Distribution of type 1 cannabinoid receptor expressing neurons in the septal-hypothalamic region of the mouse. Colocalization with GABAergic and glutamatergic markers.

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Abstract

Type 1 cannabinoid receptor (CB1) is the principal mediator of retrograde endocannabinoid signaling in the brain. In this study, we addressed the topographic distribution and amino acid neurotransmitter phenotype of endocannabinoid-sensitive hypothalamic neurons in mice. The in situ hybridization detection of CB1 mRNA revealed high levels of expression in the medial septum (MS) and the diagonal band of Broca (DBB), moderate levels in the preoptic area and the hypothalamic lateroanterior (LA), paraventricular (Pa), ventromedial (VMH), lateral mammillary (LM) and ventral premammillary (PMV) nuclei, and low levels in many other hypothalamic regions including the suprachiasmatic (SCh) and arcuate (Arc) nuclei. This regional distribution pattern was compared to location of GABAergic and glutamatergic cell groups, as identified by the expression of glutamic acid decarboxylase 65 (GAD65) and type 2 vesicular glutamate transporter (VGLUT2) mRNAs, respectively. The MS, DBB and preoptic area showed overlaps between GABAergic and CB1-expressing neurons, whereas hypothalamic sites with moderate CB1 signals, including the LA, Pa, VMH, LM and PMV, were dominated by glutamatergic neurons. Low CB1 mRNA levels were also present in other glutamatergic and GABAergic regions. Dual-label in situ hybridization experiments confirmed the cellular co-expression of CB1 with both glutamatergic and GABAergic markers. In this report we provide a detailed anatomical map of hypothalamic glutamatergic and GABAergic systems whose neurotransmitter release is controlled by retrograde endocannabinoid signaling from hypothalamic and extrahypothalamic target neurons. This neuroanatomical information contributes to the understanding of the role that the endocannabinoid system plays in the regulation of endocrine and metabolic functions.
Introduction

Endocannabinoids modulate brain neurotransmission primarily via acting on type 1 cannabinoid receptor (CB1) localized on presynaptic axon terminals (Pagotto et al., 2006; Piomelli, 2003). Activation of CB1 by endogenous lipid ligands including anandamide (Devane et al., 1992) and 2-arachidonoyl-glycerol (2-AG) (Mechoulam et al., 1995) decreases the synaptic release of GABA (Hoffman and Lupica, 2000), glutamate (Gerdeman and Lovinger, 2001; Huang et al., 2001), acetylcholine (Degroot et al., 2006), noradrenaline (Vizi et al., 2001) and possibly, other neurotransmitters. CB1 is one of the most abundant G-protein-coupled receptors which is distributed widely in the rodent brain (Bellocchio et al., 2010; Herkenham et al., 1990; Katona et al., 2001; Katona et al., 1999; Marsicano and Lutz, 1999; Matsuda et al., 1993; McDonald and Mascagni, 2001; Tsou et al., 1998).

Endocannabinoids exert profound effects on hypothalamic functions, including endocrine hormone secretion, appetite, food intake and energy balance (for a review, see: (Pagotto et al., 2006)). Somewhat conflictingly, previous studies only detected a low abundance of CB1 immunoreactive axons (Tsou et al., 1998) and a low expression level of CB1 mRNA (Cota et al., 2003; Marsicano and Lutz, 1999) in the rodent hypothalamus, leaving the central regulatory mechanisms unexplained. A more recent immunocytochemical mapping study (Wittmann et al., 2007) from our laboratory, which used a newly developed CB1 antiserum (Fukudome et al., 2004), revealed an unexpectedly dense CB1-immunoreactive fiber network in the mouse hypothalamus. The CB1-immunoreactive terminals established symmetric as well as asymmetric synapses, indicating that retrograde endocannabinoid signaling in the
hypothalamus can influence inhibitory and excitatory synaptic transmission, respectively. The lack of previous immunocytochemical proof for this surprisingly dense CB1 immunoreactive input to hypothalamic neurons and the previous evidence that CB1 can be synthesized at highly variable levels in distinct classes of neurons elsewhere in the brain (Katona et al., 2006), suggest that the detection sensitivity is critical to successfully visualize moderate or low levels of CB1. This is apparently a problem when in situ hybridization is used to visualize CB1 expression. Neurons of the rodent hypothalamus only seem to contain low levels of CB1 mRNA, observed primarily in non-GABAergic cells of the ventromedial and anterior hypothalamic nuclei (Marsicano and Lutz, 1999) and in specific subsets of peptidergic neurons in the paraventricular nucleus (Cota et al., 2003).

While partial information already exists about the topography and phenotype of hypothalamic CB1 expressing neurons (Bellocchio et al., 2010; Cota et al., 2003; Marsicano and Lutz, 1999; Matsuda et al., 1993), there is no comprehensive study available about the presence, signal level and amino acid neurotransmitter phenotype of CB1 mRNA expressing neurons in distinct septal/hypothalamic regions of the mouse. In the present study we used an improved radioisotopic in situ hybridization technique (Hrabovszky and Petersen, 2002) to establish a detailed map of CB1 mRNA-expressing neurons. To also identify the amino acid neurotransmitter phenotype of these endocannabinoid-sensitive cells, the CB1 expression map was compared to the distribution of GABAergic and glutamatergic septal/hypothalamic neurons, characterized by the expression of glutamic acid decarboxylase 65 (GAD65) and type 2 vesicular glutamate transporter (VGLUT2) mRNAs, respectively. Finally,
dual-label *in situ* hybridization experiments were carried out to confirm the cellular colocalization of CB1 mRNA with the GABAergic and glutamatergic markers.
**Methods**

**Animals**

Adult male CD1 mice (N=10; 30-35 g BW) derived from a local breeding colony at the Medical Gene Technology Unit of the Institute of Experimental Medicine were used for the experiments. They were housed under standard environmental conditions (lights on between 0600–1800 h, temperature 22±1 °C, humidity 60±10%, standard rodent chow and water *ad libitum*). All experiments were carried out in accordance with the Council Directive of 24 November 1986 of the European Communities (86/609/EEC) and were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine (47/003/2005).

