

M. Kathmann · U. Bauer · E. Schlicker

CB₁ receptor density and CB₁ receptor-mediated functional effects in rat hippocampus are decreased by an intracerebroventricularly administered antisense oligodeoxynucleotide

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Abstract We have studied (i) the effect of antisense oligodeoxynucleotides complementary to CB₁ mRNA on the CB₁ receptor binding in hippocampus, striatum and cerebral cortex of the rat; (ii) the possible mechanism of action of one of the antisense oligodeoxynucleotides; and (iii) its effect on two functional CB₁ receptor-mediated effects.

Synthetic oligodeoxynucleotides or saline were administered to male Wistar rats by the intracerebroventricular (i.c.v.) route twice daily for 3 days. Antisense oligodeoxynucleotides corresponding to the nucleotides 4 to 21 (AS1; GCCATCTAGGATCGACTT) and –8 to 12 (AS2; GATCGACTTCATAACCTCAG) and a mismatch oligodeoxynucleotide differing from AS1 in 6 positions (MM; TCCAGCTACTATGGACTG) were used. The dissociation constant (K_D) of rat CB₁ cannabinoid receptors, labelled by the radioligand [³H]-SR141716, did not differ in membranes from rats treated with saline, AS1, AS2 or MM. The density of receptor binding (B_{max}) was reduced by the antisense oligodeoxynucleotides, 10 nmol, in the hippocampus (AS1, –40%; AS2, –20%) and striatum (AS1, –29%; AS2 –6%), but not in the cerebral cortex. When the dose of AS1 was raised to 30 nmol, the reduction of B_{max} in the hippocampus and striatum was only marginally increased; a dose of 3 nmol of AS1 reduced B_{max} in both brain regions by somewhat more than the half-maximum effect. The mismatch oligodeoxynucleotide MM (3–30 nmol) did not affect B_{max} . In the second part of the study, RNA obtained from the three brain regions of rats pretreated with AS1 10 nmol, MM 10 nmol or saline was analyzed using reverse transcription-polymerase chain reaction of CB₁ receptor mRNA and of β -actin mRNA levels (used as reference value). The ratio

of CB₁ receptor mRNA over β -actin mRNA after treatment with AS1 did not differ from the ratios following treatment with saline or MM in the hippocampus, striatum and cerebral cortex. Finally, pretreatment with antisense oligodeoxynucleotide AS1 30 nmol attenuated two functional effects via CB₁ receptors, i.e., the facilitatory effect of WIN 55,212–2 on [³⁵S]-GTP γ S binding in rat hippocampus membranes and the inhibitory effect of WIN 55,212–2 on acetylcholine release in rat hippocampus slices.

In conclusion, (i) two antisense oligodeoxynucleotides reduce the density of CB₁ receptors in the rat hippocampus and striatum after i.c.v. administration. (ii) The effect of the antisense oligodeoxynucleotide AS1 does not appear to be related to breakdown of CB₁ receptor mRNA. (iii) Pretreatment with AS1 attenuated the CB₁ receptor-mediated effect in two functional models.

Key words i.c.v. administration · Antisense oligodeoxynucleotides · CB₁ · Cannabinoid receptors · [³H]-SR141716 binding studies · [³⁵S]-GTP γ S binding studies · Acetylcholine release · Rat hippocampus and striatum

Introduction

The effects of Δ^9 -tetrahydrocannabinol and chemically-related constituents of hashish and marijuana are mediated via CB₁ receptors, which were cloned by Matsuda et al. (1990), and are present in the CNS and at a lower density in the periphery, and via CB₂ receptors, which were cloned by Munro et al. (1993), and are restricted to the periphery (for review, see Pertwee 1997; Childers and Breivogel 1998). Stimulation of cannabinoid receptors, which belong to the superfamily of G protein-coupled receptors, causes activation of mitogen-activated protein kinase, inhibition of adenylyl cyclase, blockade of N and P/Q type Ca²⁺ channels and/or activation of A-type and inwardly rectifying K⁺ channels (see Pertwee 1997; Childers and Breivogel 1998). Arachidonylethanolamide

M. Kathmann (✉) · U. Bauer · E. Schlicker
Institut für Pharmakologie und Toxikologie,
Rheinische Friedrich-Wilhelms-Universität Bonn,
Reuterstrasse 2b, D-53113 Bonn, Germany
e-mail: m.kathmann@uni-bonn.de,
Tel.: +49-228-739558, Fax: +49-228-735404

("anandamide") (Devane et al. 1992), *sn*-2 arachidonyl-glycerol (Stella et al. 1997) and chemically-related compounds (Mechoulam et al. 1994) have been proposed as potential endogenous ligands. Potent cannabinoid receptor agonists (for review, see Pertwee 1997) and a potent and selective CB₁ receptor antagonist, SR141716 (Rinaldi-Carmona et al. 1994), and a CB₂ receptor antagonist, SR144528 (Rinaldi-Carmona et al. 1998), have been described as well. Recently, CB₁-receptor-mediated inhibition of acetylcholine release in superfused rat hippocampal slices (Gifford and Ashby 1996; Gifford et al. 1997) and inhibition of noradrenaline release in superfused human and guinea-pig hippocampal slices (Schlicker et al. 1997) have been shown.

