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Research report

Neuroprotective and brain edema-reducing efficacy of the novel cannabinoid receptor agonist BAY 38-7271

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Abstract

BAY 38-7271 is a new high-affinity cannabinoid receptor agonist with strong neuroprotective efficacy in a rat model of traumatic brain injury (acute subdural hematoma, SDH). In the present study we investigated CB1 receptor signal transduction by [^{35}S]GTPγS binding in situ and in vitro to assess changes in receptor functionality after SDH. Further, we continued to investigate the neuroprotective properties of BAY 38-7271 in the rat SDH and transient middle cerebral artery occlusion (tMCA-O) model as well as the efficacy with respect to SDH-induced brain edema. [^{35}S]GTPγS binding revealed minor attenuation of CB1 receptor functionality on brain membranes from injured hemispheres when compared to non-injured hemispheres or controls. In the rat SDH model, BAY 38-7271 displayed strong neuroprotective efficacy when administered immediately after SDH either as a 1 h (65% infarct volume reduction at 0.1 μg/kg) or short-duration (15 min) infusion (53% at 10 μg/kg). When administered as a 4 h infusion with a 5 h delay after injury, significant neuroprotection was observed (49% at 1.0 μg/kg/h). This was also observed when BAY 38-7271 was administered as a 5 h delayed 15 min short-duration infusion (64% at 3 μg/kg). In addition, the neuroprotective potential of BAY 38-7271 was demonstrated in the rat tMCA-O model, displaying pronounced neuroprotective efficacy in the cerebral cortex (91% at 1 ng/kg/h) and striatum (53% at 10 ng/kg/h). BAY 38-7271 also reduced intracranial pressure (28% at 250 ng/kg/h) and brain water content (20% at 250 ng/kg/h) when determined 24 h post-SDH. Based on these data it is concluded that the neuroprotective efficacy of BAY 38-7271 is mediated by multiple mechanisms triggered by cannabinoid receptors.

Theme: Disorders of the nervous system

Topic: Ischemia

Keywords: BAY 38-7271; Neuroprotection; CB1; Transient middle cerebral artery occlusion; Acute subdural hematoma; Intracranial pressure; Brain edema

1. Introduction

Scientific studies on the pharmacology of cannabis were advanced considerably by the identification of the cannabinoid Δ^9-THC as the major active constituent of cannabis, predominantly responsible for its therapeutic and psychoactive effects [11], the cloning of the centrally located CB1 receptors [25], and the peripherally located CB2 receptors [31] as well as the identification of selective CB1 and CB2 receptor agonists and antagonists [1]. Both receptors are negatively coupled to adenylate cyclase through heterotrimeric $G_{i/o}$ proteins [36]. The CB1 receptor was also found to be negatively coupled to N- and P/Q-type voltage-sensitive Ca^{2+}- and D-type K^+ channels, and positively coupled to A-type and inward-rectifying K^+ channels [30,36]. Activation of CB1 receptors led to cell hyperpolarization and inhibition of neurotransmitter release [12,20].

One of the key events caused by brain ischemia after traumatic brain injury (TBI) is the excessive release of the excitatory neurotransmitter glutamate, which triggers a cascade of intracellular processes leading finally to neuronal death [24]. As cannabinoid receptor activation may lead to inhibition of glutamate release by cell hyperpolarization, it can be hypothesized that cannabinoid receptor agonists should have neuroprotective properties [14,38]. In fact, the neuroprotective efficacy of cannabinoid receptor agonists such as Δ^9-THC, anandamide, WIN 55,212, and

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2-arachidonyl glycerol has been demonstrated in several in vivo models. Also, HU-211, a non-psychotropic cannabinoid, displayed encouraging neuroprotective efficacy in vivo. The neuroprotective efficacy of the latter compound is, in all probability, not mediated via the CB1 receptor [14]. Interestingly, CB1 receptor expression was found to be increased four-fold in the penumbra after transient middle cerebral artery occlusion (tMCA-O) [19]. This finding suggests that the cannabinoid system may become more sensitive after brain ischemia and that CB1 receptor agonists may offer a unique therapeutic opportunity under these conditions.

Recently, BAY 38-7271 has been characterized as a structurally novel, selective and highly potent cannabinoid CB1/CB2 receptor agonist in vitro and in vivo with pronounced neuroprotective properties [8,27]. BAY 38-7271 demonstrated highly potent and efficient neuroprotective properties in a rat traumatic brain injury model when administered as a 4 h infusion immediately after induction of subdural hematoma. Even when applied with a 3 h delay a significant neuroprotective efficacy could be observed. The neuroprotective efficacy of BAY 38-7271 was also shown in a rat model of focal cerebral ischemia induced by permanent occlusion of the middle cerebral artery (pMCA-O) [27].

The aim of the present study was to investigate potential changes in functional CB1 receptor expression in the SDH model and to further characterize the neuroprotective potential of BAY 38-7271 in models of traumatic brain injury and brain edema.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of the highest purity available commercially and were, if not otherwise indicated, purchased from Merck (Darmstadt, Germany). BAY 38-7271 [(−)-(*R*)-3-(2-hydroxymethylindanyl-4-oxy)phenyl-4,4,4-trifluoro-1-sulfonate] and SR 141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidehydrochloride] were synthesized by the Department of Chemical Research, Bayer Health Care (Wuppertal, Germany). [^{35}S]GTPγS was from Amersham (Dreieich, Germany) and [^{3}H]BAY 38-7271 was obtained from the Department of Radiochemistry, Bayer Health Care (Wuppertal, Germany).

2.2. Methods

2.2.1. [^{35}S]GTPγS binding autoradiography

After surgical induction of SDH (see below), rats were decapitated at 24 h or 7 days, their brains rapidly removed and frozen in 2-methylbutane on dry ice at −30 °C. Coronal sections (20 μm thick) were cut through previously defined anatomical levels using a cryostat microtome (Leica CM 3050, Leica Vertrieb GmbH, Bensheim, Germany) and were dry fixed under vacuum. Slide-mounted brain sections were incubated at 25 °C in assay buffer (50 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl, 1 mg/ml BSA) and were then transferred for 15 min at 25 °C to assay buffer containing 2 mM GDP. Slices were then transferred to assay buffer containing 40 pM [^{35}S]GTPγS and the test compounds. The incubation lasted 120 min at 25 °C and was terminated by three 30 s dips in ice-cold 50 mM Tris–HCl, pH 7.4, and a 3 s rinse in ice-cold deionized water. The sections were dried under a constant flow of cold air before exposure. Sections were exposed for 7 days to Kodak GP SO-230 Phospho-Screens (Amersham, Dreieich, Germany). Screens were scanned on a Storm 860 (Molecular Dynamics, Amersham, Dreieich, Germany). Analysis of the digitized autoradiograms was performed using the image analysis program Image Quant, v 5.0 (Molecular Dynamics, Amersham, Dreieich, Germany) by analyzing the signal intensity of intact tissue. Areas which gave no signal (necrotic areas) on slices from injured hemispheres and the corresponding areas of the non-injured hemispheres or control slices were excluded from analysis.

