European Journal of Neuroscience, Vol. 11, pp. 4213–4225, 1999

Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain

Giovanni Marsicano and Beat Lutz
Max-Planck-Institute of Psychiatry, Kraepelinstrasse 2-10, 80804 Munich, Germany

Keywords: calcium-binding proteins, cholecystokinin, GABAergic system, hippocampus, *in situ* hybridization

Abstract

Cannabinoids can modulate motor behaviour, learning and memory, cognition and pain perception. These effects correlate with the expression of the cannabinoid receptor 1 (CB1) and with the presence of endogenous cannabinoids in the brain. In trying to obtain further insights into the mechanisms underlying the modulatory effects of cannabinoids, CB1-positive neurons were determined in the murine forebrain at a single cell resolution. We performed a double *in situ* hybridization study to detect mRNA of CB1 in combination with mRNA of glutamic acid decarboxylase 65k, neuropeptide cholecystokinin (CCK), parvalbumin, calretinin and calbindin D28k, respectively. Our results revealed that CB1-expressing cells can be divided into distinct neuronal subpopulations. There is a clear distinction between neurons containing CB1 mRNA either at high levels or low levels. The majority of high CB1-expressing cells are GABAergic (γ-aminobutyric acid) neurons belonging mainly to the cholecystokinin-positive and parvalbumin-negative type of interneurons (basket cells) and, to a lower extent, to the calbindin D28k-positive mid-proximal dendritic inhibitory interneurons. Only a fraction of low CB1-expressing cells is GABAergic. In the hippocampus, amygdala and entorhinal cortex area, CB1 mRNA is present at low but significant levels in many non-GABAergic cells that can be considered as projecting principal neurons. Thus, a complex mechanism appears to underlie the modulatory effects of cannabinoids. They might act on principal glutamatergic circuits as well as modulate local GABAergic inhibitory circuits. CB1 is very highly coexpressed with CCK. It is known that cannabinoids and CCK often have opposite effects on behaviour and physiology. Therefore, we suggest that a putative cross-talk between cannabinoids and CCK might exist and will be relevant to better understanding of physiology and pharmacology of the cannabinoid system.

Introduction

The characteristic behavioural effects of cannabinoids are mediated by CB1, the 'brain type' cannabinoid receptor, a seven-transmembrane G-protein-coupled receptor (Matsuda *et al*., 1990). Biochemical studies showed that a G_i protein transduces the cannabimimetic inhibition of adenylate cyclase (Howlett, 1995). CB1 causes a cAMP-independent inhibition of N- and Q-type voltage-dependent Ca^{2+} channels (Mackie & Hille, 1992; Felder *et al*., 1995), and a cAMP-dependent stimulation of inwardly rectifying K^+ channels (Deadwyler *et al*., 1993; Mackie *et al*., 1995). These signal transduction pathways can explain some effects of cannabinoids in the central nervous system, e.g. inhibition of acetylcholine, noradrenaline and glutamate release, e.g. in the hippocampus (Gifford & Ashby, 1996; Shen *et al*., 1996; Schlicker *et al*., 1997). CB1 is also coupled to the activation of adenylate cyclase in striatal neurons upon simultaneous stimulation of the dopamine receptor D2 (Glass & Felder, 1997), possibly due to differential regulation of adenylate cyclase isozymes by CB1 (Rhee *et al*., 1998). Endogenous ligands for CB1 have been discovered (Devane *et al*., 1992; Stella *et al*., 1997), sharing many of the properties of plant-derived and synthetic cannabinoids. Therefore, a cannabinoid system has been proposed to play an important neuromodulatory role in brain physiology (for review see Di Marzo *et al*., 1998).

Correspondence: Dr B. Lutz, as above.
E-mail: lutz@mpipsykl.mpg.de

Received 10 June 1999, revised 12 August 1999, accepted 13 August 1999

Several pharmacological effects of cannabinoids (e.g. decrease of learning behaviour) are thought to be mediated by CB1 expressed in forebrain structures. *In situ* hybridization (ISH) and immunohistological studies described CB1 expression in the rat forebrain in several cortical areas and in several non-cortical regions, e.g. the striatum and some thalamic and hypothalamic nuclei (Mailleux & Vanderhaegen, 1992; Matsuda *et al*., 1993; Tsou *et al*., 1998; Pettit *et al*., 1998). The pattern of CB1 expression in the mouse forebrain, however, has not yet been described in detail.

Determination of CB1-positive neurons at a single cell resolution is a prerequisite for better understanding of the physiological role of the cannabinoid system in mouse. Based on morphological features, CB1-expressing neurons in the hippocampus and other cortical regions have been proposed to be GABAergic (Matsuda *et al*., 1993; Tsou *et al*., 1998), but conclusive evidence for this notion is still missing. GABAergic neurons contain γ-aminobutyric acid (GABA) and express glutamic acid decarboxylase (GAD), and are classified according to criteria, e.g. morphology, orientation of afferent and efferent fibres, type of innervating neurons, and presence of distinct calcium-binding proteins and neuropeptides (for review see Freund & Buszáki, 1996). Morphological criteria are difficult to evaluate in ISH experiments, but detection of coexpression of CB1 with various markers can be well accomplished using double ISH technique, as transcripts are generally localized in the soma of the cells. Thus, we investigated which CB1-expressing cells are GABAergic and whether CB1 is coexpressed with other neuronal markers.

Recent immunohistochemical studies in rats suggest that a fraction of CB1-positive cells in the hippocampus has morphological features

of basket cells, which are also called perisomatic inhibitory cells (Tsou *et al*., 1998). Typically, basket cells are forming 'basket' nets around principal cell bodies. From the neurochemical point of view, basket cells are GABAergic neurons that can further be divided into two distinct subpopulations: (i) basket cells containing the calcium-binding protein parvalbumin (PV); and (ii) basket cells containing the neuropeptide cholecystokinin (CCK, Soriano *et al*., 1990; Gulyás *et al*., 1991; Freund & Buszáki, 1996). In addition, Tsou *et al*. (1998) described CB1-positive neurons possessing fibres with orientations different from typical basket cells. Thus, CB1-expressing cells might belong to an additional subpopulation of hippocampal GABAergic neurons. Two putative candidates for these CB1-containing non-basket cells are calbindin D28k (C28)-positive mid-proximal dendritic inhibitory cells (Gulyás & Freund, 1996) and calretinin (CRT)-positive interneurons that are specialized in innervating other interneurons (Gulyás *et al*., 1996). Therefore, we performed double ISH experiments on the hippocampus, but also on other cortical and some selected non-cortical areas of the mouse forebrain, using radioactive riboprobes for GAD 65, CCK, C28, PV and CRT, respectively, together with a non-radioactive CB1 riboprobe.

Materials and methods

Tissue preparation

Adult mice (3–5 months old; FVB/N and CD1 strains) were killed by cervical dislocation. Brains were removed, snap-frozen on dry-ice and stored at –80 °C. After removing from –80 °C, brains were mounted on Tissue Tek (Polysciences, PA, USA), and 14-µm-thick coronal sections were cut from the forebrain on a cryostat Microtome HM560 (Microm, Germany). Sections were mounted onto frozen SuperFrost/Plus slides (Fisher Scientific, Germany), dried on a 42 °C warming plate and stored at –20 °C until used.

Synthesis of probes

Both radioactive (^{35}S) and non-radioactive (fluorescein isothiocyanate, FITC) labelled riboprobes were used. Probes were generated by RT-PCR from cDNA derived from total mouse brain RNA. For each probe, GenBank accession number, length and sequence of the primers are listed below; nucleotide positions are identical to those used in deposited sequences in GenBank: CB1, accession number U22948, 1530 bp from 152 to 1682 (forward primer 5′-GTT GAG CCT GGC CTA ATC AAA, reverse primer 5′-GTT GAC CGA ACC TCT GTT TTC); GAD 65, accession number D42051, 1041 bp from 1055 to 2096 (forward primer 5′-GGC GAT GGA ATC TTT TCT CCT, reverse primer 5′-CGA GGC GTT CGA TTT CTT CAA); PV, accession numbers X54613 and X67141, 802 bp from 11 of accession number X54613 to 510 of accession number X67141 (forward primer 5′-CAG CGC TGA GGA CAT CAA GAA, reverse primer 5′-GAT CTA GCT AGT CCT GAA GGA); CRT, accession number X73985, 1023 bp from 35 to 1058 (forward primer 5′-CCG ACC GAA GAG AAT TTC CTT, reverse primer 5′-GGG AAG CCA AAG AGA AAA GGA); C28, accession number M21531, 1016 bp from 166 to 1182 (forward primer 5′-GAG ATC TGG CTT CAT TTC GAC, reverse primer 5′-GAT GAA CAC TTG GAT TTC CC); CCK, accession number X59520 and X59522, 411 bp from 188 of accession number X59520 to 151 of accession number X59522 (forward primer 5′-ACT TAG CTG GAC TGC AGC TT, reverse primer 5′-GGA CTA CGA ATA CCC ATC GTA). PCR products were cloned into pBluescript KS$^-$ (Stratagene, CA, USA) and used as templates for riboprobe synthesis. The identity of all fragments was checked by sequencing. Linearized template DNA was phenol-extracted, precipitated, resuspended in diethyl pyrocarbonate (DEPC)-treated H_2O at a concentration of 1 µg/µL, and stored at –20 °C. For ^{35}S-labelled riboprobes, *in vitro* transcription was carried out for 3 h at 37 °C in a total volume of 30 µL containing 1.5 µg of linearized DNA, 1 × transcription buffer, 1 mM of rATP/rCTP/rGTP each, 16.7 mM dithiothreitol (DTT), 40 units RNasin (Promega, WI, USA), 10 µL of ^{35}S-thio-rUTP (NEN, MA, USA; 1250 Ci/mmol), and 30 units of T7 or T3 RNA polymerase (Roche Molecular Diagnostics, Germany). For FITC-labelled riboprobes, *in vitro* transcription was carried out for 3 h at 37 °C in a total volume of 50 µL containing 1.5 µg of linearized DNA, 1 × transcription buffer, 0.35 mM FITC-rUTP (Roche Molecular Diagnostics), 0.65 mM rUTP, 1 mM rATP/rCTP/rGTP each, 80 units RNasin (Promega), and 100 units of T3 or T7 RNA polymerase. Reactions were treated with 20 units of RNase-free DNaseI (Roche Molecular Diagnostics) for 15 min at 37 °C, and labelled probes were purified by ammonium acetate precipitation. Restriction enzymes (New England Biolabs, MA, USA) used for linearization and RNA polymerases used for each probe were as follows: CB1 sense, PstI, T7; CB1 antisense, BamHI, T3; GAD 65 sense, EcoRI, T7; GAD 65 antisense, BamHI, T3; PV sense, BamHI, T3; PV antisense, HindIII, T7; CRT sense, EcoRI, T7; CRT antisense, BamHI, T3; C28 sense, EcoRV, T7; C28 antisense, BamHI, T3; CCK sense, BamHI, T3; CCK antisense, EcoRI, T7. Using these probes in ISH experiments, sense controls did not give any detectable signals, and antisense probes gave distribution patterns identical to those already published in rat or mouse (data not shown).

