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IDENTIFICATION OF THE CANNABINOID RECEPTOR TYPE 1 IN SEROTONERGIC CELLS OF RAPHE NUCLEI IN MICE

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Abstract—The endocannabinoid system (ECS) possesses neuromodulatory functions by influencing the release of various neurotransmitters, including GABA, noradrenaline, dopamine, glutamate and acetylcholine. Even though there are studies indicating similar interactions between the ECS and the serotonergic system, there are no results showing clear evidence for type 1 cannabinoid receptor (CB1) location on serotonergic neurons. In this study, we show by *in situ* hybridization that a low but significant fraction of serotonergic neurons in the raphe nuclei of mice contains CB1 mRNA as illustrated by the coexpression with the serotonergic marker gene tryptophane hydroxylase 2, the rate limiting enzyme for the serotonin synthesis. Furthermore, by double immunohistochemistry and confocal microscopy, we were able to detect CB1 protein on serotonergic fibers and synapses expressing the serotonin uptake transporter in the hippocampus and the amygdala. Our findings indicate that the CB1-mediated regulation of serotonin release can depend in part on a direct cross-talk between the two systems at single cell level, which might lead to functional implications in the modulation of emotional states.

Key words: CB1, serotonin, endocannabinoid system, hippocampus, amygdala.

The endocannabinoid system (ECS) is a recently discovered neuromodulatory system implicated in a multitude of physiological and pathophysiological functions. There is an ever-growing evidence that it exerts an important control of several neuronal functions by influencing the activity of diverse neurotransmitter systems (Schlicker and Kathmann, 2001; Freund et al., 2003; Piomelli, 2003) and by regulating different behavioral processes (Valverde, 2005; Wotjak, 2005). Endogenous cannabinoids (endocannabinoids), released from the post-synapse after activation of the postsynaptic site, are believed to travel retrogradely and to bind to type 1 cannabinoid receptors (CB1), located at presynaptic level and modulate the release of several neurotransmitters (Schlicker and Kathmann, 2001; Alger, 2002; Piomelli, 2003; Chevaleyre et al., 2006; Marsicano and Lutz, 2006). To date, CB1 presence has been verified in GABAergic, dopaminergic, adrenergic, glutamatergic and cholinergic neurons (e.g. Marsicano and Lutz, 1999; Kathmann et al., 1999; Hajos and Freund, 2002; Wallmichrath and Szabo, 2002; Monory et al., 2006; Nyiri et al., 2005). Evidence for CB1 in serotonergic neurons is still missing, although the results of various studies suggest that there is a direct connection between the ECS and the serotonergic system. Thus, the lack of CB1 reduces the responsiveness of mice to the anxiolytic drug buspirone, a 5-HT receptor agonist (Uriguen et al., 2004). *In vitro* studies show that the release of 5-HT can be altered by the CB1 agonist WIN55, 212-2 and the CB1 antagonist rimonabant in mouse cortex slices (Nakazi et al., 2000). In other studies it has been found that the fatty acid amide hydrolase inhibitor URB597 increases firing of 5-HT neurons in the dorsal raphe nucleus and increases hippocampal levels of 5-HT after repeated administration (Gobbi et al., 2005).

Influencing the serotonergic system via the ECS might lead to new approaches to treat various anxiety disorders, as both systems have been shown to influence various physiological functions and to control a wide range of behaviors and emotional states (Lucki, 1998; Walther and Bader, 2003; Linthorst, 2005; Wotjak, 2005; Piomelli, 2003). Serotonin is present in many tissues, where it functions as neurotransmitter or hormone. In the brain, the highest concentrations of serotonergic neurons can be detected in midbrain and brainstem areas called raphe nuclei. Projections from the raphe nuclei were found to innervate many regions of the CNS, including spinal cord, hypothalamus, cortex, hippocampus, amygdala and striatum (Conrad et al., 1974).

