0026-895X/06/7004-1236–1245$20.00
MOLECULAR PHARMACOLOGY
Vol. 70, No. 4
24661/3142370
Mol Pharmacol 70:1236–1245, 2006
Printed in U.S.A.

CB_1 Receptor Antagonism Increases Hippocampal Acetylcholine Release: Site and Mechanism of Action

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Received April 16, 2006; accepted July 19, 2006

ABSTRACT

Evidence indicates that blockade of cannabinoid receptors increases acetylcholine (ACh) release in brain cortical regions. Although it is assumed that this type of effect is mediated through CB_1 receptor (CB_1R) antagonism, several in vitro functional studies recently have suggested non-CB_1R involvement. In addition, neither the precise neuroanatomical site nor the exact mechanisms underlying this effect are known. We thoroughly examined these issues using a combination of systemic and local administration of CB_1R antagonists, different methods of in vivo microdialysis, CB_1R knockout (KO) mice, tissue measurements of ACh, and immunochemistry. First, we showed that systemic injections of the CB_1R antagonists *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride (SR-141716A) and *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2, 4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251) dose-dependently increased hippocampal ACh efflux. Likewise, local hippocampal, but not septal, infusions of SR141716A or AM251 increased hippocampal ACh release. It is noteworthy that the stimulatory effects of systemically administered CB_1R antagonists on hippocampal ACh release were completely abolished in CB_1R KO mice. CB_1R KO mice had similar basal but higher stress-enhanced hippocampal ACh levels compared with wild-type controls. It is interesting that dopamine D_1 receptor antagonism counteracted the stimulatory effect of CB_1R blockade on hippocampal ACh levels. Finally, immunohistochemical methods revealed that a high proportion of CB_1R-positive nerve terminals were found in hippocampus and confirmed the colocalization of CB_1 receptors with cholinergic and dopaminergic nerve terminals. In conclusion, hippocampal ACh release may specifically be controlled through CB_1Rs located on both cholinergic and dopaminergic neuronal projections, and CB_1R antagonism increases hippocampal ACh release, probably through both a direct disinhibition of ACh release and an indirect increase in dopaminergic neurotransmission at the D_1 receptors.

Animal studies have shown that cannabinoid receptor activation and blockade impair and enhance cognitive performance, respectively, under certain experimental conditions (Chaperon and Thiebot, 1999). These effects on cognition have been correlated with fluctuating extracellular acetylcholine (ACh) levels in the hippocampus, in which an abundance of cannabinoid receptors and cholinergic nerve terminals reside (Tzavara et al., 2003b; Inui et al., 2004). The effect of cannabinoid receptor stimulation on hippocampal ACh efflux can vary depending on dosage and site of administration. In general, high doses of cannabinoid agonists decrease whereas lower doses increase hippocampal ACh release, and these effects are mediated through the hippocampus and septum, respectively (Tzavara et al., 2003b). Two types of cannabinoid receptors have been identified: the cannabinoid receptor type 1 (CB_1R) and type 2 (CB_2R) (Devane et al., 1988). The modulation of hippocampal ACh levels induced by cannabinoids is probably mediated through CB_1R given their distribution in the septohippocampal pathway (Howlett et al., 2004).

The effect of cannabinoid receptor stimulation on hippocampal ACh efflux has been well documented. On the other hand, limited evidence suggests that cannabinoid receptor

A.K. was supported by an International Brain Research Organization 2005 Research Fellowship.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.024661.

ABBREVIATIONS: ACh, acetylcholine; VAChT, vesicular acetylcholine transporter; CB_1R, cannabinoid receptor type 1; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2, 4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; KO, knockout; WT, wild type; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ANOVA, analysis of variance; DAT, dopamine transporter; DA, dopaminergic; SCH23390, *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine.

antagonism increases ACh release in the hippocampus (Gessa et al., 1998; Tzavara et al., 2003b). Although it is assumed that this effect is mediated through CB_1R, recent studies indicate that cannabinoids may mediate some of their neurochemical/behavioral actions through novel, yet-to-be-identified central cannabinoid receptors (Di Marzo et al., 2000; Haller et al., 2002; Köfalvi et al., 2005). As such, a putative cannabinoid receptor, which is sensitive to the CB_1R antagonist SR141716A (Rimonabant) (Rinaldi-Carmona et al., 1994) but insensitive to the CB_1R antagonist AM251 (Lan et al., 1999), has been shown to inhibit the release of glutamate in the hippocampus (Köfalvi et al., 2003). Thus, an increase in hippocampal ACh levels elicited by both antagonists would indicate that this effect is probably mediated through CB_1R antagonism.

It is also possible that cannabinoid receptor antagonism at a site other than the hippocampus may regulate hippocampal ACh release. One obvious candidate is the septum, because stimulation of septal cannabinoid receptors increases hippocampal ACh release (Tzavara et al., 2003b), and the septum provides the main cholinergic input to the hippocampus (Dutar et al., 1995). Finally, CB_1R antagonism may regulate hippocampal ACh levels through a modulation of the dopaminergic system: blockade of CB_1 receptors increases brain dopamine levels (Tzavara et al., 2003a), and stimulation of dopaminergic receptors affects ACh release (Day and Fibiger, 1994). In particular, increased dopaminergic neurotransmission through D_1 receptors increases whereas through D_2 receptors decreases hippocampal ACh efflux, respectively (Day and Fibiger, 1994). Moreover, stimulation of D_2 and D_1 receptors mediates the inhibitory and stimulatory actions of high and low doses of cannabinoids on hippocampal ACh release, respectively (Nava et al., 2001; Tzavara et al., 2003b). Thus, a facilitation of dopaminergic neurotransmission at D_1 receptors may be involved in a possible stimulatory effect of hippocampal ACh release induced by CB_1R antagonism.

The current study sought to address these issues by conducting a detailed analysis of the site and mechanism of action through which CB_1R blockade modulates hippocampal ACh efflux. For this reason, a combined neurochemical and neuropharmacological approach that included local and systemic administrations, dual and quantitative microdialysis, and studies in CB_1R knockout (KO) mice was used. In addition, a histochemical analysis of the distribution of vesicular acetylcholine transporter (VAChT), dopamine transporter, and CB_1R immunoreactivity with a novel, highly sensitive method (Köfalvi et al., 2005) within the hippocampus was used to examine the neuroanatomical interrelationship of these elements.

Materials and Methods

Animals. All studies were conducted according to the guidelines set forth by the National Institutes of Health and implemented by the Animal Care and Use Committee of Eli Lilly and Company. We used male Wistar rats (250–300 g; Harlan Sprague-Dawley, Indianapolis, IN), male CB_1R KO, and corresponding wild-type (WT) mice. All animals were housed in a vivarium for at least 7 days before use with food and water available ad libitum. Both CB_1R KO and WT mice were derived from a stock of genotyped animals received from the University of Bonn (Bonn, Germany). CB_1R KO ($CB_1^{-/-}$) mice and their homozygous controls ($CB_1^{+/+}$ mice) were developed in C57BL/6J mice by replacing most of the CB_1R coding sequence with a nonreceptor sequence through homologous recombination in MPI2 embryonic stem cells, as described previously by Zimmer et al. (1999). Both KO and WT mice used in the present study were derived from the same newly established breeding colony (by interbreeding of heterozygous mice and subsequent genotype characterization) and were matched for age and weight.

