CB₁ Receptor Antagonism Increases Hippocampal Acetylcholine Release: Site and Mechanism of Action


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VAChT, vesicular acetylcholine transporter; BAS, Bioanalytical Systems, Inc
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 CB₁ receptor (CB₁R) antagonism, recently several in vitro functional studies have suggested non-CB₁R 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 CB₁R antagonists, different methods of in vivo microdialysis, CB₁R knockout (KO) mice, tissue measurements of ACh, and immunochemistry. First, we showed that systemic injections of the CB₁R antagonists SR141716A and AM251 dose-dependently increased hippocampal ACh efflux. Similarly, local hippocampal, but not septal, infusions of SR141716A or AM251 increased hippocampal ACh release. Importantly, the stimulatory effects of systemically administered CB₁R antagonists on hippocampal ACh release were completely abolished in CB₁R KO mice. CB₁R KO mice had similar basal, but higher stress-enhanced, hippocampal ACh levels compared to wild-type controls. Interestingly, dopamine D₁ receptor antagonism counteracted the stimulatory effect of CB₁R blockade on hippocampal ACh levels. Finally, immunohistochemical methods revealed that a high proportion of CB₁R positive nerve terminals were found in hippocampus and confirmed the co-localization of CB₁ receptors with cholinergic and dopaminergic nerve terminals. In conclusion, hippocampal ACh release may specifically be controlled through CB₁Rs located on both cholinergic and dopaminergic neuronal projections, and CB₁R antagonism increases hippocampal ACh.
release probably through both a direct disinhibition of ACh release and an indirect increase in dopaminergic neurotransmission at the D₁ receptors.
It has been shown in animal studies that cannabinoid receptor activation and blockade impair and enhance cognitive performance, respectively, under certain experimental conditions (see Chaperon and Thiebot 1999). These effects on cognition have been correlated with fluctuating extracellular acetylcholine (ACh) levels in the hippocampus, where an abundance of cannabinoid receptors and cholinergic nerve terminals reside (e.g., Inui et al., 2004; Tzavara et al., 2003b). The effect of cannabinoid receptor stimulation on hippocampal ACh efflux can vary depending on dosage and site of administration. Generally, 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 (CB₁R) and type 2 (CB₂R) (Devane et al., 1988). The modulation of hippocampal ACh levels induced by cannabinoids is likely mediated through CB₁R given their distribution in the septohippocampal pathway (for review, see 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 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 CB₁R, recent studies indicate that cannabinoids may mediate some of their neurochemical/behavioral actions through novel, yet to be identified central cannabinoid receptors (e.g., Di Marzo et al., 2000; Haller et al., 2002; Köfalvi et al., 2005). As such, a putative cannabinoid receptor, which is sensitive to the CB₁R antagonist SR141716A (Rimonabant; Rinaldi-Carmona et al., 1994), but insensitive to the CB₁R 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 likely mediated through CB, R 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, since 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). Lastly, CB, R antagonism may regulate hippocampal ACh levels through a modulation of the dopaminergic system: blockade of CB, receptors increases brain dopamine levels (Tzavara et al., 2003a), and stimulation of dopaminergic receptors affects ACh release (Day and Fibiger, 1994). Specifically, increased dopaminergic neurotransmission through D₁ receptors increases, whereas through D₂ receptors decreases hippocampal ACh efflux, respectively (Day and Fibiger, 1994). Moreover, stimulation of D₂ and D₁ receptors mediates the inhibitory and stimulatory actions of high and low doses of cannabinoids on hippocampal ACh release, respectively (Nava et al., 2000; Tzavara et al., 2003b). Thus, a facilitation of dopaminergic neurotransmission at D₁ receptors may be involved in a possible stimulatory effect of hippocampal ACh release induced by CB, R antagonism.