Given that CB1 activation regulates serotonin release (Nakazi et al., 2000; Gobbi et al., 2005), it is possible that this function is exerted either by direct CB1-mediated control of serotonergic neurons and/or by indirect CB1-dependent modulation of afferent fibers contacting serotonergic neurons. A necessary prerequisite to support the first hypothesis is the presence of CB1 on serotonergic neurons at single cell level, which has not been clearly demonstrated yet (Ashton et al., 2006). We therefore used tryptophane hydroxylase type 2 (TPH2) as marker gene for serotonergic cells in the raphe nuclei and performed double *in situ* hybridization (ISH) experiments to detect coexpression of TPH2 and CB1 mRNA. TPH2 is one isoform of the rate limiting enzyme for serotonin synthesis, which can

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Abbreviations: B-Amyg, nucleus amygdalae basalis; CB1, type 1 cannabinoid receptor; ECS, endocannabinoid system; ir, immunoreactivity; ISH, *in situ* hybridization; L-Amyg, nucleus amygdalae lateralis; PBS, phosphate-buffered saline; PFA, paraformaldehyde; TBS, Tris-buffered saline; TPH2, tryptophane hydroxylase type 2; 5-HTT, 5-HT reuptake transporter.

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predominantly be found in serotonergic cells of the raphe nuclei and represent an optimal marker of these neurons (Patel et al., 2004). Furthermore, we used double fluorescence immunohistochemistry and confocal analysis to identify in detail CB1 protein expression on 5-HT reuptake transporter (5-HTT) positive fibers and synapses. 5-HTT is a serotonin reuptake transporter located in projecting fibers of serotonergic cells and represents an optimal marker for these neurons at protein level (Zhou et al., 1998). Our results indicate that a low number of serotonergic neurons in the raphe nuclei and of serotonergic fibers in the hippocampus and amygdala contain CB1 mRNA and protein, respectively. Thus, a direct influence of CB1 on the activity of serotonergic neurons is possible, although indirect mechanisms are also likely to occur.

EXPERIMENTAL PROCEDURES

Animals

This study was performed on adult (3–5 months old) C57BL/6N female mice. Animals were housed in a temperature- and humidity-controlled room with a 12-h light/dark cycle and had access to food and water *ad libitum*. As the amount of TPH2 mRNA was found to be under circadian control with the highest level 1–2 h before the ending of the light cycle (Malek et al., 2005), the animals were killed in this time point. The experimental protocols were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Ethical Committee on animal care and use of Rheinland-Pfalz, Germany. Every effort was made to minimize the number of animals used and their suffering.

ISH

ISH and double ISH procedures were as described in Marsicano and Lutz, 1999 and Hermann et al., 2002, respectively. Mice were shortly anesthetized by isoflurane and decapitated. Brains were isolated and snap-frozen on dry ice and stored at −80 °C. After removing from −80 °C, brains were mounted on Tissue Freezing Medium (Jung, Nussloch, Germany) and 18-μm thick coronal or sagittal sections were cut on a Microm HM560 cryostat (Microm, Walldorf, Germany). Sections were mounted on frozen SuperFrost Plus slides (Menzel, Braunschweig, Germany) and stored at −20 °C until use. CB1 cDNA was as described (Marsicano and Lutz, 1999) and TPH2 cDNA (AY090565; nucleotides 2036–2624) was a kind gift of Dr. Paresh D. Patel (University of Michigan Medical Center, Ann Arbor, MI, USA). The TPH2 cDNA sequence, contained in a pSportI vector (p699 TPH2-3′UT), was subcloned into a pBluescript vector using the restriction enzymes *Kpn*I and *Xba*I (New England Biolabs, Ipswich, MA, USA). After linearization with *Kpn*I, the antisense riboprobe was synthesized with T7 RNA polymerase (Roche, Basel, Switzerland). For the generation of the sense riboprobe, *Xba*I was used for linearization and T3 RNA polymerase (Roche) for the synthesis. Both radioactive (^{35}S) and non-radioactive (DIG)-labeled riboprobes were used as described in Marsicano and Lutz, 1999 and Hermann et al., 2002. The incubation with sense riboprobes did not show any signal (data not shown).