Surgical Procedures

Implantation of Microdialysis Guide Cannulae and Probe Insertions in the Hippocampus. Seven days before being used in microdialysis experiments, rats were anesthetized with a mixture of chloral hydrate and pentobarbital (170 and 36 mg/kg, respectively, in 30% propylene glycol and 14% ethanol), placed in a stereotaxic apparatus, and implanted unilaterally with guide cannulae (Bioanalytical Systems, Inc., West Lafayette, IN) in the hippocampus (coordinates: anteroposterior, −5.2; mediolateral, 5.2; dorsoventral, −3.8) according to the stereotaxic atlas of Paxinos and Watson (1998). Twenty-four hours before testing, a 4-mm concentric microdialysis probe (BAS Bioanalytical Systems, West Lafayette, IN) was inserted through the guide cannula.

Likewise, for mice, 2-mm microdialysis probes (CMA/Microdialysis, Solna, Sweden) were implanted unilaterally in the hippocampus (mediolateral, +3.1; anteroposterior, −3.3; dorsoventral, −4.2, based on the stereotaxic atlas of Franklin and Paxinos, 1997) under anesthesia with a mixture of chloral hydrate and pentobarbital. Animals were given a 48-h recovery period before being used in microdialysis experiments. The correct placement of the probes was verified histologically at the end of each experiment.

In Vivo Microdialysis of Hippocampal ACh Concentrations. Acetylcholine determination in hippocampal dialysates was performed as described previously (Damsma et al., 1988) with some modifications (Tzavara et al., 2003a,b). On the day of the experiment, a modified Ringer's solution (147.0 mM NaCl, 3.0 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mM $Na_2HPO_4{\cdot}7H_2O$, and 0.2 mM $NaH_2PO_4{\cdot}H_2O$, pH 7.25) supplemented with either 0.1 μM (rats) or 0.3 μM (mice) neostigmine was perfused at a rate of 2.4 μl/min (rats) or 1.5 μl/min (mice) in the hippocampus. Samples were collected every 15 min (unless indicated otherwise) and analyzed immediately online with high-performance liquid chromatography coupled to electrochemical detection, with a 150 × 3-mm acetylcholine-3 column (ESA, Inc., Chelmsford, MA) maintained at 35°C. The mobile phase (100 mM disodium hydrogen phosphate, 2 mM 1-octanesulfonic acid, and 50 μl/l of the microbicide reagent MB; ESA, Inc.; pH 8.0 was adjusted with phosphoric acid) was delivered by a high-performance liquid chromatography pump (ESA, Inc.) at 0.4 ml/min. The potentiostat used for electrochemical detection (ESA Coulochem II) was connected with a solid-phase reactor for ACh (ESA Inc.; ACh-SPR) and with an analytical cell with platinum target (ESA 5041). Animals were given a 3-h stabilization period before four baseline samples were collected. Thereafter, animals were systemically injected or locally infused with vehicle or drug, and an additional 6 to 12 samples (see *Results*) were collected.

Drugs and Experimental Design. In experiment 1, rats that had been implanted previously with microdialysis probes directed at the hippocampus as described were injected intraperitoneally with vehicle (0.9% NaCl containing 2% dimethyl sulfoxide and 2% cremophor EL), SR141716A (1, 3, or 10 mg/kg; synthesized at Lilly Research Laboratories), or AM251 (3 or 10 mg/kg; purchased from Tocris Cookson, Inc., Ellisville, MO). Drugs were suspended in vehicle and injected at a volume of 3 ml/kg. The doses of SR141716A and AM251 were selected on the basis of results of previous studies (Tzavara et al., 2001; Chambers et al., 2004).

In experiment 2, mice (CB_1R KO or WT) that were implanted previously with microdialysis probes as described were injected intraperitoneally with vehicle (see *Implantation of Microdialysis Guide Cannulae and Probe Insertions in the Hippocampus*), SR141716A (20 mg/kg), or AM251 (10 mg/kg). Drugs were suspended

in vehicle and injected at a volume of 10 ml/kg. In preliminary studies, it was determined that in mice, a higher dose of SR141716A but not AM251 was required than in rats to induce robust and reproducible increases in hippocampal ACh levels. This may be due to different metabolic rates or differences in receptor density between species.

In experiment 3, rats were also implanted with guide cannulae and probes in the medial septal area (Moor et al., 1994; Tzavara et al., 2003b), and they were locally infused with SR141716A or AM251 at a rate of 2.4 μl/min in either the hippocampus or the septum for 60 min after the initial basal levels of hippocampal ACh had been established. Drugs (SR141716A or AM251) were dissolved in perfusion solution containing 1% DMSO and 1% cremophor EL at a final concentration of 1 mM. A lower concentration of SR141716A (0.1 mM) has been shown previously not to affect hippocampal ACh efflux (Tzavara et al., 2003b). It should also be noted that although the in vivo recovery of the administered CB_1R antagonists was not determined, this has been shown previously to be $<1\%$ for a compound, nicotine, administered locally under similar experimental conditions, such as those used in the present study (Marshall et al., 1997). Neostigmine was omitted from the perfusion solution used for septal perfusions because the probe in the septum was only used for drug delivery (Moor et al., 1994; Tzavara et al., 2003b).

In experiment 4, the basal and stress-induced levels of hippocampal ACh efflux were compared between CB_1R KO and WT mice using different methods of microdialysis (conventional, semiquantitative/low perfusion rate, and the quantitative/zero-net-flux method), tissue level measurements, and an animal model of exposure to stress (predatory odor test). Semiquantitative microdialysis was conducted by perfusing CB_1R KO or WT mice that had been implanted previously with microdialysis probes at a very low perfusion rate (0.08 μl/min) with a perfusion solution that did not contain neostigmine; this method allows for a better estimate of the basal steady-state concentrations of neurotransmitters in the sampled extracellular fluid than conventional microdialysis, because at low perfusion rates, their in vivo recovery reaches high levels (Gerber et al., 2001). After a standard 3-h stabilization period, dialysate was collected until a final volume of 40 μl was reached (8.3 h) and analyzed offline for ACh content. In the zero-net-flux microdialysis method (Day et al., 2001), CB_1R KO or WT mice implanted with microdialysis probes were perfused with a perfusion solution (1.5 μl/min) that did not contain neostigmine but contained known concentrations of ACh (2.5, 5.0, 10, or 25 nM) instead. The premise of the method is that when the concentration of ACh in the perfusion solution ($[ACh_{in}]$) is lower than extracellular hippocampal ACh levels, ACh will flow down its concentration gradient into the probe, which increases the ACh concentration in the dialysate $[ACh_{out}]$. On the other hand, if $[ACh_{in}]$ is higher than hippocampal levels, then ACh will flow out of the probe, and $[ACh_{out}]$ will be less than $[ACh_{in}]$. By measuring the point where $[ACh_{in-out}] = 0$, we can accurately determine the basal, steady-state concentrations of ACh in the extracellular fluid of the hippocampus. After a 3-h stabilization period, dialysate samples were collected every 20 min for a total of 10 samples and analyzed offline for ACh content.

To measure hippocampal tissue levels of ACh, brains were dissected from CB_1R KO or WT mice after decapitation, and the hippocampi were removed. Hippocampi were placed on a freeze plate, and tissue samples were weighed. Frozen tissue was suspended in a 1.5-ml Eppendorf vial in 0.5 ml of 0.1 N trichloroacetic acid containing 2 μM ethyl-homocholine and sonicated. The resulting solution was left at 0°C for 1 h and centrifuged at 12,000*g*. Next, the vial was placed in an autosampler (Bio-Rad Laboratories, Hercules, CA), and the supernatant was injected (20 μl). ACh was detected electrochemically with a Bioanalytical Systems LC-4C detector using a platinum electrode at 500-mV potential. Data were subsequently collected and analyzed with EZChrome Elite (Scientific Software, Inc., Pleasanton, CA).

