μg of protein), prepared from cultures of neuronal or glial cells obtained from the cerebral cortex or the hippocampus, were incubated in assay buffer containing 0.05 nM [35S]GTPγS and 5 μM GDP in the absence or presence of 5 μM WIN-55,212-2 and/or 2 μM SR141716. Nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS. After incubation, membranes were filtered and washed and the radioactivity measured. Specific [35S]GTPγS binding in the three situations, basal, WIN-55,212-2-stimulated and SR141716-antagonized WIN-55,212-2-stimulated, was calculated as the difference between binding in the absence or presence of unlabeled GTPγS.

Analysis of cAMP production

The analysis of cannabinoid-inhibition of forskolin-stimulated cAMP production was carried out following the protocol used by Howlett et al. (1990). Neuronal or glial cells were incubated in the presence of (i) 25 μM forskolin; (ii) 3 μM Δ²-THC; (iii) 25 μM forskolin and 3 μM Δ²-THC; (iv) 25 μM forskolin, 3 μM Δ²-THC and 0.01 μM SR141716; and (v) equivalent amounts of the different vehicles (see details in Howlett, 1985; Hernández et al., 1997). After incubation, the cells were collected in absolute ethanol (500 μl/plate; 1 ml/flask), sonicated and centrifuged and the supernatants collected and evaporated. The determination of cAMP contents was carried out using an assay kit (Amersham Ibérica, Madrid, Spain). All the experiments done included data from at least three different series of cell cultures, each of them including the different experimental groups.

Statistics

Student’s t-test or analysis of variance followed by Student-Newman-Keuls test were used as required.

RESULTS

CB₁ receptor mRNA expression in the fetal brain (GD14-GD21)

CB₁ receptor mRNA levels were already measurable at GD14 (Fig. 1), although it was difficult to locate these receptors precisely in discrete regions due to the immaturity of the brain at this fetal age. CB₁ receptor mRNA levels could be located

![Fig. 1](image-url) Representative autoradiograms (10x) for the analysis of cannabinoid receptor mRNA transcripts in slide-mounted sections of rat fetuses at GD14 (left panel) and GD16 (right panel). See details in the Materials and Methods. 1. Cerebral cortex; 2. caudate-putamen; 3. hippocampus; 4. midbrain; 5. cerebellum; 6. brainstem.
and measured in progressively better-defined regions from GD16 (Table 1; Fig. 1), including the hippocampus, the cerebellum and, to a lesser extent, the caudate-putamen area, three regions that contain a marked signal for CB1 receptor mRNA and also for binding in the adult brain (see Mailleux and Vanderhaeghen, 1992; Table 1; Fig. 4). Significant levels of mRNA were also measurable at GD16 in the cerebral cortex and areas of midbrain and brainstem (Table 1; Fig. 1). It is interesting to note that these structures, in particular the areas of midbrain and brainstem, contain relatively low levels of this messenger in the adult brain (see Mailleux and Vanderhaeghen, 1992; Table 1; Fig. 4), thus indicating a potentially transient expression of cannabinoid receptor gene in atypical areas during the fetal period. The signal for CB1 receptor mRNA in the hippocampus, caudate-putamen and, in particular, cerebral cortex progressively increased from GD16 up to GD21, remaining mostly unchanged in the other areas (Table 1). From GD18, the signal for CB1 receptor mRNA in the cerebral cortex could be differentiated in the external layer (II-III: 77.8±1.4 units of optical density) and the internal layer (V -VI: 47.1±5.6). At GD18, CB1 receptor mRNA transcripts could be measured in discrete structures, such as the septum nuclei (40.1±0.2), diagonal band (41.7±5.2), medial habenula (83.0±3.5), ventromedial hypothalamic nucleus (76.1±2.0), subthalamic nucleus (76.6±7.9), zona incerta (59.6±10.6) and subventricular thalamic nucleus (63.9±7.7) (see Fig. 2). Of particular interest was the location of mRNA transcripts in some areas that exhibited the common characteristic of being proliferative areas, such as the striatal subventricular zone (61.5±6.8), neocortical subventricular zone (70.2±2.9) and subventricular zone of the nucleus accumbens (68.7±3.9) (see Fig. 3). The relevance of this fact is that CB1 receptor mRNA transcripts were not observed in all these areas at earlier fetal ages (see Fig. 2); in fact, only the striatal subventricular zone exhibited measurable mRNA levels at GD18 (see Fig. 2). This suggests again that the expression of the CB1 receptor gene in some areas might be a transient phenomenon, thus supporting a specific role of this gene during that period.