In situ *hybridization*

Slides were warmed up for 30 min at RT, fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS, containing, in mM: NaCl, 136; KCl, 2.7; Na_2HPO_4, 10; KH_2PO_4, 1.8, pH 7.4), rinsed three times in PBS, incubated for 10 min in 0.1 M triethanolamine–HCl (pH 8.0) to which 0.63 mL of acetic anhydride was added dropwise, rinsed twice in standard saline citrate (2 × SSC, where 1 × SSC contains 150 mM NaCl, 15 mM Na_3 citrate, pH 7.4), dehydrated in graded series of ethanol, delipidized in chloroform for 5 min, rinsed in 100% and 95% ethanol, and air-dried. Hybridization was carried out overnight at 64 °C in 90 µL of hybridization buffer containing ^{35}S-labelled riboprobe (35 000–70 000 c.p.m./µL) and/or FITC-labelled riboprobe (1 µg/mL). Hybridization buffer consisted of 50% formamide, 20 mM Tris–HCl, pH 8.0, 0.3 M NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, 10% dextran sulphate (D8906, Sigma, Germany), 0.02% Ficoll 400 (F2637, Sigma), 0.02% polyvinylpyrrolidone (MW 40 000, PVP40, Sigma), 0.02% bovine serum albumin (BSA, A6793, Sigma), 0.5 mg/mL tRNA (Roche Molecular Diagnostics), 0.2 mg/mL fragmented herring sperm DNA and 200 mM DTT.

After incubation in a humid chamber, slides were rinsed four times for 5 min each in 4 × SSC at RT, incubated for 30 min at 37 °C in 20 µg/mL of RNaseA in 0.5 M NaCl, 10 mM Tris–HCl, pH 8.0, 5 mM EDTA, rinsed at RT in decreasing concentrations of SSC (1, 0.5 and 0.1 × SSC) containing 1 mM DTT, washed twice for 30 min each at high stringency in 0.1 × SSC/1 mM DTT at 64 °C and washed twice for 10 min at RT in 0.1 × SSC.

At this point, ^{35}S-labelled slides were dehydrated in graded ethanol series, air-dried and exposed to Biomax MR film (Kodak, Germany). On the next day, slides were dipped in photographic emulsion (NTB-2 from Kodak, diluted 1 : 1 in distilled water). After exposition for 5–20 days at 4 °C, slides were developed for 3 min (D-19, Kodak), fixed for 6 min (Kodak fixer), rinsed for

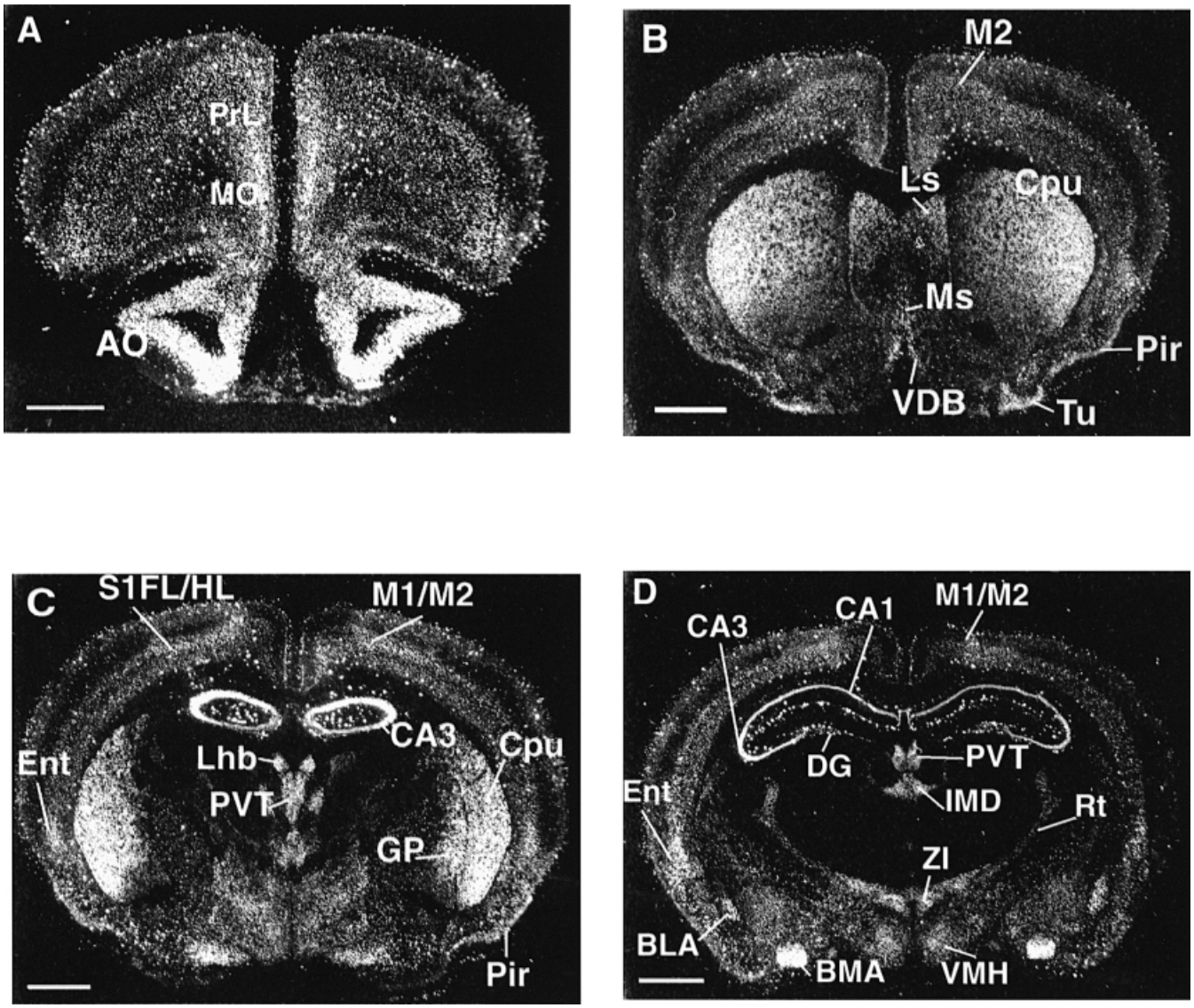

FIG. 1. Dark-field micrograph of coronal sections from adult mouse forebrain showing the distribution of CB1 mRNA, as detected by ISH with a ^{35}S-radiolabelled riboprobe for CB1. Note the high levels of CB1 expression in the anterior olfactory nucleus (A), neocortex (A–D), dorsolateral caudate putamen (B and C), hippocampus (C and D), entorhinal/perirhinal cortex area (C and D), and basolateral and basomedial amygdaloid nuclei (D). Scale bars, 1 mm.

30 min in tap water and air-dried. Slides were mounted in DPX (Prod. 360294H, BDH, England).

For non-radioactive (FITC) and double-labelled (^{35}S/FITC) ISH experiments, slides were soaked after the last 0.1 × SSC wash at RT (see above) in buffer 1 (100 mM maleic acid, pH 7.5, 150 mM NaCl) for 1 min at RT, blocked in buffer 2 (1% blocking reagent in buffer 1, Roche Molecular Diagnostics) for 30 min at RT, incubated with alkaline phosphatase-conjugated anti-FITC antibody (Roche Molecular Diagnostics) diluted 1 : 3000 in buffer 2 for 2 h at RT, washed twice in buffer 1 for 15 min at RT, washed twice in buffer 3 (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 2 min at RT. Chromogenic reaction was carried out with Vector Red kit (Vector Laboratories) at RT for 24–36 h with two changes of staining solution. The reaction was stopped by a 10-min incubation in 0.1 M Tris–HCl, pH 8.2, followed by 10 min in 2.5% glutaraldehyde in PBS and four washes for 15 min in 0.1 × SSC. In double ISH experiments, slides were treated at this point like slides from radioactive ISH, with dehydration, dipping and developing. At the end, slides were counterstained for 10 s in 0.1% aqueous toluidine blue solution, rinsed twice for 10 s in tap water, destained for 45 s in 70% ethanol (containing 1 drop of 100% acetic acid per 100 mL), rinsed for 45 s in 100% ethanol and air-dried. Slides from double ISH were mounted in Kaiser's gelatin (Merck, Germany).

Numerical evaluation of coexpression

As CB1 is expressed at various levels, cells stained with FITC-labelled riboprobe were classified according to the following criteria. Cells expressing CB1 at high levels (termed high CB1-expressing cells) were considered to be those showing a round-shaped and intense red staining surrounding the nucleus or even covering the entire nucleus (e.g. see Fig. 2D and F). Cells expressing CB1 at low levels (termed low CB1-expressing cells) were defined as cells clearly stained above background levels and in a discontinuous shape and/or at uniform and low intensity of staining (e.g. see Fig. 2D and F). Principal cells in CA1/CA3 regions of the hippocampus displayed low and uniform levels of expression, with a slightly stronger intensity in CA3. Concerning numerical evaluation of the double ISH