Next, we examined whether CB_1R KO mice had altered evoked (i.e., stress-enhanced) concentrations of hippocampal ACh efflux compared with WT mice. Mice implanted with microdialysis probes directed at the hippocampus were exposed to the predatory odor stress test. After the baseline period, mice were placed in a bucket that contained soiled bedding from rat cages for a period of 60 min, whereas four additional hippocampal dialysate samples were collected. The predatory odor stress test was used to determine the effects of stress on hippocampal ACh in WT and KO mice, because exposure of mice to predatory odors has been shown previously to result in robust increases in cortical/hippocampal ACh efflux (Smith et al., 2005).

In experiment 5, we used a novel, highly sensitive method of quantification to calculate what percentage of cholinergic and dopaminergic nerve terminals in the hippocampus contained CB_1 receptors (see *Immunochemical Analysis*).

In experiment 6, rats were injected subcutaneously with vehicle (0.9% NaCl) or the D_1 receptor antagonist SCH23390 (0.3 mg/kg; purchased from Tocris) at a volume of 1 ml/kg. These injections occurred 15 min before being injected systemically (i.p.) or infused locally in the hippocampus with SR141716A (10 mg/kg for i.p. injection and 1 mM for local infusion). Local infusions were conducted as described above for experiment 3.

Immunochemical Analysis. Immunochemical analyses were performed as described previously (Köfalvi et al., 2005). In brief, synaptosomes from hippocampi of male Wistar rats were obtained through a discontinuous Percoll gradient, following the procedure described by Díaz-Hernandez et al. (2002) with minor modifications. Hippocampi were homogenized in 0.25 M sucrose and 5 mM TES, pH 7.4. The homogenate was spun for 3 min at 2000*g* at 4°C, and the resulting supernatant was spun again at 9500*g* for 13 min. Then the pellets were resuspended in 8 ml of 0.25 M sucrose and 5 mM TES, pH 7.4. Two milliliters of this synaptosomal suspension was placed onto 3 ml of Percoll discontinuous gradients containing 0.32 M sucrose, 1 mM EDTA, 0.25 mM dithiothreitol, and 3, 10, or 23% Percoll, pH 7.4. The gradients were centrifuged at 25,000*g* for 11 min at 4°C. Synaptosomes were collected between the 10 and 23% Percoll bands and diluted in 15 ml of HEPES-buffered medium (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4). The synaptosomes were placed onto coverslips coated previously with poly(L-lysine), fixed with 4% paraformaldehyde for 15 min, and washed twice with PBS (140 mM NaCl, 3 mM KCl, 20 mM NaH_2PO_4, and 15 mM KH_2PO_4, pH 7.4). Permeabilization was performed in PBS containing 0.2% Triton X-100 for 10 min; afterward, the synaptosomes were incubated in PBS medium containing 3% bovine serum albumin and 5% normal rat serum for 1 h. The synaptosomes were then washed twice with PBS and incubated with rabbit anti-CB_1 receptor and guinea pig antivesicular acetylcholine transporter (anti-VAChT; 1:500; Chemicon International, Temecula, CA), rat antidopamine transporter (anti-DAT; 1:500, Chemicon) or mouse antisynaptophysin (1:200; Sigma, St. Louis, MO) for 1 h at room temperature. The rabbit CB_1R antibody, a generous gift of Dr. Ken Mackie, was raised against glutathione transferase corresponding to the last 15 amino acids of the rat CB_1R (1:3000). No staining with this CB_1R antibody was seen in the CB_1R homozygote null-mutant mouse, obtained from Dr. Catherine Ledent (Université Libre de Bruxelles, Brussels, Belgium) (Köfalvi et al., 2005). All antibodies gave one band in Western analysis of rat hippocampal tissue. The synaptosomes were then washed three times with PBS/bovine serum albumin (3%) and were incubated for 1 h at room temperature with a AlexaFluor-488 (green)-labeled goat antirabbit IgG antibodies (1:200; Molecular Probes, Leiden, The Netherlands) or goat anti-guinea pig or goat anti-rat or goat anti-mouse, all labeled with AlexaFluor-598 (red; 1:200 for all; Molecular Probes). After washing and mounting on slides with Prolong Antifade, the preparations were visualized in a Zeiss Axiovert 200 (Carl Zeiss AG, Jena, Germany) inverted microscope equipped with a cooled charge-coupled device camera and analyzed with MetaFluor 4.0 software (Molecular Devices, Sunnyvale, CA). To test the selectivity of the secondary antibodies, we carried out the same

procedure as described above on 2-2 coverslips from each animal but without applying primary antibodies. Under this condition, the secondary antibodies failed to label the synaptosomes, and only 0 to 3 bright spots were observed in each field (nonspecific staining).

Statistics. The microdialysis data were expressed as mean (± S.E.M.) absolute values or multifold changes from baseline, which is the average of the four basal values before vehicle or drug injection; data were also expressed as average changes from baseline over a certain period of time (overall effects). Absolute values or percentage changes were analyzed with a three-, two-, or one-way analysis of variance (ANOVA) with treatment, time, or genotype as variables. Individual time points were analyzed with a one-way ANOVA followed by Bonferroni post hoc tests. A P level of 0.05 was used for statistical significance.

Results

Experiment 1

Effects of Systemic Injections of the CB_1R Antagonists SR141716A and AM251 on Hippocampal ACh Efflux. This experiment examined the dose-dependent nature of CB_1R antagonist administration and compared the effects of two slightly different (mainly based on functional responses, see Introduction) CB_1R antagonists, SR141716A and AM251, on hippocampal ACh efflux.

SR141716A and AM251 Dose-Dependently Increase ACh Efflux in the Hippocampus. There were no statistically significant differences in the basal values of ACh among the various experimental groups in this or subsequent experiments with values ranging from 125 to 500 fmol/sample. Figure 1A indicates that SR141716A dose-dependently increases hippocampal ACh efflux. Thus, analysis with a two-way ANOVA revealed a significant interaction ($F_{45,\ 390} = 15.45$, $P < 0.0001$), treatment ($F_{3,\ 390} = 16.17$, $P < 0.001$), and time ($F_{15,\ 390} = 25.42$, $P < 0.0001$) effect. Subsequent analysis at individual time points with a one-way ANOVA indicated that rats injected with 3 or 10 but not 1 mg/kg SR141716A had significantly increased levels of hippocampal ACh. This significant increase persisted for eight samples (120 min) and reached a maximum level of almost 200% above basal values for the highest injected dose. The 3 mg/kg dose of SR141716A resulted in significantly increased hippocampal ACh efflux that persisted for two sample periods (30 min) and reached a maximum of 150% above basal values. Injections of 1 mg/kg increased hippocampal ACh efflux to a maximum of 50% above basal values; this increase failed to reach significance. In all drug-injected groups, ACh efflux followed the same kinetic profile, with the highest increases being reached at the 30-min time point before values diminished over the course of time. Vehicle-injected animals showed an initial increase at the 15-min time point; this reflects the stress-induced increase in hippocampal ACh that results from the injection.

Figure 1B shows that similarly to SR141716A, AM251 increased hippocampal ACh efflux at 3 and 10 mg/kg in a dose-dependent manner, albeit this increase was not as robust as that observed after injections of SR141716A. A two-way ANOVA yielded a significant interaction ($F_{30,\ 225} = 14.25$, $P < 0.0001$), treatment ($F_{2,\ 225} = 15.64$, $P < 0.01$), and time ($F_{15,\ 225} = 22.19$, $P < 0.0001$) effect. In particular, injections of 10 mg/kg resulted in a maximum increase of almost 100% above basal values, and this increase was significant for five samples (75 min). The lower dose caused a slightly lower increase that was maintained at statistical significance for two sample periods (30 and 45 min).