Table 1. Cannabinoid receptor mRNA levels measured by in situ hybridization in several brain regions of rat fetuses at GD16, GD18 and GD21 and of adult rats (>8 weeks)

<table>
<thead>
<tr>
<th>Brain region</th>
<th>GD16</th>
<th>GD18</th>
<th>GD21</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>cerebral cortex*</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>hippocampus†</td>
<td>++</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>caudate-putamen</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>midbrain</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>brainstem</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>pons</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>bulb</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>cerebellum</td>
<td>++++</td>
<td>++</td>
<td>++++</td>
<td>++++</td>
</tr>
</tbody>
</table>

*Corresponds to the superficial layers at GD18 and GD21 and in the adult brain
†Corresponds to dentate gyri at GD18 and GD21 and in the adult brain

Values are optical density (expressed as arbitrary units) and correspond to the mean of at least 3 animals per age. They have been assigned to the following semiquantitative table: +: optical density = 0-25; ++: optical density = 25-50; +++: optical density = 50-75; ++++: optical density = 75-100; +++++: optical density >100.

Cannabinoid receptor binding in the fetal brain (GD14-GD18)

Specific binding for cannabinoid receptors was also already
measurable in the brain of fetuses at GD14, although it was difficult to distinguish it in some areas from the nonspecific binding and, as occurred for mRNA levels, it was not possible to locate the precise regions containing these receptors due to the immaturity of the brain at this fetal age. As occurred for mRNA signal, specific binding for cannabinoid receptors could also be located in progressively better-defined regions from GD16 (Table 2; Fig. 5). We found that the binding signal was small but specific, since significantly lower levels of grey matter were found in the sections incubated with a saturating concentration of non-labelled CP-55,940 (see Fig. 5); these included the hippocampus, cerebral cortex, caudate-putamen, midbrain, brainstem and cerebellum (Table 2; Fig. 5). Binding levels significantly increased in the above areas, in particular in the hippocampus, at GD18 (Table 2; Fig. 6), which paralleled the increase found in mRNA levels (Table 1). The only exceptions were the bulb and, in particular, the cerebral cortex (see Table 2). As mentioned above, in this last region at GD18, a significant difference existed between the higher mRNA levels (Table 1) and the lower binding levels (Table 2). The same trend was previously reported for binding levels at GD21 in our earlier study (Romero et al., 1997). In addition, as we demonstrated in that earlier report for GD21 fetuses (Romero et al., 1997), cannabinoid receptor binding was also significantly detected at GD18 in white matter regions, including some transverse or commissural pathways, where this receptor is absent or scarcely distributed in the adult brain (Herkenham et al., 1991; Romero et al., 1997). Among these, the most relevant were the fasciculus retroflexum (17.4±1.1 fmol/mg of tissue) and the white matter areas of the midbrain and the brainstem (see Table 2; Fig. 6), although, at this age, the corpus callosum and the anterior commissure did not exhibit measurable binding levels (see Fig. 6).

Cannabinoid receptor activation of signal transduction mechanisms in the fetal brain (GD16 and GD18)

The activation of cannabinoid receptors with WIN-55,212-2 increased $[^{35}S]$GTP$\gamma$S binding in those areas that contain measurable $[^{3}H]$CP-55,940-binding levels in the brain at GD16 and GD18 (see Table 3; Fig. 7). This increase was specific because it was reversed by a specific antagonist, SR141716 (see Fig. 7). Interestingly, the areas where the stimulation was more marked (approx. 100%) were the midbrain and brainstem, whereas lesser increases were found in the cerebral cortex, hippocampus and caudate-putamen (stimulation was very poor in the cerebellum at both ages). This supports again a transient expression of cannabinoid receptors in atypical areas during the fetal period.

