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COEXPRESSION OF THE CANNABINOID RECEPTOR TYPE 1 WITH DOPAMINE AND SEROTONIN RECEPTORS IN DISTINCT NEURONAL SUBPOPULATIONS OF THE ADULT MOUSE FOREBRAIN

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Abstract—The cannabinoid receptor type 1 (CB_1) displays unusual properties, including the dual capacity to inhibit or stimulate adenylate cyclase and a brain density considerably higher than the majority of G protein-coupled receptors. Together with overlapping expression patterns of dopamine and serotonin receptors this suggests a potential of CB_1 to modulate the function of the dopamine and serotonin system. Indeed, pharmacological studies provide evidence for cross-talks between CB_1 and receptors of these neurotransmitter systems. In trying to obtain further insights into possible functional and/or structural interactions between CB_1 and the dopamine receptors and the serotonin receptors, we performed double-label *in situ* hybridization at the cellular level on mouse forebrain sections by combining a digoxigenin-labelled riboprobe for CB_1 with ^{35}S-labelled riboprobes for dopamine receptors D1 and D2, and for serotonin receptors 5-HT1B and 5-HT3, respectively. As a general rule, we found that CB_1 colocalizes with D1, D2 and 5-HT1B only in low-CB_1-expressing cells which are principal projecting neurons, whereas CB_1 coexpression with 5-HT3 was also observed in high-CB_1-expressing cells which are considered to be mostly GABAergic. In striatum and olfactory tubercle, CB_1 is coexpressed to a high extent with D1, D2 and 5-HT1B. Throughout the hippocampal formation, CB_1 is coexpressed with D2, 5-HT1B and 5-HT3. In the neocortex, coexpression was detected only with 5-HT1B and 5-HT3. In summary a distinct pattern is emerging for the cannabinoid system with regard to its colocalization with dopamine and serotonin receptors and, therefore, it is likely that different mechanisms underlie its cross-talk with these neurotransmitter systems.

Key words: cannabinoids, colocalization, *in situ* hybridization, cross-talk, neurotransmitter systems.

The main psychoactive component of *Cannabis sativa*, Δ^9-tetrahydrocannabinol, exerts most of its effects by interacting with cannabinoid receptors. At present, two G protein-coupled receptors have been identified; the cannabinoid receptor type 1 (CB_1) is preferentially expressed in the CNS, whereas the cannabinoid receptor type 2 (CB_2) is mainly present in immune cells (for review see Pertwee, 1997). Based on the finding of endogenous ligands (Devane et al., 1992; Sugiura et al., 1995; Mechoulam et al., 1998), the endocannabinoid system has emerged as an important neuromodulatory system in brain physiology (for review see Di Marzo et al., 1998). Recent analyses of CB_1-deficient mice have further underlined the importance of the cannabinoid system in various behaviors such as learning and memory (Reibaud et al., 1999), drug withdrawal response (Ledent et al., 1999) and locomotor activity (Zimmer et al., 1999).

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Abbreviations: CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 2; CCK, cholecystokinin; D1, dopamine receptor 1; D2, dopamine receptor 2; DIG, digoxigenin; DTT, dithiothreitol; EDTA, ethylene diaminetetra-acetate; PBS, phoshate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; SSC, saline sodium citrate; 5-HT1B, serotonin receptor 1B; 5-HT3, serotonin receptor 3.

CB_1 displays unusual properties, including the dual capacity to inhibit or stimulate adenylate cyclase via $G_{i/o}$ or G_s proteins (Bonhaus et al., 1998; Shire et al., 1999) and a brain density considerably higher than any other known G protein-coupled receptor (Herkenham et al., 1990). Implicit in these properties is the potential of CB_1 to modulate the function of other receptor systems such as the dopamine and serotonin system, and evidence to support this notion is mounting, as the following observations exemplify. Glass and Felder (1997) found that the activation of either CB_1 or dopamine receptor 2 (D2) in rat primary striatal cells resulted in an inhibition of cAMP accumulation, whereas simultaneous activation of both receptors resulted in an increase of cAMP accumulation. Pharmacological experiments in mice by Meschler et al. (2000) showed that the application of a D2 agonist was able to attenuate the motor dysfunction caused by the CB_1 agonist levonantradol. Similarly, a dopamine receptor 1 (D1) agonist attenuated the effect of levonantradol, while a D1 antagonist enhanced the effects of levonantradol. A functional interaction of the cannabinoid and the dopamine system was also suggested in memory storage (Castellano et al., 1997; Nava et al., 2000). Regarding the serotonin system, it was shown that low concentrations of cannabinoid agonists inhibit the function of the serotonin receptor 3 (5-HT3) (Fan, 1995). Similar results were shown later by another group revealing that the pharma-

cological activity of the endocannabinoid anandamide might be partially mediated through serotonin receptors (Kimura et al., 1998). Such observations lead to the notion that there are allosteric binding sites for cannabinoids on the 5-HT3 receptor or that there is a cooperative interaction between these two receptors.

The first indication to possible interactions between different receptors is given, when both receptors are expressed within the same neuron. Indeed, CB_1 in rodent forebrain structures (Marsicano and Lutz, 1999; Tsou et al., 1998; Egertová and Elphick, 2000) displays a significant extent of overlapping expression with various dopamine and serotonin receptors, among which D1 (Mansour et al., 1991), D2 (Meador-Woodruff et al., 1989), serotonin receptor 1B (5-HT1B) (Maroteaux et al., 1992) and 5-HT3 (Tecott et al., 1993) are the focus of the present work. This study provides a comparative coexpression analysis at single-cell level of the CB_1 receptor with dopamine and serotonin receptors for the first time using double-label *in situ* hybridization experiments on mouse forebrain sections, by combining ^{35}S-labelled riboprobes for D1, D2, 5-HT1B, and 5-HT3, respectively, with a digoxigenin (DIG)-labelled riboprobe for CB_1.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

Animals were housed in a temperature- and humidity-controlled room with a 12 h light-dark cycle (light from 07:00–19:00) and with access to food and water *ad libitum*. The experimental protocols were approved by the Ethical Committee on Animal Care and Use of the Government of Bavaria, Germany.