Experiment 2

Effects of Systemic Injections of the CB_1R Antagonists SR141716A and AM251 on Hippocampal ACh Efflux in CB_1R KO or WT Mice. In this experiment, we determined whether the effects of SR141716A and AM251 on hippocampal ACh efflux that were observed in experiment 1

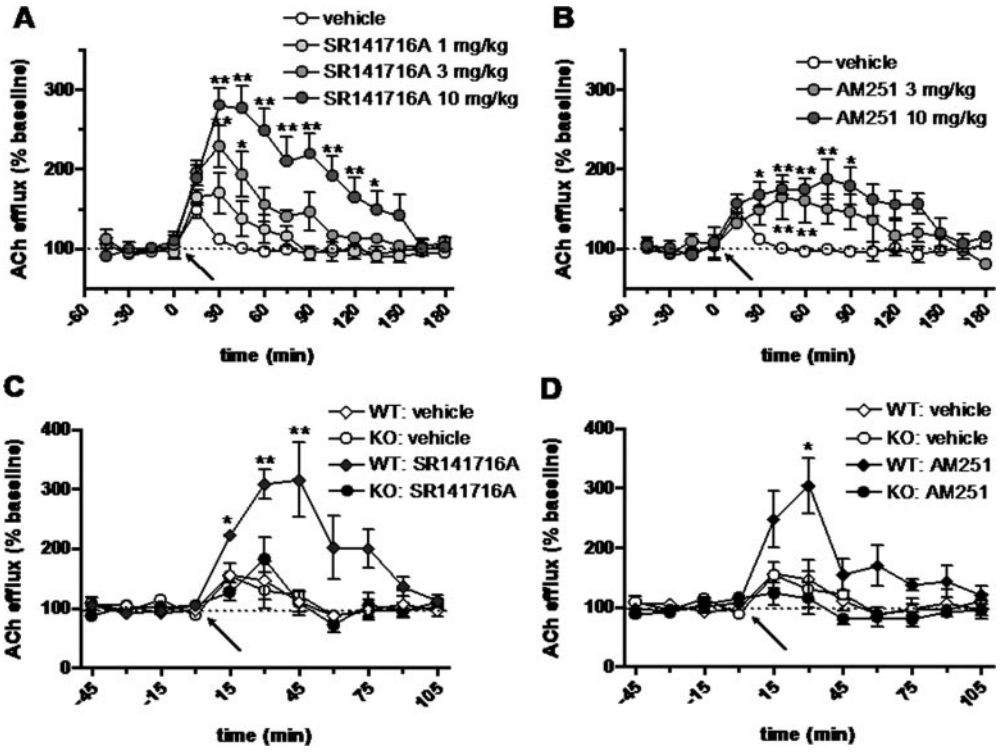

Fig. 1. Systemic administration (1, 3, and 10 mg/kg i.p., indicated by arrows) of the CB_1 receptor antagonists SR141716A (A) and AM251 (B) dose-dependently increases hippocampal ACh efflux in rats. These stimulatory effects of SR141716A and AM251 (20 and 10 mg/kg i.p., respectively, indicated by arrows) on hippocampal ACh efflux are abolished in CB_1 receptor KO mice (C and D, respectively). Data are expressed as mean (± S.E.M., n = 5–8 per group) hippocampal ACh efflux percentage of baseline). *, $P < 0.05$; **, $P < 0.01$ versus vehicle-injected rats (A and B) or vehicle-injected WT mice (C and D).

were retained in mice in which the CB_1R had been genetically deleted.

The Stimulatory Effects of Systemic Injections of SR141716A and AM251 on Hippocampal ACh Efflux Are Abolished in CB_1R KO Mice. There were no statistically significant differences in the basal values of ACh among different experimental groups and between CB_1R KO and WT mice with values ranging from 65 to 125 fmol/sample. As can be seen in Fig. 1C, injections of SR141716A increased hippocampal ACh efflux in WT but not KO mice. Thus, a two-way ANOVA yielded a significant interaction ($F_{30,\ 170} = 24.55$, $P < 0.0001$), treatment ($F_{3,\ 170} = 17.09$, $P < 0.001$), and time ($F_{10,\ 170} = 20.37$, $P < 0.0001$) effect. SR141716A caused a significant increase during three sample periods (45 min), which reached a maximum of 200% above basal values at the 45-min time point. Figure 1D shows that the same effect occurs after injections of AM251 in WT or KO mice. Therefore, whereas AM251 increased hippocampal ACh efflux in WT mice, there was no effect in CB_1R KO mice. Thus, a two-way ANOVA resulted in a significant interaction ($F_{30,\ 180} = 17.20$, $P < 0.0001$), treatment ($F_{3,\ 180} = 15.40$, $P < 0.001$), and time ($F_{10,\ 180} = 24.08$, $P < 0.0001$) effect. The increase in hippocampal ACh induced by AM251 only reached significance at one time point (30 min) but reached a maximum value of approximately 200% above basal values.

Experiment 3

Effects of Local Perfusion of CB_1R Antagonists in the Septum or the Hippocampus on Hippocampal ACh Efflux. Next, we wanted to determine through which specific neuroanatomical site in the septohippocampal pathway CB_1R antagonists modulate hippocampal ACh efflux.

Local Hippocampal Infusion of SR141716A or AM251 Increased whereas Septal Infusion Decreased Hippocampal ACh Efflux. As demonstrated in Fig. 2A, infusions of CB_1R antagonists locally in the hippocampus significantly increased hippocampal ACh efflux ($F_{2,\ 69} = 12.15$, $P < 0.0001$). In particular, local infusion of either SR141716A or AM251 significantly increased ACh release in the hippocampus (Fig. 2A) during both the 1-h infusion period ($P < 0.05$ for SR141716A and $p < 0.001$ for AM251) and the 1-h postinfusion period ($P < 0.001$ for AM251). In contrast to the effects observed after systemic injections, local infusion produced a greater increase in hippocampal ACh efflux after infusions of AM251 compared with infusions of SR141716A.

Unlike hippocampal infusions, Fig. 2B indicates that septal infusions of CB_1R antagonists decreased hippocampal ACh levels. Thus, septal infusions of AM251 or SR141716A had a tendency to decrease hippocampal ACh efflux during the 1-h infusion period and significantly ($P < 0.01$) decreased hippocampal ACh efflux during the 1-h postinfusion period (Fig. 2B; $P < 0.001$ for SR141716A and $P < 0.01$ for AM251). Local infusion of vehicle in either the hippocampus or the septum did not affect hippocampal ACh efflux (Fig. 2, A and B).

Experiment 4

Analysis of Basal and Stress-Induced Hippocampal ACh Levels in CB_1R KO or WT Mice. Based on the data obtained through pharmacological blockade of CB_1R, we sought to investigate whether genetic deletion of CB_1R would affect basal hippocampal ACh efflux and tissue content. Basal levels were quantified using conventional microdialysis, semiquantitative microdialysis, the zero-net-flux method of microdialysis, or tissue level analysis.