Antisense oligodeoxynucleotides are useful tools to reduce the density of a given receptor and, therefore, have been widely used *in vitro* and *in vivo* (for review, see Crooke 1992). Recently, two antisense oligodeoxynucleotides directed against mouse CB₁ receptor mRNA have been described. The first one (AS1), complementary to nucleotides 4 to 21, inhibits the antinociceptive effect of the cannabinoid receptor agonist CP-55,940 in mice (when administered intracerebroventricularly; *i.c.v.*) (Edsall et al. 1996) and produces hyperalgesia in mice (when administered intrathecally) (Richardson et al. 1998). The second one (AS2), complementary to nucleotides -8 to 12, blunts the CB₁ receptor-stimulated arachidonate release in N18 mouse neuroblastoma cells (Hunter and Burstein 1997).

It was the aim of the present study to compare the effects of the two antisense oligodeoxynucleotides on CB₁ receptor density in the rat CNS (note that the sequence of the rat AS1 differs from the mouse AS1 in two nucleotides, whereas the sequence of AS2 is identical for both species). We determined the effects of *i.c.v.* administration of both oligodeoxynucleotides on CB₁ receptor binding in the hippocampus, striatum and cerebral cortex. For the first oligodeoxynucleotide AS1, which proved to be the more efficient one, the dose-response relationship was established and its effect on the CB₁ receptor mRNA was determined, using the reverse transcription-polymerase chain reaction (RT-PCR). Finally, we investigated whether antisense oligodeoxynucleotide AS1 (30 nmol) also affects two functional models in rat hippocampus, *i.e.*, the CB₁ receptor-mediated stimulation of [³⁵S]-GTPγS binding and the CB₁ receptor-mediated inhibition of acetylcholine release.

Methods

Drugs. AS1 (GCC ATC TAG GAT CGA CTT), AS2 (GAT CGA CTT CAT AAC CTC AG), *i.e.*, antisense oligodeoxynucleotides corresponding to nucleotides 4 to 21 and -8 to 12 of the rat CB₁ receptor (GenBank accession no. X55812), respectively, and MM (TCC AGC TAC TAT GGA CTG), a mismatched oligodeoxynucleotide, were obtained from MWG Biotech (Ebersberg, Germany). The mismatch oligodeoxynucleotide MM differs from AS1 in 6 positions (underlined), but shows the same G/C composition. To avoid self- or cross-homologies between primers (*e.g.*, hairpins), all oligodeoxynucleotides were checked using a specific

software program (Primer-Master 1.0, St. Petersburg, Russia). The oligodeoxynucleotide sequences were tested for homology to known nucleotide sequences in the National Center for Biological Information (GenBank) data base by the BLASTN program. Only the antisense sequences of AS1 and AS2 showed significant homology to any sequence and this was to the rat CB₁ receptor. The oligodeoxynucleotides were dissolved in sterilized water, and stored at -20°C until used. Furthermore, the following drugs were used: [³H]-SR141716, [³H]-N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide, [³⁵S]-GTPγS, guanosine 5'-γ-[³⁵S] thiotriphosphate (Amersham, Braunschweig, Germany), [methyl-³H]-choline chloride (NEN, Zaventem, Belgium), hemicholinium-3 (ChemCon, Freiburg, Germany), GTPγS, GDP, guanosinediphosphate (Sigma, München, Germany), CP-55,940, (-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol (Pfizer, Groton, Conn., USA), WIN 55,212-2, R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl](1-naphthalenyl)methanone mesylate (RBI, Köln, Germany).

Intracerebroventricular injections. Male Wistar rats (220–240 g), purchased from Charles-River (Extetal, Germany), were anesthetized with pentobarbital (40 mg/kg), and mounted in a stereotaxic instrument (Stoelting, Wood Dale, Ill., USA). An intraventricular cannula was inserted in the bone for drug application (position of tip: 0.1 mm posterior to bregma, 4.0 mm ventral to it, and 1.6 mm lateral to the midline on the right side) (Paxinos and Watson 1997), according to the method described by Matthies et al. (1995). Ten days after surgery, intracerebroventricular injections of oligodeoxynucleotides (3 nmol/3 μl, 10 nmol/5 μl or 30 nmol/10 μl, injection speed 2 μl/min) or of physiological salt solution (3–10 μl, injection speed 2 μl/min) were carried out twice daily (08.00h and 18.00h) for 3 days using a 25 μl Hamilton microliter syringe with a 26-gauge needle.