Studies in neuronal and glial cell cultures

CB$_1$ receptor mRNA is present in cortical and hippocampal neuronal cells, but not in the glial cells, as revealed the data of PCR amplification (see Table 4; Fig. 8). In the Fig. 8, it can be seen that amplification only occurred with the RNA obtained from the cerebellum (positive control) or from cortical or hippocampal neuronal cells. The specificity of the amplification

| Table 2. Cannabinoid receptor binding measured by autoradiography in several brain regions of rat fetuses at GD16 and GD18 |
|-----------------|-----------------|-----------------|
| Brain region    | GD16            | GD18            |
| cerebral cortex*| 6.52±0.66       | 8.12±0.58       |
| hippocampus†    | 7.76±1.45       | 37.3±3.14       |
| caudate-putamen | 5.90±0.79       | 10.5±0.69       |
| midbrain        | 9.84±2.54       | 16.9±2.11       |
| brainstem       | 14.2±1.23       | 22.9±1.95       |
| pons            | 10.2±1.34       | 18.5±1.79       |
| bulb            | 4.45±0.73       | 10.9±1.13       |

*Corresponds to the superficial layer at GD18.
†Corresponds to dentate gyrus at GD18.
Values are fmol/mg tissue and correspond to the mean±s.e.m. of at least 3 animals per age.
process for the CB₁ receptor mRNA can be asserted because amplification did not occur in the negative control (liver), as well as in the cerebellum and neuronal cells when reverse transcriptase reaction was avoided (see Fig. 8). The activation of cannabinoid receptors with WIN-55,212-2 stimulated [³⁵S]GTP₆S binding in membrane fractions obtained from hippocampal or cortical neuronal cell cultures, although to a lesser extent in the cortical neurons (see percentages of stimulation in Table 4), and this binding was reversed by SR141716. This was not seen with hippocampal glial cell cultures, but did occur in cortical glial cells (Table 4). In addition, the activation of cannabinoid receptors with Δ⁹-THC significantly reduced forskolin-stimulated cAMP production in cortical or hippocampal neuronal and glial cell cultures (Table 4). This effect was reversed by SR141716, more markedly in the neuronal cell cultures (Table 4). All this suggests the existence in cortical glial cells of a cannabinoid receptor subtype, other than the CB₁, but coupled to GTP-binding proteins and sensitive to SR141716.

DISCUSSION

Our present results demonstrate that cannabinoid receptor binding and mRNA expression could be detected in the developing fetal brain at least from GD14, increasing progressively up to GD21. During the course of our present study, Buckley et al. (1998) reported expression of CB₁ receptor mRNA in the nervous system during the embryonic period, although these authors detailed to a much lesser extent the regions where CB₁ receptor mRNA expression occurs. Our present results extend our previous data on the presence of cannabinoid receptor binding in the fetal brain at GD21 (Romero et al., 1997). In addition, we provide here evidence that binding levels measured at GD16 and GD18 in several brain structures represent binding to functional receptors because they are coupled to GTP-binding proteins and, then, are capable of activating signal transduction mechanisms. Collectively, the signal for the cannabinoid receptor binding, gene expression and activation of signal transduction mechanism appeared located in regions that also contain this signal in the adult brain, such as the hippocampus, the cerebellum and, to a lesser extent, the caudate-putamen area. Cannabinoid receptor indices could be located in progressively better-defined nuclei as a function of brain maturation. However, the most interesting observation of our study was that, comparing the results with receptor-binding results in our earlier study (Romero et al., 1997), CB₁ receptor mRNA levels could be transiently detected in areas that do not contain or have a small signal for this messenger in the adult brain. A similar finding could be obtained from the data on the activation of signal transduction mechanisms. Three regions where this is particularly relevant are (i) areas of the midbrain and brainstem, (ii) the cerebral cortex and (iii) several proliferative areas.