CB_1R KO Mice Have Similar Basal but Higher Stress-Enhanced Hippocampal ACh Levels Compared with WT Mice. CB_1R KO mice did not have significantly different basal hippocampal ACh levels compared with WT mice, as assessed using tissue level measurements (Fig. 3A). Semiquantitative dialysis resulted in a slight tendency toward increased ACh efflux in the hippocampus in KO mice, but this tendency did not reach statistical significance (Fig. 3B). The zero-net-flux method of dialysis also failed to reveal significant differences in basal levels between KO and WT mice, with both groups having values of approximately 5 nM (Fig. 3C). However, Fig. 3D shows that CB_1R KO mice did have higher stress-induced levels of hippocampal ACh efflux. Statistical analysis with a two-way ANOVA of the data obtained in the predatory odor stress test revealed a significant interaction ($F_{13,\ 130} = 6.16$, $P < 0.01$) and time ($F_{13,\ 130} = 52.29$, $P < 0.0001$) effect. In particular, CB_1R KO mice had increased ACh levels during the 15- to 45-min time periods (Fig. 3D). This effect reached a maximum of 50% above values obtained with WT mice and approximately 150%

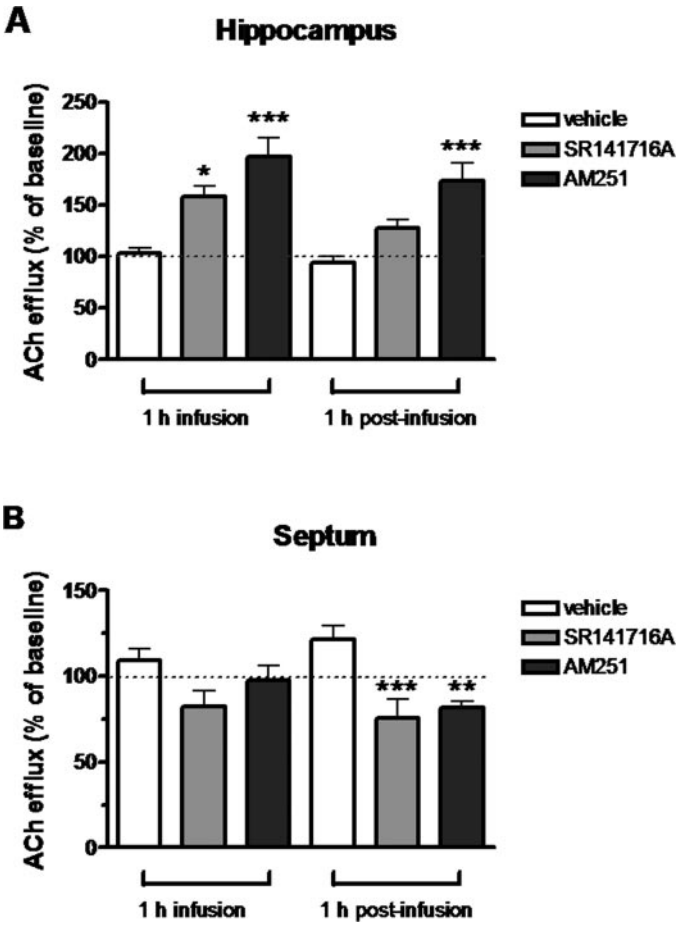

Fig. 2. Local infusion of the CB_1 receptor antagonists SR141716A and AM251 in the hippocampus (A) but not the septum (B) significantly increases hippocampal ACh efflux during both the 1-h infusion and the 1-h postinfusion periods; septal infusions decreased hippocampal ACh efflux during the 1-h postinfusion period only. Data are expressed as mean (± S.E.M., $n = 5$–8 per group) overall hippocampal ACh efflux (percentage of baseline). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus vehicle-infused rats.

above basal values. In WT mice, hippocampal ACh efflux was increased by more than 100% above basal values. For both WT and KO mice, this increase was greatest 30 min after the exposure to predatory odor. There was a second increase in hippocampal ACh that occurred at the 90-min time point. This increase was a direct result of the stress-induced increase associated with removing the animal from the test area (bucket with soiled bedding from rats) and returning it to the microdialysis bowl. An analysis (Student's t test) of the average overall ACh efflux during stress indicated that CB_1R KO mice had significantly higher overall levels compared with WT mice ($t_{1,\ 10} = 2.27$, $P < 0.05$; Fig. 3D).

Experiment 5

Quantification of CB_1R-Positive Cholinergic and Dopaminergic Nerve Terminals in the Rat Hippocampus. Based on the results obtained in experiment 2, we wanted to determine the extent to which cholinergic nerve terminals contain CB_1 receptors. In addition, given the fact that CB_1R antagonism increases brain dopamine levels (Tzavara et al., 2003a) and that fluctuations in dopamine levels can modulate hippocampal ACh efflux, the possibility that dopaminergic terminals in the hippocampus possess CB_1 receptors was explored. Other studies have reported low levels of CB_1R immunoreactivity in cell types in the hippocampus other than the GABAergic interneurons. However, this may be due to accessibility/sensitivity problems. Therefore, in this experiment we used a novel, highly sensitive method of quantification to calculate what percentage of cholinergic and dopaminergic nerve terminals in the hippocampus contained CB_1 receptors.

CB_1 Receptors Are Colocalized with Vesicular Acetylcholine Transporter and Dopamine Transporter in Nerve Terminals of the Rat Hippocampus. The protocol for separation of nerve terminals is designed to exclude contamination by postsynaptic elements. Nonetheless, we stained the nerve terminals for PSD95, a postsynaptic marker protein, and observed no PSD95 positivity in synaptophysin-costained plates of synaptosomes (data not shown), establishing the specificity of the isolation procedure. Figure 4, A and B, illustrates that 7.1% of nerve terminals/varicosities (identified by synaptophysin, 7140 dots counted) display VAChT positivity, which correlates well with a previous finding obtained through electron microscopic analysis (Towart et al., 2003). Similar results were observed with DAT (9.3% of 4310 synaptophysin-positive terminals). Finally, we observed that almost all VAChT-positive nerve terminals (91.1%) colocalize with the CB_1R immunoreactivity (6311 counted; Fig. 4, A and B), and slightly fewer, 60.1% of DAT-positive terminals, express CB_1Rs. This novel finding clearly demonstrates the high density of CB_1Rs in hippocampal cholinergic and dopaminergic nerve terminals, suggesting a substantial endocannabinoid control on cholinergic and dopaminergic neurotransmission.

Experiment 6

The Effect of D_1 Receptor Antagonism on the Stimulatory Effect of SR141716A on Hippocampal ACh Efflux. Based on the results from experiment 5, we examined whether the effect of CB_1R blockade on hippocampal ACh efflux involved changes in dopaminergic neurotransmission. As demonstrated previously, D_1 receptor agonism increases hippocampal ACh efflux (Day and Fibiger, 1994) and D_1 receptors mediate the stimulatory actions of low levels of cannabinoids on hippocampal ACh release (Nava et al., 2001; Tzavara et al., 2003b); therefore, an attempt was made to reverse the stimulatory actions of systemic injections and local hippocampal infusions of SR141716A on hippocampal ACh efflux using the D_1 receptor antagonist SCH23390.