[³H]-SR141716 binding assay. Binding experiments were carried out according to Rinaldi-Carmona et al. (1996) with slight modifications. Cerebral cortex, hippocampus or striatum was homogenized (Potter Elvehjem) in 25 volumes of ice-cold Tris-HCl buffer (Tris 50 mM, pH 7.5, EDTA 5 mM, sucrose 10.27%) and centrifuged at 1000 g for 10 min (4°C). The supernatant was centrifuged at 35,000 g for 10 min and the pellet (P2 pellet) was resuspended in 10 volumes of Tris-HCl buffer and frozen at -80°C. The binding assay was performed in Tris-HCl buffer (Tris 50 mM, pH 7.5, EDTA 5 mM) in a final volume of 0.5 ml containing 40–80 μg protein. [³H]-SR141716 was used at seven concentrations ranging from 0.05 to 8 nM. The incubation (25°C) was terminated after 60 min by filtration through polyethyleneimine (0.3%)-pretreated Whatman GF/C filters. Nonspecific binding (29% of total binding) was determined in the presence of CP 55,940 (3 μM). Protein was assayed according to the method described by Bradford (1976). Data were analyzed using the program GraphPadPrism (Prism, GraphPad Software, San Diego, Calif., USA).

[³⁵S]-GTPγS binding study. Experiments were carried out according to Breivogel et al. (1998) with slight modifications. Rat membranes (P2 pellets, see [³H]-SR141716 binding studies) were prepared in membrane buffer (50 mM Tris-HCl, 100 mM NaCl, 3 mM MgCl₂, 1 mM EGTA, pH 7.4) and stored at -80°C. All preparations were assayed for protein content according to the method described by Bradford (1976) before addition to assay tubes. Four to 15 μg of membrane preparations were incubated for 1 h at 30°C in membrane buffer containing additionally 0.5% bovine serum albumin, 30 μM GDP, and 0.05 nM [³⁵S]-GTPγS in a final volume of 0.5 ml. Nonspecific binding was determined with 10 μM GTPγS (22% of total binding). The incubation was terminated by rapid filtration through Whatman GF/B filters. Data were analyzed by nonlinear regression analysis using the program GraphPadPrism (Prism; GraphPad Software, San Diego, Calif., USA).

RT-PCR analysis. The brains from rats treated *i.c.v.* with 10 nmol antisense oligodeoxynucleotide AS1, 10 nmol mismatch oligode-

oxynucleotide MM or saline were dissected on dry ice. Total RNAs from hippocampus, striatum and cerebral cortex were obtained using Tripure reagent (Boehringer, Mannheim, Germany). RNA/DNA-containing samples were subjected to DNaseI (Boehringer, Mannheim, Germany) treatment to exclude any trace of genomic contamination from preparations. RNA was then reverse transcribed into cDNA. Reverse transcription reaction mixture (50 µl) containing 5 µg total RNA, 1× reaction buffer (50 mM Tris-HCl, 8 mM MgCl₂, 30 mM KCl, 1 mM dithiothreitol, pH 8.5; Boehringer, Mannheim, Germany), 0.4 mM of each dNTP, 2.5 U RNase inhibitor, 0.5 µg random primer p(dN)₆, and 20 U of AMV (avian myeloblastosis virus) reverse transcriptase (Boehringer, Mannheim, Germany) were incubated at 42°C for 1.5 h, heated to 95°C for 5 min, and quick-chilled at 4°C for 5 min. PCR was performed in 100 µl containing 1× PCR buffer (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, pH 8.3 and 0.01% gelatin), 0.25 µM each of the 3' and 5' primers (see below), 0.2 mM dNTP, and 0.025 U of *Taq* DNA Polymerase (AGS, Heidelberg, Germany). The mixture was amplified in a DNA Thermal Cycler (Hybaid, Heidelberg, Germany) for 25 cycles (94°C, 56°C and 72°C for 1, 1 and 1 min, respectively), followed by an additional 3 min extension at 72°C. The primers used were: CB₁ sense 24-mer primer 5'-TG CAGGCCTTCCTACCACTTCATC-3' and CB₁ antisense 24-mer primer 5'-GACGTGTGGATGATGATGCTCTTC-3' (position 600–623 and 1097–1120, respectively, from GenBank accession no. X55812); β-actin sense 24-mer primer 5'-GATG-GTGGGTATGGGTCAGAAGGA-3' and β-actin antisense 24-mer primer 5'-GCTCATTGCCGATAGTGATGACCT-3' (position 1457–1480 and 2529–2552, respectively, from GenBank accession no. J00691). The expected size of the amplicons were 520 bp for CB₁ and 650 bp for β-actin. A volume of 10 µl of the PCR products was separated by agarose (1.3%) gel electrophoresis and visualized by ethidium bromide staining. The gel-images were videoscanned (MWG Biotech, Ebersberg, Germany) and analyzed using the Bandleader program (Magnitec, Tel-Aviv, Israel).