Immunohistochemistry

For IHC, mice were deeply anesthetized with pentobarbital and trans-cardially perfused with 4% paraformaldehyde (PFA) solution. After isolation, the brains were post-fixed for 24 h in 4% PFA solution, treated with 30% sucrose/phosphate-buffered saline (PBS) solution for 48 h and stored at −80 °C until use. For section preparation, 30 μm thick brain slices were prepared on a Microm

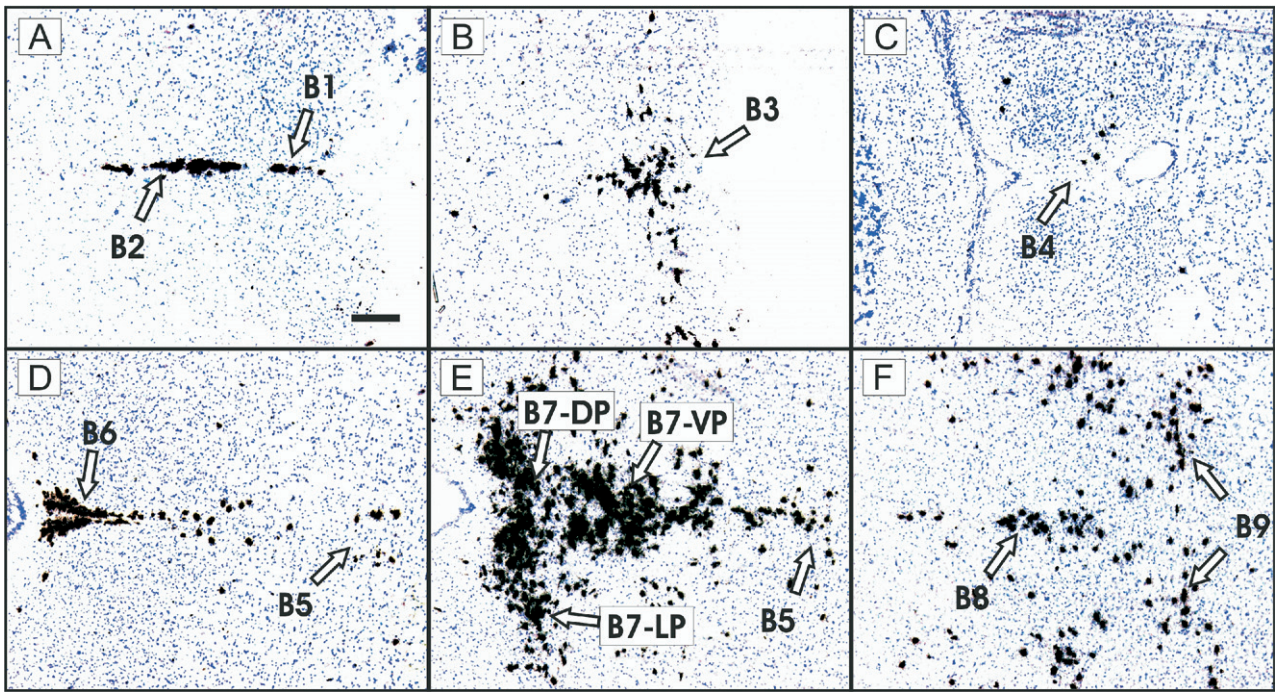

Fig. 1. Identification of serotonergic cells in the different raphe nuclei in coronal brain sections. Panels A–F show the different raphe nuclei (B1-9) identified according to Dahlström and Fuxe (1964), by detecting TPH2 mRNA (silver grains). (A) nucleus raphe pallidus (B1) and nucleus raphe obscurus (B2), (B) nucleus raphe magnus (B3), (C) nucleus paragigantocellularis (B4), (D) nucleus raphe pontis resp. medianus (B5) and nucleus dorsalis (caudal part) (B6), (E) nucleus raphe dorsalis (B7) (VP, ventral part; LP, lateral part; DP, dorsal part) and (F) nucleus centralis superior (B8) and nucleus reticularis pontis (B9). Scale bar=200 μm.

HM560 cryostat then stored at −20 °C in cryoprotection solution (25% glycerin, 25% ethylene glycol and 50% PBS) until use. To determine where CB1 is located in serotonergic cells, immunohistochemical experiments were performed using polyclonal antibodies against CB1 (rabbit L15 antiserum, directed against the last 15 amino acids of CB1, diluted 1:5000; kind gift of K. Mackie, University of Washington, Department of Anesthesiology, Seattle, WA, USA Billerica, MA, USA) and 5-HTT (guinea-pig anti-5HTT-IgG, AB1772, diluted 1:5000; Chemicon, USA) on free-floating brain sections. All incubation steps were performed in wells of a