**Preparation of the mouse CB1 probe**

To prepare a cRNA hybridization probe to mouse CB1 receptor mRNA, a 1368-bp cDNA fragment was amplified with polymerase chain reaction from mouse hypothalamic cDNA. The amplicon (corresponding to bases 579-1946 of the mouse CB1 mRNA; NM_007726.2) was inserted into plasmid vector using the PGEM T cloning kit from Promega (Madison, WI). The plasmid was grown in DH5α cells (Invitrogen, Carlsbad, CA, USA), isolated with the QIAfilter Plasmid Maxi kit (Qiagen; Valencia, CA, USA), linearized with Sal I and purified with phenol/chloroform/isoamyl alcohol (PCI), followed by chloroform/isoamyl alcohol (CI) extractions, and then, precipitation with NaCl and ethanol. The linearized transcription template was transcribed with T7 RNA polymerase in the presence of ^35^S-UTP (NEN Life Science Products, Boston, MA, USA), to yield antisense transcripts (Hrabovszky et al., 2004). To generate a sense control for specificity
testing, the insert was cleaved at an internal ApaI site and a 1 Kb sense transcript was transcribed using the SP6 promoter site.

**Preparation of the mouse GAD65 and VGLUT2 probes**

Templates to generate probes to the GABAergic and glutamatergic marker enzymes, GAD65 and VGLUT2, respectively, were prepared with the PGEM T cloning kit, as described above for the CB1 cDNA. The inserts corresponded to bases 166-994 of mGAD65 (NM_008078) and bases 1762-2390 of mVGLUT2 (NM_080853) mRNAs. Probes for single-labeling experiments were transcribed in the presence of $^{35}$S-UTP.

**Single-label in situ hybridization studies**

**Tissue preparation**

Five mice were decapitated. The brains were removed and snap-frozen on powdered dry ice. Then 12-μm-thick coronal sections were cut from the septal and hypothalamic regions of the frozen tissues using a Leica CM 3050 S cryostat (Leica Microsystems, Vienna, Austria), thaw-mounted on microscope slides coated with (3-Aminopropyl) triethoxy-silane (Sigma, St. Louis, MO, USA), and air-dried. The sections were then processed for the radioisotopic in situ hybridization detection of CB1 mRNA.

**Prehybridization tissue treatments**

Prior to hybridization, the sections were pretreated as described elsewhere (Hrabovszky et al., 2004). Briefly, they were fixed in 4% paraformaldehyde for 15 min, acetylated with 0.25% acetic anhydride in 0.9% NaCl/0.1 M triethanolamine (Sigma; pH 8.0) for 10 min, rinsed in standard saline citrate solution (2XSSC; 1X...
SSC=0.15 M NaCl/0.015 M sodium citrate, pH 7.0) for 2 min, dehydrated in 70, 80, 95 and 100% ethanol (2 min each), delipidated in chloroform for 5 min, and finally, rehydrated partially in 100%, followed by 95% ethanol (2 min each) and air dried.

**Hybridization and posthybridization**

Every tenth hypothalamic section from each of five mice, was hybridized with a $^{35}$S-labeled antisense probe against mouse CB1 mRNA. Further two series consisting of immediate neighbors of these sections were hybridized to the antisense VGLUT2 and the antisense GAD65 probes, respectively, whereas one series was Nissl-stained with cresyl violet, to facilitate anatomical analysis. Sections hybridized with sense transcripts were processed in parallel, to serve as negative controls. To prevent the formation of autoradiographic background in the hybridization experiments, high concentrations of dithiothreitol (1000 mM) were added to the hybridization buffer (Hrabovszky and Petersen, 2002). To enhance hybridization signal and allow single-cell detection of low signal levels, high concentrations of radioisotopic probe (80,000 cpm/μl) and dextran sulfate (20%) were used in the hybridization solution (Hrabovszky and Petersen, 2002). Following an overnight hybridization at 52 °C, the non-specifically bound probe was digested with 20 μg/ml ribonuclease A (Sigma; dissolved in 0.5 M NaCl/10 mMTris-HCl/1 mM EDTA; pH 7.8) for 60 min at 37 °C, followed by a stringent treatment step to further clear the background (55 °C in 0.1X SSC solution for 60 min). The slides were dipped into MQ water for 2 sec, rinsed in 70% ethanol for 5 min and air-dried.
**Autoradiography**

The slides were exposed to Kodak BioMax MR autoradiography films for 5 days and the signal was developed with standard procedures. Then the sections were coated with Kodak NTB nuclear track emulsion (Kodak; Rochester, NY; diluted 1:1 with distilled water) and exposed for 3 weeks. The autoradiographs were visualized with Kodak processing chemicals. The sections were lightly counterstained with 0.05% Toluidine blue (Sigma) to enable the distinction of hypothalamic nuclei, then dehydrated with 95%, followed by 100% ethanol (five min each), cleared with xylene (2X5 minutes), and coverslipped with DPX mounting medium (Fluka Chemie; Buchs, Switzerland).