Adult mice (3–5 months old; C57BL/6) were killed by cervical dislocation. Brains were removed, snap-frozen on dry-ice and stored at -80°C prior to sectioning. Brains were mounted on Tissue Tek (Polysciences, PA, USA), and 14-µm-thick coronal sections in consecutive series 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 (DIG)-labelled riboprobes were used. Probes were generated by reverse transcription-polymerase chain reaction (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: CB_1, accession number U22948, 1530 bp from 152 to 1682 (primers as described in Marsicano and Lutz, 1999); D1, accession number S46131, 560 bp from 4237 to 4792 (mouse probe cloned from the homologous rat sequence was a gift from Dr. Thomas Lemberger, DKFZ Heidelberg, Germany); D2, accession number X55674, 837 bp from 302 to 1139 (forward primer 5′-CTG TAT CAC GAG AGA AGG CTT; reverse primer 5′-CTG GGA TTG ACA ATC TTG GCA); 5-HT1B, accession number Z11597, 763 bp from 443 to 1206 (forward primer 5′-GCC AAA GGA GAC AAG CCT ATA, reverse primer 5′-GAG CAG GGT GGG TAA ATA GAA), and 5-HT3, accession number M74425, 1121 bp from 458 to 1579 (forward primer 5′-GGA AGT CTC CGA ACA TTC CTT, reverse primer 5′-CCC CCA TAC TTA TCC TAA CCA). 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-treated H_2O at a concentration of 1 µg/µl, and stored at −20°C until used. 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 [α-^{35}S]UTP (NEN, MA, USA; 1250 Ci/mmol), and 30 units of T7 or T3 RNA polymerase (Roche Molecular Diagnostics, Mannheim, Germany). For DIG-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, 5 µl of DIG RNA labelling mix (Roche Molecular Diagnostics), 80 units RNasin (Promega), and 100 units of T7 or T3 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: CB_1 sense, *Pst*I, T7; CB_1 antisense, *Bam*HI, T3; 5-HT1B sense, *Xba*I, T3; 5-HT1B antisense, *Eco*RI, T7; 5-HT3 sense *Eco*RI, T7; 5-HT3 antisense, *Xba*I, T3; D1 sense, *Sac*I, T3, D1 antisense, *Sal*I, T7; D2 sense, *Eco*RI, T7; D2 antisense *Bam*HI, T3. Using these probes in *in situ* hybridization experiments, sense controls did not give any detectable signals (data not shown), and antisense probes gave distribution patterns identical to those already published in rat or mouse (D2: Meador-Woodruff et al., 1989; D1: Mansour et al., 1991; 5-HT1B: Maroteaux et al., 1992; 5-HT3: Tecott et al., 1993; CB_1: Marsicano and Lutz, 1999).

In situ hybridization

Slides were warmed up for 30 min at RT and selected in such a manner that represents the whole mouse forebrain. They were 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) for 20 min, rinsed twice in PBS, quenched for 15 min in 1% H_2O_2 in 100% methanol, rinsed twice in PBS, quenched for 8 min in 0.2 M HCl, rinsed twice with PBS, treated with proteinase K 20 µg/ml (Roche Molecular Diagnostics) in 50 mM Tris–HCl, 5 mM EDTA (pH 8.0) for 10 min, rinsed once with PBS, fixed in ice-cold 4% paraformaldehyde in PBS, incubated for 10 min in 0.1 M triethanolamine (pH 8.0) to which 1.2 ml acetic anhydride was added dropwise, rinsed once with PBS, washed with 0.9% NaCl for 5 min, dehydrated in graded series of ethanol (30, 50, 70, 80, 95, 100%), and air-dried. Hybridization was carried out in 100 µl of hybridization buffer containing ^{35}S-labelled riboprobe (70 000–100 000 c.p.m./µl) and DIG-labelled riboprobe (0.2 µg/ml). Hybridization buffer consisted of 50% deionized formamide, 20 mM Tris–HCl (pH 8.0), 0.3 M NaCl, 5 mM EDTA (pH 8.0), 10% dextran sulfate (D8906, Sigma, Germany), 0.02% Ficoll 400 (F2637, Sigma), 0.02% polyvinylpyrrolidone (MW 40 000, PVP 40, Sigma), 0.02% bovine serum albumin (A6793, Sigma), 0.5 mg/ml tRNA (Roche Molecular Diagnostics), 0.2 mg/ml fragmented herring sperm DNA and 200 mM dithiothreitol. Before applying to the tissue, hybridization cocktail was denatured for 2 min at 95°C. Slides were incubated overnight at 54°C in a humidified chamber.

