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DISTRIBUTION OF CB1 CANNABINOID RECEPTORS IN THE RAT AMYGDALOID COMPLEX

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The amygdaloid complex (AK) has a very important role in the modulation of endocrine and visceral functions, in complex behavioral mechanisms such as defence, feeding, aggression, affects, reproduction, memory and learning. The aim of this study was to determine the precise distribution of cannabinoid CB1 receptors in the rat AK, using the immunohistochemical (ABC) method.

According to our results, CB1-immunoreactivity in the rat AK was highest in the medial nucleus. Slightly lower immunoreactivity was found in the basolateral nucleus. Moderate density of CB1 receptors occurred in the central, basomedial, lateral and posterior nuclei of the AK. CB1-immunoreactivity in all of these nuclei was present in the form of discrete spot-like precipitates of unequal size and appearance. These precipitates exhibited three different patterns: 1. elongated columns or lines, 2. complete or incomplete rings and 3. in a small number of AK regions CB1-immunoreactivity was separately dispersed in the form of single spot-like precipitates between more complex columns and rings of precipitates.

Considering the functional importance of amygdala and the distribution of CB1 receptors in the AK we could conclude that our findings suggest a role for cannabinoids in modulating responses of the AK to stress and fear as well as to pain.

Key words: immunohistochemistry, CB1 receptors, amygdaloid nuclei, rat

INTRODUCTION

The history of the consumption of marijuana (a representative of the exogenous cannabinoid family) is almost as long as the history of mankind. The principal active constituent of exogenous cannabinoids, Δ^9-tetrahydrocannabinol (Δ^9-THC), was isolated in the sixties (Gaoni and Mechoulam, 1964), but the mechanism of its action and the structure of the potential receptor remained unknown until the end of the eighties. Since Δ^9-THC is highly lipophilic, for a long time it was considered that this substance acts by non-specific interaction with the cellular membrane lipids, which may stimulate or inhibit membrane-

associated enzymes and alter ion channels (Hillard *et al*., 1985; Martin, 1986). However, later it was recognized that binding at the specific membrane receptor protein, CB1 receptor (Devane *et al*., 1988), exerts the central effects of this substance. Since then one more type of cannabinoid receptor has been isolated and investigated in detail. This is the CB2 receptor, which albeit intermediates the peripheral effects of Δ^9-THC (Munro *et al*., 1993).

After the discovery and cloning of CB1 receptors, the endogenous ligands of cannabinoid receptors (anandamide and 2-arachidonyl glycerol) were found (Devane *et al*., 1992; Mechoulam *et al*., 1995). The coexistence of these substances in nervous tissue indicated the plausible existence of a central cannabinoid neuromodulatory system.

The CB1 receptor is one of the most abundantly expressed neuronal receptors, and its protein structure exhibits the basic features of the G-protein-coupled receptor family (Matsuda *et al*., 1990). Cannabinoid CB1 receptors exert their effects through multiple signal transduction pathways including the inhibition of adenylate cyclase, modulation of ion channels and activation of mitogen-activated protein (MAP) kinase (Howlett and Fleming, 1984; Mackie and Hille, 1992).

Many studies suggest that the CB1 receptor has remained highly conserved during evolution. For example, at the molecular level the compatibility between man and rat nucleic acids amounts to 93 %, and at the amino acid level the degree of homology is even greater (97%). The CB1 gene in humans is located on chromosome 6 (Caenazzo *et al*., 1991) and in rats on chromosome 4 (Onaivi *et al*., 1996).

Initial research on the distribution of CB1 receptors was done using the methods of autoradiography (Herkenham *et al*., 1991) and *in situ* hybridization (Mailleux *et al*., 1992). Cloning of CB1 receptors created the opportunity to raise highly specific antibodies and enabled development of an immunohistochemical method for their precise detection and localization in the nervous system as well as in peripheral tissues.

Current immunohistochemical investigations of cannabinoid receptor distribution in rats refer mostly to their general distribution in the central nervous system. However, in this study we focused our attention on the detailed distribution of CB1 receptors in the amygdaloid complex.

MATERIALS AND METHODS

Five male adult Sprague-Dawley rats, weighing between 250-300 g, were treated with colchicine 48 hours before sacrifice. The experimental animals were anaesthetized with a cocktail of ketamine (0.3 ml) and 0.2% xylazinhydrochloride (0.2 ml).