**Dual-label in situ hybridization studies**

To demonstrate the cellular coexpression of CB1 with GAD65 or CB1 with VGLUT2 mRNAs, five mice were deeply anesthetized with a cocktail of ketamine (25 mg/kg), xylavet (5 mg/kg) and pipolphen (2.5 mg/kg) in saline and sacrificed by transcardiac perfusion with 10 ml 0.1M phosphate buffered saline (PBS) followed by 40 ml of 4% paraformaldehyde in 0.1M phosphate buffered saline. The brains were removed, postfixed for 1 h in the same fixative, infiltrated with 20% sucrose overnight and then, snap-frozen on dry-ice. Septal-hypothalamic blocks were dissected and 25-μm-thick coronal sections were cut on a freezing microtome (Leica). Every fourth section was processed to colocalize CB1 with GAD65 mRNA. A second series was processed similarly to study CB1/VGLUT2 coexpression. The floating sections were acetylated with 0.25% acetic anhydride in 0.9% NaCl/0.1 M triethanolamine-HCl for 10 min, rinsed in 2X SSC for 2 min, treated sequentially
with 50%, 70% and 50% acetone (5 min each), rinsed with 2X SSC and transferred into hybridization solution containing a cocktail of the radioisotopic CB1 probe (80,000 cpm/µl) and the digoxigenin-labeled mGAD65 or mVGLUT2 hybridization probe (1:50 dilution) in PCR tubes. The hybridization buffer included 1000 mM DTT, and unlike for single-labeling, 10%, instead of 20% dextran sulfate. The digoxigenin-labeled mGAD65 and mVGLUT2 probes were transcribed in vitro in the presence of digoxigenin-11-UTP (Roche Diagnostics Co., Indianapolis, IN, USA) as detailed previously (Hrabovszky et al., 2004). Following the use of posthybridization treatments (see under single-labeling), the floated sections were rinsed briefly with 100 mM maleate buffer (pH 7.5) and blocked for 30 min against non-specific antibody binding with 2% blocking reagent (Roche) in maleate buffer. Then anti-digoxigenin antibodies conjugated to horseradish peroxidase (anti-digoxigenin-POD; Fab fragment; 1:100; Roche) were applied to the sections overnight at 4°C. To visualize the enzyme reaction, the sections were first rinsed in TBS (0.1 M Tris-HCl with 0.9% NaCl; pH 7.8), then incubated for 30 min with biotinylated tyramide working solution (TSA kit; NEN Life Science Products, Boston, MA) according to the manufacturer’s instructions (NEN). Following biotin tyramide deposition and brief rinses in TBS, the sections were transferred into the ABC-alkaline phosphatase (AP) reagent (Vector, Burlingame, CA; 1:1000) for 1 h. The enzyme reaction was visualized with the 5-bromo-4-chloro-3-indolyl-phosphate/4-nitro blue tetrazolium (BCIP/NBT) chromogen system according to the manufacturer’s instructions (Roche). The development of purple color reaction was monitored at intervals and stopped after 120 minutes. The sections were mounted on gelatin-coated microscope
slides and dipped sequentially twice into 1% Parlodion (Mallinckrodt; St. Louis, MO, USA) in amyl acetate. This step can efficiently prevent positive chemography (high autoradiographic background) associated with the use of the BCIP/NBT chromogen in dual-label in situ hybridization experiments (Hrabovszky et al., 2006; Hrabovszky et al., 2004; Hrabovszky et al., 1995). Then, the autoradiographic signal was detected with Kodak NTB emulsion in the same way as in single-labeling experiments.

**Microscopic analysis**

Dark-field images of emulsion autoradiographs from single-labeling experiments were scanned with an AxioCam MRc 5 digital camera mounted on a Zeiss AxioImager M1 microscope, using a 10X objective lens and the MozaiX modul of the AxioVision 4.6 software (Carl Zeiss, Göttingen, Germany). Additional digital photomicrographs of single- and dual-labeled sections were prepared in bright-field mode. All digital images were processed with the Adobe Photoshop 7.1 software at 300dpi resolution. Panels within the same figure were merged into a single image in which brightness and contrast were adjusted.

**Results**

*General distribution of hybridization signals*

The autoradiographic detection of CB1, GAD65 and VGLUT2 mRNAs revealed highly patterned signal distributions in various septal-hypothalamic regions (Figs. 1 and 2). In order to be considered specifically labeled with the radioactive probes, individual neurons had to exhibit at least 5 times higher autoradiographic signal (area covered by silver grains/selected total area) than the area fraction of silver grains in a
surrounding background region, as determined with the Image J software (public domain at http://rsb.info.nih.gov/ij/download/src/). No labeling was obtained following the use of sense transcripts.

The incidences of neurons labeled for the three mRNAs varied from region to region. These variations were presented on arbitrary three point scales ("+"=low; "+"=moderate; "+++"=high; Columns 1-3 of Table 2) for each area and probe. High scores revealed the regions of extensive overlap between CB1 expressing neurons and distinct GABAergic and glutamatergic cell populations.

In addition, labeled neurons also expressed variable single-cell levels of the signal. Another three point scale was used to rate the cellular intensity of labeling ("+"=low; "+"=moderate; "+++"=high) typical of labeled neurons in each area (Columns 4-6 of Table 2). In case of CB1 mRNA, the low signal level ("+"") exceeded 5-10 times the level of non-specific background. Moderate signal ("++") was 10-20 higher than background labeling, whereas high signal level ("+++") was 20-50 times higher than non-specific background.

