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COLOCALIZATION OF CB_1 RECEPTORS WITH L1 AND GAP-43 IN FOREBRAIN WHITE MATTER REGIONS DURING FETAL RAT BRAIN DEVELOPMENT: EVIDENCE FOR A ROLE OF THESE RECEPTORS IN AXONAL GROWTH AND GUIDANCE

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Abstract—There is recent evidence supporting the notion that the cannabinoid signaling system plays a modulatory role in the regulation of cell proliferation and migration, survival of neural progenitors, neuritic elongation and guidance, and synaptogenesis. This assumption is based on the fact that cannabinoid 1-type receptors (CB_1 receptors) and their ligands emerge early in brain development and are abundantly expressed in certain brain regions that play key roles in these processes. We have recently presented *in vivo* evidence showing that this modulatory action might be exerted through regulating the synthesis of the cell adhesion molecule L1 that is also a key element for those processes. To further explore this issue, we conducted here immunohistochemical studies aimed at determining the cellular substrates of CB_1 receptor–L1 interactions in the rat brain during late fetal development. In this period, we previously found that the activation of CB_1 receptors increased L1 synthesis in several forebrain white matter regions but not in gray matter areas. Using double labeling studies, we observed here colocalization of both proteins in fiber tracts including the corpus callosum, the adjacent subcortical white matter, the internal capsule and the anterior commissure. Experiments conducted with cultures of fetal rat cortical nerve cells revealed that L1 is present mainly in neurons but not in glial cells. This fact, together with the results obtained in the double labeling studies, would indicate that L1 and CB_1 receptors should possibly be present in axons elongating through these white matter tracts, or, alternatively, in migrating neurons. Further experiments confirmed the presence of CB_1 receptors in elongating axons, since these receptors colocalized with growth-associated protein 43 (GAP-43), a marker of growth cones, but not with synaptophysin, a marker of active synaptic terminals, in the same forebrain white matter regions. Lastly, using cultured fetal rat cortical neurons, we also observed that the activation of cannabinoid receptors increased the levels of the full-length L1 and altered those of some active proteolytic fragments of this protein whose generation has been associated with specific steps in the process of neuritic elongation in cultured neurons. In summary, we have demonstrated that the effects caused by cannabinoid agonists on L1 are facilitated by the colocalization of this cell adhesion molecule with CB_1 receptors in several forebrain white matter regions during fetal brain development. We have provided strong evidence that this phenomenon occurs in axons elongating through these white matter tracts, and we have explored *in vitro* how cannabinoid receptors influence L1 levels. Considering the role played by L1 in different events related to neural development, our observations support the occurrence of a physiological mechanism by which the cannabinoid system might regulate the process of axonal growth and guidance through regulating the synthesis and function of L1.

Key words: cannabinoid, CB_1 receptors, brain development, L1, GAP-43, growth cones.

Different elements of the cannabinoid signaling system, such as the cannabinoid 1-type receptor (CB_1 receptor) and their eicosanoid-derived ligands, emerge early in brain development (Romero et al., 1997; Buckley et al., 1998; Berrendero et al., 1998, 1999). They are particularly abundant in certain brain regions. For example, the forebrain subventricular zones, which play a key role in cell proliferation, contain high levels of CB_1 receptor-mRNA (Berrendero et al., 1998) and the same happens with the cerebral cortex (Berrendero et al., 1998). By contrast, CB_1 receptor binding was high in forebrain white matter structures, in particular transverse commissural tracts (Romero et al., 1997; Berrendero et al., 1998), which are essential for cell migration and axonal elongation (see Fernández-Ruiz et al., 2000, for review). This morphological evidence has provided support to the idea that the cannabinoid signaling system is located during brain development in a position concordant with the modulation of several neurodevelopmental processes (for review, see Fernández-Ruiz et al., 2000, 2004; Fride, 2004). Also in support of this idea is the fact that the "atypical" distribution of endocannabinoid elements, mainly the CB_1 receptor, disappears coinciding with the conclusion of the establishment of synaptic communication and postsynaptic target selection (Fernández-Ruiz et al., 2000; Harkany et al., 2007, for review).

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Abbreviations: CB_1 receptor, cannabinoid 1-type receptor; CB_2 receptor, cannabinoid 2-type receptor; CDM, chemically defined medium; GAP-43, growth-associated protein 43; GD, gestational day; KPBS, potassium phosphate-buffered saline; PBS, phosphate-buffered saline; RIPA, radioimmunoprecipitation assay; SDS-PAGE, sodium dodecyl-sulfate–polyacrylamide gel; Δ^9-THC, Δ^9-tetrahydrocannabinol.