2.2.2. Agonist-stimulated [^{35}S]GTPγS binding on cell membranes

The brains (n=6–7 per group) were dissected, divided into hemispheres, and cortical tissues were prepared. In the case of injured hemispheres, necrotic tissue was removed, and the equivalent regions were also removed from the tissue of non-injured hemispheres and control animals. The tissue was pooled followed by homogenization with a Potter-Elvehjem homogenisator (1200 rpm, 15 strokes) in 15 volumes of 0.32 M sucrose. The suspension was centrifuged for 10 min at 500×g and the supernatant then transferred to a new tube and centrifuged again at 40,000×g for 10 min. The supernatant was discharged, the pellet resuspended in 50 mM Tris–HCl (pH 7.4, 25 °C) and centrifuged again at 40,000×g for 10 min. Finally, the supernatant was discharged and the pellet was homogenized in 50 mM Tris–HCl (pH 7.4, 25 °C). Aliquots of the membrane preparations were stored at −140 °C over liquid nitrogen.

Test compound, 0.04 nM [^{35}S]GTPγS and brain cell membranes (15 μg protein/tube) were incubated at 30 °C for 1 h in assay buffer (50 mM Tris–HCl, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 25 μM GDP, 1 mg/ml BSA, fatty acid free, pH 7.4) in a final volume of 1 ml. The reaction was terminated by rapid vacuum filtration through Whatman GF/B filters, and rinsed three times with 3 ml ice-cold buffer (50 mM Tris–HCl, pH 7.4) using a Brandel cell harvester. The remaining radioactivity was counted using a Packard scintillation counter.

2.2.3. Saturation studies

Membranes were prepared as described above and binding studies were performed as follows: radioligand ([^{3}H]BAY 38-7271, 0.1–13 nM), test compound and membranes were suspended in 50 mM Tris–HCl, pH 7.4, 2.5 mM EDTA, 5 mM $MgCl_2$, and 5 mg/ml BSA, fatty acid free (final volume 200 μl). The reaction mixtures were incubated for 90 min at 30 °C and terminated by rapid vacuum filtration over Whatman GF/C filters (presoaked for 90 min in 50 mM Tris–HCl, pH 7.4, 0.05% BSA, fatty acid free) using a Brandel cell harvester. To reduce non-specific binding, filters were washed 12 times with 1 ml ice-cold wash buffer (50 mM Tris–HCl, pH 7.4, 0.05% BSA, fatty acid free). The remaining radioactivity was counted using a Beckmann scintillation counter.

2.2.4. Acute subdural hematoma (SDH)

2.2.4.1. Animals. Male Wistar rats (HsdCpb:Wu, 200–300 g, Harlan-Winkelmann, Borchen, Germany) were used for the acute subdural hematoma (SDH) and brain edema experiments. The animals were allowed to adapt to the housing conditions for at least 1 week before they were subjected to the study. They were housed in groups of two individuals in macrolon cages (type III, Ebeco, Castrop-Rauxel, Germany) bedded with sawdust. The animal housing room and the laboratory for surgery were climate controlled and continuously illuminated from 6:00 a.m. until 6:00 p.m. The room temperature was about 21 °C and the relative humidity about 50%. Food (Altromin® 1324, Altromin Spezialfutterwerk, Lange, Germany; or R/M-H, V1534-00 DDb, Ssniff Spezialdiäten, Soest, Germany) and water were available ad libitum. On the day of surgery, the rats were randomly assigned to one of the treatment groups. Experimental protocols and conditions conformed to the German regulations on animal welfare.

2.2.4.2. Surgical induction of acute subdural hematoma. The animals were anesthetized with the inhalation anesthetic isofluran [Forene®, Abbott, Wiesbaden, Germany, or Isofluran-Baxter, Baxter Deutschland, Unterschleißheim, Germany, mixed with ≅28% O_2 in N_2O to 5–1.5% (v/v) concentration]. Subdural hematoma was induced according to the standard surgical procedure [28] with the following minor modifications. The top of the head was shaved, the skin was disinfected and opened with a longitudinal midline cut. From the left hemisphere a small part of the periosteum was removed and a burr hole was drilled into the skull with stereotaxic coordinates −1 mm caudal, −2.8 mm lateral to bregma [35]. The dura was carefully opened and a specially designed plastic cannula was inserted into the subdural space between the dorsal surface of the brain and the dura. The cannula was then fixed into position with tissue glue (Histoacryl®, B. Braun Surgical GmbH, Melsungen, Germany). Non-heparinized autologous blood was collected by puncture of the tail vein and injected directly via the prefixed cannula into the subdural space (total volume of 0.2 ml within 4 min). The probe was then shortened and closed with cyanacrylate glue (Histoacryl®). The skin wound was closed with suture clips. During surgery and continuous i.v. infusion of the drug (for the doses used in the different experiments, see Results) or vehicle, the body temperature was monitored and maintained within the physiological range (37.0±0.5 °C) using a warming pad. After recovery from anesthesia the animals were returned to their home cage.

2.2.4.3. Determination of infarct volume. Seven days after surgery the rats were decapitated, their brains rapidly removed and frozen in 2-methylbutane on dry ice at −30 °C. Serial coronal sections (20 μm thick) were cut throughout the entire infarcted area with a standard distance of 500 μm using a cryostat microtome (Leica CM 3050, Leica Vertrieb GmbH, Bensheim, Germany, and Microm HM 500 OM, Microm Laborgeräte GmbH, Walldorf, Germany). Slide-mounted brain sections were stained with cresyl fast violet. The volume of the cortical infarct was determined by an operator blinded to the group composition using a computer-assisted image analysis system (Optimas, BioScan, Edmonds, WA, USA). Infarct volumes are expressed in mm^3 (mean±1 S.E.M.). For comparison of individual experiments, infarct volumes of treatment groups are expressed as a percentage of the respective controls, which were set at 100%.

2.2.4.4. Determination of intracranial pressure (ICP). The method used was a slightly modified version of a published method [53] and was determined by an operator blinded to the groups. The ICP measurements were conducted 24 h after surgery. ICP was assessed with a commercially available pressure transducer (Micro-Tip 2F SPR-612, Millar Instruments, TX, USA) with an external diameter of 0.44 mm. For the quantification of ICP, animals were anaesthetized with an i.p. injection of Ketavet (72 mg/kg ketamine HCl; Pharmacia & Upjohn) and Rompun (9.6 mg/kg Xylacin; Bayer, Germany) in physiological salt solution (6 ml/kg). During ICP assessment, the body temperature was maintained at 37 °C with a warming pad.

The plastic cannula which was used for blood injection was removed from the skull and the tissue glue was cleaned from the drill hole. With fine scissors the dura matter was carefully dissected and the pressure transducer was lowered into the drill hole. The process was controlled via a microprocessor-driven and automated system, which was developed in-house. The tip of the pressure transducer was placed 2 mm into the brain parenchyma directly located under the blood clot. Due to the automated process, the procedure and the insertion speed were the same for all animals. After the sensor tip was lowered into the brain parenchyma, the system was calibrated and the sensor was allowed to adapt to the pressure in the parenchyma for 10

min. After this period, the ICP was recorded for 30 min via a microprocessor-controlled amplifier, which was developed in cooperation with the Center for Sensor Systems (ZESS, University of Siegen, Siegen, Germany). After the measurement, the animal was decapitated and the brain was removed for determination of brain water content. If gross pathological assessment of the removed brain revealed that the blood clot was too small or had drifted into the cistern, the animals were excluded from analysis.