The current study sought to address these issues by conducting a detailed analysis of the site and mechanism of action through which CB, R blockade modulates hippocampal ACh efflux. For this reason, a combined neurochemical and neuropharmacological approach that included local and systemic administrations, dual
and quantitative microdialysis, as well as studies in CB₁R knockout (KO) mice was used. In addition, a histochemical analysis of the distribution of vesicular acetylcholine transporter (VACHT), dopamine transporter, and CB₁R 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, purchased from Harlan Sprague-Dawley, Indianapolis, IN), male CB1R KO and corresponding wild-type (WT) mice. All animals were housed in a vivarium for at least seven days prior to use, with food and water available ad libitum. Both CB1R KO and WT mice were derived from a stock of genotyped animals received from the University of Bonn. CB1R KO (CB1$^{-/-}$) mice and their homozygous controls (CB1$^{+/+}$ mice) were developed in C57BL/6J mice by replacing most of the CB1R coding sequence with a non-receptor 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 anaesthetized with a mixture of chloral hydrate and pentobarbital (170 mg/kg and 36 mg/kg in 30% propylene glycol and 14% ethanol), placed in a stereotaxic apparatus, and implanted unilaterally with guide cannulae (BAS; Bioanalytical Systems, Inc., West Lafayette, IN) in the hippocampus (coordinates AP: -5.2, ML: 5.2, DV: -3.8) according to
the stereotaxic atlas of Paxinos and Watson (1998). Twenty-four hours before testing, a 4 mm concentric microdialysis probe (BAS, BR-4) was inserted through the guide cannula.

Similarly, for mice, 2 mm microdialysis probes (CMA, Stockholm, Sweden) were implanted unilaterally in the hippocampus (AP: -3.3, ML: +3.1, DV: -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 (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 CaCl₂, 1.0 mM MgCl₂, 1.0 mM Na₂HPO₄ x 7H₂O, 0.2 mM NaH₂PO₄ x H₂O, 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 on-line with high performance liquid chromatography (HPLC) coupled to electrochemical detection, with a 150 x 3 mm acetylcholine-3 column (ESA, Inc., Chelmsford, MA) maintained at 35°C. The mobile phase (100 mM di-sodium hydrogen phosphate, 2 mM 1-octanesulfonic acid and 50 µl/l of the microbicide reagent MB, ESA, Inc.; pH 8.0, adjusted with phosphoric acid) was delivered by an HPLC 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; 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. Subsequently, animals were systemically injected or locally infused with vehicle or drug and additional 6-12 samples (see Results) were collected.

Drugs and experimental design:

In Experiment 1, rats that had been previously implanted with microdialysis probes directed at the hippocampus, as described, were injected intraperitoneally (i.p.) with vehicle (0.9% NaCl containing 2% DMSO 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 (e.g., Tzavara et al., 2001; Chambers et al., 2004).

In Experiment 2, mice (CB1R KO or WT) that were previously implanted with microdialysis probes, as described, were injected i.p. with vehicle (see above), 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 (see 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 previously been shown not to affect hippocampal ACh efflux (Tzavara et al., 2003b). It should also be noted that although the \textit{in vivo} recovery of the administered CB1R antagonists was not determined, this has previously been shown to be <1% for a compound, nicotine, administered locally under similar experimental conditions, such as those used in the present study (see, e.g., Marshall et al., 1997). Neostigmine was omitted from the perfusion solution used for septal perfusions, since 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 CB1R KO and WT mice using different methods of microdialysis (conventional, semi-quantitative/low perfusion rate, and the quantitative/zero-net-flux method), tissue level measurements, and an animal model of exposure to stress (predatory odor test). Semi-quantitative microdialysis was conducted by perfusing CB1R KO or WT mice that had been previously implanted 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, as at low perfusion rates their \textit{in vivo} recovery reaches high levels (see, e.g., Gerber et al., 2001). Following a standard three h stabilization period,
dialysate was collected until a final volume of 40 µl was reached (8.3 hours) and analyzed off-line for ACh content. In the zero-net-flux microdialysis method (Day et al., 2001) CB1R 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}]. Conversely, 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} - ACh_{out}]=0, we can accurately determine the basal, steady-state concentrations of ACh in the extracellular fluid of the hippocampus. Following a three h stabilization period, dialysate samples were collected every 20 min for a total of 10 samples and analyzed off-line for ACh content.

In order to measure hippocampal tissue levels of ACh, brains were dissected from CB1R KO or WT mice following 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 0.1N TCA 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 BAS LC-4C detector utilizing a platinum electrode at 500 mV.
potential. Data were subsequently collected and analyzed with EZChrome Elite (Scientific Software, Inc., Pleasanton, CA).