Four high-stringency washes were carried out at 62°C with 5× saline sodium citrate (SSC) (1× SSC contains 150 mM NaCl, 15 mM Na_3 citrate, pH 7.4)/0.05% Tween-20 (P7949, Sigma), then with 50% formamide/2× SSC/0.05% Tween-20, with 50% formamide/1× SSC/0.05% Tween-20, and finally with 0.1× SSC/0.05% Tween-20. All of the following post-hybridization washes and incubations were carried out at 30°C. Slides were washed with 0.5 M NaCl, 10 mM Tris–HCl (pH 8.0), 5 mM EDTA (NTE)/0.05% Tween-20, incubated with 15 mM iodoacetamide in NTE/0.05% Tween-20 for destruction of intracellular alkaline phosphatase, and washed twice with NTE/

0.05% Tween-20. Slides were blocked with 4% heat-inactivated sheep serum in 100 mM Tris–HCl (pH 7.6)/150 mM NaCl/0.05% Tween-20 (TNT), which was filtered through a 0.45-μm filter, washed three times with TNT, incubated for 30 min in blocking buffer (TSA Biotin System, NEN Life Science Products, Boston, MA, USA), incubated 1.5 h with anti-DIG antibody (Roche Molecular Diagnostics) diluted 1:1200 in blocking buffer, and washed three times in TNT. After antibody treatment, slides were incubated for 12 min with biotin-labelled tyramide (TSA Biotin System, NEN Life Science Products), washed with 100 mM maleic acid/150 mM NaCl/0.05% Tween-20 (wash buffer), incubated for 1 h with streptavidin–alkaline phosphatase (Roche Molecular Diagnostics) diluted 1:1000 with 1% blocking reagent (Roche Molecular Diagnostics) in wash buffer, and washed three times with Wash buffer. Chromogenic reaction was carried out with Vector Red kit (Vector Laboratories, CA, USA) at RT for 10–30 min. The reaction was stopped with a 10-min incubation in PBS, followed by 30 min in 2.5% glutaraldehyde in PBS and three washes for 10 min in 0.1× SSC. Slides were dehydrated in graded ethanol series, air-dried and exposed to Biomax MR film (Scientific Imaging Systems, NY, USA). On the next day, slides were dipped in photographic emulsion (NTB-2 from Kodak, diluted 1:1 in distilled H_2O). After exposure for 3–6 weeks at 4°C, slides were developed for 3 min (D-19, Kodak), fixed for 6 min (Kodak fixer), rinsed for 30 min in tap water and air-dried. Counterstaining was carried out for 10 s in 0.1% aqueous Toluidine Blue. Slides were mounted in Histofluid (Marienfeld, Lauda-Königshofen, Germany).

Numerical evaluation of coexpression

In double-label *in situ* hybridization experiments CB_1 mRNA was detected with a DIG-labelled riboprobe. As CB_1 is expressed at various levels, stained cells were classified according to the following criteria. Cells expressing CB_1 at high levels (termed high-CB_1-expressing cells described as GABAergic interneurons; Marsicano and Lutz, 1999) were considered to be those showing a round-shaped and intense red staining surrounding the nucleus or even covering the entire nucleus. Cells expressing CB_1 at low levels (termed low-CB_1-expressing cells described as mainly projecting principal neurons; Marsicano and Lutz, 1999) were defined as cells clearly stained above background levels and in a discontinuous shape and/or at uniform and low intensity of staining. The absolute intensity of staining varied in different *in situ* hybridization experiments, but the relative proportion of staining intensity of high to low-CB_1-expressing cells was the same. Different regions were chosen for numerical evaluation of coexpression based on the published distribution patterns of CB_1 and D1, D2, 5-HT1B or 5-HT3, which indicate a high extent of overlapping expression throughout the murine brain. Cells positive for CB_1 and one of the four markers were counted by choosing particular fields in these regions on at least three different brain sections and coexpression values were calculated as percentages. Double-label *in situ* hybridization experiments were carried out on three animals for each marker combination, showing always the same expression patterns. Our data exemplify the results from one experiment for each marker combination. Sections were analyzed on a Leica DMRB microscope.

RESULTS

Coexpression of cannabinoid CB1 receptor with dopamine receptors

CB1 and D1 receptors. The highest levels of D1 transcripts are observed in the basal ganglia (Mansour et al., 1991; Weiner et al., 1991), including caudate putamen, nucleus accumbens, and olfactory tubercle. High levels of low-CB_1-expressing cells are detected in the dorsolateral caudate putamen, while the nucleus accumbens contains only few low-CB_1-expressing cells. The olfactory tubercle shows an intense staining due to a high density of low-CB_1-expressing cells. Coexpressing cells were counted at a single-cell resolution in the caudate putamen (Table 1, Fig. 1A) and the olfactory tubercle, but not in the nucleus accumbens due to too weak signals of CB_1-positive cells. 46% of the medium-sized, CB_1-positive neurons in the dorsolateral caudate putamen coexpressed D1. Considering all D1-positive cells containing CB_1, the percentage reached 81% (Table 1). In the olfactory tubercle, D1 mRNA is present in 90% of the CB_1-expressing cells. The fraction of D1-positive cells containing CB_1 was 76%.

D1 transcripts were observed in two regions of the neocortex, but at much lower levels than in the striatum. A striking finding was that none of the high-CB_1-expressing cells in these cortical areas described below contained D1. The highest levels of D1 transcripts were observed in the piriform cortex. This cortical region showed also a high number of low-CB_1-expressing cells and a rather low number of high-CB_1-expressing cells. Due to the uniform distribution of cells expressing low levels of D1 in this area, it was not feasible to count coexpressing cells at a single-cell resolution. Thus, the numbers reflect an estimate only. 90% of CB_1-positive cells in the piriform cortex contain D1 mRNA, whereas 70% of D1-expressing cells showed also signals for CB_1 (Table 1). Coexpression of CB_1 with D1 was also observed in the dorsal endopiriform nucleus where 89% of CB_1-positive cells contain D1 mRNA and 69% of D1-positive cells coexpress CB_1 (Table 1). In other cortical areas such as the neocortex, entorhinal/perirhinal cortex and amygdala as well as in non-cortical areas such as the hypothalamus no signals for D1 transcripts were detected.

CB1 and D2 receptors. Similarly to D1, the strongest signals of D2 transcripts were detected in the caudate putamen, nucleus accumbens, and olfactory tubercle

Table 1. Coexpression of D1 receptor in low-cannabinoid-CB1-receptor-expressing neurons of the adult mouse forebrain

	Coexpression in cells expressing low CB_1		
	CB_1 cells with D1 (%)	D1 cells with CB_1 (%)	(*n*)
Dorsolateral caudate putamen	46	81	(3798)
Olfactory tubercle	90	76	(102)
Piriform cortex	90*	70*	(n.c.)
Dorsal endopiriform nucleus	89	69	(94)

n.c., not counted; *estimated percentages.