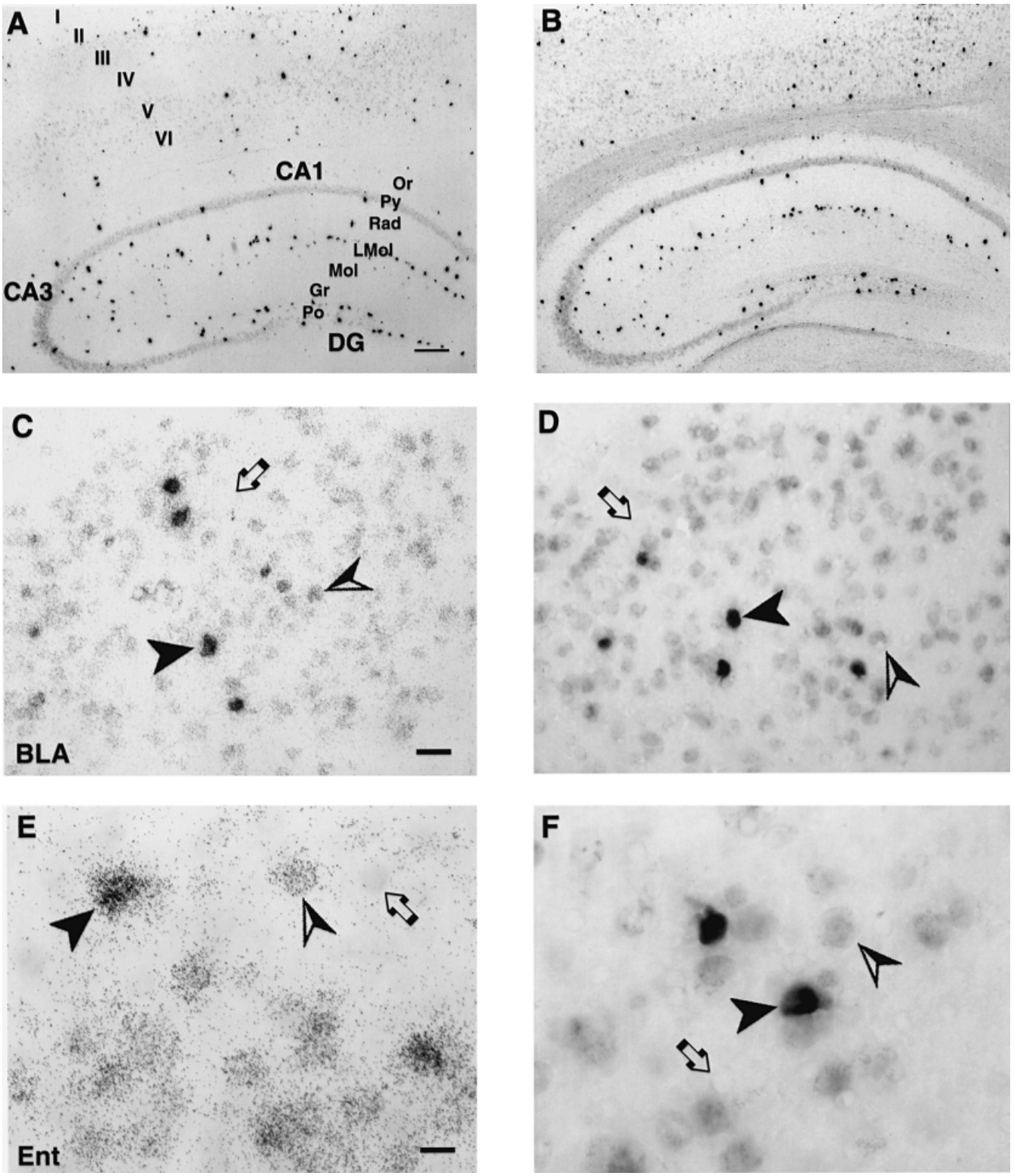

FIG. 2. Bright-field micrograph of coronal sections showing CB1 distribution and levels of expression in cortical and limbic structures. Parallel coronal sections from three different brain regions were hybridized either with a ^{35}S-radiolabelled (A, C and E) or FITC-labelled (B, D and F) riboprobe. (A and B) Hippocampus and dorsal neocortex; (C and D) basolateral amygdaloid nucleus; (E and F) entorhinal cortex. Note that the number of labelled cells and intensity of signals are the same when comparing the two ISH techniques. Both high and low CB1-expressing neurons can clearly be classified. The light grey area between CA1 and the cortex in (B) is due to unspecific staining of white matter. Filled arrowhead, high CB1-expressing cell; open arrowhead, low CB1-expressing cell; open arrow, CB1-negative cell. Scale bars, 150 µm (A and B); 40 µm (C and D); 10 µm (E and F).

experiments, these principal cells were not included. Sections were analysed on a Leica DMRB microscope. All CB1-positive cells were checked for coexpression with the following markers: GAD 65, CCK, PV, CRT or C28, respectively. Cells were evaluated and classified as

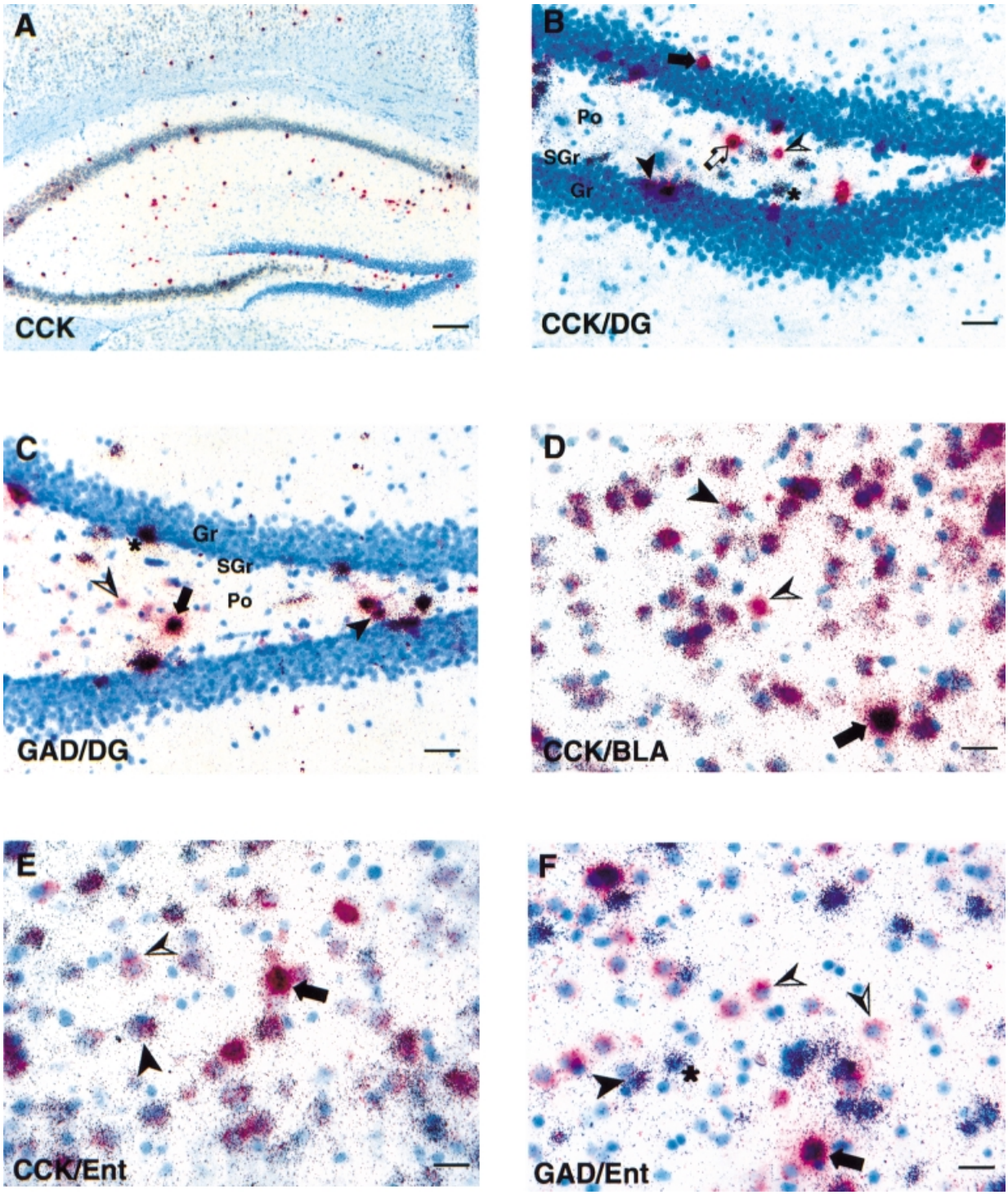

FIG. 3. Bright-field micrograph of coronal sections showing examples of coexpression of CB1 (red staining) with CCK and GAD 65 (silver grains), respectively, as detected by double ISH. All sections were counterstained with toluidine blue. (A) Overview of hippocampus double-stained for CB1 and CCK. Note the expression of CCK in pyramidal cells of CA1/CA3 regions. (B) Higher magnification of the dentate gyrus from A showing a high degree of coexpression of CB1 with CCK (filled arrow, filled arrowhead). (C) Dentate gyrus double-stained for CB1 and GAD 65, showing complete coexpression for high CB1-expressing cells. (D) Double staining for CB1 and CCK in the basolateral amygdaloid nucleus. Note that CB1 and CCK display almost complete coexpression. (E) Double staining for CB1 and CCK in the entorhinal cortex area, showing a high degree of coexpression. (F) Double staining for CB1 and GAD 65 in the entorhinal cortex area, showing that low CB1-expressing cells do colocalize partly with GAD 65. This is in contrast to CCK as shown in E. Filled arrow, high CB1-expressing cell coexpressing CCK or GAD 65; filled arrowhead, low CB1-expressing cell coexpressing CCK or GAD 65; open arrow, high CB1-expressing cell not coexpressing CCK or GAD 65; open arrowhead, low CB1-expressing cells not coexpressing GAD 65 or CCK; asterisks, cells expressing only CCK or GAD 65. Scale bars, 150 μm (A); 40 μm (B and C); 20 μm (D–F).

high CB1-expressing cells or low CB1-expressing cells with or without coexpression of the above markers, respectively. In the hippocampus, CCK- and GAD 65-positive cells were also evaluated for coexpression with CB1.

Results

CB1 expression in the forebrain

In the hippocampus (Figs 1D, and 2A and B), both low and high CB1-expressing cells were detected. Pyramidal cells (Py) express low levels of CB1, while cells with intensity of signal ranging from low to very high were observed in all layers of the hippocampus, mostly in the subgranular layer of the dentate gyrus (SGr, Fig. 3C) and the lacunosum-molecular layer of CA1 and CA3 regions (LMol, Fig. 2A). The neocortex showed the presence of scattered low and high expressing cells, located primarily in layers II–III and V–VI (Figs 1D and 2A). Few low CB1-expressing cells are scattered in layers I and IV. CB1-positive cells are more abundant in the primary and secondary motor (M1/M2) and sensory areas (S1FL/HL, Fig. 1C and D). CB1 is also highly expressed in olfactory areas (Fig. 1A). Low CB1-expressing cells are distributed uniformly in the entire anterior olfactory nucleus (AO) with the presence of many scattered cells expressing high levels of mRNA (data not shown). Rather high numbers of CB1-positive cells (both low and high CB1-expressing cells) were also observed in the piriform cortex (Pir) and olfactory tubercle (Tu, Fig. 1B and C). In the amygdaloid region, not all nuclei showed equal intensities of expression. Both anterior (Figs 1D, and 2C and D) and posterior parts of the basolateral amygdaloid nucleus (BLA, data not shown) contain a very high number of low CB1-expressing cells uniformly distributed, and a rather high number of scattered high CB1-expressing cells. The basomedial amygdaloid nucleus (BMA) showed a similar pattern of CB1 expression (Fig. 1D), while other amygdaloid nuclei contain lower levels of expression (data not shown). Entorhinal and perirhinal cortical areas (Ent) showed a very high number of both low and high CB1-expressing cells (Figs 1C and D, and 2E and F). Non-cortical areas, e.g. the striatum (dorsolateral caudate putamen, Cpu, and globus pallidus, GP) and hypothalamus (ventromedial, VMH and anterior hypothalamic nucleus) showed the presence of low CB1-expressing cells that are uniformly distributed at high cell density (Fig. 1D). Expression levels in other hypothalamic areas and in the thalamus are relatively low compared with other forebrain regions. Rather strong hybridization signals were detected in the septal region (lateral, Ls, and medial septum, Ms, and vertical and horizontal nuclei of the diagonal band, VDB, Fig. 1B). Rather high levels of expression were also observed in hypothalamic regions, e.g. the medial and lateral preoptic nucleus, magnocellular preoptic nucleus and hypothalamic nucleus (not shown). More caudally, the premammillary nucleus showed low and uniform levels of expression (not shown). Scattered, low CB1-expressing cells were observed in the lateral hypothalamus (Fig. 1D). Weak signals were also observed in thalamic regions, e.g. the paraventricular thalamic nucleus (PVT), lateral habenula (Lhb), reticular thalamic nucleus (Rt), zona incerta (ZI) and intermediodorsal thalamic (IMD, Fig. 1C and D). In summary, the pattern of CB1 expression in mouse was found to be very similar, if not identical, to that observed in rats (Matsuda *et al*., 1993).