D_1 Receptor Antagonism Prevents the Stimulatory Effect of CB_1R Blockade on Hippocampal ACh Efflux. Figure 5A shows that whereas systemic injections of

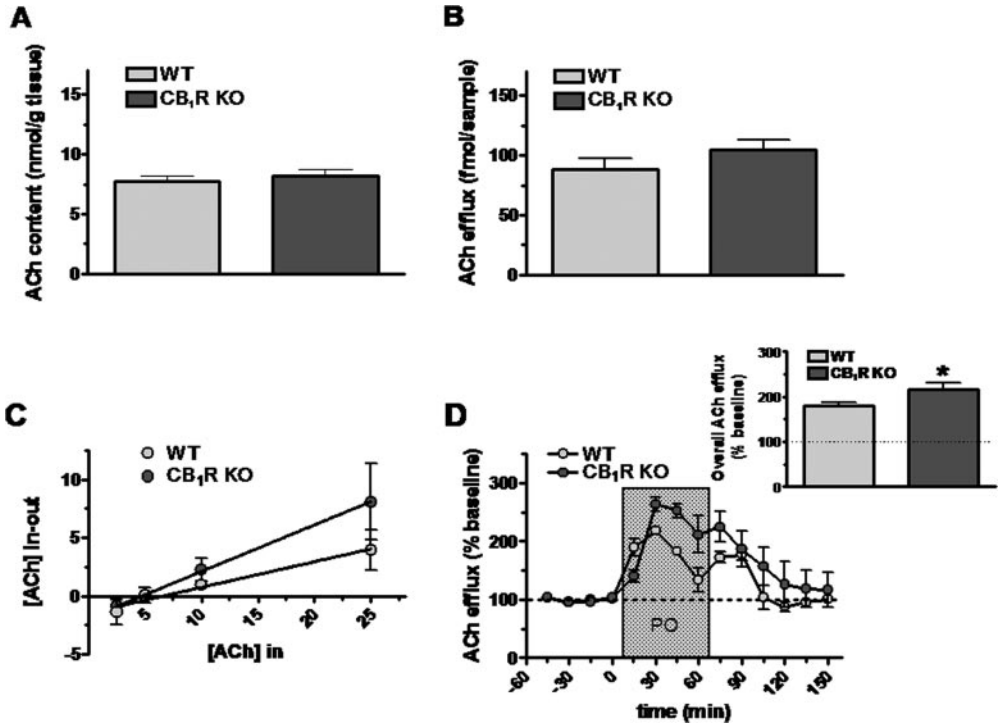

Fig. 3. CB_1R KO and WT mice have similar basal hippocampal ACh levels, as determined using tissue content analysis (A), semiquantitative microdialysis (B), and the zero-net-flux microdialysis method (C). However, CB_1R KO mice have higher stress-induced levels of hippocampal ACh, as determined using the predatory odor (PO) stress test (D, shaded area). D, inset, the average overall increase in ACh during exposure to the PO stress test over a 1-h period. Data are expressed as mean (± S.E.M., n = 5–8) ACh content (A) or efflux (B–D). *, $P < 0.05$ versus WT mice.

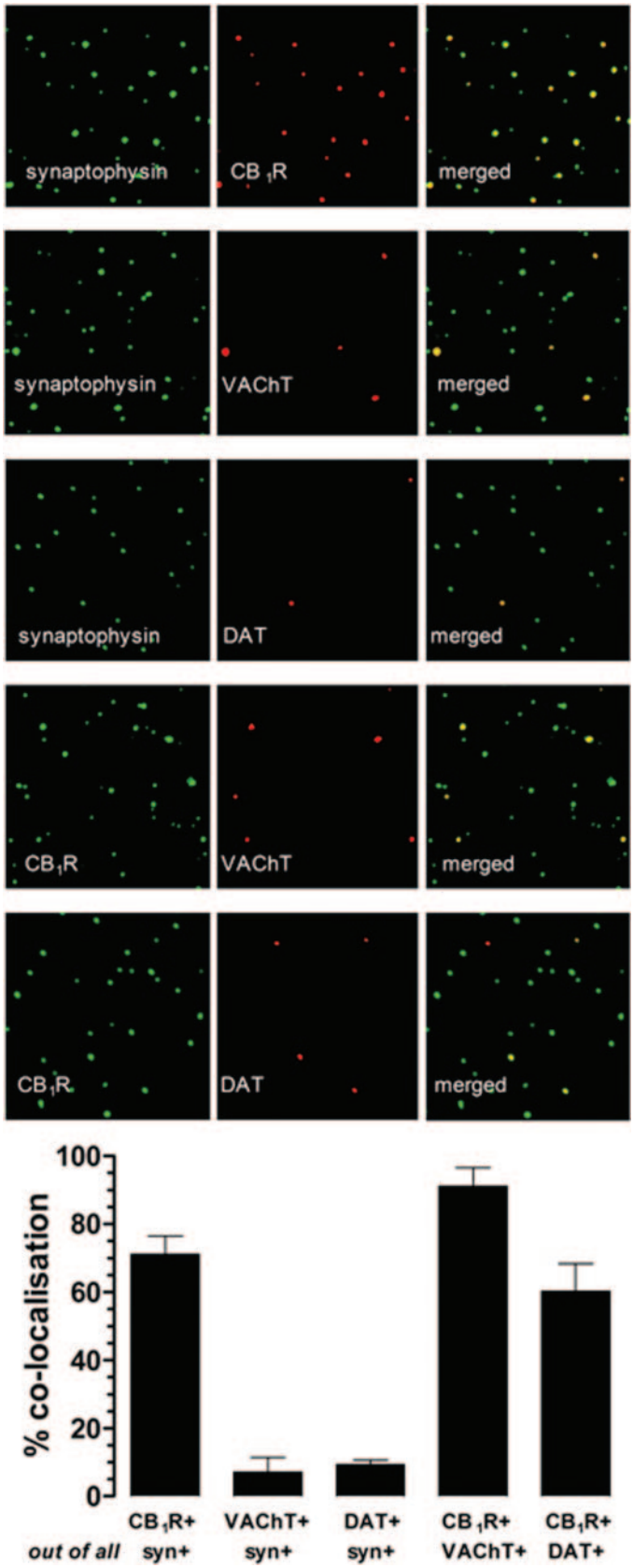

Fig. 4. Rat hippocampal cholinergic and dopaminergic nerve terminals are strongly equipped with CB_1 receptors. A, representative double-labeling images of antisynaptophysin (marker of all nerve terminals) with anti-VAChT (specific marker of cholinergic nerve terminals), with anti-DAT (specific marker of dopaminergic nerve terminals), and with anti-CB_1 receptor, and anti-CB_1 receptor with anti-VAChT and with anti-DAT. B, the extent of CB_1R, VAChT, and DAT colocalization with synaptophysin (syn, taken as 100%), CB_1 receptor colocalization with VAChT (taken as 100%), and with DAT (taken as 100%). Data represent the mean ± S.E.M. of $n = 6$ to 8 plates from three young adult rats after counting approximately 4 to 7000 terminals for each marker.

SR141716A resulted in a robust and persistent increase in hippocampal ACh, this increase was completely abolished by a prior subcutaneous injection of SCH23390 at a dose that had no effect on its own. Thereafter, a two-way ANOVA revealed a significant interaction ($F_{32,\ 208} = 19.36$, $P < 0.0001$), treatment ($F_{2,\ 208} = 18.33$, $P < 0.001$), and time ($F_{16,\ 208} = 23.07$, $P < 0.0001$) effect. The increase induced by SR141716A reached significance compared with the animals receiving both compounds at the 30-min time point, and this significant increase persisted for six samples. The maximum increase reached a level of approximately 150% above basal levels and occurred at the 45-min time point. Combined injections of SCH23390 and SR141716A resulted in a slight increase compared with animals injected with SCH23390 and vehicle, but this increase did not reach statistical significance. Animals receiving vehicle injections showed only a small increase in ACh efflux during the first 15 min after injection, as presented under *Experiment 1* (data not shown).