Superfusion experiments. Hippocampus slices (0.3 mm thick, 2 mm diameter) from rats pretreated i.c.v. with antisense oligodeoxynucleotide AS1 (30 nmol), mismatch oligodeoxynucleotide MM (30 nmol) or saline, were incubated (37°C) for 30 min with physiological salt solution (PSS) containing [³H]-choline 0.1 µM. Subsequently, the slices were superfused (1 ml/min) with PSS (37°C) containing the choline uptake inhibitor hemicholinium-3 10 µM and, when necessary, WIN 55,212-2 throughout superfusion; the superfusate was collected in 5-min samples. Tritium overflow was evoked after 40 min by one

2-min period of electrical field stimulation (3 Hz, 100 mA, 2 ms); experiments lasted for 60 min. The PSS was composed as follows (mM): NaCl 118, KCl 4.8, NaHCO₃ 25, KH₂PO₄ 1.2, MgCl₂ 1.2, CaCl₂ 1.3 (preincubation) or 2.6 (superfusion), glucose 11.1, ascorbic acid 0.06, disodium EDTA 0.03; it was aerated with 95% O₂ and 5% CO₂. Tritium efflux was calculated as the fraction of the tritium content in the slices at the beginning of the respective collection period (fractional rate of tritium efflux). To quantify the effect of WIN 55,212-2 (0.1 µM) on basal efflux, the fractional rate in the 5-min period from 55–60 min was determined. To quantify the effect of WIN 55,212-2 on the electrically evoked tritium overflow, the difference between total and basal efflux was determined and expressed as percent of the tritium present in the slice at the onset of the stimulation (basal tritium efflux was assumed to decline linearly from the 5-min period before to that 15–20 min after onset of stimulation).

Calculations and statistics. Results are given as means ± SEM of *n* experiments (RT-PCR analysis, superfusion studies) or of *n* experiments in duplicate ([³H]-SR141716 binding experiments) or triplicate ([³⁵S]-GTPγS binding experiments). Experimental data were analyzed by Student's *t* test or ANOVA followed by a post hoc test, as appropriate.

Results

Receptor binding studies

The density of rat CB₁ cannabinoid receptors was analyzed using [³H]-SR141716 binding to hippocampal, striatal and cerebral cortex membranes. In membranes prepared from untreated or saline-treated rats, [³H]-SR141716 bound saturably to an apparently homogeneous class of receptors (n_H~1) with an affinity (K_D) of 2.4–5.4 nM and a density (B_{max}) of 0.5–1.1 pmol/mg protein (Table 1). The saturation and Scatchard analysis of rat hippocampal membranes prepared from saline- and antisense AS1-pretreated rats is shown as an example in Fig. 1.

The oligodeoxynucleotides did not influence the affinity of the CB₁ receptor binding (Table 1) and affected its density in the following way. Both antisense oligodeoxynucleotides (AS1, AS2) reduced the CB₁ receptor den-

Table 1 Influence of antisense oligodeoxynucleotide (AS2), mismatch oligodeoxynucleotide (MM) and saline (NaCl) on the density and affinity of the specific [³H]-SR141716 binding to rat hippocampus, striatum and cerebral cortex membranes

Intracerebro-ventricular treatment	Concentration (nmol)	Rat hippocampus membranes		Rat striatum membranes		Rat cerebral cortex membranes	
		B _{max} ^a	pK _D ^b	B _{max} ^a	pK _D ^b	B _{max} ^a	pK _D ^b
–	–	1.07±0.02	8.40±0.02	0.95±0.01	8.37±0.02	0.50±0.01	8.60±0.02
NaCl	–	1.08±0.02	8.34±0.02	0.94±0.02	8.37±0.02	0.50±0.01	8.50±0.02
AS2	10	0.87±0.01 ^c	8.39±0.02	0.88±0.01	8.48±0.04	0.50±0.02	8.52±0.02
MM	3	1.07±0.03	8.40±0.02	0.97±0.02	8.32±0.02	–	–
	10	1.06±0.02	8.48±0.04	0.95±0.01	8.48±0.04	0.50±0.01	8.51±0.03
	30	0.99±0.03	8.36±0.02	0.94±0.02	8.27±0.04	0.49±0.04	8.55±0.06

^a The B_{max} values (pmol/mg protein) were determined from the saturation binding studies shown in Fig. 1 or similar experiments not shown. Means of 4–14 experiments

^b The pK_D values (–log of K_D [nM]) were determined from the saturation binding studies shown in Fig. 1 or similar experiments not shown. Means of 4–14 experiments

^c *P*<0.01, compared to B_{max} in hippocampus membranes from saline-treated rats