We then compared the distribution, number and intensities of ISH signals generated with radioactive and non-radioactive riboprobes specific for CB1. Representative examples are shown in Fig. 2. No differences were detected between these two techniques, and thus, non-radioactive riboprobes for CB1 were further used for double ISH experiments. For a detailed analysis of coexpression of CB1 with five neuronal markers, five cortical (hippocampus, neocortex, anterior olfactory nucleus, entorhinal cortex area and amygdaloid area) and two non-cortical areas (dorsolateral caudate putamen and ventromedial hypothalamic nucleus) were chosen because of their high expression levels and the proposed functional relevance of these regions in cannabinoid physiology and pharmacology (Breivogel & Childers, 1998).

CB1 expression in distinct interneuronal subpopulations of the hippocampus

CB1 and GAD 65 do not colocalize in pyramidal cells of CA1/CA3, as only CB1 shows expression in these cells. In CA1/CA3 regions outside the pyramidal cell layer, we observed 100% coexpression of CB1 with GAD 65, regardless of whether CB1 expression levels were high or low (Table 1). In the dentate gyrus area, colocalization was also 100% for cells expressing CB1 at high levels, while low CB1-expressing neurons only coexpress GAD 65 at 48.8% (Fig. 3C and Table 1, also see note in Table 1). Double-positive cells are located in granular and subgranular layers of the dentate gyrus. GAD 65-negative, but CB1-positive cells contain only low levels of CB1 and are located in the polymorph layer of the dentate gyrus. These cells are often mistaken for interneurons, but are actually thought to be 'misplaced' principal cells (Tóth & Freund, 1992; Freund & Buszáki, 1996). Therefore, CB1 expression in these scattered cells is consistent with the general observation that all principal cells of the CA1/CA3 area express CB1 at low levels, but lack GAD 65 expression. Table 2 shows the percentage of CB1 expression among the GABAergic cell population. In the CA1/CA3 area, 42–43% of GAD-65-expressing cells also contain mRNA coding for CB1, while the value decreases to 37.8% in the dentate gyrus. In summary, these results show that, with the exception of principal cells in CA1/CA3 and cells in the polymorph layer, all CB1-expressing cells in the hippocampus are GABAergic interneurons and CB1-positive cells represent ~40% of GABAergic neurons.

In all pyramidal cells of the CA1/CA3 region, CB1 and CCK (Figs 2A and B, and 3A) show full coexpression. In hippocampal cells outside the pyramidal cell layer, CCK mRNA is also highly coexpressed with CB1, as shown in Fig. 3A and B and Table 1. Low CB1-expressing cells show ~70% colocalization in CA1/CA3 and 47% in the dentate gyrus. In high CB1-expressing cells, values increase to 91 and 94% in CA1 and CA3, respectively, and to 73% in the dentate gyrus. Table 2 shows that the fraction of CB1-expressing cells among all CCK-containing neurons is also very high: 85 and 83% and >50% in CA1, CA3 and the dentate gyrus, respectively. CCK-containing interneurons in the hippocampus and dentate gyrus have been described as a specific subset of basket cells (Gulyás *et al*., 1991). According to our study, a large fraction of CB1-expressing cells belongs to this particular type of hippocampal interneurons. In addition, considering that pyramidal cells in CA1/CA3 do express both CB1 and CCK (Fig. 3A), a very high fraction of all CCK-positive cells in the hippocampus do also express CB1.

Colocalization experiments with calcium-binding proteins resulted in a rather different situation. In the hippocampus, PV and CRT are coexpressed with CB1 only at a very low percentage (Table 3 and Fig. 4C). Together with the results obtained with CCK, these observations showed that hippocampal CB1-positive cells can be classified as a subset of basket cells being CCK positive and PV negative (Gulyás *et al*., 1991). Because another subtype of hippocampal interneurons, the axo-axonic cells, was described to contain PV, it is clear from our study that CB1-positive cells belong only to a negligible extent to this interneuronal subtype. Instead, C28 showed a significant percentage of colocalization, in the range of 22–32% (Table 3 and Fig. 4A and B). C28-expressing cells in the

TABLE 1. Percentage of coexpression of GAD 65 and CCK with CB1 in adult mouse forebrain

	Coexpression in cells expressing low CB1 (%)				Coexpression in cells expressing high CB1 (%)			
	GAD 65	(*n*)	CCK	(*n*)	GAD 65	(*n*)	CCK	(*n*)
Hippocampal CA1 area*	100	(>200)	65.1	(235)	100	(>200)	91.1	(264)
Hippocampal CA3 area*	100	(>400)	72.6	(357)	100	(>400)	93.8	(326)
Dentate gyrus	48.8‡	(249)	47.0	(120)	100	(>100)	72.9	(86)
Layers II–III of neocortex	100	(>400)	77.1	(905)	100	(>400)	97.4	(572)
Layers V–VI of neocortex	100	(>400)	86.9	(900)	100	(>400)	99.6	(421)
Anterior olfactory nucleus	35.2	(212)	89.5	(244)	100	(>100)	100	(80)
Entorhinal cortex area	22.7	(1249)	95.0	(1211)	95.6	(114)	98.2	(114)
Amygdaloid area	See note§		89.1	(571)	95.2	(115)	98.2	(130)
Striatum†	~100		0.0					
Hypothalamus†	0.0		0.0					

Percentages indicate cells expressing low or high levels of CB1 that were also labelled with riboprobes specific for GAD 65 or CCK (*n* = number of analysed cells). For both markers, data were collected from 35 sections from two different brains. *Principal cells in CA1/CA3 areas express low levels of CB1 mRNA and are not included in coexpression data. CCK, but not GAD 65 is expressed in these cells. †Due to the uniform distribution of low CB1-expressing cells in the dorsolateral part of the striatum and the ventromedial and anterior hypothalamic nuclei, numbers in these areas reflect an estimation only. ‡Cells expressing low amounts of CB1 but not GAD 65 are present only in the polymorph layer of the dentate gyrus. In the granular layer, coexpression is 100%. §CB1/GAD 65 coexpression varies in different amygdaloid nuclei: basolateral posterior part, 42.7 (103); basolateral anterior part, 25.8 (543); basomedial anterior part, 15.0 (314).

TABLE 2. Percentage of coexpression of CB1 in GAD 65-positive and CCK-positive cells in adult mouse forebrain

	Coexpression of CB1 (%)			
	CB1/GAD	(*n*)	CB1/CCK	(*n*)
CA1 area*				
Low CB1	16.5		37.2	
High CB1	26.9		47.8	
Total	43.4	(1154)	85.0	(460)
CA3 area*				
Low CB1	20.7		37.6	
High CB1	21.3		45.3	
Total	42.0	(1366)	82.9	(683)
Dentate gyrus				
Low CB1	23.2		26.8	
High CB1	14.6		26.1	
Total	37.8	(387)	52.9	(187)

Percentages indicate the total fraction of CB1-expressing cells among the GAD 65- and CCK-positive populations (*n* = number of analysed cells). Low and high, respectively, indicate the percentages of cells expressing CB1 at low or high levels among the total number of GAD- and CCK-expressing cells. Total means the percentage of all CB1-expressing cells among the total number of GAD- and CCK-positive cells. * Principal cells in the CA1/CA3 areas express low levels of CB1 mRNA and are not included in coexpression data. GAD 65 is not expressed in these cells, but CCK is expressed.

hippocampus were described as interneurons innervating dendrites of pyramidal cells (Gulyás & Freund, 1996), but they are not considered as basket cells, as these cells innervate mostly the soma of principal neurons (Soriano *et al*., 1990).

CB1 expression in neuronal subpopulations in other cortical regions

Neocortex

In the entire neocortex, nearly all CB1-positive cells also express GAD 65 (Table 1). Similarly to the hippocampus, coexpression with CCK is >99% for high CB1-expressing cells in layers V–VI, but is decreased to 77% for low CB1-expressing cells in layers II–III (Table 1). No apparent differences in degree of coexpression were observed in various neocortical subregions. PV and CRT showed very low coexpression with CB1 (Table 3). Thus, neocortical CB1-positive cells are GABAergic neurons, expressing CCK, but neither PV nor CRT. However, C28 showed some extent of coexpression with CB1, which is higher in layers II–III than in layers IV–V (Table 3).

Entorhinal/perirhinal cortex area

The entorhinal and perirhinal cortex are the major cortical site of efferents and afferents to and from the hippocampus (Lopes da Silva *et al*., 1990; Suzuki, 1996). Layers II–III and V–VI display very high numbers of low CB1-expressing cells and many scattered high CB1-expressing cells (Figs 1C and D, and 2E and F), furthermore pointing out the modulatory functions of cannabinoids in memory processes. Figure 3F and Table 1 show that GAD 65 is expressed in nearly all high CB1-expressing cells. Much lower colocalization is observed in the population of cells expressing low levels of CB1. The degree of coexpression with CB1 is higher for CCK than for GAD 65 (Table 1 and Fig. 3E). Taken together, these results indicate that nearly all high CB1-expressing cells are GABAergic neurons containing CCK, while the cells containing low levels of CB1 mRNA are only partly GABAergic, but do express CCK. This is a striking difference to the neocortex, where all CB1-positive cells are GABAergic, regardless of the levels of CB1. As observed in the hippocampus and neocortex, coexpression of CB1 with CRT and PV is very low (Table 3), but coexpression with C28 is 16.8% for low and 39.7% for high CB1-expressing cells (Table 3 and Fig. 4F).

Olfactory system

Cells expressing low levels of CB1 were detected in the piriform cortex and in dorsal regions of the olfactory tubercle, and few high CB1-expressing cells were present in the nucleus of the lateral olfactory tract. The most prominent expression area is located in the anterior olfactory nucleus (Fig. 1A), where a high number of low CB1-expressing cells are present interspersed by a low number of strongly positive neurons. All CB1-positive cells are equally distributed throughout all parts of the anterior olfactory nucleus, where the degree of coexpression with GAD 65 and CCK is similar to that observed in the entorhinal/perirhinal cortex area (Table 1). High CB1-expressing cells were found to be positive for both markers in almost 100% of the neurons evaluated, while the degree for low CB1-expressing cells decreases to 35.2 and 89.5% for GAD 65 and CCK, respectively. Thus, as in other cortical regions, high CB1-expressing

TABLE 3. Percentage of coexpression of calcium-binding proteins with CB1 in adult mouse forebrain

	Coexpression in cells expressing low CB1 (%)						Coexpression in cells expressing high CB1 (%)					
	C28	(*n*)	PV	(*n*)	CRT	(*n*)	C28	(*n*)	PV	(*n*)	CRT	(*n*)
CA1 area*	32.4	(294)	1.3	(307)	1.1	(251)	29.1	(204)	1.3	(198)	0.0	(242)
CA3 area*	29.1	(341)	1.8	(383)	5.2	(313)	22.3	(221)	1.3	(247)	2.5	(247)
Dentate gyrus	31.5	(144)	0.0	(119)	2.0	(145)	31.5	(144)	2.6	(91)	2.3	(96)
Layers II–III neocortex	24.9	(983)	0.5	(637)	1.0	(577)	32.8	(471)	1.0	(302)	0.0	(332)
Layers V–VI neocortex	13.7	(1011)	1.1	(575)	1.3	(525)	13.7	(354)	1.5	(384)	0.0	(414)
Anterior olfactory nucleus	ND		4.1	(236)	19.2	(241)	ND		0.0	(91)	0.0	(103)
Entorhinal cortex area	16.8	(722)	3.5	(691)	2.8	(612)	39.7	(92)	1.2	(87)	1.3	(75)
Amygdaloid area	13.1	(787)	4.6	(681)	See note‡		73.5	(89)	1.7	(119)	See note‡	
Striatum†	~75		~15		0.0							
Hypothalamus†	~100		0.0		~100							