2.2.4.5. Quantification of brain water content. The wet weight/dry weight (w.w./d.w.) method was modified from a published method [51]. For the experiments, rats were decapitated 24 h after surgery and the whole brain was dissected, the olfactory bulb and the cerebellum removed, and both hemispheres separated and weighed (w.w.). After drying for 24 h at 115 °C, the hemispheres were weighed again (d.w.). The relative water content of both hemispheres was calculated according to the formula (w.w.−d.w.)/w.w.×100 by an operator blinded to the groups. The difference in relative water content between both hemispheres (%left−%right) served as a parameter to determine the severity of the brain edema.

2.2.5. Transient occlusion of the middle cerebral artery (tMCA-O)

2.2.5.1. Animals. Male Wistar rats (HsdCpb:Wu, 300–350 g, Harlan-Winkelmann, Borchen, Germany) were used for the transient middle cerebral occlusion (tMCA-O) experiments. All other conditions were the same as described in Section 2.2.4.1.

2.2.5.2. Surgical induction of transient middle cerebral artery occlusion. The animals were anesthetized with the inhalation anesthetic isofluran [Forene®, Abbott, Wiesbaden, Germany, or Isofluran-Baxter, Baxter Deutschland GmbH, Unterschleißheim, Germany, mixed with ≅28% O_2 in N_2O to 5–1.5% (v/v) concentration]. Transient middle cerebral artery occlusion was induced according to a standard surgical procedure [52] with the following modifications. After midline opening of the skin and the right lateral neck muscles, the right common carotid artery was exposed. The external carotid artery together with the pterygopalatine artery and the common carotid artery were ligated. The internal carotid artery was temporarily closed by a microvascular clip. Through a small incision made in the common carotid artery, a silicone-coated nylon monofilament thread was inserted and advanced into the internal carotid artery up to and slightly past the origin of the middle cerebral artery and secured in position by encircling sutures.

After 60 min ischemia the occluder filament was withdrawn to allow reperfusion by the ipsilateral intracranial arteries. The skin wound was closed with sutures. During the surgery and continuous i.v. infusion of the drug or vehicle the body temperature was maintained in the physiological range with a warming pad. After recovery from anesthesia the animals were returned to their home cage. Rats that did not demonstrate typical circling behavior shortly after the surgery were excluded from further study.

2.2.5.3. Determination of infarct volume. After 2 days survival, cortical and striatal infarct volume determination was carried out as described above (see Section 2.2.4.3).

2.2.6. Drug application

BAY 38-7271 was dissolved in absolute ethanol corresponding to 1% of the final volume. Thereafter, 10% cyclodextrin solution (Pharmaceutical Technology, Bayer Health Care, Leverkusen, Germany) was added to the final volume. The ready made solution was administered intravenously as a continuous infusion with an application volume of 4 ml/kg/h. When administered as a 15 min infusion, the application volume was 5 ml/kg/15 min. For brain edema experiments the administration volume was 1 ml/kg/h. In all in vivo experiments, control animals received the same volume of vehicle as the *verum* groups.

2.3. Data analysis

In situ [^{35}S]GTPγS was analyzed using the digitized autoradiograms and the image analysis program Image Quant, v 5.0 (Molecular Daymics, Amersham, Dreieich, Germany). All in vitro biochemical experiments were performed in duplicate or triplicate and were repeated at least three times. Net or stimulated [^{35}S]GTPγS binding values were calculated setting basal binding values from the corresponding controls to 0. Data analyses were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego CA, USA). Statistical analysis was performed by analysis of variance (ANOVA) followed, where appropriate, by Ducan's multiple range test (SAS version 8.02, SAS Institute, USA). Significance was accepted at the $P<0.05$ level. Significance of the differences of means for neuroprotection and brain edema studies were assessed by analysis of variance (ANOVA) followed, where appropriate, by a post-hoc least-significance difference (LSD) comparison (SYSTAT version 10, SPSS). $P\leq0.05$ was defined as the level of significance. For all in vivo experiments, $n=8–12$/group.

3. Results

3.1. [^{35}S]GTPγS binding autoradiography, [^{35}S]GTPγS binding and [^{3}H]BAY 38-7271 saturation studies

As expected, BAY 38-7271 induced [^{35}S]GTPγS binding, which could be blocked by the specific CB1 receptor

antagonist SR 141716A (Fig. 1A). Receptor activation was observed in brain regions, as reported previously [3]. When compared with the respective controls, the [^{35}S]GTPγS signal did not change after 24 h or 7 days post-SDH (Fig. 1B–E). Basal [^{35}S]GTPγS binding on membranes obtained from non-lesioned animals was 5581±416 dpm (set to 0) for the 24 h and 7433±130 dpm (set to 0) for the 7 day group. [^{35}S]GTPγS binding values induced by 100 nM BAY 38-7271 were noticeable higher at 24 h (99±16% for controls, 152±41% for non-lesioned hemispheres, and 132±26% for lesioned hemispheres) than for the 7 day group (69±14, 66±9.2, and 80±4.7%) (Fig. 2A and B). Stimulated [^{35}S]GTPγS binding could be blocked by 10 μM of the specific CB1 receptor antagonist SR 141716A in the 24 h group (−20±7.5, 1±13, and −13±7.5%) as well as in the 7 day group (−33±5, −35±2.3, and −31±0.5%) (Fig. 2A and B).

If the basal level value of the 24 h group was compared to the basal levels of the lesioned and non-lesioned groups, a significant increase of [^{35}S]GTPγS binding could be detected on membranes obtained from the non-lesioned (39±17%, $P<0.05$), but not on membranes obtained from the lesioned, hemispheres (25±13%) (Fig. 2A). The same was observed when [^{35}S]GTPγS binding was stimulated by BAY 38-7271 (Fig. 2A). In the 7 day group, a significant difference in the values for membranes of lesioned hemispheres versus non-lesioned and controls was observed (Fig. 2B). EC_{50} values (Table 1) obtained from [^{35}S]GTPγS concentration–response curves (Fig. 3A and B) as well as B_{max} and K_i values obtained from [^{3}H]BAY