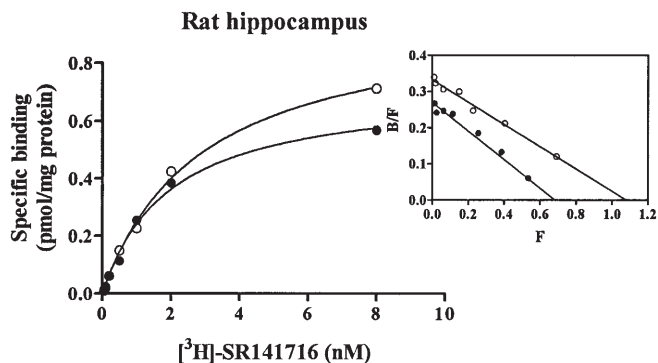


Fig. 1 Representative saturation and Scatchard (*insert*) analysis of [³H]-SR141716 binding to membranes prepared from hippocampus of rats pretreated intracerebroventricularly (i.c.v.) with saline (NaCl, ○) or 10 nmol antisense oligodeoxynucleotide (AS1, ●). The K_D values in membranes from saline- and AS1-treated animals were 3.2 and 2.4 nM, respectively; the respective B_{max} values were 1.08 and 0.67 pmol/mg protein. These data are representative of 6 experiments performed in duplicate

sity in the hippocampus more markedly than in the striatum and did not affect it at all in the cerebral cortex (Fig. 2 and Table 1). AS1 (10 nmol) reduced receptor density more markedly than the same dose of AS2, both in the hippocampus (by 40 versus 20%) and striatum (by 29 versus 6%) (Fig. 2 and Table 1). Both in the hippocampus and striatum, the effect of AS1 on receptor density was only marginally higher (by 2–4%) when its dose was increased to 30 nmol (Fig. 2). When its dose was decreased to 3 nmol, the effect amounted to slightly more than half of that obtained at 30 nmol (which may be assumed to represent the maximum effect) (Fig. 2). The mismatch oligodeoxynucleotide MM did not affect receptor binding in the three brain regions under study at 3, 10 and 30 nmol (Table 1).

RT-PCR analysis

The RT-PCR analysis was performed using a cycle PCR protocol with 25 cycles in which the amount of the PCR product is proportional to the initial amount of the tem-

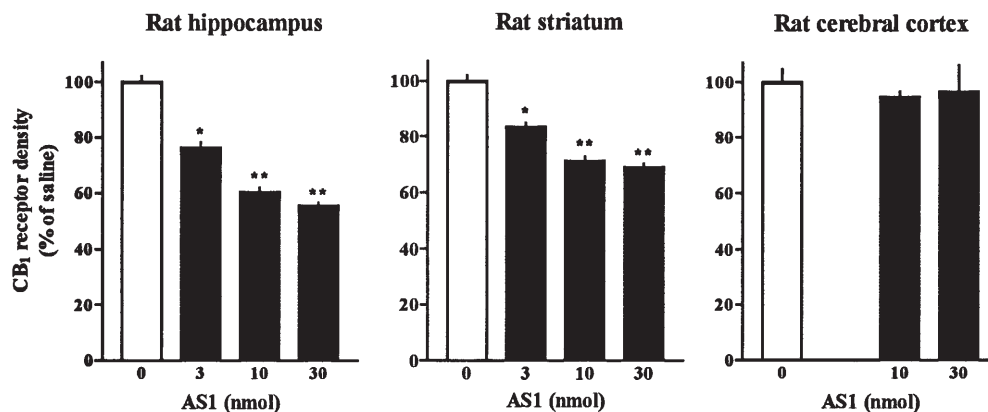
plate (exponential phase of amplification). Four different RT-PCR experiments were performed for the three brain regions of rats treated i.c.v. with 10 nmol antisense oligodeoxynucleotide AS1, 10 nmol mismatch oligodeoxynucleotide MM or saline. Primers corresponding to the CB₁ receptor and β-actin were used. The PCR products were electrophoresed (1.3% agarose) and the RT-PCR in the hippocampus is shown as an example in Fig. 3. The agarose gel images were then videoscanned and the intensity of the CB₁ amplification relative to that of β-actin after quantification with videodensitometry is shown in Table 2. There was no significant difference of the amplification signal CB₁/β-actin after i.c.v. treatment with antisense AS1 compared to saline or mismatch MM in all brain regions.

Stimulated GTPγS binding

Preliminary experiments on hippocampus membranes obtained from untreated rats revealed that WIN 55,212-2 stimulates [³⁵S]-GTPγS binding; the concentration-response curve is monophasic and the maximum effect is obtained at 10 μM (results not shown).