The coordinates for the injection of colchicine into the lateral ventricle were determined according to the atlas of Paxinos and Watson (1998). Approximately 7 μl of colchicine solution was injected into ventricle (1 μl of solution = 10 μg colchicine) and the treated animals were housed under standard conditions and sacrificed after 48 hours.

The perfusion was undertaken with Zamboni's fixative (250-300 ml per rat). Brains were removed, postfixed in the same fixative overnight and cryoprotected by immersing the tissues in 20% sucrose.

Immunohistochemical reactions for the CB1 receptor were performed on 50 μm thick floating coronal sections, obtained by slicing the tissue specimens on the cryocut. In this study we used a polyclonal antibody raised against the CB1 receptor in 1:4000 dilution, then applied the ABC technique using a rabbit Vectastatin Elite ABC-peroxidase Kit (Vector Labs). The immunoreactive regions were visualized with 3,3-diaminobenzidine (DAB) solution. Sections were then mounted on gelatin-coated slides and covered. Three control animals were processed in the same manner with the exception of antibodies which were not used.

RESULTS

In the analyzed sections of the brain CB1-immunorectivity was present in the form of nonhomogenously distributed spot-like structures. In comparison to some other regions of the brain (hippocampus, cortex) the AK exhibited a moderately dense distribution of CB1 receptors (Fig.1).

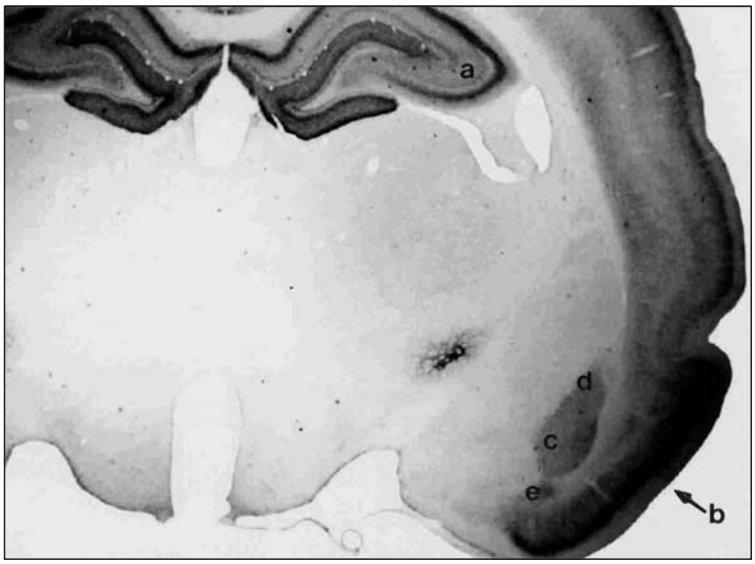

Figure 1. CB1-immunoreactivity in the rat brain (1,25x)
a) hippocampus b) cortex c) basolateral amygdaloid nucleus d) lateral mygdaloid nucleus e) basomedial amygdaloid nucleus

In general, the spot-like precipitates in the AK were organized in three ways. The greatest part of the immunoreactive precipitates was grouped in elongated columns or lines, which indicates distribution along the nerve fibers. These columns of different length were irregularly oriented, in different arrays. The second pattern of organization of immunoreactivity was in the form of complete or incomplete rings, which indicated circumferential distribution of CB1 receptors around the cell, i.e. is on the cytoplasmic membrane of the perikaryon or pericellular organization of fibers around the somata (Fig. 2). Finally, a small number of CB1 receptors was dispersed as separate spot-like structures of immunoreactivity in between the columns of precipitates.

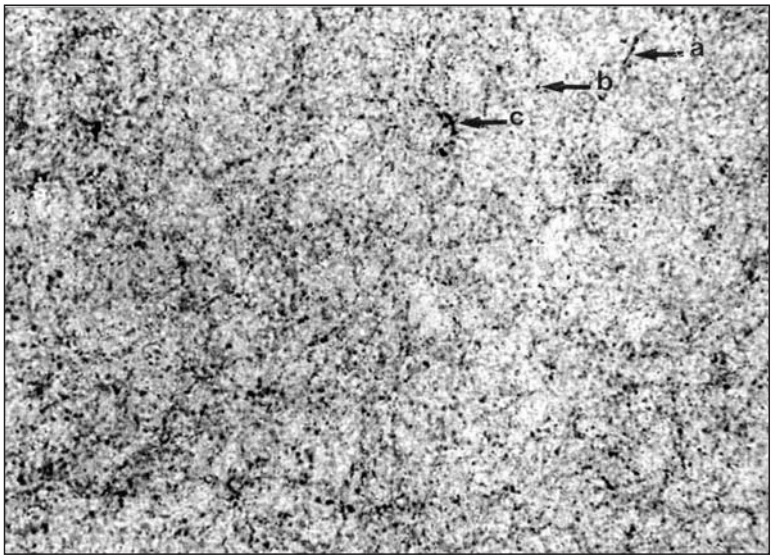

Figure 2. CB1-immunoreactivity in the basolateral amygdaloid nucleus (40x)
a) columns of immunoreactive precipitates b) immunoreactive spots
c) uncomplet ring of immunoreactive precipitates

The greatest density of CB1-immunoreactivity in the AK was present in the posterior medial nucleus (Fig. 3). CB1-immunoreactivity in this nucleus as well as in other parts of the AK was present in the form of spot-like precipitates of unequal size and appearance. High density in the ventral part of the medial nucleus prevented the observation of CB1-immunoreactivity. In other parts of this nucleus all three described patterns of immunoreactive precipitates could be seen at a moderate density.

Almost the same high density of CB1 receptors as in medial nucleus was observed in the basolateral nucleus, which is medial to the external capsule (Fig. 4).

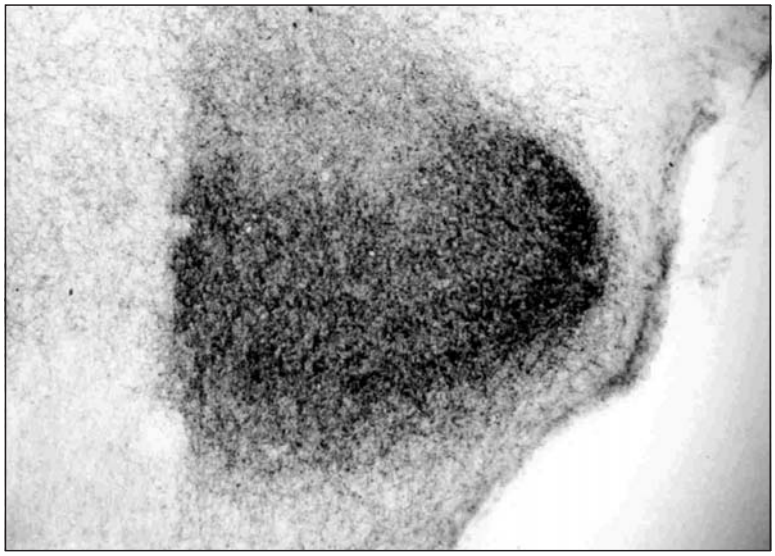

Figure 3. CB1-immunoreactivity in the medial amygdaloid nucleus (10x)

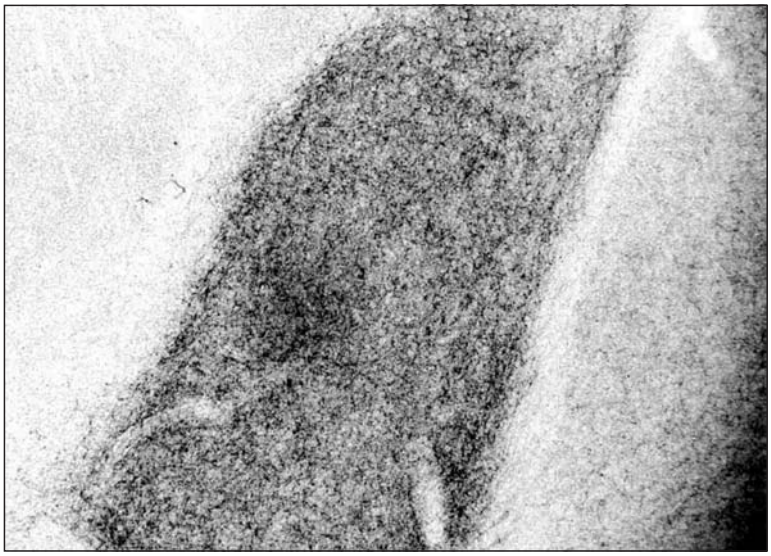

Figure 4. CB1-immunoreactivity in the basolateral amygdaloid nucleus (10x)

Compared with the basolateral nucleus, the lateral nucleus of the AK exhibited smaller number of spot-like immunoreactive precipitates. Also, less intensive CB1-immunoreactivity was observed in the basomedial nucleus. Both of these nuclei contained all three types of spot-like precipitate organization.

Moderate CB-1 immunoreactivity and all three organizational patterns of spot-like precipitates were present in the central and posterior cortical nuclei of the AK (Fig. 5).

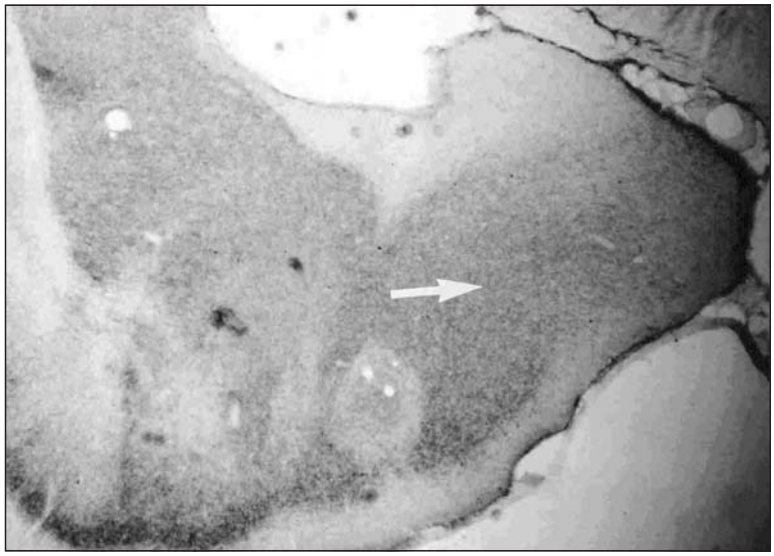

Figure 5. CB1-immunoreactivity in the posterior cortical nucleus (4x)

DISCUSSION

Knowledge about the AK collected so far indicates that it controls visceral functions, responses to stress, behavior connected with feeding and reproduction and also plays an important role in the regulation of heart action, social behaviour and memory (Davis, 1992; LeDoux, 1992; Adolphs *et al*., 1998; Cahill and McGaugh, 1998; Hamann *et al*., 1999).

Our results showed that the greatest density of CB-1 immunoreactivity is present in the medial nucleus of the AK, especially in its posterior part, which confirms earlier studies (Tsou *et al*., 1998; McDonald and Mascagni, 2001). Some authors found no CB1-immmunoreactivity in the medial amygdaloid nucleus (Moldricht and Wenger, 2000). The reason for this difference could be the application of colchicine in our study. It is thought, that transport of CB1 protein

down the axon is prevented by (colchicine) induced of microtubules leading to acumulation of CB1 protein (McDonald and Mascagni, 2001) and more intensive immunostaining.

The medial nucleus of the AK receives strong inputs from the infralimbic cortex and hypothalamus and sends moderate to heavy projections to the olfactory system, thalamus and hypothalamus (according to Pitkanen, 2001, Figs. 2.10 and 2.11) which can indicate a possible role for cannabinoids in feeding and olfaction through the medial nucleus.

It is interesting that the highest degree of labeling of estrogen receptors in the rat AK occurred in the nuclei of phylogenetically older, corticomedial part: in the medial nucleus, followed by the central, and cortical nuclei respectively (Malobabic, Drekic and Prostran, 2002). It is still unknown, if cannabinoids modulate the level of these receptors and if there is any connection between cannabinoids and sexual behaviour.

Dense distribution CB1-immunoreactivity was also observed in the basolateral amygdaloid nucleus, which confirms previous investigations (Tsou *et al*., 1998; McDonald and Mascagni, 2001; Katona *et al*., 2001). The colocalization of CB1 and cholecystokinin (CCK) in non-pyramidal GABAergic interneurons (McDonald and Mascagni, 2001; Katona *et al*., 2001) suggests that cannabinoids might increase the excitability of the basolateral nucleus by decreasing the amount of GABA released from these neurons (McDonald and Mascagni, 2001). On the other hand, cannabinoids have been shown to influence many of the neuronal systems that send afferents to the basolateral nucleus including glutamatergic projections from the cortex, dopaminergic and noradrenergic projections from the brain stem, and cholinergic projections from the basal forebrain (Brevogel and Childers, 1998).

The moderate CB1-immunoreactivity we observed in the posterior cortical nucleus of the AK, has already been described by Tsou *et al*. (2001) and McDonald and Mascagni (2001). This nucleus is strongly interconnected with the olfactory system (Canteras *et al*., 1992). Also, there are very strong outputs from the posterior cortical nucleus to the medial temporal lobe memory system (hippocampal formation and perirhinal cortex), prefrontal cortex, and bed nucleus of stria terminalis (according to Pitkanen, 2001, Figs. 2.16 and 2.17). It is evident that cannabinoids can influence memory through this nucleus.

A moderate level of CB1-immunoreactivity was found in the lateral and basomedial nucleus, which is coherent with previous results (McDonald and Mascagni, 2001; Katona *et al*, 2001).

The lateral nucleus receives substantial inputs from sensory-related lateral cortical areas, the prefrontal cortex, the hippocampal formation, the thalamus and the hypothalamus. At same time, this nucleus sends strong projections in to the medial temporal lobe memory system and the prefrontal cortex (according to Pitkanen, 2001, Figs. 2.22 and 2.23).

The basomedial nucleus is interconnected with lateral sensory-related cortical areas, the hippocampal formation, medial prefrontal cortex and the hypothalamus (according to Pitkanen, 2001, Fig. 2.6 and 2.7).

When the connections of the lateral and basomedial nuclei with other structures of the brain are taken into consideration, it is apparent that cannabis and endogenous cannabinoid receptor agonists (anandamid and 2-arachidonyl glycerol) may serve as chemical mediators in cognition, memory, learning, emotions, appetite, thermoregulation and sleep through CB1 receptors in these nuclei.

Considering the relationship of the AK to behaviour and emotions, it seems logical that it is actively involved in the stress response. Many articles imply that the central nucleus has the main role in such situations, as stimulation induces erosion of gastric mucosa (Henke, 1985), and lesion of this nucleus is followed by decreased gastric acidity (Henke, 1983).

The results of our analysis show the presence of moderate CB1-immunoreactivity in the central nucleus which confirms earlier results (Tsou *et al*., 1998; Moldricht and Wenger, 2000; McDonald and Mascagni, 2001).

The central nucleus also has an important role in the regulation of autonomicus functions. Bradycardia after the noxious influence is regulated by the activity of the central nucleus. In cats, aversive stimulation of the central nucleus induces modification of heart rate, blood pressure and respiration (Zhang *et al*., 1986).

Based on all these findings, the presence of cannabinoid receptors in the central nucleus confirms the idea that they could have a role in modulating responses to stress and fear. Also, through the amygdala (basolateral and central nuclei) cannabinoids could be included in the modulation of pain. Microinjections of morphine into the basolateral nucleus produce analgesic effects while lesion of the central nucleus reduces morphine analgesia. The presence of CB1-immunoreactivity in these regions indicates that the amygdala could be the center of the analgesic action of cannabinoids (Ameri, 1999).

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DISTRIBUCIJA CB1 KANABINOIDNIH RECEPTORA U AMIGDALOIDNOM KOMPLEKSU PACOVA

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SADRŽAJ

Cilj ovog rada bio je da se imunohistohemijskom metodom utvrdi precizna distribucija CB1 receptora u amigdaloidnom kompleksu (AK) mozga pacova zbog njegove izuzetno važne uloge u modulaciji endokrine funkcije, visceralnim efektorskim mehanizmima i kompleksnim mehanizmima ponašanja kao što su odbrana, ishrana, agresija, afekti, reprodukcija, memorija i učenje.

Dobijeni rezultati pokazuju da je CB1-imunoreaktivnost najveća u medijalnom jedru. Nešto manji nivo imunoreaktivnosti zabeležen je u bazolateralnom jedru. Umerena gustina CB1 receptora otkrivena je u centralnom, bazomedijalnom, lateralnom i zadnjem kortikalnom jedru amigdaloidnog kompleksa. CB1-imunoreaktivnost u svim ovim jedrima prisutna je u formi nehomogeno distribuiranih tačkastih precipitata neujednačenog prečnika. Ovi precipitati mogu biti trojako organizovani: u vidu dužih ili kraćih nizova orijentisanih u različitim pravcima, u vidu pojedinačno distribuiranih tačaka i u formi potpunih ili nepotpunih kružnica.

Imajući u vidu funkcionalni značaj amigdala kao i distribuciju CB1 receptora, može se zaključiti da kanabinoidi mogu imati ulogu u modulaciji odgovora na stres i strah, kao i u modulaciji bola.