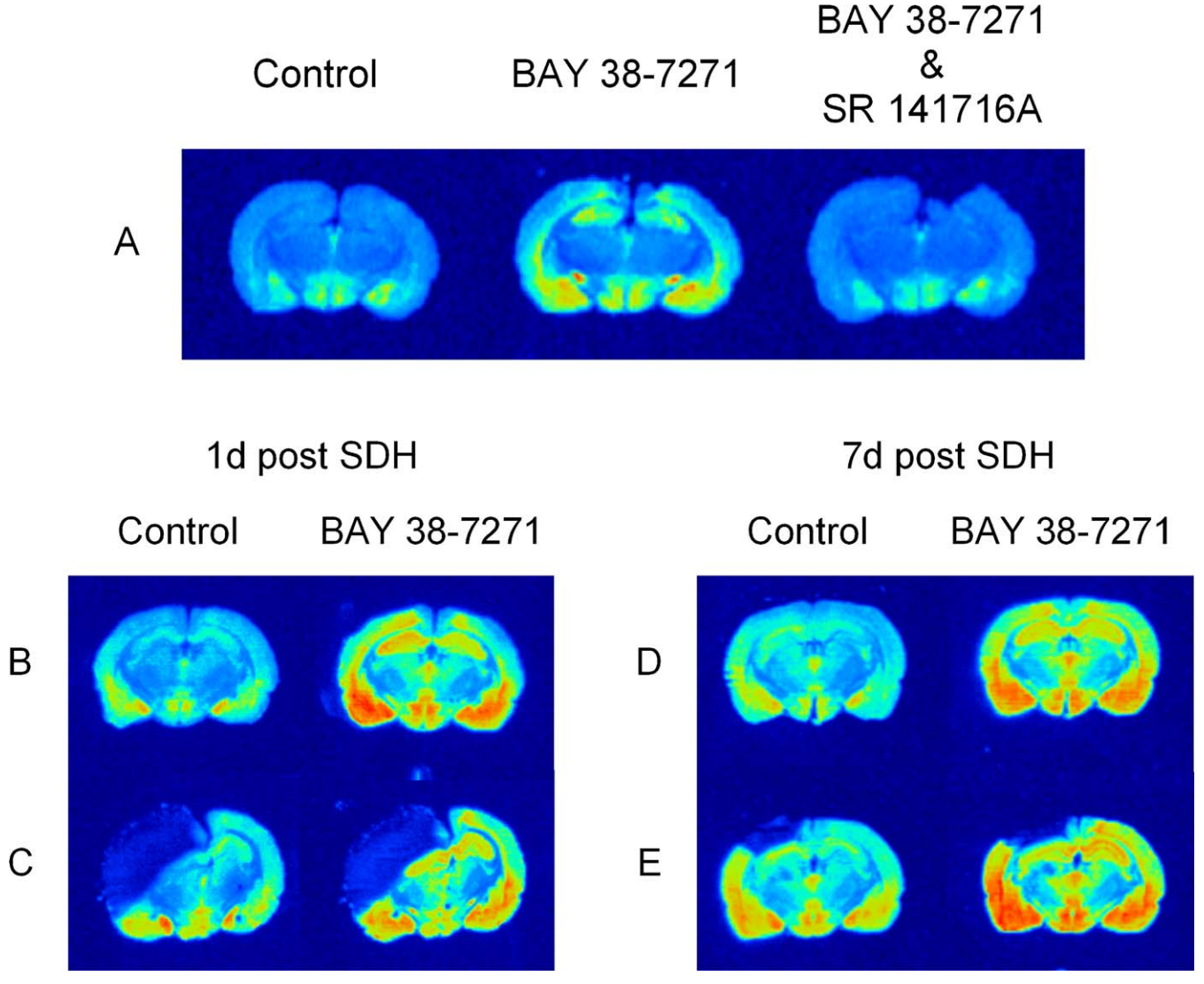

Fig. 1. [^{35}S]GTPγS autoradiography of rat brain slices. (A) Specific increase of the [^{35}S]GTPγS signal by BAY 38-7271 (100 nM), which could be blocked by SR 151716A (10 μM). (B, C) Representative slices of the [^{35}S]GTPγS autoradiography of SDH animals sacrificed 1 day or (D, E) 7 days post-SDH. Slices were incubated either with BAY 38-7271 or with buffer (control) as described in Materials and methods.

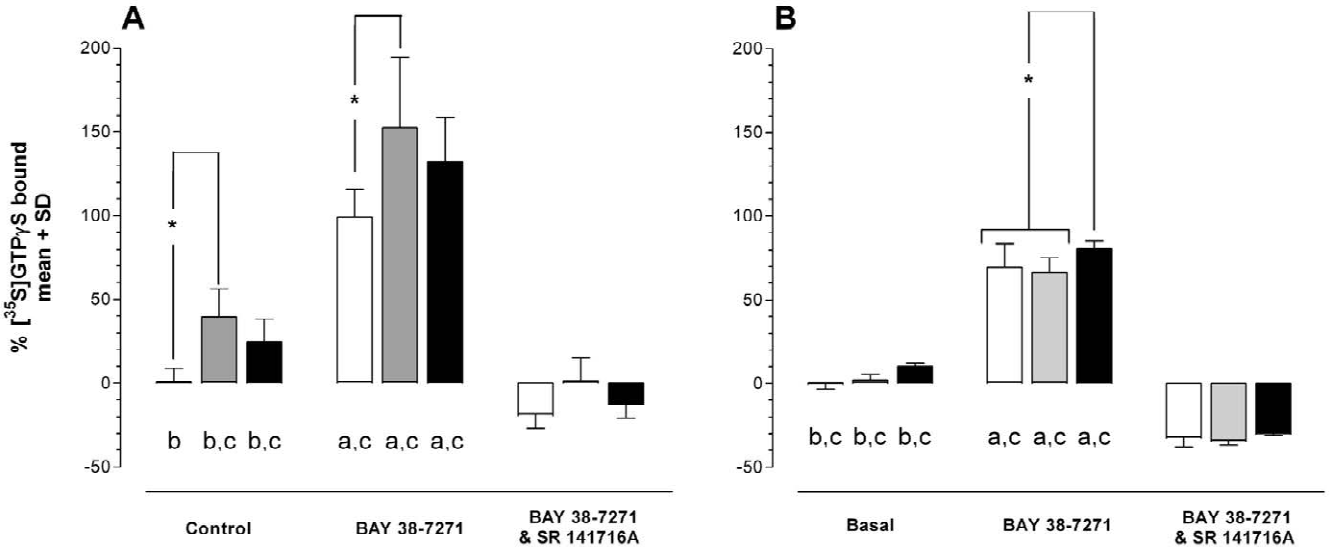

Fig. 2. [^{35}S]GTPγS binding on rat brain membranes from non-lesioned animals (□), the non-lesioned hemisphere of injured animals (▨), or the lesioned hemisphere (■) at (A) 24 h and (B) 7 days post-injury. Labels on the *x*-axis indicate experimental groups which represent either basal levels, membranes treated with 100 nM BAY 38-7271 or with 100 nM BAY 38-7271 and 10 μM SR 141716A as described in Materials and methods. Brackets indicate significant differences within experimental groups, and indices under the columns indicate significant differences between experimental groups (*$P<0.05$, ANOVA followed, where appropriate, by Ducan's multiple range test). a = $P<0.05$ versus respective control group, b = $P<0.05$ versus respective BAY 38-7271 group, c = $P<0.05$ versus respective BAY 38-7271 and SR 141716A group. Values are the mean and standard deviation of at least three independent experiments each performed in triplicate.

38-7271 binding experiments (Fig. 3C and D) were similar in all groups tested and are summarized in Table 1.

3.2. Neuroprotection studies

Pronounced neuroprotective efficacy could be observed when BAY 38-7271 was administered immediately after SDH (control values 111.26±13.22 mm^3) either as a 1 h infusion (Fig. 4A; 49, 65, and 53% infarct volume reduction at 0.01, 0.1, and 1.0 μg/kg, respectively) or a 15 min infusion (Fig. 4B; 53% infarct volume reduction at 1 μg/kg; control values 113.84±9.7 mm^3). When BAY 38-7271 was administered as a 4 h infusion with a 5 h delay after injury (infarct volumes of control animals 81.03±9.12 mm^3), a significant infarct volume reduction by 42 and 49% was observed at 0.3 (47.28±6.96 mm^3) and 1.0 μg/kg/h (41.03±6.62 mm^3), respectively (Fig. 5A). Administration of BAY 38-7271 as 15 min infusion with a 5 h delay after injury resulted in significant neuroprotection by 37, 64 and 47% at 1, 3 and 10 μg/kg (Fig. 5B; control values 96.64±10.86 mm^3). In the tMCA-O model, BAY 38-7271 also displayed strong neuroprotection over a broad dose range (0.1–100 ng/kg/h) on cortical (Fig. 6A) and striatal infarct volumes (Fig. 6B). The obtained maximal volume reduction in the cortex was 91% at 1 and 10 ng/kg/h (control values 147.62±21.16 mm^3). In the striatum, BAY 38-7271 displayed a maximal efficacy of 52% at 10 ng/kg/h (values for controls 76.01±5.43 mm^3).