In hippocampus membranes from saline-treated rats, basal [³⁵S]-GTPγS binding was 106±3 fmol/mg protein ($n=6$); the respective values in membranes from antisense AS1- and mismatch MM-treated (30 nmol each) rats were similar (not shown). WIN 55,212-2 stimulated [³⁵S]-GTPγS binding in hippocampus membranes obtained from saline-treated rats, yielding a monophasic concentration-response curve (Fig. 4). The maximum stimulation was 216±9% of basal binding and the EC_{50} was 206±26 nM. To facilitate comparisons, the increase in [³⁵S]-GTPγS binding caused by WIN 55,212-2 in membranes from AS1- and MM-treated rats was given as percent of the effect of WIN 55,212-2 (10 μM) in membranes from saline-treated rats (Fig. 4). The concentration-response curve of WIN 55,212-2 in hippocampus membranes from MM-treated rats was monophasic showing an EC_{50} of 168±33 nM and an E_{max} of 98±4% (Fig. 4). In hippocampus membranes from antisense oligodeoxynucleotide AS1 (30 nmol) pretreated rats, WIN 55,212-2 showed a

Fig. 2 Influence of intracerebroventricular (i.c.v.) pretreatment with the antisense oligodeoxynucleotide AS1 (3, 10 and 30 nmol) on the density (B_{max}) of the specific [³H]-SR141716 binding to rat hippocampus, striatum and cerebral cortex membranes, compared to saline. Means ± SEM of 4–6 saturation binding experiments. * $P<0.005$, ** $P<0.001$, compared to saline



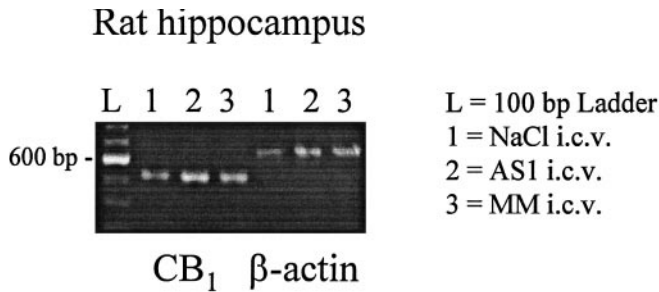


Fig. 3 Representative agarose gel showing RT-PCR analysis of RNA prepared from hippocampus of rats pretreated intracerebroventricularly (i.c.v.) with saline (NaCl; 1), 10 nmol antisense oligodeoxynucleotide (AS1; 2) or 10 nmol mismatch oligodeoxynucleotide (MM; 3). Specific primers corresponding to CB₁ receptor (520 bp) and β -actin (650 bp) were used. This type of experiment has been carried out four times

Table 2 RT-PCR analysis with RNA prepared from rat hippocampus, striatum and cerebral cortex after intracerebroventricular (i.c.v.) pretreatment with antisense oligodeoxynucleotide AS1, mismatch oligodeoxynucleotide MM and with saline. Shown is the intensity of the CB₁ receptor amplification relative to that of β -actin after quantification with videodensitometry. Means \pm SEM of 4 experiments

Intracerebroventricular treatment	Signal CB ₁ / β -actin		
	Rat hippocampus	Rat striatum	Rat cerebral cortex
NaCl	0.85 \pm 0.08	0.73 \pm 0.04	0.82 \pm 0.04
AS1 10 nmol	0.89 \pm 0.04	0.70 \pm 0.04	0.81 \pm 0.05
MM 10 nmol	0.81 \pm 0.08	0.72 \pm 0.03	0.81 \pm 0.05

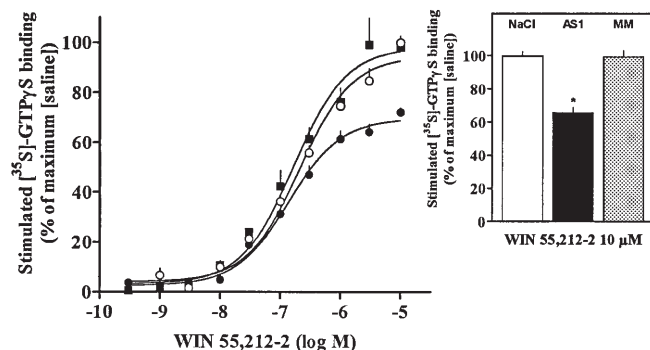


Fig. 4 Influence of intracerebroventricular (i.c.v.) treatment of rats with 30 nmol antisense oligodeoxynucleotide (AS1, ●), 30 nmol mismatch oligodeoxynucleotide (MM, ■) and saline (NaCl, ○) on stimulation of [³⁵S]-GTP γ S binding by the CB₁ receptor agonist WIN 55,212-2 in rat hippocampus membranes. Data are expressed as percent of the increase in [³⁵S]-GTP γ S binding caused by 10 μ M WIN 55,212-2 in saline-pretreated membranes. In the *insert*, the effect of 10 μ M WIN 55,212-2 is shown. Means \pm SEM of three experiments in triplicate for the concentration-response curves, and of seven experiments in triplicate for the effect of WIN 55,212-2 (10 μ M) (*insert*) are shown. * P <0.005, compared to saline

monophasic concentration-response curve which does not differ from the others with respect to the EC₅₀ value (136 \pm 21 nM), but showed a reduced maximal effect E_{max}