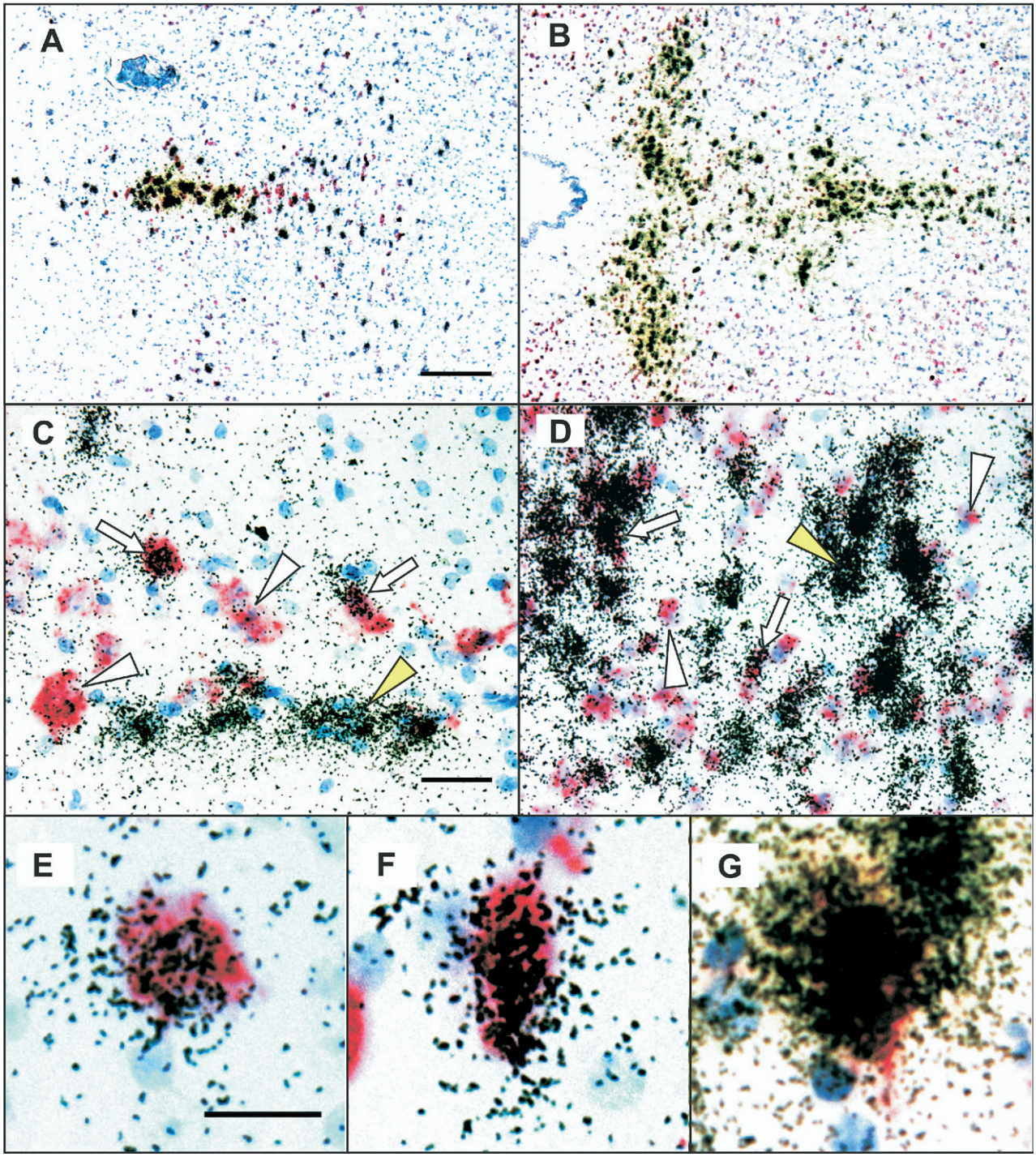

Fig. 2. Presence of CB1 mRNA in serotonergic cells. Coexpression of CB1 (red staining) and TPH2 (silver grains) in the raphe nuclei. (A) Low magnification photomicrograph of B8 and B9. (B) Low magnification photomicrograph of B7. (C) Region B9. (D) Central part of region B7. (E–G) Coexpressing cells showing different levels of TPH2 and/or CB1 expression. White arrows, CB1/TPH2 double positive serotonergic neurons; white arrowheads, single CB1-expressing neurons; yellow arrowheads, single TPH2-expressing neurons. Blue staining: Toluidine Blue counterstaining. Scale bars=250 μm (A, B), 30 μm (C, D) and 15 μm (E–G).