Next, we examined if CB₁R KO mice had altered evoked, i.e. stress-enhanced, concentrations of hippocampal ACh efflux compared to WT mice. Mice implanted with microdialysis probes directed at the hippocampus were exposed to the predatory odor stress test. Following the baseline period, mice were placed in a bucket that contained soiled bedding from rat cages for a period of 60 min while 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, as exposure of mice to predatory odors has been previously shown 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 in order to calculate what percentage of cholinergic and dopaminergic nerve terminals in the hippocampus contained CB₁ receptors (see below).

In Experiment 6, rats were injected subcutaneously (s.c.) with vehicle (0.9% NaCl) or the D₁ receptor antagonist SCH23390 (0.3 mg/kg; purchased from Tocris) at a volume of 1 ml/kg. These injections occurred 15 min prior to 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 previously described (Köfalvi et al., 2005). Briefly, 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 2,000 g at 4°C and the resulting supernatant was spun again at 9,500 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 ml 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 NaHCO3, 1.2 mM NaH2PO4, 1 mM MgCl2, 10 mM glucose, and 10 mM HEPES, pH 7.4). The synaptosomes were placed onto coverslips previously coated with poly-L-lysine, fixed with 4% paraformaldehyde for 15 min, and washed twice with PBS (140 mM NaCl, 3 mM KCl, 20 mM NaH2PO4, 15 mM KH2PO4, pH 7.4). Permeabilization was performed in PBS containing 0.2% Triton X-100 for 10 min, and afterwards the synaptosomes were incubated in PBS medium containing 3% bovine serum albumin (BSA) and 5% normal rat serum for 1 h. The synaptosomes were then washed twice with PBS and incubated with rabbit anti-CB1 receptor and guinea pig anti-vesicular acetylcholine transporter (anti-VACHT; 1:500; Chemicon International, Temecula, CA.), rat anti-dopamine transporter (anti-DAT; 1:500; Chemicon) or mouse anti-synaptophysin (1:200, Sigma, Saint Louis MI), for 1 h at room temperature. The rabbit CB1R antibody, a generous gift of Dr. Ken Mackie, was raised against a GST fusion protein corresponding to the last 15 amino acids of the rat CB1R (1:3000). No staining with this CB1R antibody was seen in the CB1R.
homozygote null-mutant mouse, obtained from Dr. Catherine Ledent (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/BSA (3%) and were incubated for 1 h at room temperature with a AlexaFluor-488 (green)-labelled goat anti-rabbit IgG antibodies (1:200; Molecular Probes, Leiden, The Netherlands) or goat anti-guinea pig or goat anti-rat or goat anti-mouse, all labelled 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, Germany) inverted microscope equipped with a cooled CCD camera and analysed with MetaFluor 4.0 software (MDC, 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 0-3 bright spots were observed in each field (non-specific 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 percent 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$_1$R antagonists SR141716A and AM251 on hippocampal AChefflux.

This experiment examined the dose-dependent nature of CB$_1$R antagonist administration and compared the effects of two slightly different (mainly based on functional responses, see Introduction) CB$_1$R antagonists, SR141716A and AM251, on hippocampal AChefflux.

SR141716A and AM251 dose-dependently increase AChefflux in the hippocampus.

There were no statistically significant differences in the basal values of AChe among the various experimental groups in this or subsequent experiments with values ranging from 125 to 500 fmoles/sample. Figure 1A indicates that SR141716A dose-dependently increases hippocampal AChefflux. 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 of SR141716A had significantly increased levels of hippocampal AChe. This significant increase persisted for 8 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 AChefflux that persisted for 2 sample periods (30 min) and reached a maximum of 150% above basal values. Injections of 1 mg/kg increased hippocampal AChefflux to a maximum of 50% above basal values; this increase failed to reach significance. In all drug-injected groups, AChefflux followed the same kinetic profile with 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 following 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. Specifically, injections of 10 mg/kg resulted in a maximum increase of almost 100% above basal values, and this increase was significant for 5 samples (75 min). The lower dose caused a slightly lower increase that was maintained statistically significant for two sample periods (30, 45 min).