Regional distribution of CB1 mRNA

CB1-expressing neurons were found in high numbers (+++ in the lateral (LS) and medial (MS) septal nuclei, in the nuclei of the horizontal (HDB) and vertical (VDB) limbs of the diagonal band of Broca (DBB), in the medial preoptic area (MPA) and in the anteroventral periventricular (AVPe), median preoptic (MnPO), medial preoptic (MPO), supraoptic (SO), ventromedial (VMH), ventral premammillary (PMV) and lateral mammillary (LM) nuclei. The abundance of labeled neurons was moderate (++) in many other regions, such as the vascular organ of the lamina terminalis.
(VOLT), the lateral preoptic area (LPO), the anterodorsal preoptic nucleus (ADP), the ventromedial preoptic nucleus (VMPO), the lateroanterior (LA), paraventricular (Pa), dorsal premammillary (PMD) and supramammillary (SuM) nuclei and the lateral hypothalamus (LH). Only few cells (+) contained CB1 mRNA in the arcuate (Arc; typically in the posterior part), suprachiasmatic (SCh), dorsomedial (DM) or medial mammillary (medial and lateral parts; MM, ML) nuclei. For results of the detailed topographic analysis, see column 1-3 in Table 2. For photographic illustrations, see Figs. 1 and 2.

**Cellular intensity of CB1 labeling**

The CB1 hybridization signal strength was low (+), moderate (++) or high (+++) in labeled septal and hypothalamic neurons and did not reach the heaviest (++++) CB1 hybridization signal within the brain which was present in scattered interneurons of the neocortex (Fig. 3A), amygdala (Fig. 3B) and hippocampal formation (Fig. 3C) and in the majority of neurons of the lateral caudate putamen (CPu; Fig. 3D). Variations in the labeling intensity of septal-hypothalamic CB1-expressing neurons are illustrated in Fig. 3. Specifically, high single-cell CB1 mRNA levels (+++) were observed in the DBB (Figs. 1B, F and 3F) and the MS (Figs. 1B, F and 3E). Moderate levels (++) were typical of labeled neurons in several subdivisions of the preoptic region (Figs. 1F and J), including the medial preoptic area (MPA; Fig. 3G) and the medial preoptic nucleus (MPO; Fig. 3H). Moderate single-cell levels (++) were also observed in several hypothalamic nuclei, including the LA (Fig. 1N), Pa (Fig. 3L), VMH (Figs. 1R, 2B and 3J), LM (Figs. 2N, R and 3K), PMV (Figs. 2F and J), PMD (Fig. 2J), and LM (Figs. 2N and R). Neurons in the AVPe (Fig. 3I) and in numerous
hypothalamic regions including the SO (Fig. 3M) contained low single-cell levels (+) of the CB1 hybridization signal (although most neurons were labeled), which was sometimes only slightly above background labeling (+/-). If labeled, neurons of the Arc (especially caudally; Fig. 3N) and the Pe (Fig. 3O) also exhibited low signal levels only.

**Distribution of the GABAergic and glutamatergic marker mRNAs**

Typically, GAD65-expressing GABAergic and VGLUT2-expressing glutamatergic neurons exhibited distribution patterns complementary to one another (Figs. 1 and 2). GABA represented the dominant amino acid neurotransmitter phenotype in neurons of the MS (Figs. 1C, G), LS (Fig. 1C), HDB (Figs. 1C, G), VDB (Fig. 1C), in most subdivisions of the preoptic region (Figs. 1G and K), in the anterior hypothalamic area (Figs. 1O, S) and in the hypothalamic SCh (Fig. 1Q), Arc (Figs. 2C, G) and DM (Fig. 2C) nuclei. Predominantly glutamatergic sites included the Pa (Figs. 1P, T), SO (Fig. 1P), VMH (Figs. 1T and 2D), LM (Figs. 2P, T), PMV (Figs. 2H, L), PMD (Fig. 2L), medial mammillary (MM and ML; Figs. 2P, T), LM (Figs. 2P, T) and SuM (Figs. 2P, T) nuclei. Both GABAergic and glutamatergic neurons were present in high numbers in the AVPe (Figs. 1G, H). The regional abundance of GABAergic and glutamatergic neurons as well as the cellular intensity of labeling were scored on similar three point scales as for CB1 mRNA (For detailed results, see Table 2 and Figs. 1 and 2).
Overlaps between the distribution of CB1 mRNA with GABAergic and glutamatergic marker mRNAs

The distribution of neurons with the highest cellular levels of CB1 mRNA (+++) in the MS, HDB and VDB overlapped with that of GAD65-expressing neurons (Figs. 1A-D). Similarly, neurons with moderate single-cell CB1 mRNA levels (++) in the preoptic region, including the LPO, MPA and MPO, corresponded to dominantly GABAergic sites (Figs. 1E-L). Interestingly, all three mRNAs showed overlapping distributions in the AVPe (Figs. 1E-H). In contrast with the dominance of the CB1/GABA overlap at septal and preoptic sites, hypothalamic neurons exhibiting moderate (++) or low (+) cellular levels of CB1 mRNA rather tended to occur in areas populated by glutamatergic cells. Such CB1/glutamate regions included the VMH (Figs. 1R, T and 2B, D), LM (Figs. 2N, P and 2R, T), PMV (Figs. 2F, H and 2J, L), PMD (Figs. 2J, L), SuM (Figs. 2N, P and 2R, T), SO (Figs. 1N, P) and Pa (Figs. 1N, P and 1R, T). Low levels of CB1 mRNA expression were detected in many other GABAergic and glutamatergic regions throughout the mouse hypothalamus (Figs. 1, 2).

Dual-labeling experiments

Dual-labeling experiments confirmed the colocalization of CB1 mRNA with the GABAergic (Fig. 4) and glutamatergic (Fig. 5) markers in regions where single-labeling experiments revealed overlaps between CB1 with GAD65 and VGLUT2 mRNAs, respectively.