Table 1. Quantification of CB1 expression in serotonergic and non-serotonergic cells in the rostral raphe nuclei

Raphe nucleus	Synonym	Cells expressing CB1 only	Cells expressing TPH2 only	Cells expressing TPH2+CB1	CB1 expressing cells (% of serotonergic cells)
N. raphe dorsalis (CP)	B6	30±6	32±7	17±8	22±6.2
N. raphe dorsalis (VP)	B7	27±3	85±10	6±1	7±1
N. raphe dorsalis (LP)	B7	71±8	72±9	6±1	8±2
N. raphe dorsalis (DP)	B7	45±3	63±8	16±2	22±3.5
N. raphe centralis sup.	B8	39±4	56±4	4±1	7±1
N. raphe reticul. pontis	B9	48±10	61±11	5±1	8±1.7

Counting of single TPH2, single CB1 or coexpressing cells was performed on 6–14 serial sections for each nucleus. Number of cells counted for both lateral parts of the N. raphe dorsalis per section was added together in this analysis. CP, caudal part; VP, ventral part; LP, lateral part; DP, dorsal part.

six-well-plate (5–6 ml solution per well) on a wave shaker (Heidolph, Schwabach, Germany) at RT. Sections were first rinsed from cryoprotection solution in Tris-buffered saline (TBS) (25 mM Tris/HCl, 150 mM NaCl, pH 7.6) (10 min) and then pre-incubated in blocking solution (5% normal donkey serum, 2.5% bovine serum albumin, 0.3% Triton X-100 in TBS) for 1 h. After blocking, the sections were treated o/n with the primary antibodies which were diluted in blocking solution. On the next day, the sections were washed in 1× TBS for 5×5 min at RT and then incubated for 2 h with the matching secondary antibodies diluted in blocking solution; Cy3-labeled anti-rabbit-IgG from goat 1:200 (Jackson ImmunoResearch, West Grove, PA, USA) for anti-CB1-antibody, and FITC labeled anti-guinea-pig-IgG from donkey 1:100 (Jackson ImmunoResearch) for anti-5HTT-IgG. The incubation was followed by five washing steps in 1× TBS-T (1× TBS/0.1% Triton X-100). Sections were counterstained with Hoechst 33258 (2 μg/μl). After the counterstaining the sections were washed for 2×2 min in distilled water, then carefully transferred into a Petri dish filled with 1× TBS. Sections were then mounted onto glass slides to dry for 2–4 h at 37 °C. The remaining salt was washed away by dipping the slides for 2 s into distilled water. Finally the sections were dried overnight in a dust free environment at RT and covered with Citifluor mounting medium (Agar Scientific, Stansted, UK). Omission of primary anti-sera resulted in no detectable signal (data not shown).

Confocal laser scanning microscopy

Sections were inspected using the confocal laser-scanning microscope Leica TCS SP2 (Leica Microsystems Wetzlar, Germany), equipped with appropriate excitation and emission filters for maximum separation of Cy3 and FITC signals. Applying the Leica Confocal Software and Adobe Photoshop (version 7.0, Adobe Inc., San Jose, CA, USA), images were saved and processed.

RESULTS

Single ISH

TPH2 transcripts were detected in mid- and hindbrain areas identified as the raphe nuclei in agreement with previously published data (Patel et al., 2004), thus confirming TPH2 as a marker gene for serotonergic neurons (Fig. 1). Following the classification of Dahlström and Fuxe (1964), all nine raphe nuclei, nucleus raphe pallidus (B1), nucleus raphe obscurus (B2), nucleus raphe magnus (B3), nucleus paragigantocellularis (B4), nucleus raphe pontis resp. medianus (B5), nucleus dorsalis (caudal part) (B6), nucleus raphe dorsalis (B7), nucleus centralis superior (B8) and nucleus reticularis pontis (B9), were identified.

As shown in Fig. 1, the highest number of TPH2-expressing cells was found in the dorsal raphe nuclei (B7), which is the largest raphe nucleus. The raphe nuclei B6 and B5 were difficult to identify, because they merge as caudal elongations into the much larger raphe nuclei B7 and B8, respectively. In B4, located at the caudal end of B6, serotonergic cells have a scattered distribution.