Experiment 2: Effects of systemic injections of the CB1R antagonists SR141716A and AM251 on hippocampal ACh efflux in CB1R KO or WT mice.

In this experiment we determined if the effects of SR141716A and AM251 on hippocampal ACh efflux that were observed in Experiment 1 were retained in mice in which the CB1R had been genetically deleted.

The stimulatory effects of systemic injections of SR141716A and AM251 on hippocampal ACh efflux are abolished in CB1R KO mice.

There were no statistically significant differences in the basal values of ACh among different experimental groups and between CB1R KO and WT mice with values ranging from 65 to 125 fmoles/sample. As can be seen in figure 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,}
SR141716A caused a significant increase during 3 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 following injections of AM251 in WT or KO mice. Consequently, whereas AM251 increased hippocampal ACh efflux in WT mice, there was no effect in CB,R KO mice. Thus, a two-way ANOVA resulted in a significant interaction (F30, 180 = 17.20, P<0.0001), treatment (F3, 180= 15.40, P<0.001), and time (F10, 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,R 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,R antagonists modulate hippocampal ACh efflux.

Local hippocampal infusion of SR141716A or AM251 increased, whereas septal infusion decreased, hippocampal ACh efflux.

As demonstrated in figure 2A, infusions of CB,R antagonists locally in the hippocampus significantly increased hippocampal ACh efflux (F2, 69 = 12.15, P<0.0001). Specifically, local infusion of either SR141716A or AM251 significantly increased ACh release in the hippocampus (figure 2A) during both the 1 h infusion period (P<0.05 for SR141716A and p<0.001 for AM251) and the 1 h post-infusion period (P<0.001 for AM251). In contrast to the effects observed following systemic injections, local infusion
produced a greater increase in hippocampal ACh efflux following infusions of AM251 compared to infusions with SR141716A.

Unlike hippocampal infusions, figure 2B indicates that septal infusions of CB1R 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 post-infusion period (figure 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 (figure 2A,B).

**Experiment 4: Analysis of basal and stress-induced hippocampal ACh levels in CB1R KO or WT mice.**

Based on the data obtained through pharmacological blockade of CB1R, we sought to investigate whether genetic deletion of CB1R would affect basal hippocampal ACh efflux and tissue content. Basal levels were quantified using conventional microdialysis, semi-quantitative microdialysis, the zero-net-flux method of microdialysis, or tissue level analysis.

**CB1R KO mice have similar basal, but higher stress-enhanced, hippocampal ACh levels compared to WT mice.**

CB1R KO mice did not have significantly different basal hippocampal ACh levels compared to WT mice as assessed using tissue level measurements (figure 3A). Semi-quantitative dialysis resulted in a slight tendency towards increased ACh efflux in the hippocampus in KO mice, but this tendency did not reach statistical significance (figure 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 (figure 3C). However, figure 3D shows that CB1R 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 (F13, 130 = 6.16, P<0.01) and time (F13, 130 = 52.29, P<0.0001) effect. Specifically, CB1R KO mice had increased ACh levels during the 15-45 time periods (figure 3D). This effect reached a maximum of 50% above values obtained with WT mice and approximately 150% 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 following 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 CB1R KO mice had significantly higher overall levels compared to WT mice (t1, 10 = 2.27, P<0.05; figure 3D).

Experiment 5: Quantification of CB1R-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 CB1 receptors. In addition, given the fact that CB1R 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 CB1 receptors was explored.
Other studies have reported low levels of CB1R 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 in order to calculate what percentage of cholinergic and dopaminergic nerve terminals in the hippocampus contained CB1 receptors.