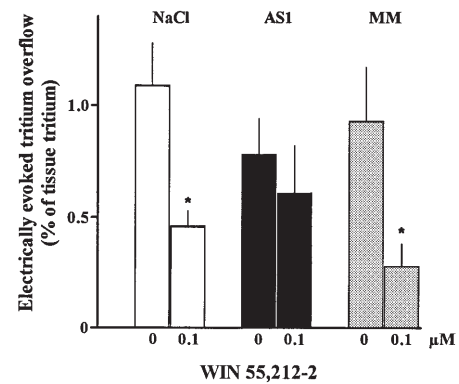


Fig. 5 Influence of intracerebroventricular (i.c.v.) treatment of rats with antisense oligodeoxynucleotide AS1 (30 nmol), mismatch oligodeoxynucleotide MM (30 nmol) and saline (NaCl) on the electrically evoked overflow from superfused rat hippocampus slices preincubated with [³H]-choline. The slices were superfused with medium containing hemicholinium-3 (10 μ M) and, when necessary, WIN 55,212-2 throughout superfusion (60 min). Tritium overflow was evoked after 40 min by electrical field stimulation (3 Hz, 100 mA, 2 ms; 2 min). Tritium overflow was calculated as percent of the tritium content at the beginning of stimulation. Means \pm SEM of 8–9 experiments. * P <0.025, compared to the corresponding control

of 69 \pm 2 (Fig. 4). For statistical analysis, additional experiments with WIN 55,212-2 at the maximal effective concentration of 10 μ M ($n=7$) were performed. [³⁵S]-GTP γ S binding at this concentration in AS1 pretreated hippocampus was significantly (P <0.005) reduced to 66% compared to saline, whereas in the MM pretreated hippocampus no change in maximum binding was observed (Fig. 4, *insert*).

Superfusion studies

Basal tritium efflux, expressed as fractional rate, was 0.0019 \pm 0.0002 min⁻¹ in hippocampus slices from saline-treated rats; similar values were obtained in slices from antisense AS1- and mismatch MM-treated (30 nmol each) rats (not shown). WIN 55,212-2 (0.1 μ M) did not affect basal tritium efflux in slices from each of the three treatment groups (not shown). The electrically (3 Hz) evoked tritium overflow was expressed as percent of tissue tritium and did not differ between the three treatment groups (Fig. 5). WIN 55,212-2 (0.1 μ M) inhibited the evoked tritium overflow in slices from saline- and MM-treated rats by 60–70% (Fig. 5). In slices from AS1-treated rats, WIN 55,212-2 only tended to inhibit the evoked overflow (Fig. 5).

Discussion

In the present study, CB₁ receptor binding was determined using the selective CB₁ receptor radioligand [³H]-SR141716. Saturation binding studies in the hippocampus, striatum and cerebral cortex membranes from un-

treated or saline-treated rats revealed CB₁ receptor densities and affinities which are in good agreement with those previously reported (Rinaldi-Carmona et al. 1996). Both antisense oligodeoxynucleotides (AS1 and AS2) reduced CB₁ receptor density in the hippocampus and striatum but failed to do so in the cerebral cortex (for discussion of regional differences and dose-response relationship, see below). AS1 reduced CB₁ receptor density more markedly than AS2, both in the hippocampus and striatum. The more pronounced effect of antisense AS1 might be explained as follows: antisense AS1, showing a G-C content of 50%, might be fixed to the CB₁ receptor mRNA more tightly than antisense AS2 (G-C content 45%) because of its higher G-C content. It is known that a G-C content of approximately 50–65% spread out evenly across the sequence is considered optimal (Hunter et al. 1995). A second reason may be that the length of antisense AS2 of 20 nucleotides is too high, leading to a reduction in uptake into the cell (Hunter et al. 1995).

The regional differences in inhibition of CB₁ receptor density by the antisense oligodeoxynucleotides might be due to the very localized effect of the intracerebroventricular administration. Zhang et al. (1996) reported the uptake and distribution of fluorescein-labelled D₂ dopamine receptor antisense oligodeoxynucleotide (of the phosphorothioate type) in the mouse brain. Their results show that i.c.v. injections of antisense oligodeoxynucleotide resulted in a signal most intense in brain areas in direct or close proximity to the ventricles, including the hippocampus and striatum. Although they found a significant signal also in the cerebral cortex, the fact that this huge brain region is far from the lateral ventricle and that the oligodeoxynucleotides show limited tissue penetration may explain why the antisense oligodeoxynucleotides did not reduce CB₁ receptor density in the cerebral cortex in our study.

We used antisense oligodeoxynucleotide AS1 for further experiments to determine its dose-response relationship and its mechanism of action. Antisense AS1 reduced CB₁ receptor density dose-dependently both in hippocampus and striatum. A dose of 10 nmol caused virtually the maximum effect since a threefold rise in dose only marginally increased the effect, both in hippocampus (40 versus 44% inhibition of CB₁ receptor density) and in the striatum (29 versus 31%). A dose of 3 nmol caused somewhat more than the half-maximum effect, i.e., 24% in hippocampus and 17% in striatum. The maximum inhibitory effect of 44% in hippocampus resembles other studies showing a maximum inhibitory effect of antisense oligodeoxynucleotides on receptor density by about 50% (Richardson et al. 1998; Wahlestedt et al. 1993a; Wahlestedt et al. 1993b).