Percentages indicate cells expressing low levels or high levels of CB1 that were also labelled with riboprobes specific for C28, PV or CRT. (*n* = number of analysed cells.) Data were collected from 35 (CB1 + PV, CB1 + CRT) and 30 (CB1 + C28) sections from two different brains. ND, not determined. *Principal cells in CA1/CA3 areas express low levels of CB1 mRNA and are not included in coexpression data. C28 is expressed in CA1 principal cells, PV and CRT are not expressed. †Due to the uniform distribution of low CB1-expressing cells in the dorsolateral part of the striatum and the ventromedial and anterior hypothalamic nuclei, numbers in these areas reflect an estimate only. ‡CB1/CRT coexpression varies in different amygdaloid nuclei: basolateral posterior part, 31.7 (184, low CB1), 14.3 (42, high CB1); basolateral anterior part, 8.8 (274, low CB1), 2.5 (40, high CB1); basomedial anterior part, 4.5 (220, low CB1), 0.0 (37, high CB1).

cells seem to belong to a population of GABAergic neurons containing CCK, while the cells containing low levels of CB1 mRNA partly belong to a CCK/GAD 65-double positive population and partly to another population that is CCK-positive, but GAD 65-negative. For PV and CRT, there is nearly a lack of coexpression with CB1 (Table 3). Only a few CRT-positive cells (19.2%) were observed in the low CB1-expressing cell population. Together with some nuclei of the amygdaloid region (see below), this is the only cortical area in which a significant coexpression of CB1 with CRT was observed.

Amygdaloid region

CB1 is expressed in several nuclei of the amygdala. Only low levels were detected in the bed nucleus of the stria terminalis and the central amygdaloid nucleus (data not shown). High numbers of both low and high CB1-expressing cells were found, however, in the anterior and posterior parts of the basolateral amygdaloid nuclei and the anterior part of the basomedial nucleus (Figs 1D, and 2C and D). In these latter nuclei, high CB1-expressing cells also contain GAD 65 (Table 1). Instead, low CB1-expressing cells have different levels of GAD 65 coexpression, depending on the nuclei. A gradual decrease along the posterolateral to anteromedial direction was apparent (Table 1, see note). In the posterior part of the basolateral nucleus, the percentage of colocalization is 42.7%, while it decreases to 15.0% in the basomedial anterior part. Taken together, almost all high CB1-expressing cells are GABAergic, while a fraction ranging from 57 to 85% for low CB1-expressing neurons is non-GABAergic. CCK highly colocalizes with CB1 in a uniform manner in the amygdaloid nuclei examined (Fig. 3D and Table 1). Approximately 90% of low CB1-expressing cells and nearly all high CB1-expressing cells contain CCK mRNA. As in other cortical areas, the amygdala contains low CB1-expressing cells that are CCK positive, but GAD 65 negative. A distinct population comprising all high CB1-expressing cells and a fraction of low CB1-expressing cells express both GAD 65 and CCK. As Table 3 and Fig. 4E show, PV is present in very few CB1-positive cells. Similarly to GAD 65, coexpression of CRT also decreases along the posterolateral to anteromedial direction (Table 3, see note). C28 displays uniform coexpression throughout the amygdaloid nuclei analysed. A considerable percentage is observed for low CB1-expressing cells (13.1%), but is increased to 73.5% in high CB1-expressing cells. Thus, in the amygdaloid region, ~70–75% of high CB1-expressing cells belong to a population of GABAergic neurons that contains both CCK and C28.

CB1 expression in neuronal subpopulations in non-cortical regions

Striatum

CB1 hybridization signals were detected at low levels in many cells throughout the striatum. The nucleus accumbens, ventromedial caudate putamen, globus pallidus and entopeduncular nucleus contain cells expressing quite low levels of CB1. In contrast, the dorsolateral caudate putamen showed an intense staining due to a very compact and uniform, but low level expression (Fig. 1B and C). Nearly all medium-sized cells in the dorsolateral caudate putamen are CB1-positive. The number of labelled cells and the intensity of the signal decrease along the medioventral axis. Due to uniform distribution of low CB1-expressing cells in this area, it was not feasible to count coexpressing cells at a single cell resolution. Thus, the numbers reflect an estimate only. Table 1 shows the values of coexpression of CB1 with GAD 65 and CCK. Basically all CB1-positive cells in the dorsolateral caudate putamen contain GAD 65 mRNA, while none are CCK positive. Spiny neurons in the caudate putamen express GAD 65, and they constitute ~95% of the cells in this region (Ottersen & Storm-Mathisen, 1984). As shown in Table 3, many CB1-positive cells (~75%) coexpress C28. Scattered cells (~15%) coexpress PV, while there is a lack of colocalization of CB1 with CRT. These results revealed that CB1-expressing cells in the dorsolateral caudate putamen are GABAergic neurons that do not express CCK. They might belong to two distinct or partly overlapping populations, which express C28 and PV, respectively.

Hypothalamus

Several hypothalamic nuclei display low levels of CB1 mRNA. The strongest hybridization signals were detected in ventromedial (Fig. 1D) and anterior hypothalamic nuclei (not shown), where CB1-positive cells are uniformly distributed. There is a lack of coexpression of CB1 and GAD 65 in these hypothalamic nuclei (Table 1), as GAD 65 is expressed mainly in hypothalamic areas surrounding the ventromedial and anterior hypothalamic nuclei. Remarkably, there is also a lack of coexpression with CCK (Table 1,

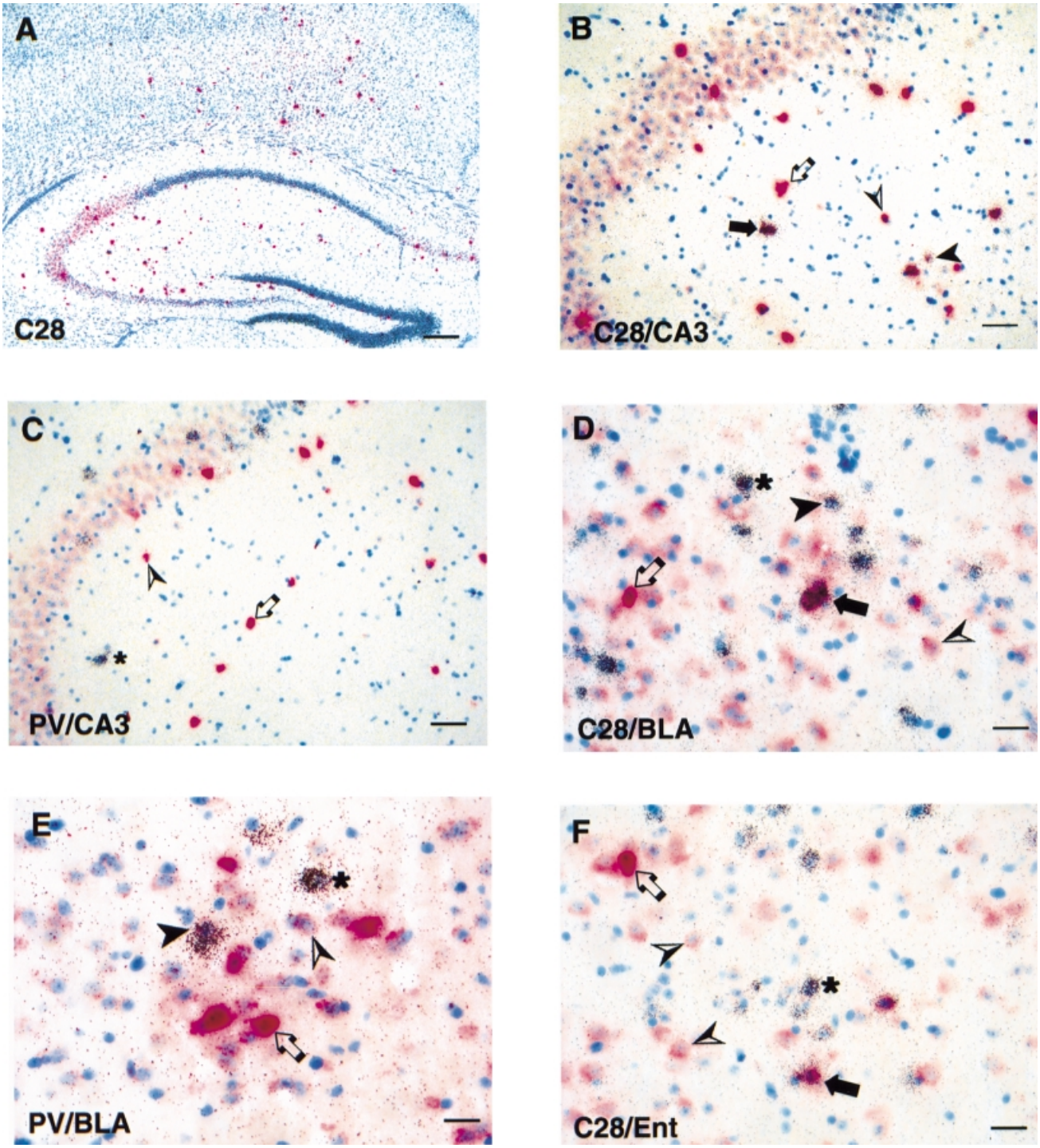

FIG. 4. Bright-field micrograph of coronal forebrain sections showing examples of coexpression of CB1 (red staining) with C28 and PV (silver grains), respectively. All sections were counterstained with toluidine blue. (A) Overview of hippocampus double-stained for CB1 and C28. Note the expression of C28 in pyramidal cells of the CA1 region and in granular cells of the dentate gyrus. (B) Higher magnification of CA3 from A showing some coexpression of CB1 and C28 in the CA3 area (filled arrow, filled arrowhead). (C) Lack of coexpression of CB1 with PV in the CA3 area (open arrow, open arrowhead, asterisks). (D) In the basolateral amygdaloid nucleus, C28 is present in many high CB1-expressing cells (filled arrow), but only in a few low CB1-expressing cells (filled arrowhead). (E) Double staining for CB1 and PV in the basolateral amygdaloid nucleus, showing only a small fraction of colocalization (filled arrowhead). (F) Double staining of CB1 and C28 in the entorhinal cortex area, showing some colocalization (filled arrow). Filled arrows, high CB1-expressing cells coexpressing C28; filled arrowhead, low CB1-expressing cells coexpressing C28 or PV; open arrows, high CB1-expressing cells not coexpressing C28 or PV; open arrowheads, low CB1-expressing cells not coexpressing C28 or PV; asterisks, cells expressing only C28 or PV. Scale bars, 150 μm (A); 40 μm (B and C); 20 μm (D–F).

see note). Among all the areas analysed, this is the only forebrain region in which neither GAD 65 nor CCK colocalize with CB1. C28 and CRT are highly enriched in the cell bodies of the ventromedial and anteromedial hypothalamus. Thus, they coexpress with CB1 to a high degree (Table 3). PV is not expressed in the hypothalamus (Celio, 1990).