Midbrain and brainstem

A first group of structures that exhibited an atypical location

Table 3. WIN-55,212-2-stimulated [³⁵S]GTP₆S binding measured by autoradiography in several brain regions of rat fetuses at GD16 and GD18

<table>
<thead>
<tr>
<th>Brain region</th>
<th>GD16</th>
<th>GD18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>basal + agonist %</td>
<td>basal + agonist %</td>
</tr>
<tr>
<td>cerebral cortex†</td>
<td>58.3±10.1</td>
<td>84.7±19.3</td>
</tr>
<tr>
<td>hippocampus‡</td>
<td>59.0±14.0</td>
<td>68.3±17.5</td>
</tr>
<tr>
<td>caudate-putamen</td>
<td>43.0±7.8</td>
<td>54.3±15.0</td>
</tr>
<tr>
<td>midbrain</td>
<td>59.5±4.7</td>
<td>145.7±39.0</td>
</tr>
<tr>
<td>brainstem</td>
<td>137.8±7.2**</td>
<td>335.7±56.2*</td>
</tr>
<tr>
<td>pons</td>
<td>82.0±17.7</td>
<td>130.7±24.9</td>
</tr>
<tr>
<td>bulb</td>
<td>82.1±16.4</td>
<td>113.7±20.3</td>
</tr>
</tbody>
</table>

†Corresponds to the superficial layer at GD18.
‡Corresponds to dentate gyrus at GD18.
Values are means±s.e.m. of at least 3 animals per age. Data were assessed by Student’s t-test (*P<0.05; **P<0.005 versus the corresponding basal group).

Table 4. Analysis of CB₁ receptor-mRNA by PCR amplification, WIN-55,212-2-stimulated [³⁵S]GTP₆S binding and Δ⁹-THC inhibition of forskolin-stimulated cAMP production in cortical or hippocampal neuronal and glial cell cultures

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>PCR amplification of CB₁ receptor-mRNA</th>
<th>WIN-55,212-2-stimulated [³⁵S]GTP₆S binding</th>
<th>Δ⁹-THC inhibition of forskolin-stimulated cAMP production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>stimulation %*</td>
<td>reversed by SR141716</td>
<td>%†</td>
</tr>
<tr>
<td>Cortical glial cells</td>
<td>NO</td>
<td>YES</td>
<td>134.1</td>
</tr>
<tr>
<td>Cortical neuronal cells</td>
<td>YES</td>
<td>YES (slight)</td>
<td>116.2</td>
</tr>
<tr>
<td>Hippocampal glial cells</td>
<td>NO</td>
<td>NO</td>
<td>–</td>
</tr>
<tr>
<td>Hippocampal neuronal cells</td>
<td>YES</td>
<td>YES</td>
<td>156.7</td>
</tr>
</tbody>
</table>

*Values of agonist stimulation over 100% of basal binding.
†% of reduction in cAMP contents over the values after forskolin stimulation.
of cannabinoid receptors includes the midbrain and the brainstem. An important signal for CB$_1$ receptor mRNA was detected in different areas of these two structures during the different fetal ages studied (these areas contain a very small signal in the adult brain; Mailleux and Vanderhaeghen, 1992). In addition, binding levels were moderately detectable in these areas at GD16 and GD18, in concordance with that previously reported at GD21 (Romero et al., 1997), whereas the highest stimulation by WIN-55,212-2 of [$_{35}$S]GTP$^\gamma$S binding was observed in these areas rather than in other classic regions such as the hippocampus, cerebellum and caudate-putamen. As these areas are rich in white matter, it might be argued that CB$_1$ receptor mRNA signal is located in glial cells, either oligodendrocytes or astrocytes, associated with neuronal fibers rather than in neuronal elements. This hypothesis was formulated in our earlier report (Romero et al., 1997) on the basis that cannabinoid receptor binding appeared associated with transverse or commissural pathways from GD21 up to PND5, in particular in the corpus callosum, anterior commissure, stria terminalis, stria medullaris and others. This will be discussed in detail later, but it should be noted here that binding levels were detected in transverse or commissural pathways in the present study at earlier ages, GD16 and GD18 (e.g. fasciculus retroflexum), although the number of structures was more limited, thus indicating that this would be a process particularly relevant at GD21 (Romero et al., 1997). As we did not find mRNA levels in these transverse or commissural pathways in the fetal brain, it is possible to conclude that, for these regions, the synthesis of receptors is occurring at sites distant from the location of functioning receptors.