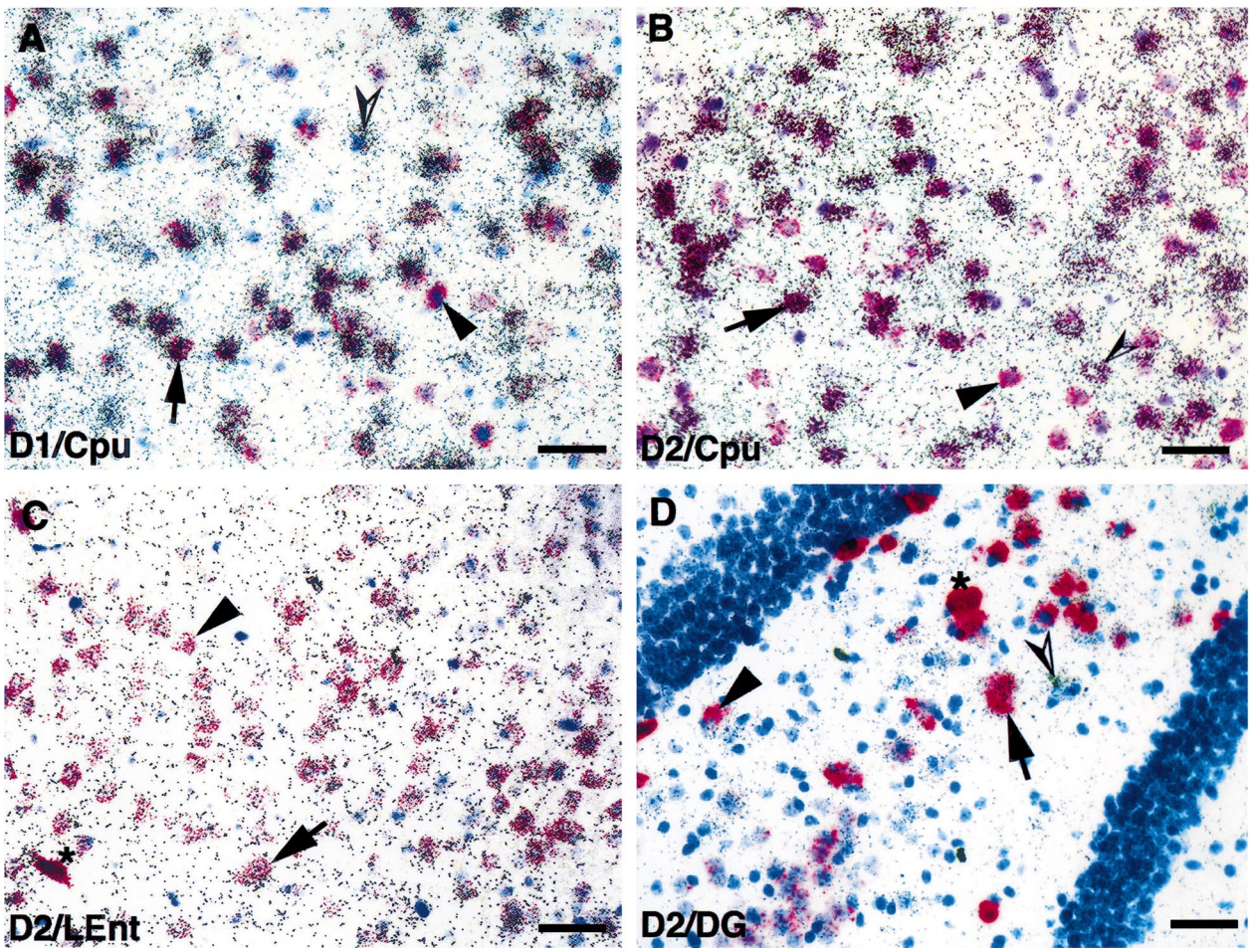

Fig. 1. Bright field micrographs of coronal sections showing examples of coexpression of CB_1 (red staining) with D1 and D2 (silver grains), respectively, as detected by double-*in situ* hybridization. All sections were counterstained with Toluidine Blue. (A, B) Coexpression of CB_1 with D1 and D2, respectively, in the caudate putamen (Cpu). (C) Coexpression of CB_1 with D2 in the lateral entorhinal cortex (LEnt). (D) Coexpression of CB_1 with D2 in the dentate gyrus (DG). Filled arrow, low-CB_1-expressing cell that coexpresses D1 and D2, respectively; filled arrowhead, low-CB_1-expressing cell; open arrowhead, D1- or D2-expressing cell; *high-CB_1-expressing cell. Scale bars = 200 μm.

(Meador-Woodruff et al., 1989; Weiner et al., 1991). In the dorsolateral caudate putamen and in the olfactory tubercle, 38% of the CB_1-positive neurons coexpress D2 (Table 2, Fig. 1B). Higher percentages of coexpression in these two areas were evaluated considering all D2-positive cells containing CB_1 with values of 73% in the striatum and 74% in the olfactory tubercle (Table 2).