Discussion

The aim of this study was to define in detail the distribution pattern and neurochemical characteristics of CB1-expressing neurons in the adult mouse forebrain. We thus performed both single ISH experiments using ^{35}S-labelled or FITC-labelled CB1 riboprobes, and double ISH experiments using a FITC-labelled CB1 riboprobe in combination with ^{35}S-labelled riboprobes to GAD 65, CCK, PV, C28 and CRT, respectively. CB1-expressing cells can clearly be discriminated into two different populations: one expressing high levels and the other expressing low levels. High CB1-expressing cells are in the great majority GABAergic, characterized mostly by the expression of CCK, the lack of PV and CRT, and rarely, the presence of C28. Low CB1-expressing cells belong only partly to the same subpopulations of GABAergic cells as high CB1-expressing cells. Remarkably, for low CB1-expressing cells, the presence of CCK also characterizes non-GABAergic neurons. In cortical areas, they can be considered as principal projecting cells.

The discrimination between high and low CB1-expressing cells is also in agreement with immunohistochemical observations in rats (Tsou *et al*., 1998), indicating that the levels of mRNA probably correlate with the levels of protein expression. The different neurochemical properties of CB1-expressing cells that we described in various forebrain areas are likely to underlie different functional properties.

CB1 and the GABAergic system

Previous immunohistochemical and ISH studies carried out in rats (Matsuda *et al*., 1993; Tsou *et al*., 1998) suggested that in cortical structures CB1 receptor is present mainly in GABAergic neurons. Our results indicate that this notion is true in the murine forebrain only for high CB1-expressing cells, where GAD 65 colocalizes with CB1 at a degree close to 100%. However, low CB1-expressing cells appear to be less correlated with the GABAergic system. Pyramidal cells of CA1/CA3 areas in the hippocampus express low levels of CB1, but do not express GAD 65. The same is true for many cells in the polymorph layer of the dentate gyrus that probably represent misplaced pyramidal cells. From immunohistochemical studies in rats, it is not clear whether CB1 protein is expressed in pyramidal cells of the hippocampus. Some authors (Tsou *et al*., 1998; Katona *et al*., 1999) report negligible levels of expression of CB1 protein in pyramidal cells of the CA1/CA3 region. In contrast, using a different antiserum and different fixation procedures, Pettit *et al*. (1998) observed a clear expression of CB1 in these cells. This discrepancy might be explained by the fact that different fixation procedures might mask different epitopes or can destroy particular subcellular organelles in which the protein is localized. However, in agreement with results obtained in rats (Matsuda *et al*., 1993), our ISH data in mouse clearly revealed that a low but significant amount of CB1 mRNA is expressed in pyramidal cells of the CA1/CA3 hippocampal regions. Similarly to the hippocampus, in the anterior olfactory nucleus, entorhinal/perirhinal cortex and amygdaloid nuclei, the majority of low CB1-expressing cells are not GABAergic. Only in the neocortex does CB1 seem to be completely correlated with the GABAergic system, regardless of the levels of CB1 expression.

The hippocampus, amygdala and entorhinal/perirhinal cortex comprise the so-called limbic system, which is considered to be a central circuit for important brain functions, e.g. learning and memory, as well as cognition (Suzuki, 1996; Miller *et al*., 1998). The effects of cannabinoids on memory processes are generally believed to be due to the interaction of CB1 with the GABAergic system (Terranova *et al*., 1995). Recently, Paton *et al*. (1998) analysed the involvement of GABA transmission in the mechanism by which cannabinoids are able to inhibit long-term potentiation (LTP), an electrophysiological correlate of learning and memory in hippocampal slices. Unexpectedly, the authors observed a decrease of GABAergic transmission in the presence of CB1 agonists. Therefore, they concluded that increased GABA activity is not directly involved in the inhibition of LTP by cannabinoids. Moreover, they speculate that presynaptic CB1 receptors located specifically on terminals of principal glutamatergic neurons, which synapse onto inhibitory interneurons, may mediate the reduction of the excitatory drive of these cells. This is in agreement with our observation that pyramidal cells of CA1/CA3 express low levels of CB1, but lack expression of GAD 65.

CB1 expression in distinct neuronal subpopulations

In the hippocampus, neurochemical characterization of GABAergic neurons defines at least four distinct subpopulations (Freund & Buszáki, 1996). (i) PV-positive GABAergic interneurons are considered as basket cells that innervate the perisoma of pyramidal cells, and as axo-axonic cells innervating the proximal part of axons of pyramidal cells (Soriano *et al*., 1990). (ii) CCK-positive GABAergic interneurons represent a different population of basket cells that does not overlap with PV-positive GABAergic neurons**,** but also innervates the soma of pyramidal cells (Gulyás *et al*., 1991). (iii) C28 is present in cells innervating mid-proximal dendrites of pyramidal cells (Gulyás & Freund, 1996). (iv) CRT is characteristic for interneurons specialized in innervating other interneurons (Gulyás *et al*., 1996). We found a high degree of coexpression of CB1 with CCK, some coexpression with C28, and nearly a lack of coexpression with PV and CRT, indicating that CB1-expressing cells are mostly basket cells of the CCK-positive and PV-negative type and, to a lower extent, C28-positive interneurons innervating mid-proximal dendrites of pyramidal cells. As almost no coexpression of CB1 was observed with CRT and PV, CB1 is also not present in axo-axonic cells, innervating the proximal part of the axons of pyramidal cells or in interneurons specialized in innervating other interneurons. Interestingly, for low CB1-expressing cells in CA1/CA3 regions of the hippocampus, the percentages of coexpression with C28 and CCK, respectively, appear to be complementary (30 versus 70%). Thus, low CB1-expressing cells that are GABAergic might belong to two distinct interneuronal subpopulations: basket cells (CCK coexpression) and, to a lower extent, cells innervating mid-proximal dendrites (C28 coexpression). For high CB1-expressing cells there must be an overlap of at least 10–20%, as CB1/C28 coexpression is ~20%, whereas CB1/CCK coexpression is >90%.

In the limbic system (e.g. the entorhinal/perirhinal cortex, amygdala and hippocampus), a significant percentage of low CB1-expressing cells are not GABAergic, but express CCK. In the neocortex, many CCK-positive pyramidal cells were described as corticostriatal- and corticothalamic-projecting neurons (Burgunder & Young, 1988; Ingram *et al*., 1989; Morino *et al*., 1994; Senatorov *et al*., 1997). Many CCK-positive cells also appear to be projecting neurons in the entorhinal/perirhinal cortex and amygdala, as, e.g. CCK was shown to be present in fibres connecting the entorhinal cortex and hippocampus, and in fibres crossing the amygdalo-hippocampal border (Greenwood *et al*., 1981; Roberts *et al*., 1984; Fredens *et al*., 1984). Considering the high levels of coexpression between CB1 and CCK in GAD-negative cells, these observations might indicate an involvement of CB1 in the limbic system that is not only due to an influence on GABAergic inhibitory control, but also to a direct effect on CCK-positive projecting neurons (see below and Fig. 5). It is interesting to note that neurons expressing CCK and low

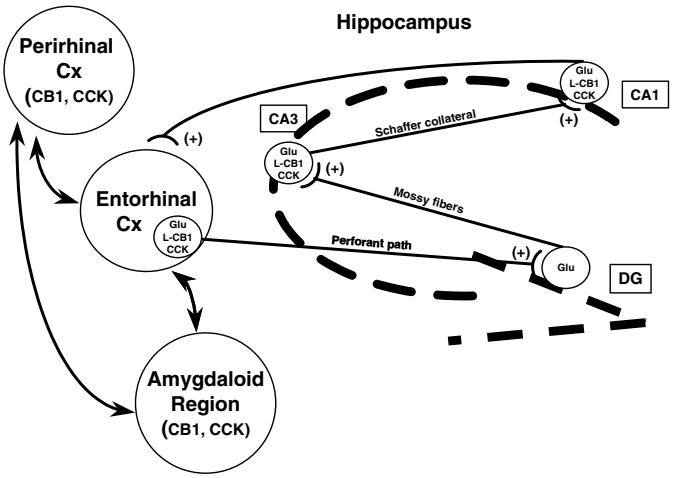

FIG. 5. Schematic representation of the connectivity of hippocampus and cortical areas that are proposed to be modulated by the cannabinoid system. CB1 is present at low but significant levels (L-CB1) in principal neurons participating in the 'trisynaptic loop' between the entorhinal cortex and hippocampus. Due to the high number of low CB1-expressing cells (that are GAD negative and CCK positive) in the perirhinal and amygdaloid areas, it appears likely that cannabinoid signalling might also modulate neuronal transmission between perirhinal, entorhinal and amygdaloid areas. Note the high degree of CB1/CCK coexpression in projecting neurons, suggesting an involvement of the neuropeptide in cannabinoid action.

levels of CB1, but no GAD 65, were not observed in the neocortex, where nearly all CB1-expressing cells are GABAergic and, thus, are considered as interneurons.

In the caudate putamen, CB1 is coexpressed with GAD 65 and, partly, with C28 and PV, but not with CCK. CB1 was shown to be important for the expression of GAD and neuropeptides, e.g. substance P, dynorphin and enkephalin (Steiner *et al*., 1999). This is an indication that the relation between neuropeptides and cannabinoids is very important in physiology and pharmacology of the cannabinoid system, and that different neuropeptides are involved in different brain areas.

CB1 and CCK

A striking finding of our study is the very high coexpression of CB1 with CCK in cortical areas. Excluding the dentate gyrus, in which coexpression is slightly lower, in all cortical areas analysed, mRNA encoding the neuropeptide is present in ~70–90% of the low CB1-expressing cells and 90–100% of the high CB1-expressing cells. CCK, originally characterized as a gastrointestinal hormone, is one of the most abundant neuropeptides in the brain. The active forms, derived from the precursor pre-procholecystokinin, range from four to 58 amino acids, but the most abundant peptide is the sulphated octapeptide CCK-8S (Fink *et al*., 1998). They exert their actions through two receptors, CCK-A, present mostly in the gastrointestinal tract, and CCK-B, expressed predominantly in the brain. CCK is involved in feeding, learning and memory, behavioural expression of anxiety, mediation of painful stimuli, and is also involved in functions controlled by the dopaminergic, serotoninergic and opioid systems (Crawley & Corwin, 1994). It is interesting to note that many functions, in which CCK is involved, are also modulated by cannabinoids. In some cases, the two systems seem to act in an antagonistic fashion. For example, CCK was shown to be hyperalgesic, and CCK receptor antagonists are used to increase the analgesic effects of opioids (Faris *et al*., 1983; Kellstein & Mayer, 1991; Duggan, 1992), while the antinociceptive effects of cannabinoids are very well known (for a review, see Martin & Lichtman, 1998). CCK is also considered to be a satiety factor (Reidelberger, 1994), while CB1 was proposed to induce feeding behaviour (Mattes *et al*., 1994). Also, many effects of cannabinoids seem to be mediated by interaction with the dopaminergic and opioid systems (e.g. Castellano *et al*., 1997; Tanda *et al*., 1997; Giuffrida *et al*., 1999; Ledent *et al*., 1999), both of which are interacting with CCK as well (Crawley, 1991; Duggan, 1992). Given these findings and given the high degree of coexpression of CB1 with CCK, it is tempting to speculate about a possible functional cross-talk between the two systems; e.g. cannabinoids might have an effect on production, processing or release of CCK peptides. Depolarization of the cell membrane was shown to induce release of CCK both *in vitro* and *in vivo* (Raiteri *et al*., 1993). Compounds that are able to increase the intracellular concentration of cAMP enhance depolarization-induced release of CCK, and a decrease of cAMP inhibits CCK release (Beinfeld, 1996). Considering that activation of CB1 can inhibit cAMP production, it appears probable that cannabinoids may modulate the release of CCK.