The mechanism(s) of action of antisense oligodeoxynucleotides may differ from cell type to cell type and may depend upon the exact nature of the target RNA and the oligodeoxynucleotides (Branch 1998). Antisense oligodeoxynucleotides can inhibit several steps in the processing of the primary transcript (capping, methylation and splicing), the nucleo-cytoplasmic transport of the primary tran-

script or directly the translation by hybridization arrest; furthermore they can activate the degradation of the RNA/DNA hybrids by RNase H (Davidkova and Weiss 1998). The latter mechanism was observed in several systems, including *Xenopus* oocytes and permeabilized cells (Branch 1998). In order to investigate whether this possibility also holds true for antisense AS1 in the present study we used a RT-PCR analysis with RNA prepared from rat hippocampus, striatum and cerebral cortex after pretreatment with oligodeoxynucleotide AS1 or MM and with saline. Primers corresponding to the CB₁ receptor and to β -actin were used; the latter was used since this is a ubiquitously expressed housekeeping gene. The amplicon of the CB₁ receptor was expressed as a fraction of the amplicon of β -actin. The intensity of the CB₁ amplification relative to that of β -actin did not differ after i.c.v. treatment with antisense AS1 compared to saline or mismatch MM in all brain regions. This result suggests that the reduction of CB₁ receptor density is possibly mediated through the binding of the antisense oligodeoxynucleotide to the mRNA in the translation phase followed by a halt of protein synthesis.

We used two functional studies to evaluate whether the CB₁ receptor down regulation also affects the two more distal events within the cascade: receptor binding – G-protein coupling – modulation of transmitter release. Consequently, we have investigated the effect of antisense oligodeoxynucleotide AS1 in rat hippocampus by studying the stimulation of [³⁵S]-GTP γ S binding and the modulation of acetylcholine release. In both models the dose of 30 nmol AS1 was chosen because it maximally reduces CB₁ receptor density and the hippocampus was used because the maximum effect was observed in this brain region.

The actions of cannabinoid agonists have been shown to be mediated through the activation of pertussis toxin-sensitive G-proteins. Breivogel et al. (1998) have shown that the agonist WIN 55,212-2 acts as a full agonist in their [³⁵S]-GTP γ S binding assay. We used the same assay and the agonist WIN 55,212-2 to investigate the possible effect of the antisense oligodeoxynucleotide. In hippocampus membranes from antisense oligodeoxynucleotide AS1 (30 nmol)-pretreated rats, no change in the EC₅₀ of WIN 55,212-2 was obtained as compared to hippocampus membranes from saline- and mismatch oligodeoxynucleotide-treated animals, indicating that AS1 had no influence on the potency of the agonist. Interestingly, in hippocampus membranes from rats pretreated with antisense oligodeoxynucleotide AS1, a reduced E_{max} value of WIN 55,212-2 of 69% was observed. To further characterize this effect, experiments with WIN 55,212-2 (10 μ M) were performed, in which the maximum [³⁵S]-GTP γ S binding was significantly reduced to 66% compared to saline. Note that the extent of the latter effect (34%) is very similar to the extent of the AS1-mediated reduction of CB₁ receptor density by 44%.

CB₁-receptor-mediated inhibition of acetylcholine release has been shown in superfused rat hippocampal slices (Gifford and Ashby 1996; Gifford et al. 1997). We

used the same functional model to investigate whether the pretreatment with antisense oligodeoxynucleotide AS1 modifies the CB₁-receptor-mediated inhibition of acetylcholine release in rat hippocampus. The electrically evoked tritium overflow (which is Ca²⁺-dependent and tetrodotoxin-sensitive, unpublished results; and represents quasi-physiological acetylcholine release) was inhibited by the cannabinoid receptor agonist WIN 55,212-2 in hippocampus slices from saline- and mismatch oligodeoxynucleotide-pretreated rats by 60–70%. However, in hippocampal slices from AS1-treated rats only a tendency towards an inhibition was found for WIN 55,212-2. These results suggest that a reduction of CB₁ receptor density in hippocampus by about 50% is sufficient to affect CB₁-receptor-mediated modulation of acetylcholine release.

In conclusion, using the antisense 'knock-down' approach we have shown that two antisense oligodeoxynucleotides reduce the density of CB₁ receptors in the rat hippocampus and striatum after i.c.v. administration. Furthermore, using RT-PCR analysis we showed that the antisense 'knock-down' approach had no effect on the level of the CB₁ receptor mRNA. Finally, the i.c.v. treatment with 30nmol antisense oligodeoxynucleotide AS1 markedly attenuated the effect of WIN 55,212-2 in two functional CB₁ receptor models.

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