Examples for heavy (+++) CB1 signal in GABAergic cells were observed in the HDB (Fig. 4A). Moderate CB1 signal levels (+) were typical for GABAergic cells
of the MPA (Fig. 4C), MPO (Fig. 4D) and the different subdivisions of the anterior hypothalamic area (Figs. 4E, F). A small subset of GABAergic neurons in the SCh contained low levels of CB1 mRNA, whereas the majority of neurons were unlabeled (Fig. 4G). GABAergic neurons in many other regions including the AVPe (Fig. 4B), the DM (Fig. 4H) and the Arc (Fig. 4I) expressed CB1 mRNA at low levels (+) only.

Examples for glutamatergic neurons with moderate levels of CB1 mRNA (++) were found in the HDB (Fig. 5A), LA (Fig. 5B), VMH (Fig. 5F), PMV (Fig. 5H) and LM (Fig. 5J). Low-to-moderate signal levels were also found in VGLUT2-expressing neurons of the AVPe (Fig. 5C), MPA (Fig. 5D), Pa (Fig. 5E), PMD (Fig. 5F) and SuM nuclei (Fig. 5K). Labeled glutamatergic neurons were also found in the Arc, most typically in its posterior part (Fig. 5G).
Discussion

In the present report we provide a detailed map of septal-hypothalamic CB1 mRNA-expressing neurons and demonstrate the differential colocalization of CB1 with GAD65 and VGLUT2 mRNAs in distinct subsets of GABAergic and glutamatergic neurons, respectively.

Differential expression of CB1 mRNA

In this study, we present evidence for a wide and differential expression of CB1 mRNA in septal-hypothalamic areas of the mouse. Overall, we found a much wider distribution of CB1 mRNA in the hypothalamus than reported earlier (Cota et al., 2003; Marsicano and Lutz, 1999). To provide semiquantitative measures of regional differences in hybridization signal strength, we characterized distinct septal-hypothalamic areas with both the regional abundance of CB1-expressing neurons and the typical single-cell level of CB1 hybridization signal, on three point scales, each. The CB1 signal strength of individual septal-hypothalamic neurons was always lower than the CB1 labeling intensity of a subset of GABAergic neurons in the basolateral amygdala (Herkenham et al., 1990; Katona et al., 2001; Katona et al., 1999; Marsicano and Lutz, 1999; Matsuda et al., 1993; McDonald and Mascagni, 2001; Tsou et al., 1998), hippocampus (Herkenham et al., 1990; Katona et al., 2001; Katona et al., 1999; Marsicano and Lutz, 1999; Matsuda et al., 1993; McDonald and Mascagni, 2001; Tsou et al., 1998) or cerebral cortex (Bodor et al., 2005). In comparison with the very heavy (++++) CB1 signal in cholecystokinin containing basket cells of these sites (Cota et al., 2003; Marsicano and Lutz, 1999), the labeling intensity of septal-hypothalamic neurons ranged from low (−) to high (+++).
**GABAergic neurons expressing CB1 mRNA**

The highest cellular levels of CB1 mRNA expression (+++) in our regions of interest were observed in GABAergic neurons in the MS, HDB and VDB; these GABAergic cells have been implicated in spatial learning and memory (Pang et al.). Various cognitive processes of the hippocampus depend on the theta rhythm which is generated and propagated to the hippocampus by GABAergic neurons located in the MS-DBB complex (Borhegyi et al., 2004; Varga et al., 2008) which selectively innervate GABAergic interneurons in the hippocampus (Freund and Antal, 1988). There is evidence that CB1 receptor activation reduces hippocampal theta oscillations, with concomitant memory impairment in hippocampus-dependent tasks. We found additional evidence for the presence of somewhat lower levels of CB1 mRNA in glutamatergic neurons in the MS-DBB region. Further, cholinergic neurons also appear to contain CB1 at this site (Nyiri et al., 2005) and the CB1 agonist WIN 55212-2 directly inhibits their acetylcholine release into the hippocampus (Gifford and Ashby, 1996).

In our studies to analyze the coexpression of CB1 and GAD65 mRNAs, we found moderate CB1 mRNA levels in GABAergic neurons of several preoptic regions, including the MPO, LPA and the MnPO and somewhat lower levels in the AVPe. From a neuroendocrine aspect, a particularly interesting observation was the presence of CB1 expression in the AVPe which represents an important source for neuronal inputs to gonadotropin-releasing hormone (GnRH) neurons (Herbison, 2008). The presence of CB1 in this putative GABAergic input system to GnRH neurons may explain our recent electrophysiological and morphological data that retrograde
endocannabinoid signaling reduces GABAergic afferent drive onto GnRH neurons via the activation of presynaptic CB1 receptors (Farkas et al., 2010). In that study we have provided evidence that GnRH neurons produce 2-arachidonoylglycerol (2-AG). Its presynaptic action via CB1 decreases GABA release, which, in turn, reduces postsynaptic GABA_A-R signaling and inhibits the firing activity of GnRH neurons (Farkas et al., 2010).

In contrast with the high and moderate levels of CB1 mRNA expression in septal and preoptic GABAergic neurons, GABAergic cells in the hypothalamus tended to express low levels of CB1 mRNA, only. Where present, moderate levels of CB1 signal were more typical for hypothalamic glutamatergic neurons. The colocalization of hypothalamic CB1 and GAD65 mRNAs in our study somewhat conflicts with the lack of evidence for CB1 mRNA in hypothalamic GABAergic cells (Cota et al., 2003; Marsicano and Lutz, 1999). It is likely that these previous studies failed to reveal the relatively low levels of CB1 expression in GABAergic cells, given that CB1 mRNA expression tends to be heavier in glutamatergic vs. GABAergic systems of the hypothalamus,

*Glutamatergic neurons with the highest cellular levels of CB1 mRNA expression*

Glutamatergic neurons also occurred in the MS, HDB and DBB and expressed CB1 mRNA, albeit at slightly lower levels than did GABAergic neurons.