Possible interactions between CB1 and CCK may also involve the regulation of CCK synthesis by cannabinoids. In this regard, it is interesting to note a possible involvement of both the receptor and the peptide in some pathophysiological conditions, e.g. schizophrenia. Based on similar scores in psychodiagnostic tests between schizophrenic patients and healthy volunteers to which cannabinoids were administered, and based on the interaction of cannabinoids with the dopaminergic and glutamatergic system, a 'cannabinoid hypothesis' of schizophrenia has recently been proposed, suggesting an increased pathological cannabinoid tone in schizophrenic patients (Emrich *et al*., 1997; Schneider *et al*., 1998). On the other hand, abnormally decreased levels of CCK mRNA were observed in schizophrenic subjects, but not in Alzheimer's patients in discrete cortical areas, e.g. the entorhinal cortex (Gabriel *et al*., 1996; Bachus *et al*., 1997). It would be tempting to relate these observations with our CB1/CCK coexpression study and to propose that an increased stimulation by cannabinoids might lead to decreased levels of CCK in distinct cortical brain areas.

From anatomy to function: putative sites of action of cannabinoids in cortical areas

CB1 is distributed throughout the central nervous system of mammals in greater abundance than most of the other known G-protein-coupled receptors (Herkenham *et al*., 1990). Therefore, it would be an error to underestimate the physiological importance of cannabinoids in cells that express CB1 at low levels, because such expression is in any case very high as compared with other identified receptors in the brain. For example, despite the rather low levels of CB1 mRNA expressed by single cells in the caudate putamen and other subcortical areas (e.g. the preoptic area in the hypothalamus), the CB1 receptor seems to exert essential functions in locomotor activity and hypothermia, respectively (Breivogel & Childers, 1998; Steiner *et al*., 1999; Giuffrida *et al*., 1999; Zimmer *et al*., 1999). CB1 is present in many cells belonging to the so-called 'trisynaptic loop' (Knowles, 1992) between the entorhinal cortex and hippocampus (Fig. 5). Our results show that a high percentage of low CB1-expressing cells are GAD 65-negative and CCK-positive, and thus, are likely to be principal projecting neurons. Cannabinoid activity on these receptors, therefore, might result in a direct modulation of the 'trisynaptic loop'. Other probable sites of action of cannabinoids in the hippocampus and probably also in other forebrain areas (e.g. the neocortex) are the local GABAergic inhibitory circuits (Fig. 6). In hippocampal slices, cannabinoids were indeed shown to cause a reduction of release of several neurotransmitters, including

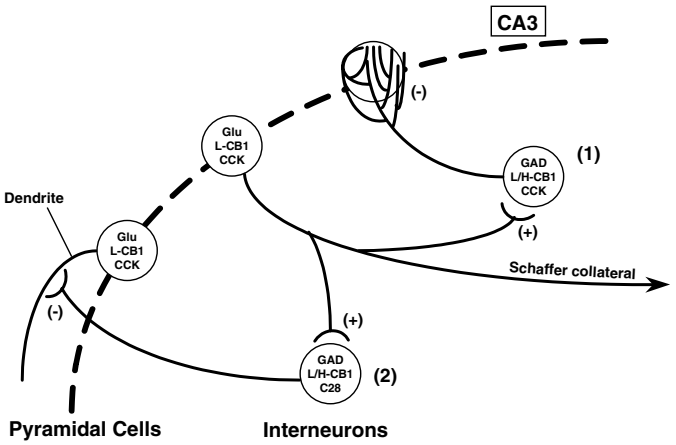

FIG. 6. Schematic representation of local hippocampal circuits in the CA3 area that are proposed to be modulated by the cannabinoid system. (1 and 2) CB1 is expressed both at low and high levels (L/H-CB1) in GAD 65-positive interneurons. (1) The majority of neurons (70–80%) belongs to the CCK-positive, PV-negative subfamily of basket cells, innervating the soma of pyramidal cells. (2) Twenty to 30% of CB1-positive cells belong to the C28-positive subfamily of interneurons that innervate the mid-proximal tract of dendrites of pyramidal cells. For clarity, pyramidal cells are represented as three different cells, but a direct feedback between one pyramidal cell and one interneuron is also possible. (+) and (–) indicate excitatory and inhibitory transmission, respectively. In summary, the cannabinoid system might be involved in local inhibitory GABAergic circuits, by modulation of basket and mid-proximal dendritic inhibitory cells.

glutamate and GABA (Shen *et al*., 1996; Katona *et al*., 1999), possibly suggesting differential modulatory effects on different cell types.

In summary, these diverse sites of action could indicate a balanced modulatory effect of cannabinoids on principal projecting neurons and interneurons. Endocannabinoids are believed to be locally produced, released and rapidly inactivated (for reviews, see Di Marzo & Deutsch, 1998; Di Marzo *et al*., 1998; Piomelli *et al*., 1998). Anandamide and 2-arachidonylglycerol, the two major endocannabinoids described so far, were shown to be released upon chemical or electrical stimulation of neurons (Stella *et al*., 1997; Piomelli *et al*., 1998; Giuffrida *et al*., 1999). This locally induced release and the rapid inactivation of endocannabinoids, together with the differential expression of CB1, could suggest that the cannabinoid system has different modulatory functions depending on particular physiological or pathophysiological conditions.

Acknowledgements

We would like to thank Michaela Wiedemann, Katja Finkl and René Schadowski for their excellent technical assistance, Drs Shahnaz Christina Azad and Uberto Pagotto for critical reading of the manuscript and valuable considerations, Dr Adelheid Kresse for anatomical expertise, and Elisabeth Güll for secretarial work.

Abbreviations

I–VI, layers of cerebral cortex; AO, anterior olfactory nucleus; BLA, basolateral amygdaloid nucleus; BMA, basomedial amygdaloid nucleus; BSA, bovine serum albumin; C28, calbindin D28k; CA1/CA3, CA1 and CA3 fields of hippocampus; CB1, cannabinoid receptor 1; CCK, cholecystokinin; Cpu, caudate putamen; CRT, calretinin; Cx, cortex; DEPC, diethyl pyrocarbonate; DG, dentate gyrus; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Ent, entorhinal/perirhinal cortex; GABA, γ-aminobutyric acid; GAD 65, glutamic acid decarboxylase 65k; Glu, glutamate; GP, globus pallidus; Gr, granular layer of dentate gyrus; FITC, fluorescein isothiocyanate; IMD, intermediodorsal thalamic nucleus; ISH, *in situ* hybridization; Lhb, lateral habenula; LMol, lacunosum-molecular layer of hippocampus; Ls, lateral septum; LTP, long-term potentiation; M1/M2, primary and secondary motor cortex; MO, medial orbital cortex; Mol, molecular layer of hippocampus; Ms, medial septum; Or, oriens layer of hippocampus; PBS, phosphate-buffered saline; Pir, piriform cortex; Po, polymorph layer of dentate gyrus; Prl, prelimbic cortex; PV, parvalbumin; PVT, paraventricular thalamic nucleus; Py, pyramidal cell layer of hippocampus; Rad, stratum radiatum of hippocampus; Rt, reticular thalamic nucleus; SSC, standard saline citrate; S1FL/HL, somatosensory cortical areas, forelimb/hindlimb regions; SGr, subgranular layer of dentate gyrus; Tu, olfactory tubercle; VDB, nucleus vertical limb diagonal band; VMH, ventromedial hypothalamic nucleus; ZI, zona incerta.

References

Bachus, S.E., Hyde, T.M., Herman, M.M., Egan, M.F. & Kleinman, J.E. (1997) Abnormal cholecystokinin mRNA levels in entorhinal cortex of schizophrenics. *J. Psychiat. Res.*, **31**, 233–256.

Beinfeld, M.C. (1996) Cholecystokinin (CCK) release from rat brain slices *in vitro* is enhanced by agents which elevate intracellular cAMP. *Regul. Pept.*, **67**, 75–77.

Breivogel, C.S. & Childers, S.R. (1998) The functional neuroanatomy of brain cannabinoid receptors. *Neurobiol. Dis.*, **5**, 417–431.

Burgunder, J.-M. & Young, W.S. III (1988) The distribution of thalamic projection neurons containing cholecystokinin messenger RNA, using *in situ* hybridization histochemistry and retrograde labeling. *Brain Res.*, **464**, 179–189.

Castellano, C., Cabib, S., Palmisano, A., Di Marzo, V. & Puglisi-Allegra, S. (1997) The effects of anandamide on memory consolidation in mice involve both D_1 and D_2 dopamine receptors. *Behav. Pharmacol.*, **8**, 707–712.

Celio, M.R. (1990) Calbindin D-28k and parvalbumin in the rat nervous system. *Neuroscience*, **35**, 375–475.

Crawley, J.N. (1991) Cholecystokinin–dopamine interactions. *Trends Pharmacol. Sci.*, **12**, 232–236.

Crawley, J.N. & Corwin, R.L. (1994) Biological actions of cholecystokinin. *Peptides*, **15**, 731–755.

Deadwyler, S.A., Hampson, R.E., Bennett, B.A., Edwards, T.A., Mu, J., Pacheco, M.A., Ward, S.J. & Childers, S.R. (1993) Cannabinoids modulate potassium current in cultured hippocampal neurons. *Receptors Channels*, **1**, 121–134.

Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A. & Mechoulam, R. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science*, **258**, 1946–1949.

Di Marzo, V. & Deutsch, D.G. (1998) Biochemistry of the endogenous ligands of cannabinoid receptors. *Neurobiol. Dis.*, **5**, 386–404.

Di Marzo, V., Melck, D., Bisogno, T. & De Petrocellis, L. (1998) Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends Neurosci.*, **21**, 521–528.

Duggan, A.W. (1992) Neuropharmacology of pain. *Curr. Opin. Neurol. Neurosurg.*, **5**, 503–507.

Emrich, H.M., Leweke, F.M. & Schneider, U. (1997) Towards a cannabinoid hypothesis of schizophrenia: cognitive impairments due to dysregulation of the endogenous cannabinoid system. *Pharmacol. Biochem. Behav.*, **56**, 803–807.

Faris, P.L., Komisaruk, B.R., Watkins, L.R. & Mayer, D.J. (1983) Evidence for the neuropeptide cholecystokinin as an antagonist of opiate analgesia. *Science*, **219**, 310–312.