3.3. Brain edema studies

3.3.1. Intracranial pressure (ICP)

An increase in brain water content was reflected by an increase in the intracranial pressure (Fig. 7A). The parenchymal intracerebral pressure in healthy animals was about 5–8 mmHg, in line with published data [10,45]. SDH surgery led to an increase in ICP from basal levels (7.5

Table 1
EC_{50} values from the [^{35}S]GTPγS binding concentration response curves, B_{max} (pM/mg) and K_d (nM) values of BAY 38-7271 for rat brain membranes derived from control and injured animals (non-lesioned and lesioned hemispheres). Values are the mean and standard deviation of at least three independent experiments (radioligand [^{3}H]BAY 38-7271)

	24 h post injury			7 days post injury		
	Control	Non-lesioned hemisphere	Lesioned hemisphere	Control	Non-lesioned hemisphere	Lesioned hemisphere
EC_{50}	19.6±4.6	19.2±7.7	19.7±11.2	27.6±3.6	25.8±3.8	33±9.5
B_{max}	2.36±0.19	2.60±0.03	2.44±0.12	2.33±0.17	2.63±0.53	2.35±0.11
K_d	0.51±0.07	0.49±0.01	0.53±0.07	0.43±0.09	0.53±0.21	0.52±0.06

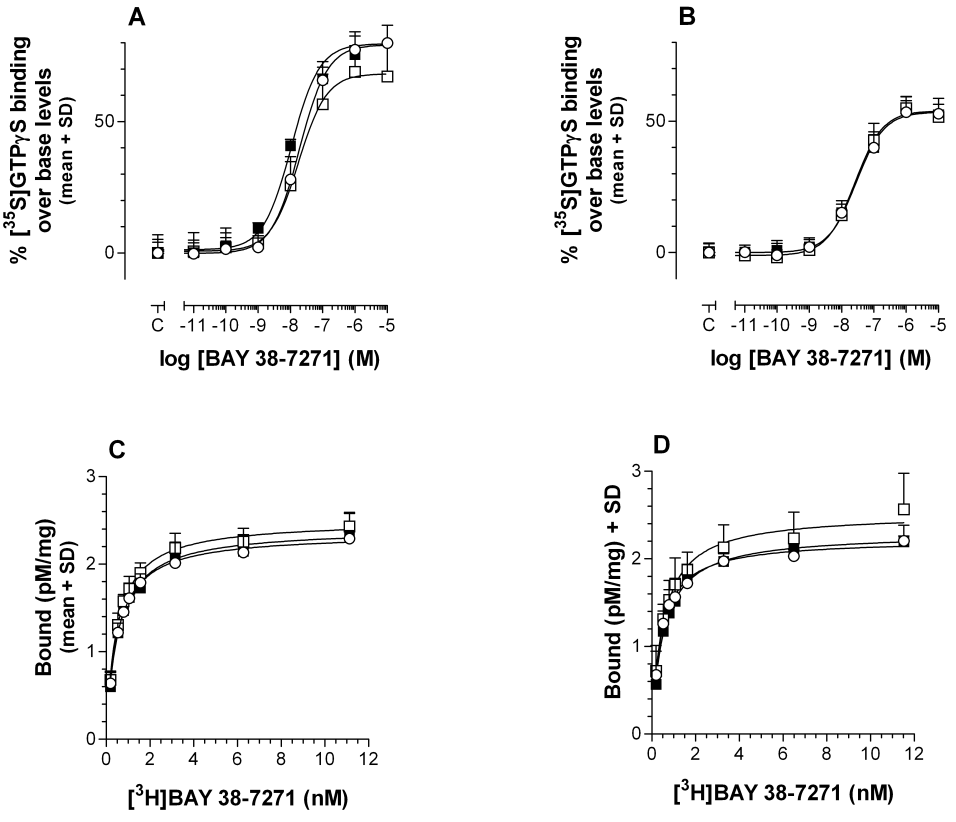

Fig. 3. BAY 38-7271-induced [^{35}S]GTPγS binding on rat cortex membranes derived from SDH and control animals after (A) 1 day or (B) 7 days, and saturation curves of [^{3}H]BAY 38-7271 on rat cortex brain membranes obtained from animals (C) 1 day or (D) 7 days post-SDH. EC_{50}, B_{max} and K_i values are shown in Table 1. Values are the mean and standard deviation of at least three independent experiments each performed in triplicate. (○) Control, (■) lesioned hemisphere, (□) intact hemisphere.

mmHg) to 20 mmHg. The strong increase in ICP during the first 10 min of the registration period was due to the calibration process of the pressure transducer after insertion into the brain parenchyma. After 10 min, the pressure was stable and the recording period lasted for 30 min. BAY 38-7271 was able to significantly ameliorate by 28% the

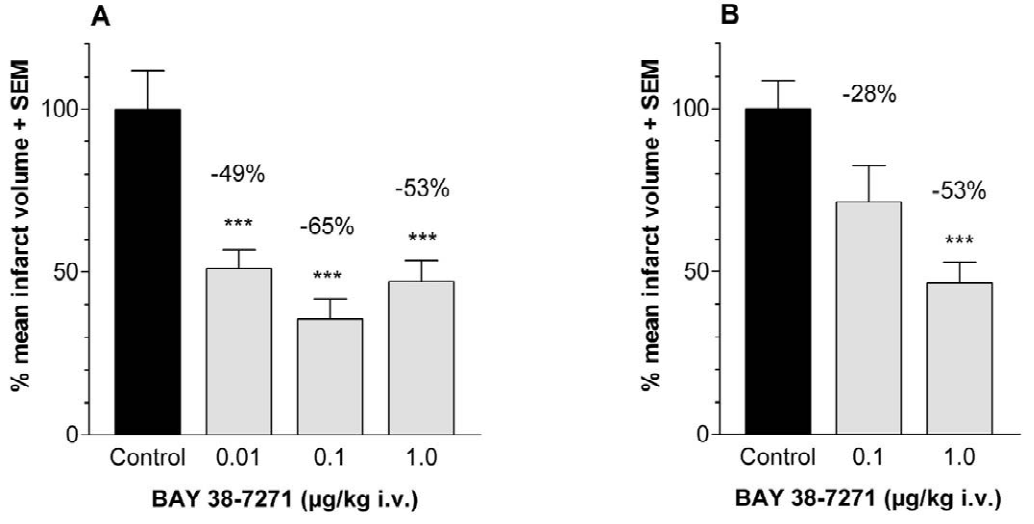

Fig. 4. Neuroprotective efficacy of BAY 38-7271 in the rat SDH model, administered (A) as a 1 h infusion or (B) as a 15 min short-duration infusion immediately after injury. Infarct volumes were determined 7 days after SDH and were calculated as a percentage of the infarct volumes of the control group, which was set at 100%. Values above the bars indicate the percent infarct volume reduction compared to controls. Control values were 111.26±13.22 mm^3 for (A) and 113.84±9.7 mm^3 for (B). ***$P<0.001$, $n=8$–12.