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Among the processes susceptible to modulation by the cannabinoid signaling, the first one would be the phenotypic differentiation of specific subpopulations of neurons. This effect would be exerted, for example, through the regulation of genes encoding for the enzyme tyrosine hydroxylase (Bonnin et al., 1994, 1996; Hernández et al., 2000), for opioid peptide precursors (Pérez-Rosado et al., 2000, 2002) or for other key proteins in the development of specific neurotransmitters. A second process would be the regulation of cell death/survival processes that occur, in a physiological way, during brain development. This includes, for example, the survival of neural progenitors (Aguado et al., 2005) and, in particular, the apoptotic events that operate to eliminate exceeding nerve cells or specific groups of neurons with a neurotrophic role (Fernández-Ruiz et al., 2004). This assumption is strongly supported by the location of CB_1 receptors during brain development in subpopulations of neuronal cells (Hernández et al., 2000) or in specific regions (Romero et al., 1997; Berrendero et al., 1998) that do not contain these receptors in the adult brain. Lastly, cannabinoids might also play a role in the regulation of neuronal and glial cell proliferation and migration, axonal elongation, synaptogenesis and formation of myelin (Fernández-Ruiz et al., 2000, 2004; Rueda et al., 2002; Jiang et al., 2005; Galve-Roperh et al., 2006; Harkany et al., 2007).

Although the molecular substrates involved in this last action remain to be completely determined, there are already some recent proposals. For instance, Harkany and coworkers (Berghuis et al., 2005) found that endocannabinoids regulate interneuron migration through TrkB receptor–dependent signaling pathways. In our laboratory, we have obtained recent *in vivo* evidence that the role of the cannabinoid signaling in the regulation of cell proliferation, migration, differentiation and synaptic communication might be exerted by influencing the synthesis of the neural cell adhesion molecule L1 in the fetal (Gómez et al., 2003) and early postnatal (Gómez et al., 2007) rat brain. L1 protein is a member of the immunoglobulin superfamily (Hortsch, 1996, 2000). This protein seems to play an important role in cell proliferation, migration and differentiation, neuritic elongation and guidance, synaptogenesis, neuron–glia interactions and myelinogenesis (for review, see Hortsch, 1996; Burden-Galley et al., 1997; Panicker et al., 2003), based on its capability to mediate cell–cell and cell–matrix interactions. In fact, mutations in the L1 gene in humans (Wong et al., 1995; Hortsch, 1996) or disruption of this gene in mice (Dahme et al., 1997) results in several brain abnormalities related to the development of specific nervous tracts. In our previous reports, we found that the nonselective cannabinoid agonist Δ^9-tetrahydrocannabinol (Δ^9-THC), administered during the prenatal period, increased mRNA levels of L1 in rat fetuses (Gómez et al., 2003). This effect was observed in the same white matter regions where CB_1 receptors were "atypically" located during brain development [i.e. corpus callosum, stria terminalis, stria medullaris and fasciculum retroflexum (Romero et al., 1997; Berrendero et al., 1998)], but not in gray matter areas (Gómez et al., 2003). However, the effects of Δ^9-THC depended on the time of the brain development, since, at the postnatal day 1, the effects observed in white matter areas disappeared. Conversely, various gray matter regions, such as the cerebral cortex, septum nuclei, striatum, and various hippocampal structures, showed a decrease in mRNA levels for L1 at the postnatal day 1, although all these effects tended to disappear in the following days (Gómez et al., 2007). This would indicate that the modulatory role played by the cannabinoid system would be restricted to specific periods (late gestation and early postnatal life) of brain development, but that, later on, the cannabinoid system would lose its capability to influence L1 synthesis.

The present study was designed to further explore the influence of cannabinoids during the fetal period on the neural adhesion molecule L1, using *in vivo* and *in vitro* strategies. First, we studied the cellular location of CB_1 receptor–L1 interactions in the fetal rat brain using immunohistochemical analyses. These studies were aimed: (i) at identifying the distribution of CB_1 receptors in comparison with previous data obtained with autoradiographic methods (Romero et al., 1997; Berrendero et al., 1998), and (ii) at elucidating whether CB_1 receptors and L1 colocalize in those regions, such as some white matter areas of the forebrain, where the activation of these receptors influences L1 synthesis during fetal ages (Gómez et al., 2003). This first study was completed with an *in vitro* approach using cultures of fetal cortical neuronal or glial cells. In these cells, we studied the location of L1 protein and the changes in the levels of this protein, including the analysis of its posttranslational processing, when these cells are exposed to the cannabinoid agonist HU-210. As the first set of studies pointed that the modulatory action exerted by the cannabinoid signaling on L1 occurred preferentially in neuronal elements, we also wanted to elucidate the degree of maturation of developing neurons where CB_1 receptors are located. To this end, we compared the cellular and subcellular location of CB_1 receptors in forebrain white matter structures with the distribution of other proteins representative of key events in neuronal development. We used growth-associated protein 43 (GAP-43), a marker of growth cones, and synaptophysin, a protein present in synaptic vesicles that may label active synaptic terminals.

EXPERIMENTAL PROCEDURES

Animals, treatment and sampling

Female virgin Wistar rats were housed from birth in a room with a controlled 12-h light/dark photoperiod (08:00–20:00 h light) and temperature (23±1 °C), and with free access to standard food (Panlab, Barcelona, Spain) and water. At adult age (>8 weeks of life; 200–250 g), daily vaginal smears were taken between 10:00–12:00 h, and only those animals exhibiting three or more consistent 4-day cycles were used in this study. Females in the proestrus phase were allowed to stay with a male for mating, and a new vaginal smear was taken on the next day. Those animals showing the presence of sperm cells were accepted as probably pregnant and used for experimental studies. The day on which sperm plugs were found was designated the 1st day of gestation. Experimental procedures and care of animals conformed to local