**CB1 receptors are co-localized 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-co-stained plates of synaptosomes (data not shown), establishing the specificity of the isolation procedure. Figure 4A,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% out of 4310 synaptophysin-positive terminals). Finally, we observed that almost all VAChT-positive nerve terminals (91.1%) co-localize with the CB1R immunoreactivity (6311 counted; figure 4A,B), and slightly fewer, 60.1% of DAT-positive terminals express CB1Rs. This novel finding clearly demonstrates the high density of CB1Rs in hippocampal cholinergic and dopaminergic nerve terminals, suggesting a substantial endocannabinoid control on cholinergic and dopaminergic neurotransmission.
Experiment 6: The effect of D₁ 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 CB₁R blockade on hippocampal ACh efflux involved changes in dopaminergic neurotransmission. As it has been previously demonstrated that D₁ receptor agonism increases hippocampal ACh efflux (Day and Fibiger, 1994) and that D₁ receptors mediate the stimulatory actions of low levels of cannabinoids on hippocampal ACh release (Nava et al., 2000; Tzavara et al., 2003b), an attempt was made to reverse the stimulatory actions of systemic injections and local hippocampal infusions of SR141716A on hippocampal ACh efflux using the D₁ receptor antagonist SCH23390. 

**D₁ receptor antagonism prevents the stimulatory effect of CB₁R blockade on hippocampal ACh efflux.**

Figure 5A shows that whereas systemic injections of SR141716A resulted in a robust and persistent increase in hippocampal ACh, this increase was completely abolished by a prior s.c. injection of SCH23390 at a dose that did not have an effect on its own. Subsequently, a two-way ANOVA revealed a significant interaction (F₁₆, 20₈ = 19.36, P<0.0001), treatment (F₂, 20₈ = 18.33, P<0.001), and time (F₁₆, 20₈ = 23.07, P<0.0001) effect. The increase induced by SR141716A reached significance compared to the animals receiving both compounds at the 30 min time point and this significant increase persisted for 6 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 to 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 post-injection, as presented above (data not shown).

Similar results to those reported in figure 5A also occurred following local infusion of SR141716A (figure 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 to 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 CB₁R antagonism increases the efflux of ACh in the hippocampus through both a direct and an indirect mechanism. The direct mechanism involves CB₁Rs 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 CB₁R agonists (Tzavara et al. 2003b), and through which endogenous cannabinoids modulate ACh release. Thus, CB₁R antagonists administered systemically or locally in the hippocampus increase ACh release through disinhibition of a primarily inhibitory action through CB₁R overarching any other opposing action that is mediated elsewhere (e.g., in the septum, see below). The indirect mechanism, on the other hand, is mediated through CB₁Rs that could be positioned on hippocampal dopaminergic nerve terminals and can also be genetically invalidated; still, an extra-hippocampal localization is not excluded from the present study. Stimulation of these CB₁Rs by endogenous or exogenous cannabinomimetics could potentially decrease dopamine release in the hippocampus, and, accordingly, CB₁R antagonists could increase ACh release through disinhibition and a stimulatory action of DA through D₁ receptor activation. This notion is supported by the fact that the stimulatory effects of CB₁R antagonists administered systemically or locally in the hippocampus on hippocampal ACh efflux can be counteracted by pharmacological blockade of D₁ receptors. Overall, these direct and indirect mechanisms combine to produce a robust and dose-dependent increase in hippocampal ACh efflux following pharmacological blockade of CB₁Rs. The increase in hippocampal ACh efflux induced by CB₁R antagonism could potentially explain why
CB₁R inactivation enhances cognitive performance in relevant animal models (see Chaperon and Thiebot, 1999).

The higher density of CB₁Rs in the hippocampus vs. striatum may explain why CB₁R 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 (Köfalvi et al., 2005; for review see van der Stelt and Di Marzo, 2003), we found evidence in the current study that CB₁R antagonism might directly modulate dopaminergic levels in the hippocampus. It should also be underlined, that although it is clear that D₁ receptor agonists increase ACh efflux in the hippocampus (see, e.g., Day and Fibiger, 1994) the exact role of D₃ receptors and the precise neuroanatomical elements partaking in this response remain to be elucidated (see Wade and Nomikos, 2005).