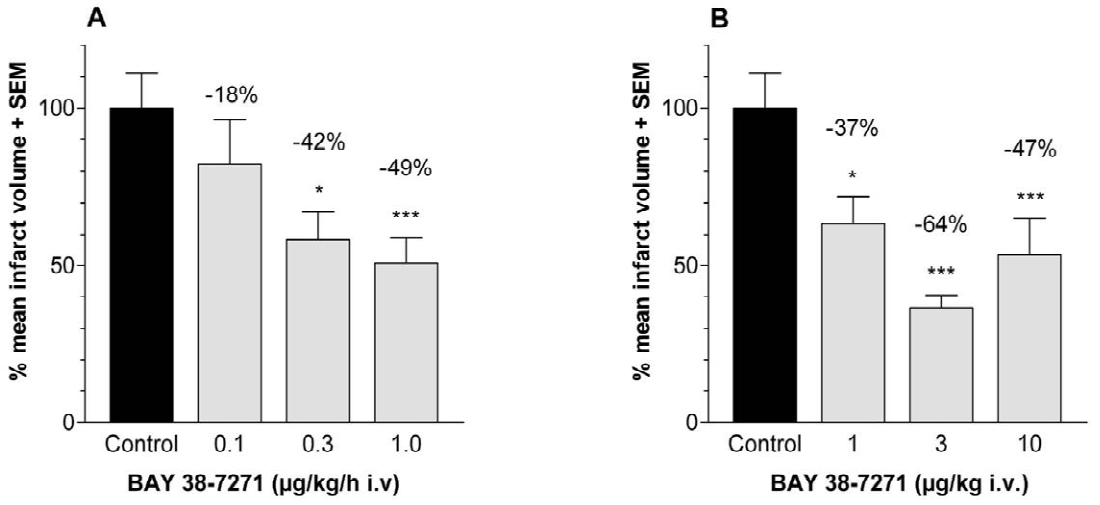

Fig. 5. Neuroprotective efficacy of BAY 38-7271 in the rat SDH model, administered with a 5 h delay after injury either as (A) a 4 h infusion or (B) as a 15 min short-duration infusion. Infarct volumes were determined 7 days after SDH and were calculated as a percentage of the infarct volume of the control group, which was set at 100%. Values above bars indicate the percent infarct volume reduction compared to controls. Control values for (A) were 81.03±9.12 mm^3 and for (B) 96.64±10.86 mm^3. *$P<0.05$, ***$P<0.001$, $n=8–12$.

SDH-induced increase in the intracranial pressure in rats 24 h post-injury at a dose of 250 ng/kg/h (Fig. 7B). A dose of 2500 ng/kg/h showed a strong tendency ($P=0.054$) to reduce ICP, but failed to reach the level of significance due to the greater variability in this treatment group.

3.3.2. Brain water content

In the SDH model, the edema reached its maximum (1.75±0.10% increase in water content versus the non-lesioned brain hemisphere) after 24 h (Fig. 8A), which corresponds to other models of brain damage [16,32,41]. In sham-operated animals, no difference between lesioned and intact brain hemispheres was detectable, whereas in vehicle-treated controls an increase of brain water content of about 2% on the lesioned side was measured (Fig. 8A). Application of BAY 38-7271 resulted in a dose-dependent reduction of brain water content versus vehicle-treated controls. A significant reduction in brain water content by 20% versus vehicle-treated controls was achieved at 250 ng/kg/h (Fig. 8B).

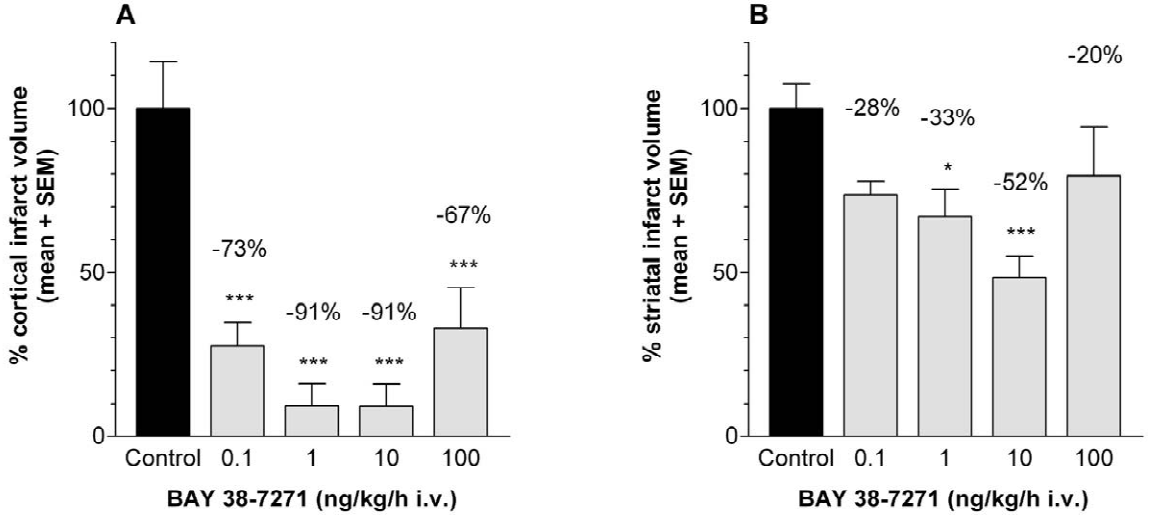

Fig. 6. Neuroprotective efficacy of BAY 38-7271 in the rat tMCA-O model with respect to (A) cortical and (B) striatal infarct volume. The MCA was occluded for 1 h and the compound was administered after reopening as a 4 h infusion. Control values for (A) were 147.62±21.16 mm^3 and for (B) 73.76±5.43 mm^3. *$P<0.05$, **$P<0.01$, ***$P<0.001$. $n=9–10$.