Double ISH

Cells coexpressing TPH2 and CB1 mRNA were identified in all raphe nuclei except in the pons nucleus B4 and the medullary nucleus B1. Most coexpressing neurons were identified in the different parts of the dorsal and median raphe nuclei (Fig. 2A, B), mostly in the dorsal part of B7 and the rostral part of B6 (Table 1). For B2, B3 and B5, only scattered cells expressing both genes were detected. Apart from the coexpressing cells (Fig. 2C, D; white arrows) and the single TPH2 expressing cells (Fig. 2C, D; yellow arrowheads), a significant number of cells expressing low levels of CB1 were identified (Fig. 2C, D; white arrowheads). The coexpressing cells show different levels of expression for TPH2 and CB1 (Fig. 2E–G). Most coexpressing cells were found to contain low levels of CB1 (Fig. 2). In contrast, the expression levels of TPH2 showed higher variability (Fig. 2E–G).

Immunohistochemistry

Based on the findings of the double ISH, showing that the large caudal nuclei B6–B9 contain a significant number of serotonergic cells expressing CB1, and on published work on CB1 and 5-HTT localization, several brain areas were studied for colocalization of CB1 and 5-HTT proteins. In all the forebrain areas analyzed, strong 5-HTT and CB1 immunoreactivity (ir) was detected in accordance with previous findings on the distribution of these two proteins (Bengel et al., 1997; Tsou et al., 1998, 1999; Owashi et al., 2004; O'Rourke and Fudge, 2006). For all regions studied, scattered dot-like overlapping signals were detected (Fig. 3, yellow arrowheads), likely indicating synaptic structures.

CB1 and 5-HTT ir was identified in isolated fibers in parts of hippocampus and amygdala (Fig. 3, white arrows); for example in the boundary between the stratum radiatum and the stratum lacunosum moleculare (CA3-BRM) as well as in the stratum pyramidale (CA3-PyL). Interestingly, the co-staining was detected only in the CA3 subregion. In