The increase in hippocampal ACh release, induced by pharmacological blockade of CB₁Rs, is specifically mediated through CB₁Rs within the hippocampal, but not the septal brain region. In fact, CB₁R antagonism in the septum induces a decrease in hippocampal ACh efflux. This is not surprising, given that we have recently reported that CB₁R agonists administered locally in the septum increase hippocampal ACh release (Tzavara et al., 2003a). Thus, endocannabinoids acting through CB₁Rs 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, CB₁R antagonism might exert a blocking effect at the septal brain region and an ensuing decrease in hippocampal ACh. It should be noted that CB₁R is a general marker of
GABAergic nerve terminals in the brain (Irving et al., 2000; Köfalvi et al., 2005; for review, see Freund et al., 2003), 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<sub>B</sub> receptors and CB<sub>1</sub>Rs (Nyiri et al., 2005). Thus, topical CB<sub>1</sub>R antagonism at the level of septum could result in a decrease in hippocampal ACh release through a mechanism that involves stimulation of GABA<sub>B</sub> receptors (possibly through an enhanced release of GABA) localized on septohippocampal cholinergic/GABA-ergic projecting neurons (see Figure 6).

In contrast to pharmacological blockade, genetic deletion of CB<sub>1</sub>Rs did not modulate basal, steady-state hippocampal ACh efflux as assessed using different methods of quantification. Thus, it appears that there may be a compensatory mechanism following chronic inactivation of CB<sub>1</sub>Rs. This is partially supported by, e.g., our own recent data, where pharmacological blockade of CB<sub>1</sub>R increased plasma corticosterone levels; still, genetic deletion of CB<sub>1</sub>R did not influence basal corticosterone concentrations, although the stimulatory effects of CB<sub>1</sub>R antagonists were completely abolished (Wade et al. unpublished findings). In spite of the negative findings on basal hippocampal ACh levels following genetic deletion of CB<sub>1</sub> receptors, CB<sub>1</sub>R KO mice had higher stress-induced hippocampal ACh compared to WT mice. Thus, even though there seems to be a compensatory response following chronic inactivation of CB<sub>1</sub> receptors, CB<sub>1</sub>R 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 (see Degroot and Nomikos, 2005), this ACh hyper-responsiveness could explain why the CB₁R 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 CB₁R antagonism modulates anxiety levels by enhancing the ability of the animals to perceive aversive stimuli and respond accordingly through active avoidance (see, e.g., Degroot and Nomikos, 2004).

Unlike the bimodal effect seen following the administration of a CB₁R agonist, CB₁R antagonists dose-dependently and uniformly increased hippocampal ACh efflux. In part, this differentiation may result from neuroanatomical specificity of the effects obtained with CB₁R antagonists and agonists, the level of the endogenous endocannabinoid tone and its disruptions by these compounds. Whereas previous data from our laboratory indicated that CB₁R 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 CB₁R antagonism in the hippocampus that appeared to play a protagonist role. Nevertheless, septal perfusions of CB₁R antagonists did suppress hippocampal ACh efflux, an effect which likely involved stimulation of the septal GABAergic system (see above). Thus, CB₁R antagonism at the level of hippocampus appears to override an inhibitory action of CB₁R antagonism at the level of septum resulting in a prevailing
stimulatory action on hippocampal ACh release after systemic administration of CB₁R antagonists.

Since D₁ receptor antagonism completely counteracted the increase in hippocampal ACh efflux that was observed following pharmacological blockade of CB₁Rs, it could be argued that CB₁R antagonism increases hippocampal ACh efflux solely through an indirect mechanism. However, as depicted in Figure 6, and as demonstrated with septal infusions of SR141716A and AM251, CB₁R 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 following inactivation of CB₁R, then removing this mechanism through D₁ receptor blockade should actually result in ACh levels below basal values, since now only the inhibitory mechanism at the level of septum remains operating. However, D₁ receptor blockade simply counteracted the increase in hippocampal ACh efflux induced by CB₁R 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 (see also above). This notion is supported by the present data demonstrating a high density of CB₁ receptors on hippocampal cholinergic nerve terminals.

Although previously we and others described that CB₁ receptors 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 CB₁ receptors (e.g., Marsicano et al., 2003). The low levels of CB₁R 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 CB₁R, indicating an even more widespread and extensive role for the endocannabinoid system in the regulation of hippocampal function.