Compared to the basal ganglia, the level of D2 transcripts in cortical areas is much lower. D2-CB_1 coexpression was detected only in low-CB_1-expressing cells. Rather high levels of D2 mRNA were observed in the piriform cortex. Due to the uniform distribution of D2-expressing cells in this area, the coexpression with CB_1 was estimated to 90% considering all CB_1-positive cells and 70% considering all D2-positive cells, respectively (Table 2). In the entorhinal/perirhinal cortex area,

Table 2. Coexpression of dopamine D2 receptor in low-cannabinoid-CB1-receptor-expressing neurons of the adult mouse forebrain

	Coexpression in cells expressing low CB_1		
	CB_1 cells with D2 (%)	D2 cells with CB_1 (%)	(*n*)
Dorsolateral caudate putamen	38	73	(4260)
Olfactory tubercle	38	74	(96)
Piriform cortex	90*	70*	(n.c.)
Entorhinal/perirhinal cortex area	80	77	(104)
Dentate gyrus (polymorph layer)	88	48	(376)

n.c., not counted; *estimated percentages.

which contains high numbers of low- and high-CB_1-expressing cells, neurons could be counted at a single-cell resolution. 80% of CB_1-positive cells coexpress D2, and 77% of D2-positive cells express CB_1 (Fig. 1C, Table 2).

In the hippocampus, CB_1 signals with intensities ranging from low to very high were observed in all layers. Coexpression with D2 was detected in the polymorph layer of the dentate gyrus (Fig. 1D), where D2 hybridization signals were detected in 88% of the low-CB_1-expressing cells, but only 48% of all D2-positive cells do coexpress CB_1 (Table 2). In other cortical areas such as the neocortex and amygdala D2 signals were not detected.

Coexpression of cannabinoid CB1 receptors with serotonin receptors

CB1 and 5-HT1B receptors. High expression levels of 5-HT1B mRNA were detected in striatum and olfactory tubercle, in agreement with the described expression pattern (Maroteaux et al., 1992). Percentages of coexpression in these regions are illustrated in Table 3. Evidently, the majority of CB_1- and 5-HT1B-expressing cells in the dorsolateral part of the caudate putamen (Fig. 2A) and the olfactory tubercle show coexpression. Intense signals for 5-HT1B were also observed in the nucleus accumbens, where most of the cells express 5-HT1B (data not shown). Due to the low expression levels of CB_1 in this area, coexpression could not be numerically evaluated by double-label *in situ* hybridization, but CB_1 is expressed in approximately 20% of the cells (Moldrich and Wenger, 2000; B. Lutz, data not shown). Thus, also in the nucleus accumbens, an estimate of 90% CB_1-expressing cells coexpress 5-HT1B.

Weaker signals for 5-HT1B were detected in the hippocampus, neocortex and hypothalamus, consistent with the known expression pattern (Maroteaux et al., 1992). In the pyramidal cells of hippocampal CA1 region, which express low levels of CB_1 mRNA (Marsicano and Lutz, 1999), 100% coexpression was observed. 5-HT1B mRNA was observed in a scattered manner throughout layers II–III of the neocortex, whereas both low- and high-CB_1-expressing cells were located primarily in layers II–III and V–VI. In layers II–III, at least 70% of all low-CB_1- and 5-HT1B-expressing cells show coexpression (Table 3, Fig. 2B), whereas high-CB_1-expressing neurons never express 5-HT1B. The ventromedial hypothalamic nuclei showed the presence of low-CB_1- and 5-HT1B-expressing cells that are uniformly distributed at high cell density. The coexpression rate was estimated to be more than 90% (Fig. 2C).

CB1 and 5-HT3 receptors. Coexpression of CB_1 and 5-HT3 was observed in several cortical regions for both low- and high-CB_1-expressing cells. As compared to CB_1 and all other markers described above, the number of 5-HT3-expressing cells in the mouse forebrain is rather low (Tecott et al., 1993). Therefore, the percentages of coexpression considering low-CB_1-expressing cells that coexpress 5-HT3 are very low, in the range of 0.9–3.6%, for all described regions except for the hippocampal formation (Table 4). In the hippocampal CA1 and CA3 areas (excluding the pyramidal cells, which do express CB_1 but not 5-HT3), the majority of both high and low-CB_1-expressing cells shows coexpression with 5-HT3, the extent being higher in CA3 (Fig. 3A). In the dentate gyrus, coexpression of 5-HT3 with low-CB_1-expressing cells is only 17% (Fig. 3B). In all parts of hippocampus, the fraction of 5-HT3-expressing cells containing CB_1 is between 35% and 39% for high-CB_1-expressing cells, thus, it is much higher than for low-CB_1-expressing cells (9–14%). This characteristic is also observed in all other forebrain regions analyzed, including neocortex (Fig. 3C), anterior olfactory nucleus, piriform cortex and entorhinal/perirhinal cortex. Regarding this feature, the basolateral amygdaloid nucleus is peculiar, as the extent of 5-HT3-expressing cells containing CB_1 was approximately the same for high (21%) and low (24%) CB_1-expressing cells (Table 4, Fig. 3D).

DISCUSSION

Increasing evidence indicates that a single receptor subtype may be linked to the formation of multiple, intracellular signals. However, it is unlikely that all signals driven by a single receptor subtype are equally operative under all circumstances, but it seems that the functional weight of one pathway relative to another can be altered by interactions with other receptors. The first indication for possible interactions between different receptors is given, when both receptors are expressed within the same neuron. Therefore, the aim of this study was to define in detail coexpression patterns of CB_1 with dopamine and serotonin receptors in distinct neuronal subpopulations of the adult mouse forebrain. We thus performed double-label *in situ* hybridization

Table 3. Coexpression of 5-HT1B receptor in low-CB_1-expressing neurons of the adult mouse forebrain

	Coexpression in cells expressing low CB_1		
	CB_1 cells with 5-HT1B (%)	5-HT1B cells with CB_1 (%)	(*n*)
Dorsolateral caudate putamen	72	81	(2983)
Olfactory tubercle	70	77	(226)
Hippocampal CA1 area**	100*	100*	(n.c.)
Layers II–III of neocortex	70	74	(1084)
Ventromedial hypothalamic nuclei	>90*	>90*	(n.c.)

n.c., not counted; *estimated percentages; **principal neurons only.