Results similar to those reported in Fig. 5A also occurred after local infusion of SR141716A (Fig. 5B). Thus, local infusion of SR141716A increased hippocampal ACh efflux during the 60-min infusion period, and again, this increase could be counteracted by a prior injection of SCH23390. Analysis of the data with a two-way ANOVA resulted in a significant interaction ($F_{26,\ 143} = 12.51$, $P < 0.05$), treatment ($F_{2,\ 143} = 15.20$, $P < 0.01$), and time ($F_{13,\ 143} = 10.83$, $P < 0.001$) effect. The increase observed with local infusion of SR141716A reached significance compared with the group receiving a combined treatment at the 30-, 60-, and 75-min time points with a maximum increase of almost 75% above basal values. As reported above, vehicle infusion in the hippocampus did not affect hippocampal ACh efflux (data not shown).

Discussion

The results from the present study suggest that CB_1R antagonism increases the efflux of ACh in the hippocampus through both a direct and an indirect mechanism. The direct mechanism involves CB_1Rs that are located on hippocampal cholinergic nerve terminals and can be invalidated by genetic deletion. These are the cannabinoid receptors that are most likely responsible for the decrease in hippocampal ACh efflux elicited by CB_1R agonists (Tzavara et al., 2003b) and through which endogenous cannabinoids modulate ACh release. Thus, CB_1R antagonists administered systemically or locally in the hippocampus increase ACh release through disinhibition of a primarily inhibitory action through CB_1R overarching any other opposing action that is mediated elsewhere (e.g., in the septum; see Fig. 6). The indirect mechanism, on the other hand, is mediated through CB_1Rs that could be positioned on hippocampal dopaminergic nerve terminals and can also be genetically invalidated; still, an extrahippocampal localization is not excluded from the present study. Stimulation of these CB_1Rs by endogenous or exogenous cannabinomimetics could potentially decrease dopaminergic (DA) release in the hippocampus, and accordingly, CB_1R

antagonists could increase ACh release through disinhibition and a stimulatory action of dopamine through D_1 receptor activation. This notion is supported by the fact that the stimulatory effects of CB_1R antagonists administered systemically or locally in the hippocampus on hippocampal ACh efflux can be counteracted by pharmacological blockade of D_1 receptors. Overall, these direct and indirect mechanisms combine to produce a robust and dose-dependent increase in hippocampal ACh efflux after pharmacological blockade of CB_1Rs. The increase in hippocampal ACh efflux induced by CB_1R antagonism could potentially explain why CB_1R inactivation enhances cognitive performance in relevant animal models (Chaperon and Thiebot, 1999).

The higher density of CB_1Rs in the hippocampus versus striatum may explain why CB_1R antagonists can affect the release of DA in the hippocampus and the neocortex but not the striatum and the nucleus accumbens, as we recently indicated (Tzavara et al., 2003a; Köfalvi et al., 2005). Whereas previous studies suggested a lack of direct modulation by cannabinoids of DA release in the brain (van der Stelt and Di Marzo, 2003; Köfalvi et al., 2005), we found evidence in the current study that CB_1R antagonism might directly modulate dopaminergic levels in the hippocampus. It should also be noted that although it is clear that D_1 receptor agonists increase ACh efflux in the hippocampus (Day and Fibiger, 1994), the exact role of D_5 receptors and the precise neuroanatomical elements partaking in this response remain to be elucidated (Wade and Nomikos, 2005).

The increase in hippocampal ACh release, induced by pharmacological blockade of CB_1Rs, is specifically mediated through CB_1Rs within the hippocampal but not the septal brain region. In fact, CB_1R antagonism in the septum induces a decrease in hippocampal ACh efflux. This is not surprising, given that we have recently reported that CB_1R agonists administered locally in the septum increase hippocampal ACh release (Tzavara et al., 2003a). Thus, endocannabinoids acting through CB_1Rs at the level of septum through a yet-unidentified mechanism could stimulate the septohippocampal cholinergic projections eliciting ACh release in the nerve terminal region; accordingly, CB_1R antagonism might exert a blocking effect at the septal brain region and an ensuing decrease in hippocampal ACh. It should be noted that CB_1R is a general marker of GABAergic nerve terminals in the brain (Irving et al., 2000; Freund et al., 2003; Köfalvi et al., 2005), and their blockade can increase brain GABA levels. Facilitation of GABAergic neurotransmission in the septum, in turn, decreases hippocampal ACh efflux (Dutar et al., 1995). In further support of this notion, a recent study indicated that cholinergic cell bodies in the septum that project to the hippocampus express both $GABA_b$ receptors and CB_1Rs (Nyiri et al., 2005). Thus, topical CB_1R antagonism at the level of septum could result in a decrease in hippocampal ACh release through a mechanism that involves stimulation of $GABA_b$ receptors (possibly through an enhanced release of GABA) localized on septohippocampal cholinergic/GABAergic-projecting neurons (Fig. 6).

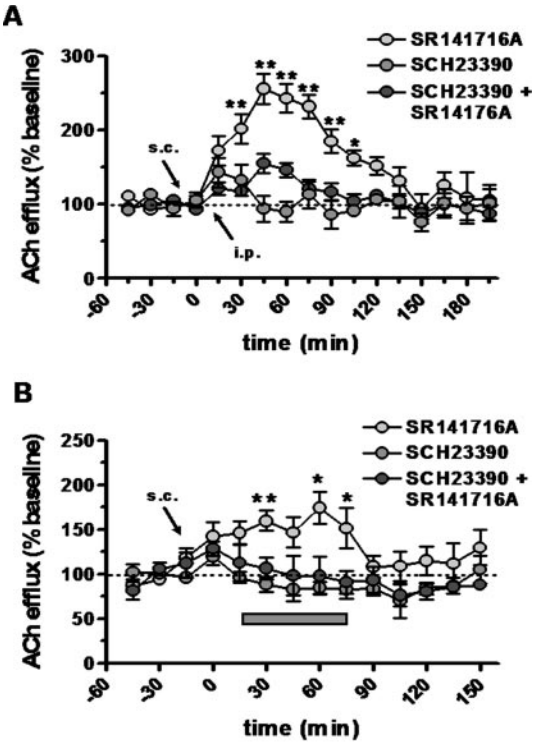

Fig. 5. The increase in hippocampal ACh efflux induced by either a systemic (10 mg/kg i.p.; A) injection or a local (1 mM; B) perfusion of SR141716A is completely abolished by administration of the dopamine D_1 receptor antagonist SCH23390 (0.3 mg/kg, s.c.) 15 min before administration of SR141716A. Data are expressed as mean (± S.E.M., $n = 4–7$ per group). *, $P < 0.05$; **, $P < 0.01$ versus SCH23390 +SR141716A-treated rats.

In contrast to pharmacological blockade, genetic deletion of CB_1Rs did not modulate basal steady-state hippocampal ACh efflux as assessed using different methods of quantification. Thus, it seems that there may be a compensatory mechanism after long-term inactivation of CB_1Rs. This is partially supported by our own recent data, in which pharmacological blockade of CB_1R increased plasma corticosterone levels; still, genetic deletion of CB_1 R did not influence basal corticosterone concentrations, although the stimulatory effects of CB_1R antagonists were completely abolished (Wade et al., 2006). Despite the negative findings on basal hippocampal ACh levels after genetic deletion of CB_1 receptors, CB_1R KO mice had higher stress-induced hippocampal ACh compared with WT mice. Thus, even though there seems to be a compensatory response after long-term inactivation of CB_1 receptors, CB_1R KO mice still have higher hippocampal ACh levels when the hippocampal cholinergic system is actively recruited. To the extent that an increase in hippocampal ACh efflux is associated with an enhanced coping ability and an improvement in cognitive performance (Degroot and Nomikos, 2005), this ACh hyper-responsiveness could explain why the CB_1R KO mice perform better in learning and memory tasks and can even experience "impaired forgetting" (i.e., inability to forget a previously learned response), even if it would be beneficial to the animals to neutralize/forget this response (Reibaud et al., 1999; Varvel and Lichtman, 2002). In addition, it could explain why CB_1R antagonism modulates anxiety levels by enhancing the ability of the animals to perceive aversive stimuli and respond accordingly through active avoidance (Degroot and Nomikos, 2004).