Among the various compartments of the preoptic region which contained glutamatergic, in addition to GABAergic neurons, the AVPe showed an interesting overlap of CB1, GAD65 and VGLUT2 mRNAs; Usually low levels of CB1 expression were detectable here in glutamatergic, in addition to GABAergic neurons.
From a neuroendocrine aspect, it is interesting to note that the AVPe contains GABAergic/glutamatergic dual-phenotype neurons in female rats, which innervate GnRH cells (Ottem et al., 2004) and mediate positive sex steroid feedback effects (Herbison, 2008).

Regarding the hypothalamus, the highest regional abundance of CB1 mRNA expression and the highest single-cell levels of CB1 mRNA tended to occur in glutamatergic regions. As we have shown in a previous (Hrabovszky et al., 2005) and the present studies, the bulk of the VMH consists of VGLUT2-expressing glutamatergic neurons in rodents. Presence of CB1 mRNA in these cells is in accordance with previous observations by others (Marsicano and Lutz, 1999) and suggests that glutamate release from hypothalamic and extrahypothalamic synapses of these neurons is modulated by endocannabinoids synthesized in postsynaptic target cells. The VMH establishes terminal fields in other parts of the medial hypothalamus, sends ascending projections to the zona incerta, the midline thalamus, the bed nuclei of the stria terminalis, the amygdala, the nucleus accumbens and the prefrontal cortex. Its descending projections reach the periaqueductal gray, the superior colliculus, peripeduncular area, locus coeruleus, Barrington's nucleus, parabrachial nucleus, nucleus of the solitary tract, and the mesencephalic, pontine, gigantocellular, paragigantocellular and parvicellular reticular nuclei (Canteras et al., 1994; Krieger et al., 1979; Saper et al., 1976). The VMH possesses massive intrinsic connections (Canteras et al., 1994; Millhouse, 1973; Nishizuka and Pfaff, 1989). Therefore, a major site of action on CB1 that is synthesized in the nucleus may be within the VMH.
itself. It is likely that the massive CB1-immunoreactive innervation of the VMH we reported earlier (Wittmann et al., 2007) arises partly from local neurons.

From a functional aspect, the VMH is involved heavily in the integration of forebrain neural influences with ascending information from the brainstem, and distributing the resulting output to regions involved in the control of visceral and behavioral mechanisms (Swanson and Mogenson, 1981). Functions associated with the VMH include the control of pituitary hormone release (Blache et al., 1991; Chateau et al., 1984; Kato et al., 1983; Okada et al., 1991; Pan and Gala, 1985). Furthermore, VMH lesions interfere with the diurnal rhythms of plasma corticosterone levels and food intake (Balagura and Devenport, 1970; Bellinger et al., 1976; Bernardis, 1973). There is also general agreement that the VMH plays important roles in the regulation of feeding (Brooks et al., 1946; Minano and Myers, 1991; Perkins et al., 1981; Shimizu et al., 1987), defensive (Fuchs et al., 1985a; b), and feminine copulatory behaviors and the modulation of autonomic responses associated with these behaviors.

The LH also contained moderate numbers of CB1-expressing glutamatergic neurons. This anatomical site has been strongly implicated in the regulation of energy balance (Richard et al., 2009). A subset of CB1-expressing neurons in the LH may correspond to melanin concentrating hormone producing neurons that were previously shown to express CB1 (Cota et al., 2003).

The LA contained a glutamate/CB1 cell population that was distinct from the glutamate/CB1 neurons of the LH. In the rat, this anatomical region forms part of the
hypothalamic attack area and contains VGLUT2-expressing neurons that co-synthesize thyrotropin-releasing hormone (Hrabovszky et al., 2005).

The medial and lateral mammillary nuclei play a crucial role in episodic memory. Some degree of parallelism exists both in the inputs and the outputs of these nuclei (Vann and Aggleton, 2004). Significant afferent sources include the hippocampal formation and the tegmental nuclei, whereas the excitatory efferents target the anterior thalamic nuclei via the mammillothalamic tract and the tegmental nuclei via the mammillotegmental tract (Hayakawa and Zyo, 1989). Both the medial and the lateral mammillary nuclei comprised VGLUT2-expressing glutamatergic neurons in our study, but only cells in the LM showed detectable levels of CB1 mRNA. The functional significance of this differential CB1 expression requires clarification.

Other nuclei with high levels of CB1 and VGLUT2 mRNAs included the PMV. This site has been heavily implicated in reproductive regulation. Its neurons expressing cocaine- and amphetamine-regulated transcript project to the preoptic area (Rondini et al., 2010). Cells of the PMV express a dense concentration of leptin receptors which appear to play a role in linking changing levels of leptin and coordinated control of reproduction. Lesions of the PMV disrupt estrous cyclicity in rats and preclude the stimulation of luteinizing hormone secretion by leptin during fasting (Donato et al., 2009). Other regions where CB1 and VGLUT2 mRNAs were frequently coexpressed included the LM, PMD, SuM, SO and Pa nuclei.

*Cells expressing low levels or no CB1*

Neurons expressing low levels or no CB1 mRNA should also be noted. Accordingly, the large GABAergic cell mass in the lateral septum, the majority of
GABAergic neurons in the SCh and the Arc exhibited either no or only low levels of CB1 hybridization signal. Glutamatergic neurons in the medial mammillary nucleus were similarly devoid of CB1 mRNA. While the activity of such neurons may also regulate endocannabinoid production in their postsynaptic targets, it is likely that their own neurotransmitter release is independent of retrograde endocannabinoid signaling by the postsynaptic neurons.

**CB1 in neuroendocrine cells**

Parvicellular and magnocellular neuroendocrine cells project outside the blood-brain barrier and release their neurohormone contents in the blood stream to regulate endocrine functions. Previous studies from our laboratory found that most of the classical neuroendocrine cells in the rat, with the exception of dopaminergic and growth hormone-releasing hormone neurons in the Arc nucleus, express VGLUT2 mRNA (Hrabovszky and Liposits, 2008). In the present studies we identified large numbers of CB1/VGLUT2 dual-labeled neurons in the SO, although individually, these cells contained low CB1 mRNA levels only. These neurons are exclusively magnocellular neurosecretory cells which secrete oxytocin and vasopressin into the peripheral circulation. Similarly, the Pa also contained many parvicellular CB1/VGLUT2 neurons some of which could also be neuroendocrine cells. Accordingly, a previous dual-label *in situ* hybridization study by Cota and co-workers provided evidence that a subset of CB1 neurons in this nucleus expresses corticotropin-releasing hormone mRNA (Cota et al., 2003). Furthermore, our previous immunocytochemical study (Wittmann et al., 2007) identified CB1-immunoreactive axon terminals in the external layer of the median eminence where
parvicellular neurosecretory systems project. Future dual-label immunofluorescent experiments will need to clear the neuropeptide phenotypes of these CB1 containing neuroendocrine terminals. From a conceptual viewpoint, it will be important to identify the glial, endothelial, or neuronal sources of endogenous cannabinoids at these neuroendocrine secretion sites. Tanyocytes, which exhibit immunoreactivity for the endocannabinoid biosynthetic enzyme diacylglycerol lipase, represent one likely source for 2-AG that affects CB1-containing parvicellular neurosecretory axon terminals (Suarez et al., 2010). Functional studies will need to determine the physiological significance of CB1 in parvicellular and magnocellular neurosecretory systems.

**Technical considerations**

Because hypothalamic neurons express relatively low levels of CB1 mRNA (Marsicano and Lutz, 1999), in the present study we chose to use an improved radioisotopic hybridization procedure (Hrabovszky and Petersen, 2002). With this method, a low autoradiographic background can be achieved via the use of high dithiothreitol concentrations (1000mM) in the hybridization solution. This modification allows the use of enhanced radioisotopic probe (80,000 cpm/ml) and dextran sulfate (20%) concentrations in the hybridization solution, increasing thus the specific hybridization signal. This method has also been adapted successfully to dual-label *in situ* hybridization (Hrabovszky et al., 2004; Hrabovszky et al., 2006). In the present study we have noticed that while increased concentration of dextran sulfate can, indeed, significantly enhance the isotopic signal for CB1 mRNA, it actually weakens the signal using the digoxigenin-labeled probes for GAD65 and VGLUT2.
Therefore, in order to achieve the sufficient visualization of GABAergic and glutamatergic neurons, we have modified the dual-labeling procedure by using high isotopic probe, but not dextran sulfate, concentrations. This necessary compromise could somewhat reduce the sensitivity of CB1 mRNA detection.

As we stated and discussed in a previous technical report (Hrabovszky and Petersen, 2002), the finding of increased hybridization signal following the use of enhanced probe and/or dextran sulfate concentrations in the hybridization solution, or an enhanced hybridization time, collectively suggest that the “saturation” of mRNA targets can not be achieved using routine in situ hybridization procedures with isotopic cRNA probes. This also implies that the in situ hybridization reaction does not follow pseudo-first order kinetics, as postulated for nucleic acid hybridization to immobilized targets (Hrabovszky and Petersen, 2002). Use of an improved hybridization method could be important in the present study to visualize low levels of CB1 mRNA in neurons previously reported not to have significant levels of CB1 mRNA, including GABAergic hypothalamic neurons (Marsicano and Lutz, 1999). It is still important to note that practical limits exist in enhancing the hybridization signal via using improved methods. Increasing probe and/or dextran sulfate concentration beyond some point will also result in increased background. Also, the visualization of isotopic probes at the single-cell level can be quite insensitive and in case of rare mRNA targets, grain clusters might not appear in the emulsion even after long exposures. Furthermore, while the in situ formed probe RNA/mRNA hybrids are considered stable based on theoretical considerations, in our experience a quite robust decrease of the specific hybridization signal can also result from too harsh
posthybridization RNase A- and stringent treatments. Therefore, negative results including the lack of CB1 hybridization signal in significant subsets of hypothalamic neurons should be interpreted with caution and not as a functional proof for the insensitivity of these cells to endocannabinoids.

In summary, in this report we present a detailed neuroanatomical map of GABAergic and glutamatergic septal-hypothalamic neurons that express CB1 mRNA. Neurotransmitter release from these cells is controlled by retrograde endocannabinoid signaling from target neurons. This neuroanatomical information may help to understand the role of the endocannabinoid system in the regulation of endocrine and metabolic functions.
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Legends

Figure 1. Comparative distribution of CB1, GAD65 and VGLUT2 hybridization signals in septal-rostral hypothalamic areas of the mouse.

The radioisotopic in situ hybridization detection of CB1 (B, F, J, N, R), GAD65 (C, G, K, O, S) and VGLUT2 (D, H, L, P, T) mRNAs in autoradiographic emulsion reveals CB1 mRNA expression in areas populated by GABAergic (GAD65 mRNA-expressing) as well as glutamatergic (VGLUT2 mRNA-expressing) neurons. The regional abundance of CB1 mRNA is high (+++) in the HDB (B, F), VDB (B) and the MS (B, F); these regions are dominated by GABAergic neurons (C, G). Similarly, CB1 labeling in most subdivisions of the preoptic region (F, J) also tends to overlap with the distribution of GABAergic neurons (G, K). Note that the AVPe contains overlapping CB1, GAD65 and VGLUT2 signals (F-H). The highest regional expression of CB1 mRNA in the rostral hypothalamus can be observed in glutamate-dominated nuclei, including the LA, SO, Pa, and VMH (N-T). GABAergic hypothalamic sites, including the AHA and AHP, express lower levels of the CB1 hybridization signal (N-T). Note the well-patterned and complementary distributions of GABAergic and glutamatergic neurons. For a list of abbreviations and the results of the detailed regional analysis, see Tables 1 and 2, respectively. Scale bar=200µm.
Figure 2. Comparative distribution of CB1, GAD65 and VGLUT2 hybridization signals in the caudal hypothalamus of the mouse.

Autoradiographic images of neighboring sections from the caudal hypothalamus illustrate the distributions of CB1 (B, F, J, N, R), GAD65 (C, G, K, O, S) and VGLUT2 (D, H, L, P, T) mRNAs. Similarly to the rostral hypothalamus, CB1 expression tends to be more abundant in glutamatergic vs. GABAergic regions, including the VMH (B), PMV (F, J), PMD (J), SuM (N, R) and LM (N, R) nuclei. CB1 mRNA expression can be observed in the DM (B) where both GABAergic (C) and glutamatergic (D) neurons occur. Several sites with GABAergic dominance, including the Arc (B, F, J), exhibit lower levels of CB1 expression, with somewhat more labeled neurons caudally (F, J). For a list of abbreviations and the results of the detailed regional analysis, see Tables 1 and 2, respectively. Scale bar=200µm.
Fig. 3. Representative autoradiographic images of septal-hypothalamic neurons with different single-cell levels of CB1 mRNA. In situ hybridization experiments reveal the most intense CB1 labeling (++++) in subsets of interneurons in the neocortex (Ctx; A), amygdala (B) and hippocampal formation (C). CB1 is also expressed very heavily (++++) in the majority of neurons in the lateral caudate putamen (CPu; D). Such heavy expression is never observed in septal-hypothalamic neurons; if labeled, these neurons exhibit CB1 signal levels that range from low (+) to high (+++). High signal (+++) is present in a subset of neurons in the diagonal band of Broca (E) and the medial septum (F). Moderate signal levels (++) are typical for neurons in several subdivisions of the preoptic region, including the medial preoptic area (MPA; G) and the medial preoptic nucleus (MPO; H). These sites are dominated by GABAergic cells (see Fig. 1). Most neurons in the anteroventral periventricular nucleus (AVPe; I) express CB1 mRNA at low levels. Moderate signal levels characterize neurons in the hypothalamic ventromedial (VMH; J), lateral mammillary (LM, K), and paraventricular nuclei (Pa; L). These hypothalamic sites are dominated by glutamatergic cells (see Figs. 1 and 2). Low levels (+) of CB1 mRNA are expressed at many other sites. Accordingly, low levels are typical for neurons of the supraoptic (SO; M), posterior arcuate (arc post; N) and periventricular (Pe; O) nuclei, among other sites. Arrows in all panels point to representative neurons used for scaling for the cellular intensity of labeling. In addition, note that the regional abundance of labeled neurons varies significantly. For scoring, see Table 2. Abbreviations: BMA, basomedial amygdaloid nucleus, anterior part; Ctx,
(retrosplenial granular) cortex; DG, dentate gyrus; HDB, nucleus of the horizontal limb of the diagonal band. Scale bar=50 µm.
Fig. 4. Representative dual-label *in situ* hybridization images of GABAergic neurons expressing CB1 mRNA from different coronal planes of the mouse septal-hypothalamic region. GAD65-expressing GABAergic neurons (purple alkaline phosphatase substrate) exhibit heavy (+++) hybridization signal (clusters of autoradiographic grains) in the HDB (A). Panels B-F illustrate the low to moderate intensity of CB1 labeling (+, ++) in large populations of GABAergic neurons in the AVPe (B), MPA (C), MPO (D), the AHC (E) and AHP (F). Note the small subset of GABAergic neurons in the suprachiasmatic nucleus (SCh; G) that express low levels of CB1 mRNA, whereas most cells are devoid of any CB1 labeling. GABAergic cells of the DM (H) and Arc (I) nuclei contain low levels of signal, if any. Arrows in all panels point to dual-labeled neurons. See Table 1, for a list of abbreviations. Scale bar=50µm.
Fig. 5. Representative dual-label \textit{in situ} hybridization images of glutamatergic neurons expressing CB1 mRNA at different coronal planes of the mouse septal-hypothalamic region. Glutamatergic neurons (purple perikarya) express moderate levels (++) of CB1 mRNA (clusters of silver grains) in the HDB (A). Compare to the heavier CB1 signal over GABAergic neurons in 3A. Moderate levels of CB1 signal can be observed over hypothalamic glutamatergic neurons of the LA (B), VMH (F), PMV (H) and LM (J) nuclei. Low to moderate levels of CB1 mRNA expression can be observed in glutamatergic cells of the AVPe (C) nucleus, the MPA (D), the Pa (E), posterior Arc (Arc post; G), PMD (I) and SuM (K) nuclei. Arrows point to dual-labeled neurons. See Table 1, for a list of abbreviations. Scale bar=50 μm.
Table 1. List of abbreviations used in text and figures.
Table 2. Regional and single-cell abundances of radioisotopic in situ hybridization signals for CB1, GAD65 and VGLUT2 mRNAs in septal-preoptic-hypothalamic regions of the mouse.