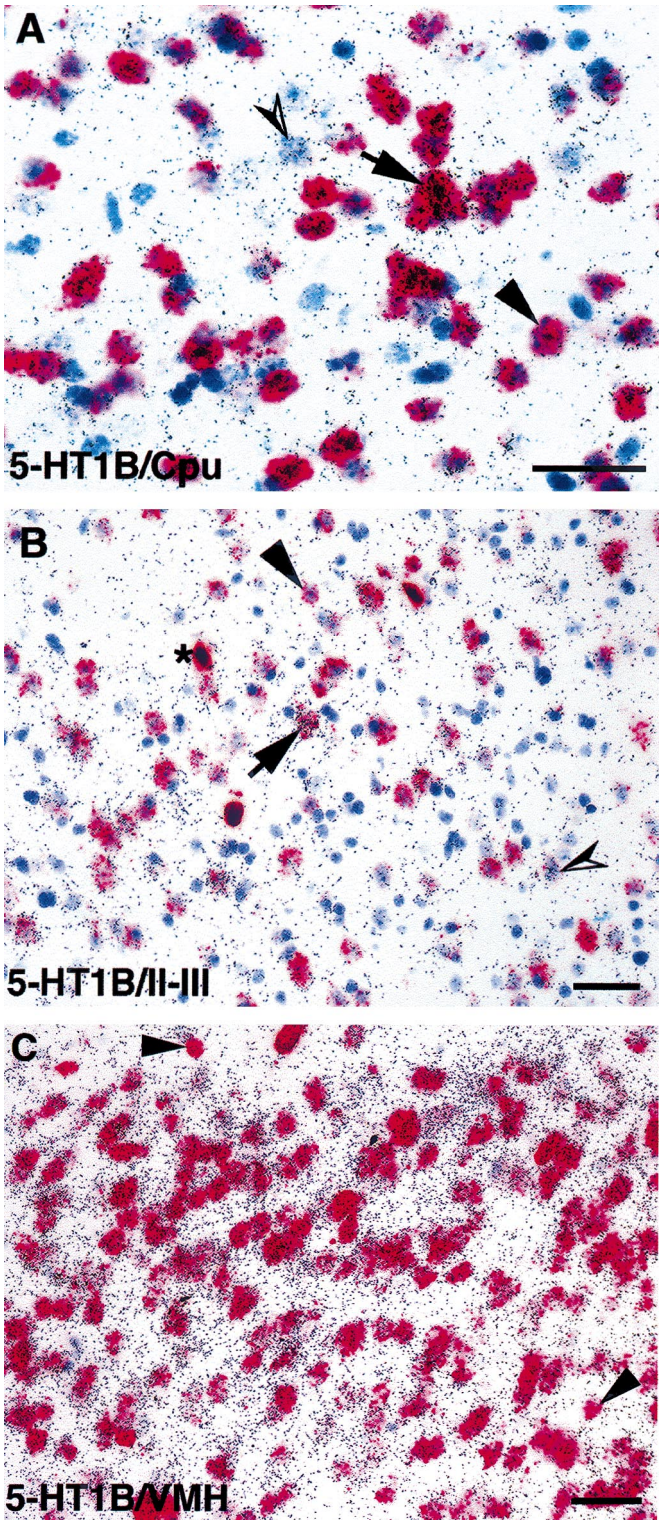

Fig. 2. Bright field micrographs of coronal sections showing examples of coexpression of CB_1 (red staining) with 5-HT1B (silver grains) as detected by double-*in situ* hybridization. All sections were counterstained with Toluidine Blue. (A) Coexpression of CB_1 with 5-HT1B in the caudate putamen (Cpu). (B) Coexpression of CB_1 with 5-HT1B in layers II and III of neocortex (II–III). (C) Coexpression of CB_1 with 5-HT1B in the ventromedial hypothalamic nuclei (VMH). Filled arrow, low-CB_1-expressing cell that coexpresses 5-HT1B; filled arrowhead, low-CB_1-expressing cell; open arrowhead, 5-HT1B-expressing cell; *high-CB_1-expressing cell. Scale bars = 200 μm.

Table 4. Coexpression of 5-HT3 receptor in cannabinoid-CB1-receptor-expressing cells of the adult mouse forebrain

	Coexpression in cells expressing low CB_1			Coexpression in cells expressing high CB_1		
	CB_1 cells with 5-HT3 (%)	5-HT3 cells with CB_1 (%)	(*n*)	CB_1 cells with 5-HT3 (%)	5-HT3 cells with CB_1 (%)	(*n*)
Anterior olfactory nucleus	1.0	19	(1134)	42	36	(97)
Piriform cortex	2.1	26	(1056)	41	32	(117)
Entorhinal/perirhinal cortex area	1.0	20	(1715)	40	31	(165)
Neocortex, layers I–II	2.2	22	(1691)	46	30	(336)
Neocortex, layers III–IV	0.9	20	(1573)	40	35	(160)
Neocortex, layers V–VI	3.6	18	(609)	41	35	(187)
Hippocampus, CA1*	77	13	(313)	73	35	(480)
Hippocampus, CA3*	96	9	(275)	82	35	(409)
Dentate gyrus	17	14	(151)	77	39	(134)
Basolateral amygdaloid nuclei (anterior)	2.8	24	(767)	31	21	(129)

*Excluding principal neurons, which do not coexpress 5-HT3.

experiments using a DIG-labelled CB_1 riboprobe in combination with ^{35}S-labelled riboprobes for D1, D2, 5-HT1B and 5-HT3, respectively.