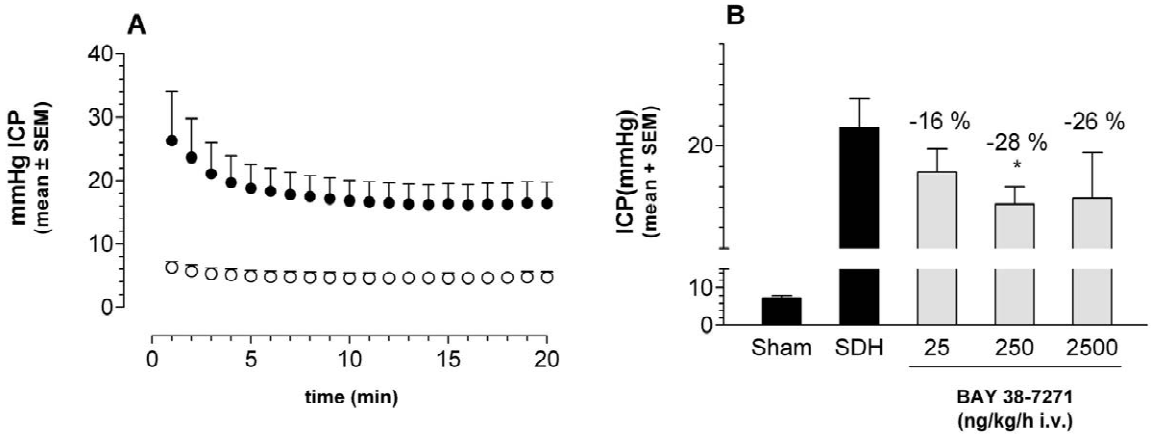

Fig. 7. (A) SDH-induced increase in ICP (●) compared with ICP of sham-operated animals (○) when measured 24 h post-surgery in animals which did not receive either vehicle or drug. (B) Reduction of ICP by BAY 38-7271. BAY 38-7271 or vehicle (sham and SDH group) was administered as described in Materials and methods. ICP values of sham-operated animals were 7.47±0.53 mmHg, and for animals belonging to the SDH group (control) 20.91±1.41 mmHg. *$P<0.05$. $n=9–10$.

4. Discussion

The diarylether sulfonylester derivative BAY 38-7271 is a novel cannabinoid receptor ligand that has previously been characterized as a highly potent and selective CB1/CB2 receptor full agonist which binds to rat and human receptors with similar affinity [27]. As previously reported, BAY 38-7271 displayed strong neuroprotective efficacy in a rat TBI model even if administered in doses below 1 ng/kg/h [27]. For further elucidation of the full neuroprotective potential of BAY 38-7271 we investigated receptor functionality in situ and in vitro, and neuroprotective efficacy in several administration schemes in vivo.

As reported recently, CB1 receptor expression was shown to be increased four-fold in the penumbra after 20 min transient middle cerebral artery occlusion, and persists for up to 72 h [19]. The increase in functional CB1 receptors could amplify the pharmacological impact of drugs acting via this receptor and could explain the high potency of BAY 38-7271 in vivo. Using [^{35}S]GTPγS binding autoradiography on brain slices prepared from animals 1 or 7 days after SDH did not show obvious

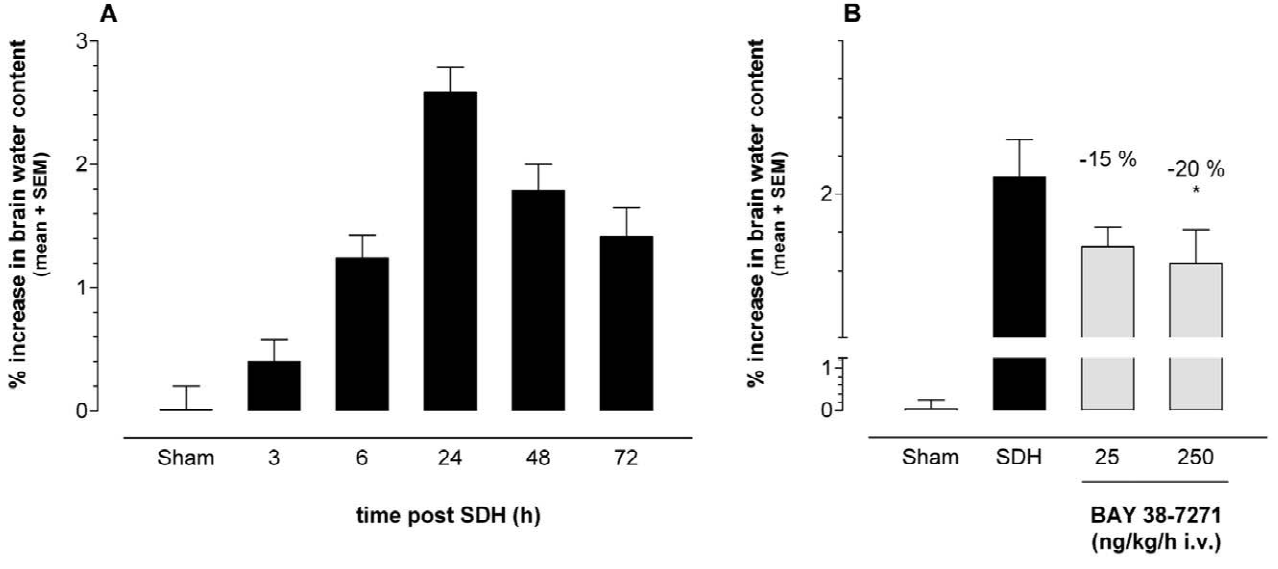

Fig. 8. (A) Time course of SDH-induced increase in brain water content in animals which did not receive either vehicle or drug. (B) Attenuation of brain water content 24 h post-surgery by BAY 38-7271. BAY 38-7271 or vehicle (sham and SDH group) was administered as described in Materials and methods. The water content of sham-operated animals was increased slightly by 0.05% compared to healthy animals. For animals belonging to the SDH group (control), brain water content increased by 2.09±0.2%. *$P<0.05$. $n=9–12$.

differences between injured and non-injured controls either in base levels or in BAY 38-7271-induced [^{35}S]GTPγS binding, although the [^{35}S]GTPγS signal tended to be more intensive on slices from injured animals than on those from the corresponding controls (Fig. 1B–E). For further clarification we investigated [^{35}S]GTPγS binding on membranes prepared from injured and non-injured hemispheres.

The data obtained from statistical analysis were inconsistent and the main restriction for a clear interpretation is the fact that, in the 24 h control group, values were significantly different only for membranes of the non-injured hemisphere, but were not different for membranes of the injured hemisphere. It was also expected that a CB1-mediated signal could be blocked by the specific CB1 receptor antagonist SR 141716A to similar values as seen for controls. As shown in Fig. 2A, this is not the case, leading to the assumption that, in this group, generally higher signal values and not an over-expression of CB1 receptors was the cause of the significant findings. Also supportive of this assumption is the fact that the EC_{50} values obtained from the [^{35}S]GTPγS concentration–response curves were not different within the 24 h groups, again suggesting no specific CB1 receptor over-expression. However, on day 7 post-injury, a weak but statistically significant signal was obtained when the lesioned group was compared to the non-lesioned group and controls (Fig. 2B). In addition, this signal could be blocked by SR 141716A to control levels, strongly indicating to be specifically CB1 receptor mediated. EC_{50} values for BAY 38-7271-induced [^{35}S]GTPγS binding were somewhat shifted to the right in the 7 day group when compared to the 24 h group. This is thought not to be linked to the particular group, but reflects naturally occurring variability. Further, we investigated whether the number of CB1 receptor binding sites was attenuated under ischemic conditions, assuming that over-expressed CB1 receptors might not be linked to the appropriate signal transduction system. However, this approach did not reveal clear differences within membrane preparations from the controls, lesioned, and non-lesioned hemispheres (Table 1). Taken together, these results suggest that, in the SDH model, functional CB1 receptor over-expression is either not present or detectable in the used rat strain when determined after 24 h. Although a weak signal could be observed after 7 days, this result remains doubtful because no corresponding signal, such as changes in binding sites or changes in EC_{50}, could be observed. That the methods we used were suitable to detect changes in signal transduction or binding sites has been demonstrated by several authors [4,39,43,46,49].