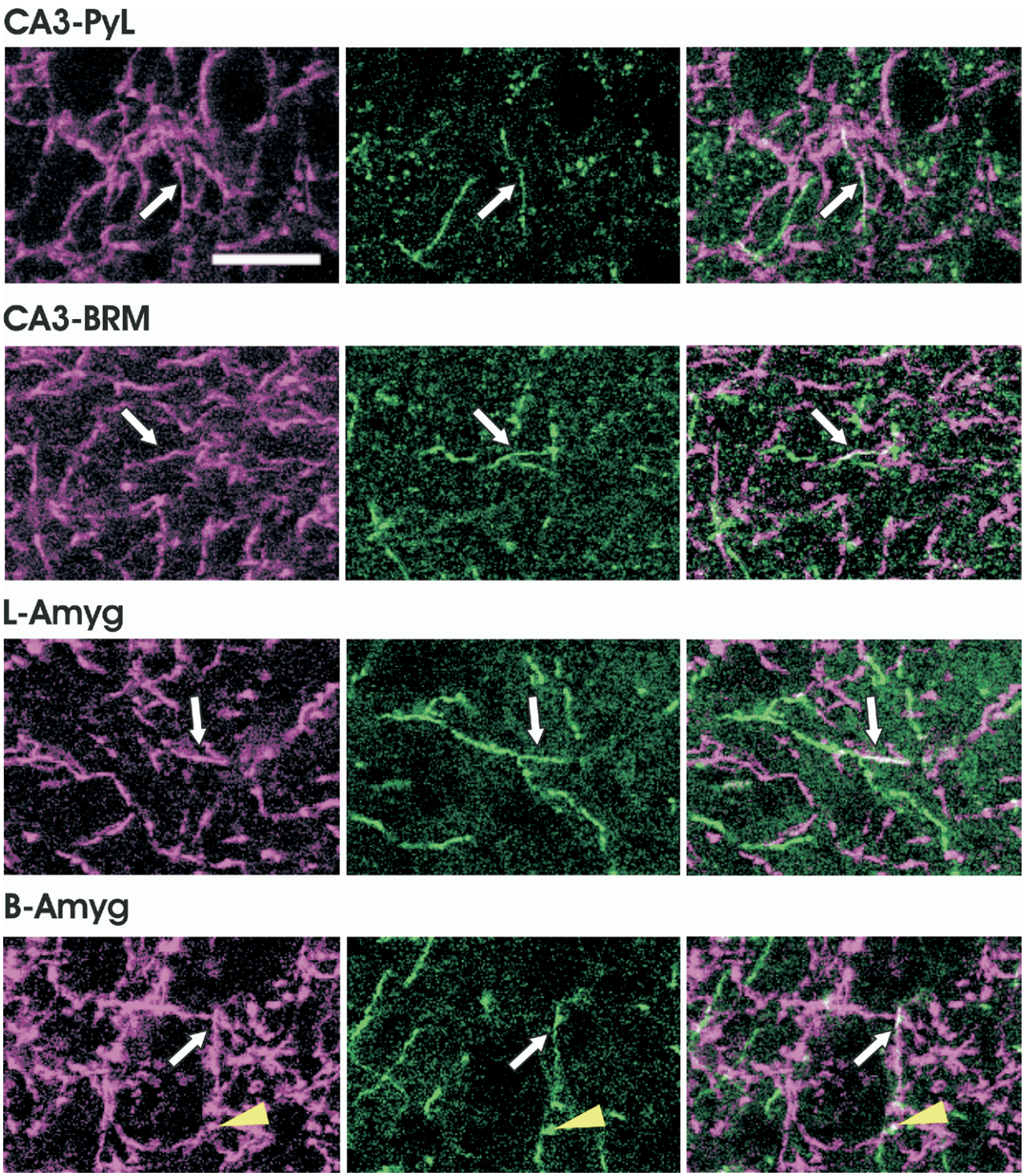

Fig. 3. Colocalization of 5-HTT and CB1 protein in amygdala and hippocampus. Representative false color images showing expression of CB1 (magenta, left column), 5-HTT (green, middle column) and merged staining (white, right column) in different regions of hippocampus and amygdala. CA3-PyL, stratum pyramidale of the hippocampal CA3 region; CA3-BRM, boundary between the stratum radiatum and the stratum lacunosum moleculare. White arrows, fibers positive for both proteins; yellow arrowheads, dot-like signals positive for both proteins. Scale bar=15 μm.

amygdaloidal areas, the nucleus amygdalae basalis (B-Amyg) and the nucleus amygdalae lateralis (L-Amyg) were found to contain fibers positive for both (5-HTT and CB1) proteins.

To prove the specificity of the CB1 ir, we performed an immunostaining with the CB1 antibody on coronal brain sections of a CB1 knockout mouse and a wild-type littermate (Marsicano et al., 2002). Fig. 4 shows part of the

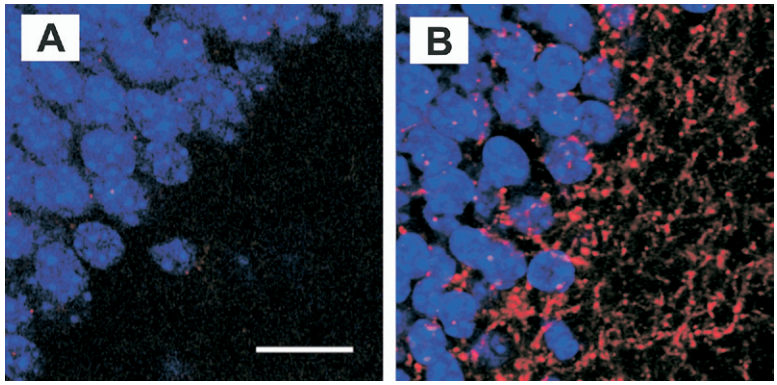

Fig. 4. CB1 ir in hippocampal sections. Photomicrographs showing the inner molecular layer of the dentate gyrus of CB1 knockout (A) and wild type (B) mice. Red staining: CB1 ir. Blue staining: nuclear counterstaining. Note the lack of ir in the CB1 knockout section. Scale bar=20 μm.

hippocampus of these mice. In Fig. 4B, the typical wild-type staining for CB1 can be seen with rich filamental staining. The lack of staining in Fig. 4A demonstrates that the CB1 ir seen in the double immunolabeling experiments is indeed specific.