Both the significant extent of overlapping expression of CB_1 with various dopamine and serotonin receptors and several investigations observing functional interactions between the cannabinoid system and other neurotransmitter systems gave reason to chose the markers mentioned above. Previous immunohistochemical and *in situ* hybridization studies carried out in rats observed high levels of D1 and D2 mRNA (Weiner et al., 1991; Levey et al., 1993) and 5-HT1B mRNA (Maroteaux et al., 1992) in the striatum. CB_1 is also expressed in many neurons throughout the striatum (Herkenham et al., 1991; Egertová and Elphick, 2000; Tsou et al., 1998; Marsicano and Lutz, 1999). These observations are in agreement with our results which revealed a high density of hybridization signals of these probes. Particularly in the dorsolateral caudate putamen, a high degree of colocalization of CB_1 with D1, D2 and 5-HT1B, respectively, was detected. The striatum is a key component of the forebrain system that controls planning and execution of motor behaviors (for a review see Nakano et al., 2000). CB_1 agonists can markedly affect the function of these systems, producing alterations of locomotion and catalepsy (Howlett, 1995; Martellotta et al., 1998). Also dopamine stimulates motor activity in the basal ganglia (Alexander and Crutcher, 1990; Parent and Hazrati, 1995). A mechanism for influencing striatal function through cannabinoids and/or dopamine could be an interaction between the cannabinoid and dopamine system which is suggested by the high coexpression rates of CB_1 and D2 shown in this study. Cell culture experiments on striatal neurons revealed clear evidence for an interaction between CB_1 and D2. Activation of either CB_1 or D2 resulted in an inhibition of cAMP accumulation, whereas simultaneous activation of both receptors resulted in an augmentation of cAMP accumulation (Glass and Felder, 1997). Also *in vivo* studies with mice treated with different combinations of cannabinoid agonists and dopamine agonists/antagonists revealed that the two receptor systems appear to interact by exerting opposing influences in regulating motor activity which also hinted to a coexistence of CB_1 and D1/D2 on the same striatal neurons (Meschler et al., 2000) as evidenced also in our study.

Immunocytochemical investigations of Aizman et al. (2000) demonstrate the coexistence of D1- and D2-like receptors in all virtually striatal neurons which can be divided in two different populations responsible either for substance P release or enkephalin release (Graybiel, 1990). CB_1 was also detected in these two neuronal subpopulations and is involved in the regulation of expression of these neuropeptides (Mailleux and Vanderhaeghen, 1992). CB_1-deficient mice display significantly increased levels of substance P and enkephalin mRNAs in these striatal neurons (Steiner et al., 1999). Therefore, colocalization of CB_1 with D1 and D2 in the caudate putamen suggests a putative regulation system of the two receptor systems in controlling expression of these neuropeptides.

5-HT1B was also shown to be involved in motor behavior. Administration of the 5-HT1B agonist RU 24969 to rats resulted in an increase of locomotor activity (Oberlander et al., 1987), whereas application of the 5-HT1B antagonists GR 127935 could block the RU 24969-induced hyperactivity in rodents (O'Neill et al. et al., 1996). In our study, high coexpression levels of 5-HT1B and CB_1 were detected in the striatum assuming a modulatory role for 5-HT1B together with CB_1 in motor function.

CB_1 is differentially coexpressed with all four markers in several cortical regions (hippocampus, neocortex, entorhinal/perirhinal cortex, amygdala) which contribute to important brain functions, e.g. learning and memory (Suzuki, 1996; Miller et al., 1998). Considering this, modulation of cognitive processes could be mediated through the interaction of CB_1 with dopamine or serotonin receptors. Recently, it was shown that Δ^9-tetrahydrocannabinol-induced impairment of working memory was reversed by a D2 antagonist and potentiated by a D2 agonist, concluding that this effect is mediated by the simultaneous activation of CB_1 and D2 (Nava et al., 2000). The concurrent activation of both receptors might produce an accumulation of cellular cyclic AMP in neurons where these receptors are colocalized.