Felder, C.C., Joyce, K.E., Briley, E.M., Mansouri, J., Mackie, K., Blond, O., Lai, Y., Ma, A.L. & Mitchell, R. (1995) Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Mol. Pharmacol.*, **48**, 443–450.

Fink, H., Rex, A., Voits, M. & Voigt, J.P. (1998) Major biological actions of CCK – a critical evaluation of research findings. *Exp. Brain Res.*, **123**, 77–83.

Fredens, K., Stengaard-Pedersen, K. & Larsson, L.I. (1984) Localization of enkephalin and cholecystokinin immunoreactivities in the perforant path terminal fields of the rat hippocampal formation. *Brain Res.*, **304**, 255–263.

Freund, T.F. & Buszáki, G. (1996) Interneurons of the hippocampus. *Hippocampus*, **6**, 347–470.

Gabriel, S.M., Davidson, M., Haroutunian, V., Powchik, P., Bierer, L.M., Purohit, D.P., Perl, D.P. & Davis, K.L. (1996) Neuropeptide deficits in schizophrenia vs. Alzheimer's disease cerebral cortex. *Biol. Psychiatry*, **39**, 82–91.

Gifford, A.N. & Ashby, C.R. Jr (1996) Electrically evoked acetylcholine

release from hippocampal slices is inhibited by the cannabinoid receptor agonist, WIN 55212-2, and is potentiated by the cannabinoid antagonist, SR 141716A. *J. Pharmacol. Exp. Ther.*, **277**, 1431–1436.

Giuffrida, A., Parsons, L.H., Kerr, T.M., Rodriguez de Fonseca, F., Navarro, M. & Piomelli, D. (1999) Dopamine activation of endogenous cannabinoid signaling in dorsal striatum. *Nature Neurosci.*, **2**, 358–363.

Glass, M. & Felder, C.C. (1997) Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a G_s linkage to the CB1 receptor. *J. Neurosci.*, **17**, 5327–5333.

Greenwood, R.S., Godar, S.E., Reaves, T.A. & Hayward, J.N. (1981) Cholecystokinin in hippocampal pathways. *J. Comp. Neurol.*, **203**, 335–350.

Gulyás, A.I. & Freund, T.F. (1996) Pyramidal cell dendrites are the primary targets of calbindin D28k-immunoreactive interneurons in the hippocampus. *Hippocampus*, **6**, 525–534.

Gulyás, A.I., Hájos, N. & Freund, T.F. (1996) Interneurons containing calretinin are specialized to control other interneurons in the rat hippocampus. *J. Neurosci.*, **16**, 3397–3411.

Gulyás, A.I., Tóth, K., Dános, P. & Freund, T.F. (1991) Subpopulations of GABAergic neurons containing parvalbumin, calbindin D28k, and cholecystokinin in the rat hippocampus. *J. Comp. Neurol.*, **312**, 371–378.

Herkenham, M., Lynn, A.B., Little, M.D., Johnson, M.R., Melvin, L.S., De Costa, B.R. & Rice, K.C. (1990) Cannabinoid receptor localization in brain. *Proc. Natl Acad. Sci. USA*, **87**, 1932–1936.

Howlett, A.C. (1995) Pharmacology of cannabinoid receptors. *Annu. Rev. Pharmacol. Toxicol.*, **35**, 607–634.

Ingram, S.M., Krause, R.G. II., Baldino, F. Jr, Skeen, L.C. & Lewis, M.E. (1989) Neuronal localization of cholecystokinin mRNA in the rat brain by using *in situ* hybridization histochemistry. *J. Comp. Neurol.*, **287**, 260–272.

Katona, I., Sperlagh, B., Sik, A., Kafalvi, A., Vizi, E.S., Mackie, K. & Freund, T.F. (1999) Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. *J. Neurosci.*, **19**, 4544–4558.

Kellstein, D.E. & Mayer, D.J. (1991) Spinal co-administration of cholecystokinin antagonists with morphine prevents the development of opioid tolerance. *Pain*, **47**, 221–229.

Knowles, W.D. (1992) Normal anatomy and neurophysiology of the hippocampal formation. *J. Clin. Neurophysiol.*, **9**, 252–263.

Ledent, C., Valverde, O., Cossu, G., Petitet, F., Aubert, J.F., Beslot, F., Bohme, G.A., Imperato, A., Pedrazzini, T., Roques, B.P., Vassart, G., Fratta, W. & Parmentier, M. (1999) Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science*, **283**, 401–404.

Lopes da Silva, F.H., Witter, M.P., Boeijinga, P.H. & Lohman, A.H.M. (1990) Anatomic organization and physiology of the limbic cortex. *Physiol. Rev.*, **70**, 453–511.

Mackie, K. & Hille, B. (1992) Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. *Proc. Natl Acad. Sci. USA*, **89**, 3825–3829.

Mackie, K., Lai, Y., Westenbroek, R. & Mitchell, R. (1995) Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. *J. Neurosci.*, **15**, 6552–6561.

Mailleux, P. & Vanderhaeghen, J.J. (1992) Distribution of neuronal cannabinoid receptor in the adult rat brain: a comparative receptor binding radioautography and *in situ* hybridization histochemistry. *Neuroscience*, **48**, 655–668.

Martin, B.R. & Lichtman, A.H. (1998) Cannabinoid transmission and pain perception. *Neurobiol. Dis.*, **5**, 447–461.

Matsuda, L.A., Bonner, T.I. & Lolait, S.J. (1993) Localization of cannabinoid receptor mRNA in rat brain. *J. Comp. Neurol.*, **327**, 535–550.

Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C. & Bonner, T.I. (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*, **346**, 561–564.

Mattes, R.D., Engelman, K., Shaw, L.M. & Elsohly, M.A. (1994) Cannabinoid and appetite stimulation. *Pharmacol. Biochem. Behav.*, **49**, 187–195.

Miller, L.A., Lai, R. & Munoz, D.G. (1998) Contributions of entorhinal cortex, amygdala and hippocampus to human memory. *Neuropsychologia*, **36**, 1247–1256.

Morino, P., Herrera-Marschitz, M., Castel, M.N., Ungerstedt, U., Varro, A., Dockray, G. & Hökfelt, T. (1994) Cholecystokinin in cortico-striatal neurons in the rat: immunohistochemical studies at the light and electron microscopical level. *Eur. J. Neurosci.*, **6**, 681–692.

Ottersen, O.P. & Storm-Mathiesen, J. (1984) Neurons containing and accumulating transmitter amino acids. In Björklund, A., Hökfelt, T. & Kuhar, M.J. (eds), *Handbook of Chemical Neuroanatomy*, *Vol. 3: Classical Transmitters and Transmitters Receptors in the CNS, P.II.* Elsevier Science, Amsterdam, pp. 141–246.

Paton, G.S., Pertwee, R.G. & Davies, S.N. (1998) Correlation between cannabinoid mediated effects on paired pulse depression and induction of long-term potentiation in the rat hippocampal slice. *Neuropharmacology.*, **37**, 1123–1130.

Pettit, D.A., Harrison, M.P., Olson, J.M., Spencer, R.F. & Cabral, G.A. (1998) Immunohistochemical localization of the neural cannabinoid receptor in rat brain. *J. Neurosci. Res.*, **51**, 391–402.

Piomelli, D., Beltramo, M., Giuffrida, A. & Stella, N. (1998) Endogenous cannabinoid signaling. *Neurobiol. Dis.*, **5**, 462–473.

Raiteri, M., Paudice, P. & Vallebuona, F. (1993) Release of cholecystokinin in the central nervous system. *Neurochem. Int.*, **22**, 519–527.

Reidelberger, R.D. (1994) Cholecystokinin and control of food intake. *J. Nutr.*, **124** (Suppl.), 1327–1333.

Rhee, M.-H., Bayewitch, M., Avidor-Reiss, T., Levy, R. & Vogel, Z. (1998) Cannabinoid receptor activation differentially regulates the various adenylyl cyclase isozymes. *J. Neurochem.*, **71**, 1525–1534.

Roberts, G.W., Woodhams, P.L., Polak, J.M. & Crow, T.J. (1984) Distribution of neuropeptides in the limbic system of the rat: the hippocampus. *Neuroscience*, **11**, 35–77.

Schlicker, E., Timm, J., Zentner, J. & Göthert, M. (1997) Cannabinoid CB1 receptor-mediated inhibition of noradrenaline release in the human and guinea-pig hippocampus. *Naunyn Schmiedebergs Arch. Pharmacol.*, **356**, 583–589.

Schneider, U., Leweke, F.M., Mueller-Vahl, K.R. & Emrich, H.M. (1998) Cannabinoid/anandamide system and schizophrenia: is there evidence for association? *Pharmacopsychiatry*, **31**, 110–113.

Senatorov, V.V., Trudeau, V.L. & Hu, B. (1997) Expression of cholecystokinin messenger RNA in reciprocally connected auditory thalamus and cortex in the rat. *Neuroscience*, **79**, 915–921.

Shen, M., Piser, T.M., Seybold, V.S. & Thayer, S.A. (1996) Cannabinoid receptor agonists inhibit glutamatergic synaptic transmission in rat hippocampal cultures. *J. Neurosci.*, **16**, 4322–4334.

Soriano, E., Nitsch, R. & Frotscher, M. (1990) Axo-axonic chandelier cells in the rat fascia dentata: Golgi-electron microscopy and immunocytochemical studies. *J. Comp. Neurol.*, **293**, 1–25.

Steiner, H., Bonner, T., Zimmer, A.M., Kitai, S.T. & Zimmer, A. (1999) Altered gene expression in striatal projection neurons in CB1 cannabinoid receptor knockout mice. *Proc. Natl Acad. Sci. USA*, **96**, 5786–5790.

Stella, N., Schweitzer, P. & Piomelli, D. (1997) A second endogenous cannabinoid that modulates long-term potentiation. *Nature*, **388**, 773–778.

Suzuki, W.A. (1996) The anatomy, physiology and functions of the perirhinal cortex. *Curr. Opin. Neurobiol.*, **6**, 179–186.

Tanda, G., Pontieri, F.E. & Di Chiara, G. (1997) Cannabinoid and heroin activation of mesolimbic dopamine transmission by a common mu1 opioid receptor mechanism. *Science*, **276**, 2048–2050.

Terranova, J.-P., Michaud, J.-C., Le Fur, G. & Soubrié, P. (1995) Inhibition of long-term potentiation in rat hippocampal slices by anandamide and WIN55212-2: reversal by SR141716A, a selective antagonist of CB1 cannabinoid receptors. *Naunyn Schmiedebergs Arch. Pharmacol.*, **352**, 576–579.

Tóth, K. & Freund, T.F. (1992) Calbindin D28k-containing nonpyramidal cells in the rat hippocampus: their immunoreactivity for GABA and projection to the medial septum. *Neuroscience*, **49**, 793–805.

Tsou, K., Brown, S., Sanudo-Pena, M.C., Mackie, K. & Walker, J.M. (1998) Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience*, **83**, 393–411.

Zimmer, A., Zimmer, A.M., Hohmann, A.G., Herkenham, M. & Bonner, T.I. (1999) Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. *Proc. Natl Acad. Sci. USA*, **96**, 5780–5785.