Unlike the bimodal effect seen after the administration of a CB_1R agonist, CB_1R antagonists dose-dependently and uniformly increased hippocampal ACh efflux. In part, this differentiation may result from neuroanatomical specificity of the effects obtained with CB_1R antagonists and agonists, the

level of the endogenous endocannabinoid tone, and its disruptions by these compounds. Whereas previous data from our laboratory indicated that CB_1R agonism differentially controls hippocampal ACh release through both the septum and the hippocampus, depending on which dose was used, the uniform effect seen in the current study was specifically controlled through CB_1R antagonism in the hippocampus that seemed to play a protagonist role. Nevertheless, septal perfusions of CB_1R antagonists did suppress hippocampal ACh efflux, an effect which probably involved stimulation of the septal GABAergic system (see above). Thus, CB_1R antagonism at the level of hippocampus seems to override an inhibitory action of CB_1R antagonism at the level of septum, resulting in a prevailing stimulatory action on hippocampal ACh release after systemic administration of CB_1R antagonists.

Because D_1 receptor antagonism completely counteracted the increase in hippocampal ACh efflux that was observed after pharmacological blockade of CB_1Rs, it could be argued that CB_1R antagonism increases hippocampal ACh efflux solely through an indirect mechanism. However, as depicted in Fig. 6 and as demonstrated with septal infusions of SR141716A and AM251, CB_1R antagonism at the level of septum could also have an inhibitory effect on hippocampal ACh efflux. Therefore, if only the indirect mechanism is involved in increased hippocampal ACh efflux observed after inactivation of CB_1R, then removing this mechanism through D_1 receptor blockade should actually result in ACh levels below basal values, because now only the inhibitory mechanism at the level of septum remains operating. However, D_1 receptor blockade simply counteracted the increase in hippocampal ACh efflux induced by CB_1R blockade. Thus, it seems more plausible that a direct mechanism at the level of the hippocampus is also involved, which partially offsets the inhibitory mechanism that remains active once the indirect stimulatory mechanism is removed. This notion is supported by the present data demonstrating a high density of CB_1Rs on hippocampal cholinergic nerve terminals.

Although we and others described previously that CB_1Rs are primarily located on the nerve terminals of cholecystokinin-positive GABAergic interneurons in the rat and human hippocampus (Katona et al., 2000), it is now generally believed that other neuron types also express CB_1Rs (Marsicano et al., 2003). The low levels of CB_1R immunoreactivity in other cell types in electron microscopic assays might be due to accessibility/sensitivity problems. Here, using a much more sensitive method, we report that a relatively high percentage of cholinergic and dopaminergic hippocampal nerve terminals and varicosities are equipped with the CB_1R, indicating an even more widespread and extensive role for the endocannabinoid system in the regulation of hippocampal function.

Conclusions

Pharmacological antagonism of CB_1Rs increases hippocampal ACh efflux through both a direct mechanism through CB_1Rs only at the level of the hippocampus and an indirect mechanism that involves both CB_1R disinhibition and D_1 receptor stimulation most likely at the level of hippocampus, although other sites cannot be convincingly excluded. The increase in hippocampal ACh levels in response to CB_1R antagonists is abolished after inactivation of either mechanism. It is possible that when combined, both mechanisms can override a demonstrated suppression of hippocampal ACh release induced by blockade of CB_1Rs in the septum. Genetic invalidation of CB_1Rs engenders a compensated normalization of basal steady-state hippocampal ACh levels, although exposure to an aversive stimulus renders the animals more susceptible to mobilization of the cholinergic sys-

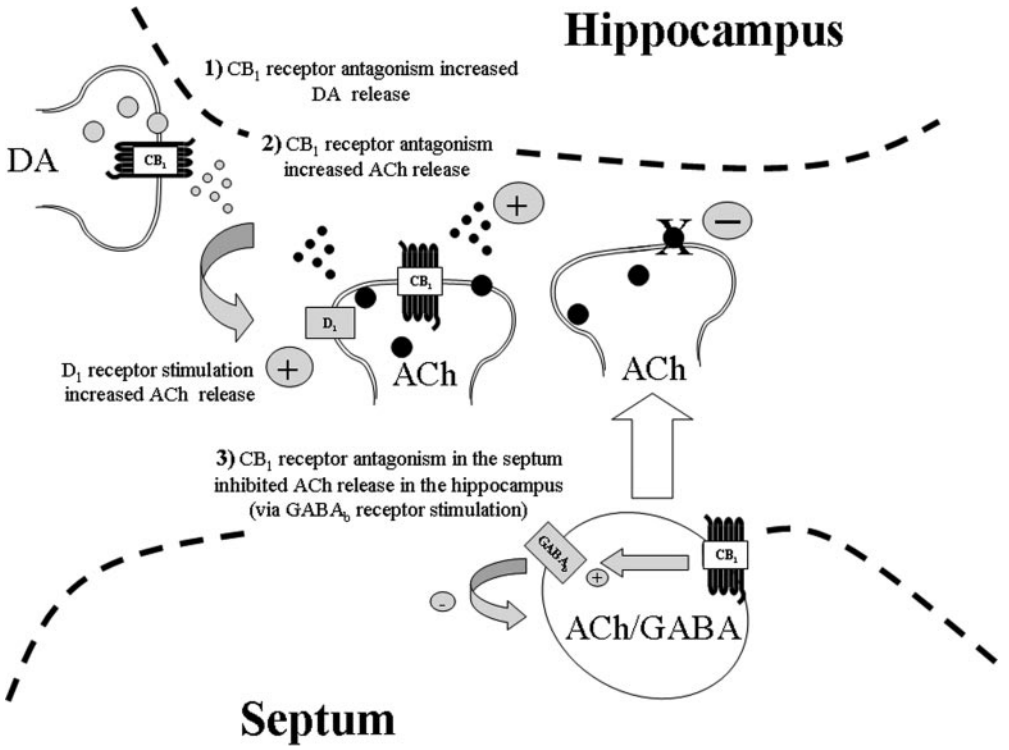

Fig. 6. Schematic diagram of suggested direct and indirect mechanisms by which CB_1R antagonism affects ACh efflux in the hippocampus. Antagonism of CB_1R can increase hippocampal ACh efflux indirectly through the DA system (D_1 receptor-mediated stimulation) (1) and directly through CB_1R located on cholinergic nerve terminals (2). Blockade of CB_1R in the septum can also have an inhibitory effect on hippocampal ACh efflux through an interaction with $GABA_b$ receptors (possibly via an increase in GABA release in the septum) located on septohippocampal cholinergic/GABAergic neurons (3).

tem and its neurophysiological consequences. This increase in hippocampal ACh efflux probably accounts for the enhanced cognitive effect observed in preclinical models (Chaperon and Thiebot, 1999). In addition, it could account for the modulation of anxiety responses, as described by Degroot and Nomikos (2004). Our study sheds light onto the mechanism of action through which CB_1R antagonism increases hippocampal ACh efflux, which further helps us understand the role of the brain endocannabinoid system in regulating cholinergic function in the brain, and, consequently, neuroadaptive, both cognitive and affective, responses of the organism.

Acknowledgments

We are grateful to Ken Mackie for generously supplying us with the antibody.

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