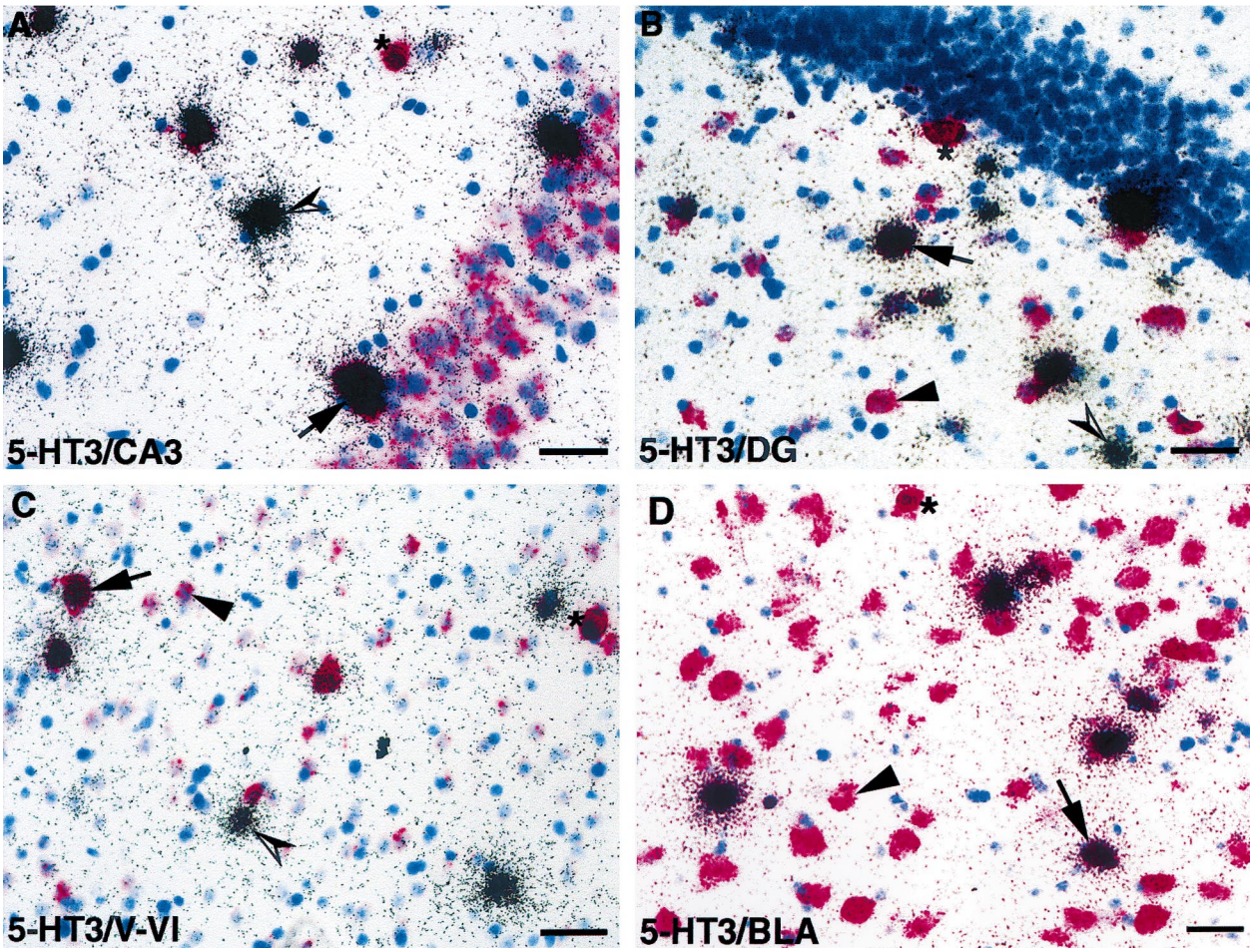

Fig. 3. Bright field micrographs of coronal sections showing examples of coexpression of CB_1 (red staining) with 5-HT3 (silver grains) as detected by double-*in situ* hybridization. All sections were counterstained with Toluidine Blue. (A) Coexpression of CB_1 with 5-HT3 in the CA3 area of hippocampus (CA3). (B) Coexpression of CB_1 with 5-HT1B in the dentate gyrus (DG). (C) Coexpression of CB_1 with 5-HT1B in layers V and VI of neocortex (V–VI). (D) Coexpression of CB_1 with 5-HT3 in the basolateral amygdala (BLA). Filled arrow, high-CB_1-expressing cell that coexpresses 5-HT3; filled arrowhead, low-CB_1-expressing cell; open arrowhead, 5-HT3-expressing cell; *high-CB_1-expressing cell. Scale bars = 200 µm.

A striking finding of our study was that the coexpression of CB_1 with 5-HT3 in several forebrain regions (hippocampus, neocortex, anterior olfactory nucleus, amygdala) was mainly detected in high-CB_1-expressing cells, which are considered as GABAergic neurons belonging predominantly to the cholecystokinin (CCK)-positive type of interneurons (Marsicano and Lutz, 1999; Tsou et al., 1999; Katona et al., 1999). In line with our observation, *in situ* hybridization and immunocytochemistry experiments by Morales and Bloom (1997) showed that 5-HT3-expressing neurons in the neocortex, olfactory cortex, hippocampus and amygdala are mainly GABA-containing cells with CCK immunoreactivity. The widespread colocalization of 5-HT3 with CB_1 in GABAergic neurons suggests the participation of these two receptors in the modulation of inhibitory neurons. As these cells contain CCK, a role of 5-HT3 and CB_1 in regulating CCK neurotransmission might be put forward.

The mechanism of such interactions remains to be investigated, but recently, direct evidences for heterodimerization between different neurotransmitter receptors have been reported (for review see Bouvier, 2001). However, interaction could also take place at the level of the intracellular signalling pathways. Considering the high levels of expression of CB_1 in mammalian brain (Herkenham et al., 1990), it is therefore plausible to hypothesize a putative mechanism explaining the functional interaction of the cannabinoid system with other receptor systems. Coexpression of CB_1 and other receptors in the same neurons provides the first condition for such direct, functional interactions.

CB_1 and its colocalization with receptors for other neurotransmitters can contribute to understanding certain neurodegenerative diseases, caused by multiple neurotransmitter and receptor alterations. In reserpine-treated rats, an animal model for Parkinson's disease, increased levels of endocannabinoids in basal ganglia were found (Di Marzo et al., 2000), which might be correlated to significantly reduced levels of CB_1 expression in striatum in this disease (Silverdale et al., 2001). Moreover, Di Marzo et al. (2000) could show that coadministration of a D2 agonist and a CB_1 antagonist leads to full restoration of normal locomotor behavior in

reserpine-treated rats suggesting a close functional relationship between the cannabinoid and the dopamine system. These data support the idea that modulation of the endocannabinoid signalling system provide a useful treatment for the symptoms of Parkinson's disease or other basal ganglia-related movement disorders.

In summary, CB_1 is differentially coexpressed in the mouse forebrain with dopamine and serotonin receptors either in principal projecting neurons (mainly with D1, D2 and 5-HT1B) or in interneurons (mainly with 5-HT3). Together, these receptor systems might be involved in modulating excitatory circuits as well as inhibitory GABAergic circuits. Particularly in the striatum, high coexpression extent of CB_1 with D1, D2 and 5-HT1B, respectively, were observed, suggesting putative cross-talks between the cannabinoid system and other neurotransmitter systems regulating locomotor activity. High levels of coexpressing cells in cortical areas might be an indication for a functional interaction of CB_1 with dopamine and serotonin receptors, respectively, having modulatory effects on cannabinoid-induced impairment of working memory and cognitive functions.

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