For further in vivo evaluation of the neuroprotective potential of BAY 38-7271 we followed four approaches. (i) To ascertain the neuroprotective potential in the SDH model at more appropriate times, we reduced the infusion time to either 1 h or 15 min. (ii) To investigate further the therapeutical window we administered BAY 38-7271 with a 5 h delay after injury as a 4 h long- or a 15 min short-duration infusion. (iii) We investigated the neuroprotective efficacy in the rat tMCA-O model, which represents a preclinical model of reperfusion injury. (iv) We examined the efficacy of BAY 38-7271 with respect to brain edema in the SDH model.

In general, BAY 38-7271 displayed strong neuroprotective efficacy in the SDH and tMCA-O models, whereas the efficacy in the rat brain edema model was comparably moderate for both ICP and brain water content. In some experiments the dose–response curve was wide and U-shaped, which has already been reported for this compound [27].

When administered as a 1 h (Fig. 4A) or 15 min short-duration infusion immediately after injury the efficacy observed was similar to results reported previously [27]. Although we did not carry out a complete dose–response curve in the latter experiment (Fig. 4B), approximately 10-fold higher doses were needed to obtain similar neuroprotection levels as found for the 1 or 4 h infusion scheme. These results support the assumption that BAY 38-7271 attenuates the initial processes involved in the pathogenesis of TBI, e.g. inhibition of glutamate release. This has been shown for cannabinoids in vitro [12,20] and other compounds such as 5-HT_{1A} receptor agonists in vivo [26].

To determine the therapeutical window between injury and drug administration we further investigated the time window of the neuroprotective efficacy of BAY 38-7271. As shown in Fig. 5A and B, the efficacy, but not the potency, of BAY 38-7271 appears to be similar in the SDH model, independent of infusion duration (4 h versus 15 min) when administered with a 5 h delay after injury. The potency itself was 10- (4 h infusion) to 30-fold (15 min infusion) lower when compared with data obtained when the compound was administered immediately after injury. A decreasing efficacy occurring under ‘extended conditions’ has been reported for several pharmacological approaches [18] and is thought to reflect upcoming involvement of processes which are not attenuated by the specific treatment and/or mechanism [9]. Moreover, the fact that BAY 38-7271 displayed similar neuroprotective efficacy over a wide range of different application schemes suggests that cannabinoids interact via CB1 receptor activation with several different mechanisms during the development of infarction (see below).

As reported previously [27], BAY 38-7271 is more effective in the rat SDH than in the rat pMCA-O model, suggesting that inflammatory processes are predominantly attenuated. We have provided support for this assumption by demonstrating a strong neuroprotective efficacy of BAY 38-7271 in the rat tMCA-O model, which mimics reperfusion injury and the following inflammatory processes [2].

An increase in ICP could deteriorate infarct volume formation by reducing intracranial perfusion pressure or cranial perfusion. In turn, compounds strongly reducing

ICP could simulate neuroprotective efficacy when the infarct volume is determined after 7 days. From recent reports it has become evident that cannabinoids might reduce brain edema [33], which can result in a reduced infarct volume. Thus, we investigated whether the compound attenuates ICP and/or brain water content after SDH. As shown in Figs. 7B and 8B, BAY 38-7271 significantly reduced ICP and brain water content when measured 24 h after induction of SDH, whereby the efficacy was somewhat weak with respect to both parameters. We conclude that these effects cannot explain the overall neuroprotective efficacy of BAY 38-7271, but might have some beneficial impact.

Our data suggest that cannabinoid receptor activation triggers either a mechanism which can attenuate pathophysiological processes over a long time period or, more probably, the CB1 receptor triggers several processes, which then attenuate infarct formation at varying time points. As mentioned above, activation of CB1 receptors leads to cell hyperpolarization and inhibition of glutamate release [12,20]. This could explain the neuroprotective efficacy of BAY 38-7271 per se, but not necessarily the efficacy observed when administration is delayed.

Thus, it is more likely that other processes, such as inflammation, are also attenuated by cannabinoid receptor activation. Inflammation following injury could enforce pathogenesis through the release of cytokines, such as IL-1, IL-6 and TNF, which were found to be elevated for several hours after traumatic brain injury [21,44] and acted as perpetrators. It has been found that cannabinoids attenuate directly or indirectly a multiplicity of cytokines (for a review, see Ref. [15] and references therein). Further, it has been reported that cannabinoids attenuate microglia. In the adult brain, the microglia are relatively quiescent and ramified in appearance [23], whereas, after brain injury, they become activated, ameboidal, phagocytose tissue debris [22], and produce cytokines such as IL-1 [13], IL-6 [50], and TNFα [40]. Under physiological conditions, the release of nitric oxide (NO) from microglial cells is negligible, but upon stimulation with cytokines such as TNFα and γ-interferon, brain microglial cells release substantial amounts of the free radical NO [6]. nNOS as well as iNOS mRNA were shown to rapidly increase after brain injury and persisted for at least 6 h at elevated levels [17,37]. NO itself acts as an amplifier of acute excitotoxicity [34,42]. Recent report have shown that Δ^9-THC and CP 55,940 were able to block CB1 receptor-mediated TNFα-induced NO production on murine macrophages [7], rat brain microglia [46], and activated mouse brain astrocytes [29]. Recently, it has been shown that peripherally occurring CB2 receptors are expressed in neonatal rat brain cortex microglia upon IFN-γ stimulation [5]. Because BAY 38-7271 also binds to CB2 receptors, its neuroprotective efficacy could partially be meditated via CB2 receptor activation at later time points of pathogenesis. Supportive to this hypothesis are very recent findings suggesting that CB2 receptors regulate microglial cell migration [47]. However, it is demanding to form all these data into one clear picture because, depending on the specific experimental conditions, controversial results were reported. Systematic investigations regarding the expression pattern of the above factors in brain injured animals are lacking and, in addition, cytokines such as TNFα and IL-1 might also have neurotrophic effects when hold at a particular level (for a review, see Ref. [48]).

Finally, BAY 38-7271 is able to induce hypothermia [27] and it can be argued that hypothermia itself is responsible for the observed neuroprotective effects. Thus, special care was taken during the neuroprotection experiments that the body temperature of the animals was maintained at 37 ± 0.5 °C during the whole experiment.

In conclusion, BAY 38-7271 displays pronounced neuroprotective efficacy in a wide variety of brain ischemia models and administration schemes. Based on the wide therapeutical window, strong efficacy and potency we assume that BAY 38-7271 acts via the cannabinoid receptor-mediated attenuation of different pathways involved in the pathogenesis of infarct generation in TBI.

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References

[1] F. Barth, Cannabinoid receptor agonists and antagonists, Expert Opin. Ther. Patents 8 (1998) 301–313.
[2] R. Berti, A.J. Williams, J.R. Moffett, S.L. Hale, L.C. Velarde, P.J. Elliott, C. Yao, J.R. Dave, F.C. Tortella, Quantitative real-time RT-PCR analysis of inflammatory gene expression associated with ischemia–reperfusion brain injury, J. Cereb. Blood Flow Metab. 22 (2002) 1068–1079.
[3] C.S. Breivogel, L.J. Sim, S.R. Childers, Regional differences in cannabinoid receptor/G-protein coupling in rat brain, J. Pharmacol. Exp. Ther. 282 (1997) 1632–1642.
[4] G. