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LOCALIZATION OF THE CB1 TYPE CANNABINOID RECEPTOR IN THE RAT BASOLATERAL AMYGDALA: HIGH CONCENTRATIONS IN A SUBPOPULATION OF CHOLECYSTOKININ-CONTAINING INTERNEURONS

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Abstract—The neuronal localization of the CB1 cannabinoid receptor in the rat basolateral amygdala was studied using peroxidase and fluorescence immunohistochemical techniques. All nuclei of the basolateral amygdala contained a large number of lightly stained pyramidal neurons and a small number of more intensely stained non-pyramidal neurons. Most of the latter cells had medium-sized to large multipolar somata and three to four aspiny dendrites, but some exhibited smaller oval somata. The axon initial segments of some of these non-pyramidal neurons exhibited large swollen varicosities in colchicine-injected animals, suggesting that much of the CB1 receptor protein is transported down the axons of these cells. Double-labeling studies using immunofluorescence histochemistry combined with confocal laser scanning microscopy revealed that the great majority of non-pyramidal neurons with CB1 receptor immunoreactivity belonged to a cholecystokinin-containing subpopulation. Whereas none of the other subpopulations of non-pyramidal neurons (exhibiting immunoreactivity for calretinin, parvalbumin, or somatostatin) expressed high levels of CB1 receptor immunoreactivity, a small percentage of these cells exhibited low levels of immunoreactivity.

The results indicate that cannabinoids may modulate the activity of pyramidal projection neurons as well as a subpopulation of cholecystokinin-containing non-pyramidal neurons in the basolateral amygdala. Previous studies indicate that most of the latter are inhibitory interneurons that utilize GABA as a neurotransmitter. The intense staining of the cholecystokinin-containing interneurons and the evidence that large amounts of CB1 receptor protein are transported down the axons of these cells suggests that, as in the hippocampus, cannabinoids may inhibit the release of GABA from the axon terminals of these neurons.

Key words: immunohistochemistry, neuropeptides, interneurons, GABA.

Marijuana (*Cannabis sativa*) has been used for spiritual, therapeutic, and recreational purposes for thousands of years. However, it was not until the last decade that significant progress was made in determining how its active ingredient, Δ^9-tetrahydrocannabinol, affects brain function. These recent studies have demonstrated that there is an endogenous cannabinoid system in the brain that is widespread and very important for modulating synaptic activity (Pertwee, 1997; Breivogel and Childers, 1998; Di Marzo et al., 1998). Thus, similar to the opioid system, the fortuitous finding that a particular plant has psychotropic effects has eventually led to the discovery of an important and powerful neuromodulator system of the nervous system. Analogous to the opioid system, several endogenous cannabinoid neuromodulators, termed endocannabinoids, have been found in the brain. These include two eicosanoids: (1) *N*-arachidonoylethanolamine (anandamide), and (2) 2-arachidonoylglycerol (Di Marzo et al., 1998). In addition, two major classes of cannabinoid receptors have been discovered. Only the CB1 receptor is found in the CNS (Pertwee, 1997).

The cannabinoid system is involved in numerous functions including regulation of movement, endocrine function, nociception, thermoregulation, sensory perception, memory, and mood (Breivogel and Childers, 1998; Chaperon and Thiébot, 1999). Since the amygdala is one of the main brain regions involved in emotion and memory, it is a likely candidate structure for the affective and mnemonic alterations produced by cannabinoids. Indeed, Herkenham and co-workers, using receptor binding autoradiography, found cannabinoid receptor binding in the amygdala that was most dense in its basolateral portion and of lower density in the centromedial and cortical nuclei (Herkenham et al., 1990, 1991).

To date there have been no detailed immunohistochemical studies of CB1+ neurons in the rat basolateral amygdala (ABL). As a result there is little information

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Abbreviations: ABC, avidin–biotin–peroxidase; ABL, basolateral amygdala; BLa, anterior subdivision of the basolateral amygdalar nucleus; BLp, posterior subdivision of the basolateral amygdalar nucleus; CB, calbindin-D28k; CB1, type 1 cannabinoid receptor; CCK, cholecystokinin; CR, calretinin; GST, glutathione *S*-transferase; Lvm, ventromedial subdivision of the lateral amygdalar nucleus; NPY, neuropeptide Y; PBS, phosphate-buffered saline; PV, parvalbumin; SOM, somatostatin; VIP, vasoactive intestinal polypeptide.

regarding the anatomical localization of CB1 protein in the ABL of this species. One of the most interesting aspects of the cytology of the ABL is that although it is a subcortical structure, its two major cell types, the pyramidal and non-pyramidal neurons, are very similar to their counterparts in the cerebral cortex (Hall, 1972; Millhouse and DeOlmos, 1983; McDonald, 1992a). In addition, the synaptology and pharmacology of cortical and ABL neurons are very similar (Carlsen and Heimer, 1986; Carlsen, 1988; Washburn and Moises, 1992; Rainnie et al., 1993). The principal neurons in the ABL are large pyramidal-like projection neurons with spiny dendrites that utilize glutamate as a neurotransmitter (McDonald, 1982, 1992b, 1996; Millhouse and DeOlmos, 1983). Most non-pyramidal neurons in the ABL are spine-sparse interneurons that utilize GABA as an inhibitory neurotransmitter (McDonald, 1982, 1985a; Millhouse and DeOlmos, 1983; Carlsen, 1988).

As in the cerebral cortex, distinct subpopulations of non-pyramidal neurons in the rat ABL can be distinguished on the basis of their content of calcium-binding proteins (parvalbumin [PV], calbindin-D28k [CB], and calretinin [CR]) and neuropeptides (somatostatin [SOM], cholecystokinin [CCK], neuropeptide Y [NPY], vasoactive intestinal polypeptide [VIP]) (McDonald, 1985b, 1989, 1994, 1997; Kemppainen and Pitkänen, 2000; McDonald and Betette, 2001; McDonald and Mascagni, 2001). The results of these investigations, in conjunction with several unpublished double-labeling studies recently performed in the authors' laboratory, suggest that there may be at least four major subpopulations of GABAergic interneurons in the rat ABL that have different combinations of calcium-binding proteins and neuropeptides (although there is not 100% overlap within each subpopulation): (1) SOM/CB/NPY neurons, (2) PV/CB neurons, (3) large CCK/CB neurons, and (4) small CCK/CR/VIP neurons.

In the present investigation immunoperoxidase techniques were used to investigate the morphology of CB1-expressing neurons in the ABL, and a double-labeling immunofluorescence technique combined with confocal laser scanning microscopy was used to determine whether any of the four major subpopulations of non-pyramidal neurons exhibit CB1 immunoreactivity.

EXPERIMENTAL PROCEDURES

Tissue preparation

A total of 10 male Sprague–Dawley rats (250–350 g; Harlan) were used in this study. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to produce reliable scientific data. Rats were anesthetized with chloral hydrate (350 mg/kg) and perfused intracardially with phosphate-buffered saline (PBS; pH 7.4) containing 1% sodium nitrite (50 ml) followed by 4.0% paraformaldehyde–0.2% picric acid in 0.1 M phosphate buffer at pH 7.4 (500 ml). One rat received bilateral injections of colchicine (100 μg total) into the lateral cerebral ventricles 1 day before perfusion. Following perfusion, brains were removed and postfixed for 4 h in 4.0% paraformaldehyde.

All brains were sectioned on a vibratome at a thickness of 50 μm in the coronal plane. Sections were processed for immunohistochemistry in wells of tissue culture plates. All antibodies were diluted in 0.1 M Tris-buffered saline (pH 7.6) containing Triton X-100 (0.1–0.3%) and 1% normal goat serum. All sections were incubated in a blocking solution (1% bovine serum albumin, 3% normal goat serum, Triton X-100 [0.1–0.3%], in Tris-buffered saline) for 30 min prior to incubation in primary antibodies.

Immunoperoxidase experiments

Localization of CB1-like immunoreactivity was performed in six rats using the avidin–biotin peroxidase (ABC) technique. The primary antibody to CB1 (1:4000; generously donated by K. Mackie, University of Washington) was raised in rabbit using the N-terminal 77 amino acid residues of the rat CB1 receptor fused to glutathione *S*-transferase (GST) as an antigen (Tsou et al., 1998a). Sections were incubated in primary antibody overnight at 4°C and then processed for the ABC technique using a rabbit Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Nickel-enhanced 3,3′-diaminobenzidine-4HCl (Sigma Chemical Co., St. Louis, MO, USA) was used as a chromogen to generate a black reaction product (Hancock, 1986). Following the immunohistochemical procedures, sections were mounted on gelatinized slides, dried overnight, dehydrated in ethanols followed by xylenes, and coverslipped in Permount (Fisher). In one animal some sections were counterstained with a Nissl stain (pyronin Y) before coverslipping. Sections were analyzed and photographed using an Olympus BHA microscope. Negatives were scanned, and brightness and contrast were adjusted using Photoshop 5.5 software.

In one of the six rats processed for the ABC technique, alternate sections were stained with antisera to CB1 (1:4000) or gastrin/CCK (1:500; mouse monoclonal antibody 9303 generously donated by Dr. J.H. Walsh and H.C. Wong, UCLA) in order to compare the perikaryal sizes of these two neuronal populations. The gastrin/CCK antiserum was raised against gastrin but recognizes CCK due to homologies in the terminal pentapeptide shared by these peptides. Gastrin is not found in the telencephalon (Rehfeld, 1978) and this antibody has been used in numerous previous studies of the forebrain to study the distribution of CCK (Oeth and Lewis, 1990; Tsou et al., 1999; Snyder et al., 1993). Sections were processed as described above using ABC kits appropriate for the species in which each primary antibody was raised. Neuronal somata exhibiting CB1 or CCK immunoreactivity were drawn at 1000× magnification using a camera lucida. Cross-sectional areas of immunoreactive somata were measured using a Summagraphics II digitizing tablet interfaced with an IBM computer. The morphometry software for this analysis (The Morphometer) was obtained from Woods Hole Educational Associates (Woods Hole, MA, USA).

Immunofluorescence experiments

Colocalization of CB1 with markers for each of the four major subpopulations of non-pyramidal neurons (CCK, PV, SOM, CR) was investigated in four rats. The following primary antibodies were used for these studies: rabbit anti-CB1 (1:4000), mouse anti-gastrin/CCK (1:500), mouse anti-PV (1:1500, Sigma), mouse anti-SOM (1:500; generously donated by the MRC Regulatory Peptide Group, University of British Columbia), and mouse anti-CR (1:3000, Chemicon).

Sections were incubated in a cocktail of two primary antibodies (the CB1 antibody and one of the neuropeptide or calcium-binding protein antibodies) overnight at 4°C, rinsed in three changes of PBS (10 min each), and then incubated sequentially in Alexa 568-labeled goat anti-rabbit IgG (1:400, Molecular Probes) followed by Alexa 488-labeled goat anti-mouse IgG (1:400, Molecular Probes), each for 2.5 h at room temperature. Both secondary antibodies were highly cross-adsorbed by the manufacturer to insure specificity for primary antibodies raised in rabbit or mouse, respectively. Sections were then rinsed in three changes of PBS (10 min each) and mounted on glass slides

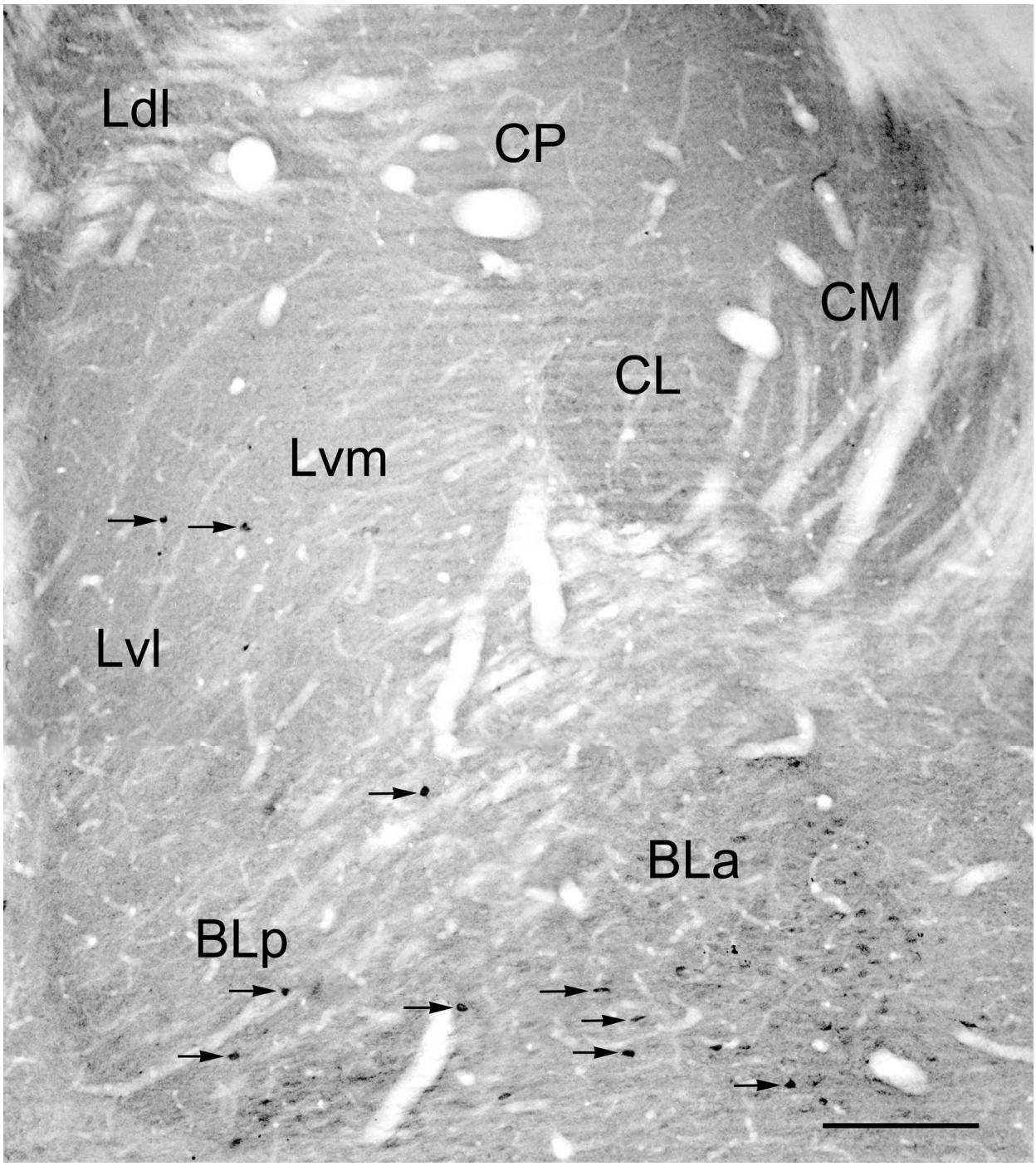

Fig. 1. Photomontage of CB1 cannabinoid receptor immunoreactivity in the basolateral and central amygdalar nuclei in a coronal section (bregma level −3.14 of the atlas by Paxinos and Watson, 1986). Note that there is a small number of intensely stained CB1+ non-pyramidal neurons (arrows) in the nuclei of the basolateral nuclear complex (Ldl, dorsolateral subdivision of the lateral nucleus; Lvl, ventrolateral subdivision of the lateral nucleus; Lvm, ventromedial subdivision of the lateral nucleus; BLa, anterior subdivision of the basolateral nucleus; BLp, posterior subdivision of the basolateral nucleus). In addition, there are numerous lightly stained pyramidal cells in the ventromedial (lower right) portions of the BLa and BLp. Other abbreviations: CP, caudate–putamen; CL, lateral subdivision of the central amygdalar nucleus; CM, medial subdivision of the central amygdalar nucleus. Scale bar = 200 μm.

using Vectashield (Vector) mounting medium. Sections were examined with a Bio-Rad MRC-1024 confocal laser scanning microscope equipped with an argon–krypton laser attached to a Nikon Optiphot fluorescence microscope. Fluorescence of Alexa 488 (green) and Alexa 568 (red) dyes was analyzed using filter configurations for sequential excitation/imaging via 488-nm and 568-nm channels. Digital images were adjusted for brightness and contrast using Photoshop 5.5 software.

The rat ABL consists of three main nuclei: (1) lateral nucleus, (2) basolateral nucleus, and (3) basomedial nucleus. Each nucleus can be divided into two or more subdivisions. The present semi-quantitative confocal analysis focused on two adja-

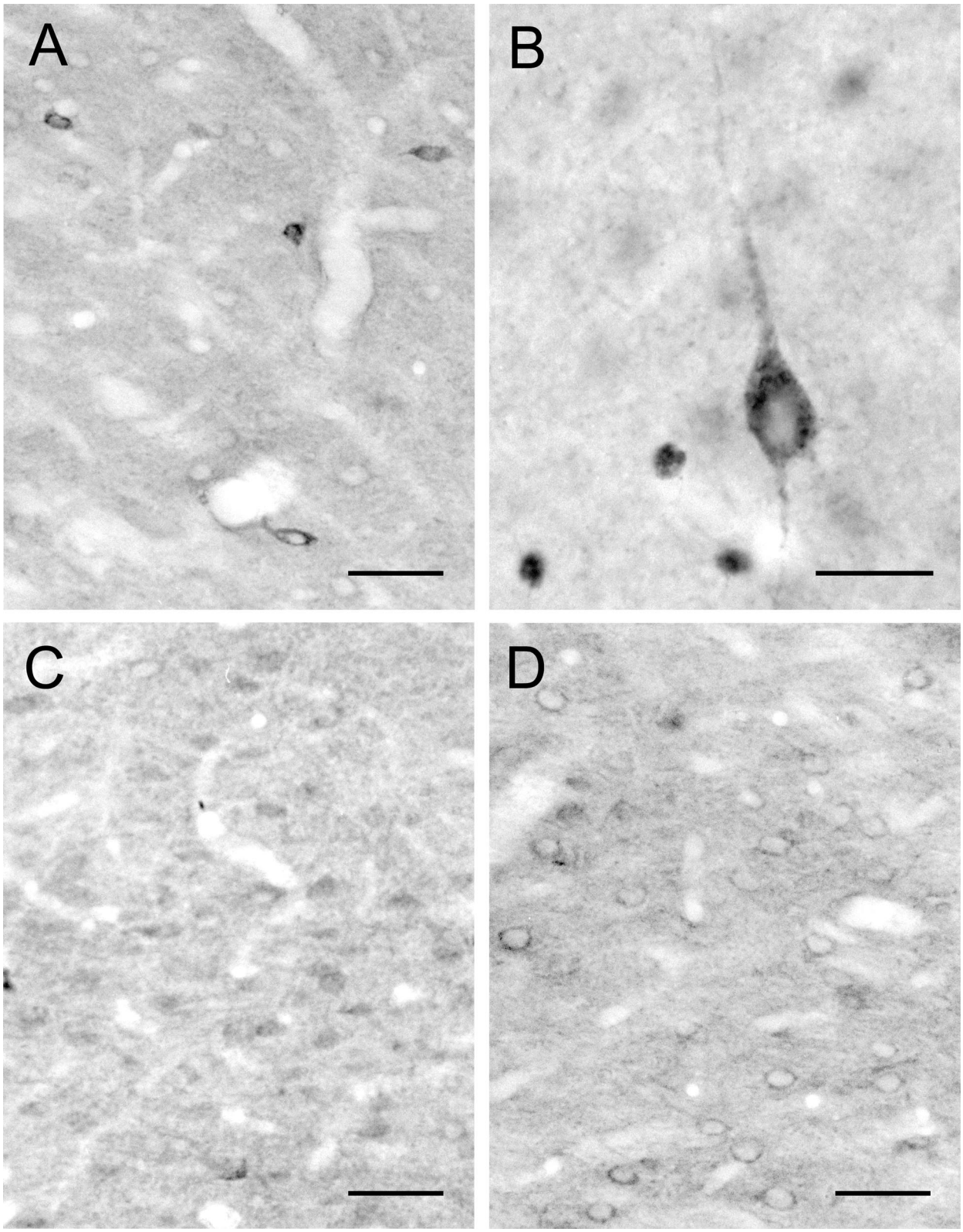

Fig. 2. Photomicrographs of CB1 cannabinoid receptor immunoreactivity in the ABL. (A) Four intensely stained CB1+ non-pyramidal neurons located along the border separating the lateral and basolateral nuclei. Scale bar = 50 μm. (B) One large CB1+ neuron and three astrocytes in the BLa immunostained using non-GST-absorbed CB1 antiserum. Astrocyte staining is non-specific (see Experimental procedures). Scale bar = 20 μm. (C and D) Numerous lightly stained CB1+ pyramidal neurons in the BLa (C) and BLp (D). Scale bars = 50 μm.

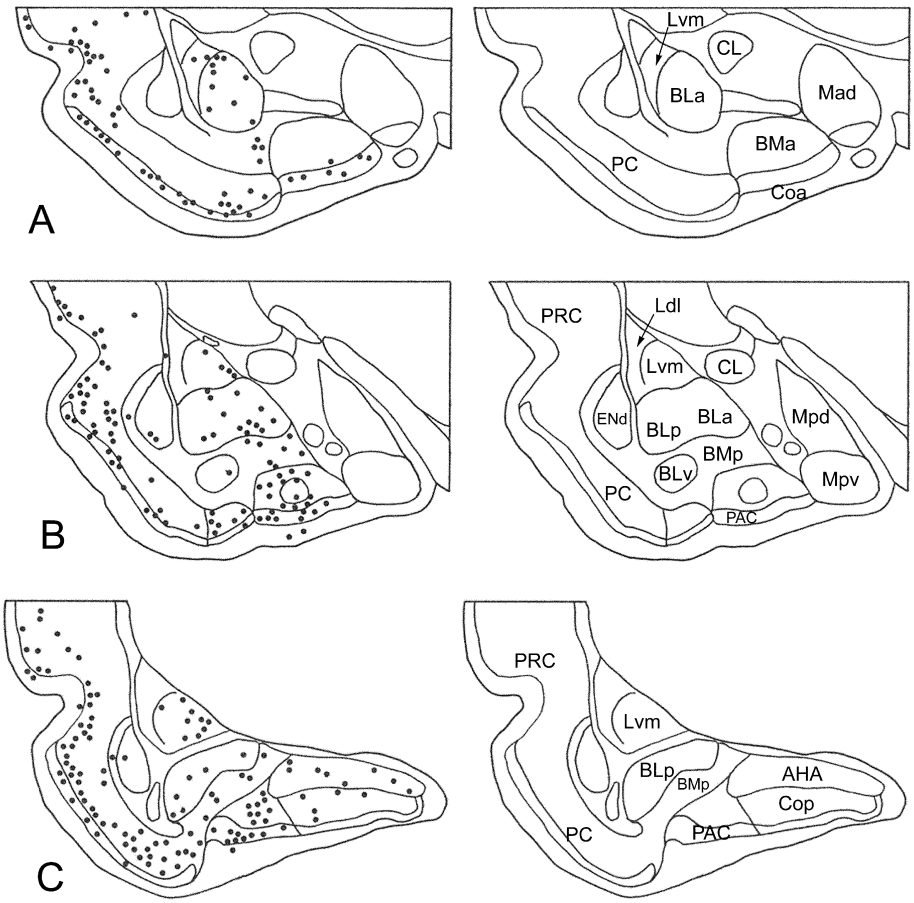

Fig. 3. Distribution of CB1+ non-pyramidal neurons in the amygdala and adjacent piriform/perirhinal cortices at three rostrocaudal levels: (A) bregma level −2.1; (B) bregma level −3.1; (C) bregma level −4.0. Neurons were plotted from two adjacent sections, one of which was Nissl-counterstained to reveal nuclear borders. Only neurons with levels of CB1 immunoreactivity significantly above background were plotted. Comparisons with other brains revealed that the distribution and density of CB1+ neurons depicted in this map are representative. Abbreviations: AHA, amygdalohippocampal area; BLa, anterior subdivision of the basolateral nucleus; BLp, posterior subdivision of the basolateral nucleus; BLv, ventral subdivision of the basolateral nucleus; BMa, anterior subdivision of the basomedial nucleus; BMp, posterior subdivision of the basomedial nucleus; CL, lateral subdivision of the central nucleus; Coa, anterior subdivision of the cortical nucleus; Cop, posterior subdivision of the cortical nucleus; ENd, dorsal endopiriform nucleus; Ldl, dorsolateral subdivision of the lateral nucleus; Lvm, ventromedial subdivision of the lateral nucleus; Mad, anterodorsal subdivision of the medial nucleus; Mpd, posterodorsal subdivision of the medial nucleus; Mpv, posteroventral subdivision of the medial nucleus; PAC, periamygdaloid cortex; PC, piriform cortex; PRC, perirhinal cortex.

cent nuclear subdivisions that form the core of the ABL, the anterior subdivision of the basolateral nucleus (BLa) and the ventromedial subdivision of the lateral nucleus (Lvm). Cell counts of single-labeled and double-labeled somata were made in these regions and were pooled from two animals. Since dendrites of ABL non-pyramidal neurons are less than 3 μm wide, it was not difficult to distinguish somata from dendrites. Counts were made from the image of a 400 μm×400 μm field (200× magnification) displayed for merged (red/green) channels on the computer screen (double-labeled cells appear yellow). Images of the non-merged red and green channels were also displayed. Depending on the size of the nuclear subdivision at different levels of the amygdala, counts were made from either one such field positioned in the center of the subdivision (and involving about 80–90% of its cross-sectional area), or two adjacent non-overlapping fields. A total of 91–169 neurons of each type (CCK, PV, SOM, and CR) from each nucleus was analyzed for colocalization of CB1.

In each of the confocal immunofluorescence cases some control sections were processed with one of the two primary antibodies omitted. In all cases only the color of the corresponding secondary fluorescent antibody was observed, and only on the appropriate channel. These results indicated that the secondary antibodies were specific for rabbit or mouse IgGs and that there was no 'cross-talk' between the red and green channels (Wouterlood et al., 1998).

Controls for antibody specificity

Absorption experiments were performed to test the specificity of the CB1 antiserum. The diluted antiserum was preabsorbed with its immunizing fusion protein (N-terminal 77 amino acid residues of the rat CB1 receptor fused to GST; 1 μg/ml) or with GST alone (10 μg/ml; generously donated by K. Mackie, University of Washington) overnight at 4°C. The non-absorbed antiserum stained discrete neuronal subpopulations in particular brain regions (e.g., the ABL and cerebral cortex), as well as astrocytes throughout the brain. The neuronal staining pattern appeared to be identical to that described by Tsou et al. (1998a), who used affinity-purified antibodies obtained from the whole serum used in the present study. However, Tsou and coworkers did not stain astrocytes with their affinity-purified antibodies. In our absorption studies preabsorption of the CB1 antiserum with the immunizing fusion protein abolished all tissue staining, both neuronal and astrocytic. However, preabsorption of the CB1

antiserum with GST alone abolished all of the astrocytic staining, but none of the neuronal staining. These results indicate that the astrocyte staining was due to anti-GST antibodies in the CB1 whole antiserum. Since the immunostained astrocytes had very small cell bodies that were easily distinguishable from neuronal somata, both absorbed and non-absorbed CB1 antisera were used in the present study. All photomicrographs were taken from preparations using GST-preabsorbed antisera except Figs. 2B and 5.

Preabsorption of the gastrin/CCK antibody with CCK-8 (25 μg/ml; Sigma) and the SOM antibody with somatostatin-14 (100 μg/ml; Sigma) abolished all immunostaining. Previous absorption studies have shown that the monoclonal PV antibody (Celio et al., 1988) recognizes PV, but not other calcium-binding proteins. For all of these antibodies, as well as the monoclonal CR antibody, sections incubated in diluent in place of the primary antibody exhibited no immunostaining.

RESULTS

CB1-like immunoreactivity in the basolateral amygdala

In the ABL CB1 immunoreactivity was observed in many pyramidal neurons and in a small number of non-pyramidal neurons. The immunostaining in most pyramidal neurons was very light. The immunostaining in many of the non-pyramidal neurons was intense, but in others it was light to moderate.

All portions of the ABL contained non-pyramidal CB1+ neurons (Figs. 1–5). However, usually only three to six immunostained cells were seen in each nucleus per section. The most intensely stained cells had relatively large multipolar somata that were 12–16 μm long and 9–11 μm wide, and exhibited three to four aspiny primary dendrites (Figs. 2A, B, 4 and 5). Some neurons, particularly in the lateral nucleus, were smaller (Figs. 7 and 8). Most of these smaller neurons were more lightly stained than the larger CB1+ neurons. In non-colchicine-injected brains only the somata and proximal portions of the primary dendrites were stained. Even in the colchicine-injected brain, dendrites could only be followed for 50–75 μm and showed very sparse branching. In the colchicine-injected brain many neurons had one process, presumably the axon, that extended 10–30 μm from the cell body and exhibited one to five large, intensely stained varicosities along its distal portion (Figs. 4 and 5). These intensely stained varicosities may represent accumulations of CB1 protein whose transport down the axon had been prevented by the disruption of microtubules caused by the colchicine.

Each nucleus of the ABL also contained a large number of lightly stained large somata that appeared to correspond to the principal neurons of the ABL, the pyramidal cells (Fig. 2C, D). In most portions of the ABL the staining intensity was just above background levels. However, pyramidal cell staining was more intense in the ventral and medial portions of the basolateral nucleus (Fig. 1). Many lightly stained dendrites were seen in all nuclei of the ABL. Since their relative number and staining intensity correlated with the relative number and staining intensity of the surrounding pyramidal cell somata, they are probably dendrites of pyramidal cells. In caudal portions of the posterior division of the basolateral nucleus (BLp), lightly stained CB1+ dendrites extended ventrally toward the rhinal fissure. Previous Golgi studies have shown that the apical dendrites of pyramidal cells in that region have a similar orientation (McDonald, 1984).

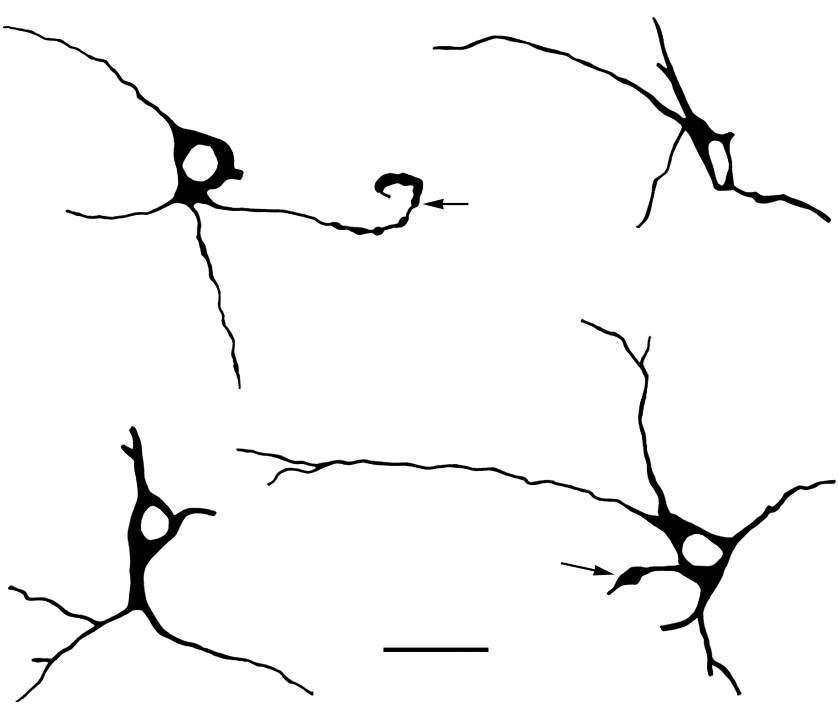

Fig. 4. Camera lucida drawings of four CB1 cannabinoid receptor-positive non-pyramidal neurons in the ABL of a colchicine-injected rat. Arrows indicate large CB1+ varicosities found along the initial segment of the axon. These were only seen in the colchicine-injected animal. Scale bar = 20 μm.

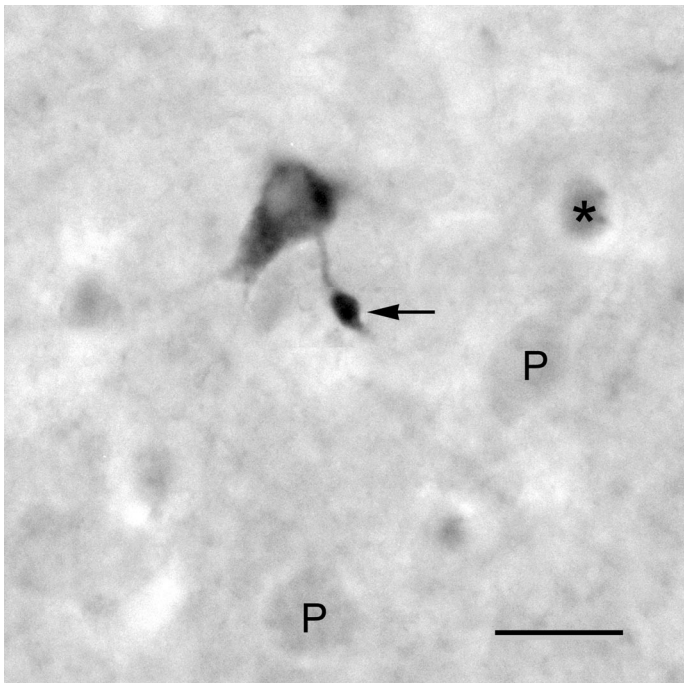

Fig. 5. Photomontage of an intensely stained CB1+ non-pyramidal neuron and two lightly stained pyramidal cells (P) in the ABL of a colchicine-injected rat. Arrow indicates a large CB1+ varicosity found along the initial segment of the axon of the non-pyramidal cell. Asterisk indicates a lightly stained astrocyte located out of the plane of focus. Section was stained using non-GST-absorbed antiserum. Astrocyte staining is non-specific (see Experimental procedures). Scale bar = 20 μm.

CB1-like immunoreactivity in the cortical and centromedial amygdalar nuclei

The pattern of CB1 staining seen in the ABL was also observed in the cortical amygdalar nuclei and the amygdalohippocampal amygdalar nucleus. These regions contained numerous lightly stained pyramidal neurons, whose dendrites often extended into the molecular layer, and a small number of more intensely stained non-pyramidal neurons (Fig. 3). Similar staining was also seen in the adjacent piriform and perirhinal cortices. There were no intensely stained neurons in the central and medial amygdalar nuclei, but almost all of the principal neurons exhibited light CB1 immunoreactivity (Fig. 1). The staining of principal neurons was more intense in the lateral subdivision of the central nucleus and anteroventral subdivision of the medial nucleus than in other portions of the centromedial amygdala.

Colocalization studies of the basolateral amygdala using confocal immunofluorescence microscopy

The immunoperoxidase studies demonstrated that the CB1+ neurons with the more robust levels of immunoreactivity belong to the non-pyramidal neuronal population of the ABL. As discussed in the Introduction, different subpopulations of non-pyramidal neurons can be recognized on the basis of their content of particular neuropeptides and calcium-binding proteins. The extent of colocalization of CB1 with markers for the four main subpopulations of non-pyramidal cells (CCK, CR, PV, and SOM) was investigated in the BLa and the Lvm to

Table 1. Colocalization of cannabinoid receptor CB1 with markers for different subpopulations of interneurons in the BLa and Lvm using immunofluorescence confocal laser scanning microscopy

Marker	Nucleus	Neurons[a] counted	Single-labeled[b] marker	Marker/CB1 double-labeled	% Marker double-labeled
CCK	BLa	99	18	81	81.8
	Lvm	91	36	55	60.4
CR	BLa	105	103	2*	1.9*
	Lvm	112	102	10*	8.9*
PV	BLa	169	153	16*	9.5*
	Lvm	102	97	5*	4.9*
SOM	BLa	110	109	1*	0.9*
	Lvm	105	105	0	0.0

Asterisks indicate double-labeled neuronal populations that exhibited low levels of CB1 immunoreactivity.
[a]Total number of neurons that were either single-labeled for the marker (CCK, CR, PV, or SOM) or double-labeled (marker+/CB1+). Only these neurons were counted (see text).
[b]Number of neurons that were single-labeled for the marker (CCK, CR, PV, or SOM) but which did *not* exhibit CB1 immunoreactivity.

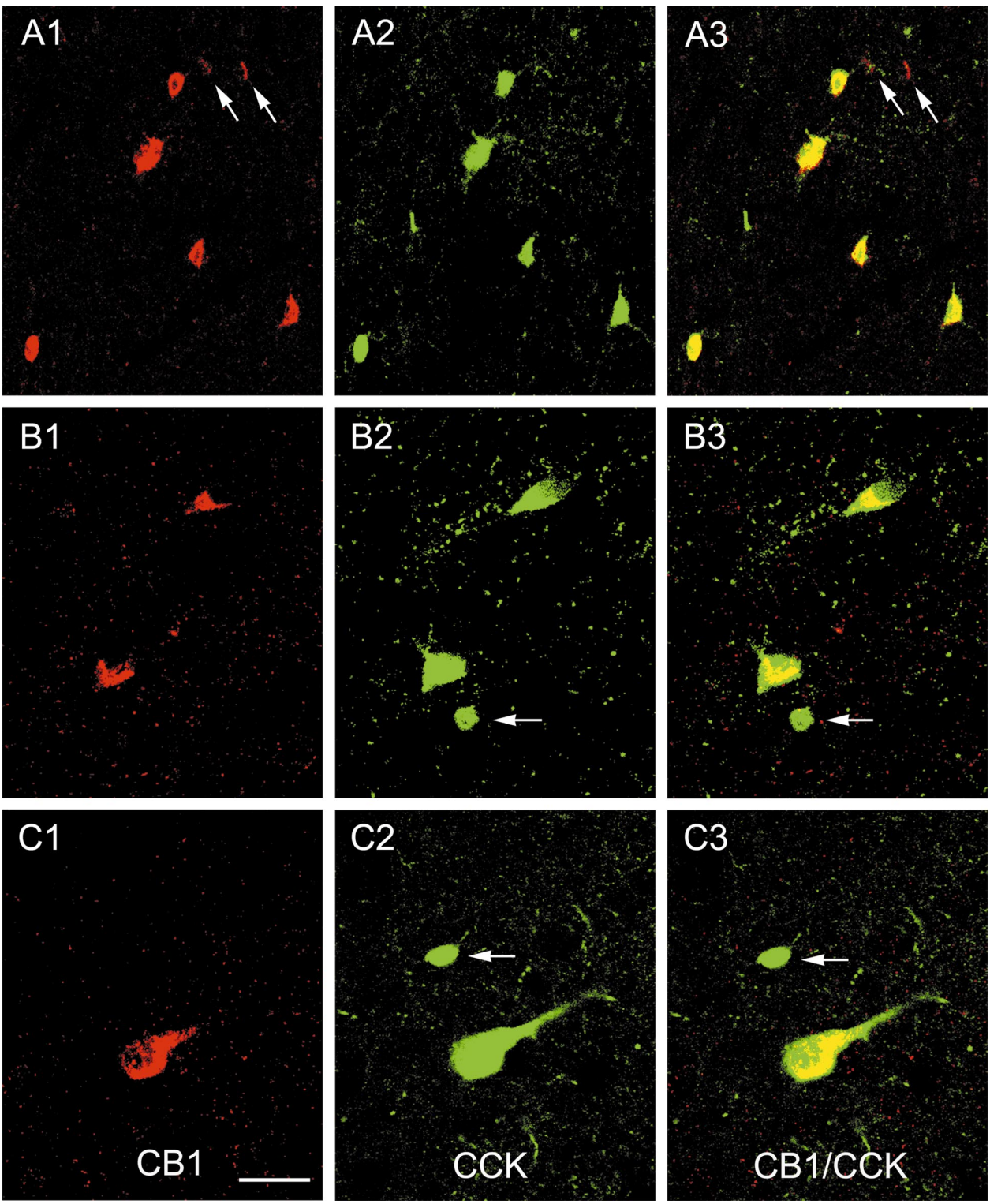

Fig. 6. Colocalization of CB1 cannabinoid receptor with CCK in different nuclei of the ABL using immunofluorescence confocal laser scanning microscopy. (A) Colocalization of CB1 and CCK in the lateral nucleus. (A1) Five intensely stained CB1+ somata and two less intensely labeled somata (arrows) in the lateral nucleus (red). (A2) Five CCK+ somata (green) in the same field. (A3) Merging of the red and green channels indicates that the five intensely stained CB1+ neurons exhibit CCK immunoreactivity (overlapping red and green is indicated in yellow), but that the two less intensely labeled CB1+ neurons do not contain CCK (arrows). (B) Colocalization of CB1 and CCK in the BLa. (B1) Two CB1+ somata (red) in the BLa. (B2) Two large CCK+ somata and one small CCK+ soma (arrow) in the same field (green). (B3) Merging of the red and green channels indicates that the two large CCK+ neurons (yellow), but not the small CCK+ neuron (green), exhibit CB1 immunoreactivity. (C) Colocalization of CB1 and CCK in the BLp. (C1) One CB1+ soma (red) in the BLp. (C2) One large CCK+ soma and one small CCK+ soma (arrow) in the same field (green). (C3) Merging of the red and green channels indicates that the large CCK+ neuron (yellow), but not the small CCK+ neuron (green), exhibits CB1 immunoreactivity. Scale bar = 50 μm in A, 25 μm in B and C.

determine which non-pyramidal subpopulation(s) express CB1.

The pattern of CB1 immunoreactivity in the ABL seen in the immunofluorescence investigations was similar to that observed in the immunoperoxidase studies. As in the latter studies, the most conspicuous CB1+ neurons were large multipolar non-pyramidal neurons. With the antibody dilution used in these immunofluorescence investigations, CB1 immunoreactivity in most pyramidal cells was not above background levels.

The pattern of immunoreactivity for CCK, CR, PV, and SOM matched that reported in previous immunoperoxidase studies of the rat ABL (McDonald, 1985b, 1994; Kemppainen and Pitkänen, 2000; McDonald and Betette, 2001). Most intensely stained CR+ non-pyramidal neurons were bipolar or bitufted neurons with small oval somata. Some lightly stained CR+ pyramidal neurons were observed but were easily distinguished from the intensely stained non-pyramidal CR+ cells at the antibody dilutions used in this study (McDonald and Mascagni, 2001). Only the non-pyramidal CR+ neurons were examined for CB1 immunoreactivity. Most SOM+ neurons were medium-sized multipolar or fusiform neurons. PV+ neurons were morphologically heterogeneous, but most were large to medium-sized multipolar neurons. Two subpopulations of CCK+ neurons were seen: (1) small oval neurons that closely resembled CR+ neurons (which will be termed type S neurons), and (2) large to medium-sized multipolar neurons (which will be termed type L neurons).

The main non-pyramidal neuronal subpopulation exhibiting CB1 immunoreactivity was the CCK+ population (Table 1, Fig. 6). Because some CB1+ neurons exhibited immunoreactivity that was barely above background levels, accurate counts of single-labeled CB1+ neurons could not be obtained. However, only about 10% of CB1+ neurons with moderate to high levels of CB1 immunoreactivity were not CCK+ in these preparations (BLa: 6.9%; Lvm: 11.3%) (Fig. 6A). Since the small oval type S CCK+ neurons were generally distinguishable from the larger multipolar type L CCK+ neurons, separate counts of colocalization were performed for each type. In both the BLa and Lvm, 100% of the neurons judged to be type L neurons exhibited intense CB1 immunoreactivity (Fig. 6). Although some type S CCK+ neurons were double-labeled (e.g., BLa: 14.3% of type S neurons [constituting 3.7% of all double-labeled CCK+ cells in the BLa]; Lvm: 10.0% of type S neurons [constituting 7.3% of all double-labeled CCK+ cells in the Lvm]) the level of CB1 immunoreactivity in these cells was usually lower than in the double-labeled type L CCK+ neurons. All of the single-labeled CCK+ neurons (CCK+/CB1−) in the lateral and basolateral nuclei were type S (Fig. 6). Since the lateral nucleus has a higher ratio of type S to type L CCK+ neurons, the percentage of CCK+ neurons exhibiting double labeling was lower than in the basolateral nucleus (Table 1).

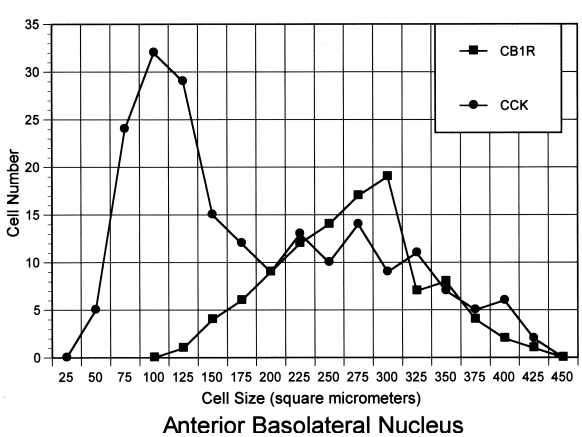

Fig. 7. Graph depicting the perikaryal cross-sectional areas of CCK+ neurons (circles; $n = 150$) and non-pyramidal CB1 receptor-immunoreactive neurons (CB1R; squares; $n = 100$) in the anterior subdivision of the basolateral nucleus. Note that the size distribution of CB1+ neurons appears to match that of the large CCK+ neurons, but not the small CCK+ neurons.

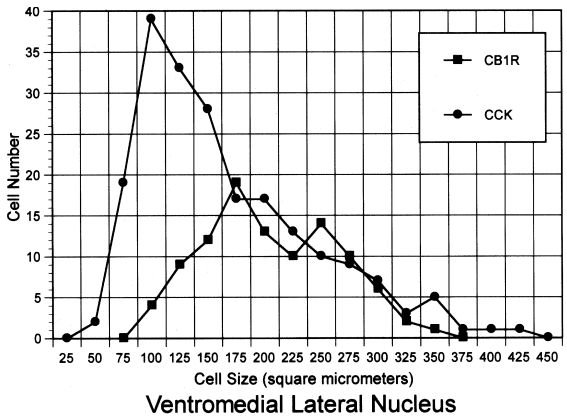

Fig. 8. Graph depicting the perikaryal cross-sectional areas of CCK+ neurons (circles; $n = 150$) and non-pyramidal CB1 receptor-immunoreactive neurons (CB1R; squares; $n = 100$) in the ventromedial subdivision of the lateral nucleus. Note that the size distribution of CB1+ neurons appears to match that of the large CCK+ neurons, but not the small CCK+ neurons.

Whereas none of the CR+, PV+, or SOM+ neurons expressed high levels of CB1 immunoreactivity, a moderate number of CR+ neurons in the Lvm (8.9%) and PV+ neurons in the BLa (9.5%) expressed low levels of immunoreactivity (Table 1).

Analysis of perikaryal sizes of CCK+ and CB1+ neurons in the basolateral amygdala

The confocal immunofluorescence studies suggested that CB1 immunoreactivity is mainly associated with the large to medium-sized CCK+ neurons, whereas most small CCK+ neurons are CB1-negative. In order to more objectively evaluate this observation, cross-sectional areas of CCK+ and CB1+ neurons were measured in immunoperoxidase-stained preparations. Consistent with the results of the subjective evaluation of neuronal morphology in the colocalization experiments, most CB1+ neurons were similar in size to the larger CCK+ cells (Figs. 7 and 8).

DISCUSSION

This investigation demonstrates that many pyramidal neurons in the ABL exhibit low levels of CB1 immunoreactivity and that there is a subpopulation of non-pyramidal neurons that has higher levels of immunoreactivity. In colchicine-injected brains there is a large accumulation of CB1+ material in the initial portion of the axon of the non-pyramidal neurons indicating that substantial amounts of the CB1 protein are transported down the axons of these cells. Dual localization immunohistochemical studies indicate that the great majority of these CB1+ non-pyramidal neurons are a subset of the CCK+ non-pyramidal neuronal population. Whereas none of the CR+, PV+, or SOM+ non-pyramidal neurons expressed high levels of CB1 immunoreactivity, a moderate number of CR+ neurons in the Lvm and PV+ neurons in the BLa expressed low levels of immunoreactivity.

Neuronal localization of CB1 immunoreactivity in the ABL

Although previous immunohistochemical studies of the rat forebrain have reported CB1+ neurons in the ABL (Pettit et al., 1998; Tsou et al., 1998a), these investigations did not provide sufficient morphological detail to permit the cell types to be identified. In the monkey ABL, however, Ong and Mackie (1999) reported that both pyramidal and non-pyramidal neurons were CB1+. The present study demonstrated that a population of medium-sized to large CCK+ non-pyramidal neurons (type L) exhibit robust CB1 immunoreactivity, and that lower levels of immunoreactivity were seen in a small percentage of other non-pyramidal cells, including small CCK+ neurons (type S), and in pyramidal projection neurons. These results are consistent with recent *in situ* hybridization studies that have found high levels of CB1 mRNA in a small number of ABL neurons and lower levels in the principal neurons of the ABL (Mailleux and Vanderhaeghen, 1992; Matsuda et al., 1993; Marsicano and Lutz, 1999).

The findings of the present investigation suggest that there are two distinct subpopulations of CCK+ non-pyramidal neurons that can be distinguished on the basis of CB1 immunoreactivity. Additional dual labeling immunofluorescence studies performed in the authors' laboratory (unpublished observations) indicate that these two subpopulations can also be distinguished on the basis on their content of calcium-binding proteins. Many type L CCK+ neurons contain calbindin, but not CR, whereas many type S CCK+ neurons contain CR, but not calbindin. The latter result is consistent with the finding that similar percentages of CR+ neurons and type S CCK+ neurons in the BLa and Lvm exhibit low levels of CB1 immunoreactivity.

When the results of these unpublished observations are taken into consideration, it is apparent that the recent findings of a dual localization *in situ* hybridization study of the mouse amygdala (Marsicano and Lutz, 1999) closely match the results of the present study. Thus, Marsicano and Lutz found that in the interneuronal (non-pyramidal) population expressing high CB1 mRNA levels, 98% expressed CCK mRNA, 73% expressed calbindin mRNA, but very few expressed PV or CR mRNA. Since previous immunohistochemical studies have shown that the great majority of the non-pyramidal CCK+ and CB+ neurons in the ABL are GABAergic (McDonald and Pearson, 1989; McDonald and Mascagni, 2001), it is not surprising that Marsicano and Lutz (1999) found that almost all of the neurons expressing high CB1 mRNA levels in the ABL also expressed GAD mRNA. In summary, both immunohistochemical and *in situ* hybridization studies agree that most robustly labeled CB1 neurons in the ABL are a discrete subpopulation of medium-sized to large GABAergic interneurons that contain CCK, and often calbindin.

Marsicano and Lutz (1999) also detected colocalization of CCK and CB1 mRNA in ABL pyramidal cells. We observed light CB1 immunoreactivity in ABL pyramidal neurons but no CCK immunoreactivity. Like cortical pyramidal cells, ABL pyramidal cells contain CCK mRNA but do not exhibit CCK-like immunoreactivity in their cell bodies (Schiffmann and Vanderhaeghen, 1991; Ingram et al., 1989). It has been suggested that there may be differential processing of the precursor peptide or rapid transport of CCK in these neurons (Schiffmann and Vanderhaeghen, 1991). Somata of cortical pyramidal cells can be stained with antibodies to pro-CCK, and with colchicine injections accumulation of CCK immunoreactivity is observed in their axon initial segments (Morino et al., 1994). Likewise, we have observed CCK immunoreactivity in the axon initial segments of ABL pyramidal cells in colchicine-injected animals (unpublished findings). However, no CCK immunoreactivity was seen in ABL pyramidal cells in the present study since none of the animals processed for CCK immunohistochemistry received colchicine injections and because the antibody used recognizes only the first five amino acids of CCK-8 rather than pro-CCK (Oeth and Lewis, 1990). As a result, colocalization of CCK and CB1 was only observed in non-pyramidal neurons.

Functional significance

Studies using changes in regional blood flow as an index of neuronal activity suggest that the ABL is the brain structure most affected by administration of the endocannabinoid anandamide (Stein et al., 1998). However, surprisingly little is known about how cannabinoids affect synaptic function in this region. Since previous anatomical, electrophysiological, and pharmacological studies have shown that the cell types of the ABL closely resemble those of the neocortex and hippocampus (Hall, 1972; Millhouse and DeOlmos, 1983; Carlsen and Heimer, 1986; Carlsen, 1988; Washburn and Moises, 1992; McDonald, 1992a; Rainnie et al., 1993), it seems likely that cannabinoids may act in similar ways in these regions. *In situ* hybridization studies in both the ABL and hippocampus have shown that high levels of CB1 mRNA are found in non-pyramidal CCK+ neurons and that low levels are found in pyramidal cells. While there is some disagreement as to whether there is CB1

immunoreactivity in hippocampal pyramidal neurons (Twitchell et al., 1997; Tsou et al., 1998a; Katona et al., 1999), it is clear that the cell population with the highest levels of CB1 immunoreactivity in the hippocampus (Tsou et al., 1998a; Katona et al., 1999) and ABL (present study) is a subpopulation of interneurons that contain CCK.

In the ABL there is little CB1 immunoreactivity in the dendrites of these CB1+/CCK+ neurons. The finding that there are accumulations of CB1+ material in the axon initial segments of the CB1+ neuronal population in the ABL in colchicine-injected brains indicates that most of the CB1 receptor protein is transported down the axons of these cells. In the present study CB1+ axon terminals could not be identified in the ABL. However, using a different antibody, Egertová and Elphick (2000) reported that the amygdala contained a network of CB1+ fibers that surrounded neuronal somata. This is consistent with the localization of the CB1 in CCK+ neurons, and the observation that CCK+ terminals are frequently found forming pericellular baskets around the somata of ABL pyramidal cells (unpublished observations). As in the hippocampus (Katona et al., 1999), these presynaptic CB1 receptors may reduce GABA release upon postsynaptic pyramidal cells. It is also of interest in this regard that recent immunohistochemical studies have demonstrated that the somata of ABL pyramidal neurons contain fatty acid amide hydrolase, an enzyme that catalyzes the synthesis and breakdown of anandamide (Tsou et al., 1998b).

There is also evidence that glutamate release is reduced in hippocampal pyramidal cells by similar cannabinoid-mediated presynaptic mechanisms (Shen et al., 1996; Twitchell et al., 1997). This finding suggests that the CB1 receptor found in pyramidal cells (Mailleux and Vanderhaeghen, 1992; Matsuda et al., 1993; Twitchell et al., 1997; Marsicano and Lutz, 1999) may be associated with the axon terminals of these neurons in the hippocampus, and perhaps in the ABL. In fact, Ong and Mackie (1999) observed CB1+ axon terminals forming asymmetric (presumably glutamatergic) synapses in the primate ABL, although it is possible that such axons could actually represent cortical or thalamic afferents.

In general, treatments that increase the excitability of the ABL facilitate emotional learning and increase anxiety, whereas treatments that decrease the excitability of the ABL impair emotional learning and produce anxiolytic effects (Davis et al., 1994). The putative presence of CB1 receptors on the axon terminals of GABAergic CCK+ non-pyramidal interneurons suggests that cannabinoids might increase the excitability of the ABL by decreasing the amount of GABA released from these neurons. On the other hand, the possible existence of CB1 receptors on the axon terminals of glutamatergic pyramidal cells suggests that cannabinoids might decrease the excitability of the ABL by decreasing glutamatergic neurotransmission. Thus, it is difficult to predict the overall effect of cannabinoid administration on the function of the ABL. It also has to be taken into consideration that cannabinoids have been shown to influence many of the neural systems that send afferents to the ABL including glutamatergic projections from the cortex, dopaminergic and noradrenergic projections from the brain stem, and cholinergic projections from the basal forebrain (Breivogel and Childers, 1998). Because of these diverse targets of cannabinoids in the ABL, as well as in other intra-amygdaloid (e.g., the central nucleus: McGregor et al., 1998) and extra-amygdaloid targets, it is perhaps not surprising that anxiogenic, anxiolytic, or biphasic effects that are dose- and test-dependent have been described in animals and humans upon cannabinoid administration (Chaperon and Thiébot, 1999).

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