DISCUSSION

In this study, we show that CB1 receptors are present in a subset of serotonergic neurons in the brain. These results support the concept that a direct cross-talk between the ECS and serotonergic neurons exists and that the observed effects of exogenous and/or endogenous activation of CB1 receptors on serotonin release (Nakazi et al., 2000; Egashira et al., 2002; Darmani et al., 2003; Tzavara et al., 2003) can be at least in part due to a direct action of CB1 receptors on serotonergic terminals.

Although in several raphe nuclei there is a low level of TPH2-CB1 coexpression, in the caudal and dorsal part of nucleus raphe dorsalis (B6 and B7, respectively) we found that more than 20% of serotonergic cells express CB1. Importantly, this area receives intense glutamatergic innervation from the prefrontal cortex (Hajos et al., 1998), a brain region expressing a significant amount of CB1 (Marsicano and Lutz, 1999). Some of the fibers from prefrontal cortex innervate directly the serotonergic neurons while the majority of them synapse onto GABAergic interneurons (Jankowski and Sesack, 2004), which maintain a tonic inhibition of the 5-HT neurons (Tao et al., 1996). Among other important functions, the prefrontal cortex is also involved in stress controllability decisions (Amat et al., 2005, 2006), which in turn plays a role in the development of several psychological disorders, such as depression and PTSD.

The relatively low number of serotonergic fibers expressing CB1 protein detected might be due to technical and/or neurochemical reasons. In fact, it is possible that our counting procedure underestimates the actual levels of CB1 on serotonergic neurons as sensitivity of available antibodies for CB1 receptors might represent a limit for the detection of CB1 on serotonergic neurons at the protein level. Indeed, the high variability in the intensity of CB1 expression in different neuronal subpopulations made it difficult in the past to detect the receptor in some subpopulations of neurons. For instance, the presence of CB1 protein on glutamatergic fibers and terminals of the hippocampus has not been shown (Freund et al., 2003) until recently (Monory et al., 2006; Kawamura et al., 2006; Katona et al., 2006). More sensitive CB1 antisera have recently been developed, but they showed a certain degree of background staining in CB1-defficient mice (Katona et al., 2006). It is, therefore, possible that the amount of CB1 mRNA and protein in serotonergic neurons might be higher than estimated in the present study, thereby reinforcing the concept of a direct effect of CB1 receptors on the release of serotonin from serotonergic terminals.

Nevertheless, it is very likely that the ECS interferes with serotonergic neurons also by indirect means. For instance, retrograde endocannabinoid signaling has recently been shown to modulate orexin B-dependent release of glutamate onto serotonergic neurons in the raphe nuclei (Haj-Dahmane and Shen, 2005). Moreover, cannabinoids could alter the responses of serotonin receptors (Hill et al., 2006) and the ECS could, under some circumstances, increase the firing rate of serotonergic neurons in the raphe nucleus via still unidentified mechanisms (Gobbi et al., 2005). Indeed, CB1 mRNA is present in a significant number of non-TPH2-positive neurons in the raphe nuclei (Table 1). These non-serotonergic neurons are very likely to be GABAergic interneurons, which could mediate an indirect CB1-dependent enhancement of serotonergic activity in specific conditions (Gobbi et al., 2005).

Most papers published so far addressing the interaction between the ECS and serotonin in the brain have used systemic administration of drugs, making it difficult to interpret the data in terms of site of action (Egashira et al., 2002; Darmani et al., 2003; Tzavara et al., 2003). However, our data are in agreement with the study by Nakazi et al. (2000), showing that cannabinoid agonists are able to decrease serotonin release in isolated mouse cortex slices, indicating the possible direct control by CB1 receptors of serotonin release at presynaptic level.

Our data support the existence of a functional interaction between the endocannabinoid and the serotonergic systems in the brain. This interaction might be important in

the regulation of several functions controlled by these two systems, including anxiety, mood and affective disorders (Gobbi et al., 2005; Arevalo et al., 2001; Tzavara et al., 2003).

The mechanisms of these functional interactions might be of multiple nature. However, our data support the concept that a direct control of serotonin release by CB1 receptors is among the possible means of such interactions and might represent a future promising therapeutic target for the treatment of brain diseases involving both the endocannabinoid and the serotonergic systems.

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