Cerebral cortex

The cerebral cortex exhibited levels of CB$_1$ receptor mRNA, in particular at GD21, significantly higher than those found in the adult brain. It is possible that this transient phenomenon indicates the presence of CB$_1$ receptor mRNA transcripts in neuronal cells migrating from the cortex to their final target sites. As will be discussed afterwards, mRNA transcripts were detected in proliferative areas, such as the subventricular zone, and it is known that neuronal or glial cells originating in this area migrate out through the white matter, in particular through the corpus callosum (for review, see Jacobson, 1993). It would then be reasonable to assume that migrating cells also make their way through the cerebral cortex. Another possibility would be, as in the above case, the presence of these receptors in the different subpopulations of glial cells (mainly astrocytes) located in this region. The use of cell cultures of either glial cells or neurons obtained from the cerebral cortex and PCR amplification allowed demonstration of the presence of the CB$_1$ receptor subtype only in neuronal cells. The same finding was obtained with neurons or glial cells isolated from the fetal hippocampus. Glial cells, in both cases, were unresponsive to CB$_1$ receptor-mRNA amplification; however, we provide here evidence for the possible presence of a cannabinoid receptor subtype, other than the CB$_1$, in cortical glial cells, but not in hippocampal glial cells. This new receptor subtype presumably located in cortical glial cells would share some characteristics with the CB$_1$ subtype, since both would be (i) coupled to GTP-binding proteins, (ii) able to diminish cAMP production and (iii) sensitive to SR141716 as antagonist. However, this receptor subtype would be molecularly different to the CB$_1$ receptor since there was no PCR amplification in cortical glial cells. The fact that WIN-55,212-2-stimulated [$^{35}$S]GTP$^\gamma$S binding and $\Delta^2$-THC-inhibited forskolin-stimulated cAMP production were both reversed by SR141716 in these cells refutes the involvement of CB$_2$ receptor subtype and supports the idea that a new receptor subtype, still undiscovered, might be involved.

Hence, although it appears well demonstrated (Howlett et al., 1990) that cannabinoid receptors are located in neuronal elements in the adult brain, these receptors might be transiently expressed in non-neuronal cells (oligodendrocytes and/or astrocytes) during brain development. It is possible that this receptor might be a subtype other than CB$_1$, as revealed by the data obtained in cultures of cortical glial cells. This would