**Conclusions**

Pharmacological antagonism of CB₁Rs increases hippocampal ACh efflux through both a direct mechanism through CB₁ receptors only at the level of the hippocampus and an indirect mechanism that involves both CB₁R disinhibition and D₁ 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 CB₁R antagonists is abolished following 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 CB₁Rs in the septum. Genetic invalidation of CB₁Rs 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 system and its neurophysiological consequences. This increase in hippocampal ACh efflux likely accounts for the enhanced cognitive effect observed in preclinical models (see 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 CB₁R 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.
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References


Footnotes

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Figure Legends

**Figure 1:** Systemic administration (1, 3 and 10 mg/kg, i.p., indicated by arrows) of the CB₁ receptor antagonists SR141716A (A) and AM251 (B) dose-dependently increases hippocampal ACh efflux in rats. These stimulatory effects of SR141716A and AM251 (20 mg/kg and 10 mg/kg, respectively, i.p., indicated by arrows) on hippocampal ACh efflux are abolished in CB₁ receptor knockout (KO) mice (C and D, respectively). Data are expressed as mean (± S.E.M., n=5-8/group) hippocampal ACh efflux (% of baseline). *P<0.05, **P<0.01 vs. vehicle-injected rats (A and B) or vehicle-injected wild-type (WT) mice (C and D).

**Figure 2:** Local infusion of the CB₁ 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 post-infusion periods; septal infusions decreased hippocampal ACh efflux during the 1h post-infusion period only. Data are expressed as mean (± S.E.M., n=5-8/group) overall hippocampal ACh efflux (% of baseline). *P<0.05, **P<0.01, ***P<0.001 vs. vehicle-infused rats.
Figure 3: CB₁ receptor (CB₁R) knockout (KO) and wild-type (WT) mice have similar basal hippocampal ACh levels, as determined using tissue content analysis (A), semi-quantitative microdialysis (B), and the zero-net-flux microdialysis method (C). However, CB₁R KO mice have higher stress-induced levels of hippocampal ACh, as determined using the predatory odor (PO) stress test (D, shaded area). Insert in (D) shows 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 vs. WT mice.

Figure 4: Rat hippocampal cholinergic and dopaminergic nerve terminals are strongly equipped with CB₁ receptors. (A) Representative double labeling images of anti-synaptophysin (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-CB₁ receptor receptor, and anti-CB₁ receptor receptor with anti-VAcHT and with anti-DAT. (B) The extent of CB₁R, VAcHT and DAT co-localization with synaptophysin (syn, taken as 100%), CB₁ receptor co-localization with VAcHT (taken as 100%), and with DAT (taken as 100%). Data represent the mean ± S.E.M of n = 6-8 plates from 3 young adult rats after counting ca. 4-7000 terminals for each marker.
**Figure 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 D₁ receptor antagonist SCH23390 (0.3 mg/kg, s.c.) 15 min prior to administration of SR141716A. Data are expressed as mean (± S.E.M., n=4-7/group). * P<0.05, **P<0.01 vs. SCH23390+SR141716A-treated rats.

**Figure 6:** Schematic diagram of suggested direct and indirect mechanisms by which CB₁R antagonism affects ACh efflux in the hippocampus. Antagonism of CB₁R can increase hippocampal ACh efflux indirectly through the dopaminergic (DA) system (D₁ receptor mediated stimulation) (1) as well as directly through CB₁R located on cholinergic nerve terminals (2). Blockade of CB₁R in the septum can also have an inhibitory effect on hippocampal ACh efflux through an interaction with GABA₉ receptors (possibly via an increase in GABA release in the septum) located on septohippocampal cholinergic/GABAergic neurons (3).
Figure 2

A  Infusion in Hippocampus

B  Infusion in Septum

α-Ch efflux (% baseline)
Figure 3

Panel A: Graph showing the ACh content (nmol/g tissue) for WT and CB1R KO.

Panel B: Graph showing the ACh efflux (pmol/sample) for WT and CB1R KO.

Panel C: Line graph comparing [ACh] in-out and [ACh] in for WT and CB1R KO.

Panel D: Graph showing the ACh efflux (% baseline) over time (min) for WT and CB1R KO. The inset shows the average ACh efflux (pmol/min).

PO indicates the period of observation.
1) CB<sub>1</sub> receptor antagonism increased DA release

2) CB<sub>1</sub> receptor antagonism increased ACh release

3) CB<sub>1</sub> receptor antagonism in the septum inhibited ACh release in the hippocampus (via GABA<sub>B</sub> receptor stimulation)