![Fig. 4. Representative autoradiograms (10×) for the analysis of cannabinoid receptor mRNA transcripts in slide-mounted brain sections of adult rats (>70 days old). See details in the Materials and Methods. These autoradiograms correspond to sections consecutive to those included in a previous report (Romero et al., 1998) and have been included here only for comparative purposes. 1, cerebral cortex; 2, caudate-putamen; 3, hippocampus; 4, ventromedial hypothalamic nucleus; 5, basolateral amygdaloid nucleus; 6, cerebellum; 7, brainstem.](image-url)
mean that cannabinoid receptors could participate in the important roles played by glial cells in the neural development. For instance, oligodendrocytes form the myelin, whereas astrocytes serve not only for the metabolic and trophic support of neurons, but also for the guidance of neuronal migration and axonal elongation and for the direct signaling to neurons (Nedergaard, 1994). Considering these possibilities, Shivachar et al. (1996) have recently proposed that the endogenous cannabinoid system might play a role in neuronal-glial signaling in the brain, so that anandamide released from neuronal cells might act on the astrocyte function via the activation of cannabinoid receptors located in these cells. As reviewed in our earlier report (Romero et al., 1997), some recent studies support the presence of cannabinoid receptors in glial cells. Thus, it has been demonstrated that the cannabinoid-receptor-agonist-induced arachidonic acid mobilization (Shivachar et al., 1996) and kro-24 expression (Bouaboula et al., 1995) in cultures of astrocytes were both blocked by SR141716. In the same line, Di Marzo et al. (1994) reported that astrocytes can bind \(^{[3]}\text{H}\)anandamide, and Bouaboula et al. (1995) proved CB\(_1\) receptor gene expression in cultured astrocytes using PCR amplification. Using also glial cell cultures, Guzmán et al. (personal communication) have observed that cannabinoids increased both basal and forskolin-inhibited glucose oxidation and phospholipid synthesis in cultured neonatal cortical and hippocampal astrocytes. The involvement of cannabinoid receptors in these effects seems likely because they were blocked by pertussis toxin and SR141716, although the receptor subtype involved might be other than the CB\(_1\), because we were unable to find mRNA expression for this receptor subtype using northern blot in those cells (J. J. F.-R. et al., unpublished results). In any case, although further research would be required to complete these initial results, it is important to note that these effects of cannabinoids increasing glucose metabolism indicate a metabolic activation in glial cells that would be concordant with the necessary metabolic support of these cells in the process of neuronal proliferation and migration, axonal development and, particularly, in the myelination.

**Proliferative areas**

mRNA transcripts for the CB\(_1\) receptor could be detected at GD21 in some areas, such as the striatal and neocortical subventricular zones and the subventricular zone of the nucleus accumbens. These three regions have the common characteristic of being proliferative areas for both neurons and glial cells (for review, see Jacobson, 1993), thus indicating that CB\(_1\) receptor gene expression might play an important role in the cell proliferating process. In addition, it is known that neuronal or glial cells originating in these areas migrate out through the fiber tracts, in particular through the corpus callosum (for review, see Jacobson, 1993). This might indicate that the important binding levels found in transverse or
commissural fiber tracts in our earlier report (Romero et al., 1997), and also in this study, would correspond to binding to receptors located in migrating cells. The same finding could be obtained from the data on binding and activation of signal transduction mechanisms in the midbrain and the brainstem, since these areas are also rich in fiber tracts.

Hence, it would be possible to suggest a role for CB1 receptors in the processes of cell proliferation and migration. The relevance of this hypothesis is greater considering the fact that mRNA transcripts were mostly not observed in these areas at earlier fetal ages, GD16 and GD18 (at GD18, mRNA transcripts were seen only in the striatal subventricular zone, but not in the other zones), or in the adult brain, which may indicate that this is a transient phenomenon. A possible interpretation of this transiency is that the cannabinoid receptor plays a specific role in the molecular events occurring during proliferation and migration of specific groups of cells.

Conclusion
In summary, we have detected cannabinoid receptor binding, mRNA expression and activation of signal transduction mechanisms in the fetal rat brain (GD14-GD21), which support the view that the system constituted by these receptors and their putative endogenous ligands might play a role in specific molecular events of the brain development. Of relevance is the atypical distribution of cannabinoid receptor binding and mRNA expression in the fetal brain as compared with the adult brain and their presence in white-matter-enriched areas might presumably indicate their location in non-neuronal cells. Studies with cell cultures suggest that the CB1 receptor subtype is located in neuronal cells obtained from fetal brain, although evidence is provided of the existence of another receptor subtype operative in glial cells obtained from the cerebral cortex. This last observation, namely their location in non-neuronal cells, supports a role of the endogenous cannabinoid system in the molecular functions associated with these cells during neural development, including metabolic and trophic support, guidance of neuronal migration and axonal elongation, and formation of myelin.

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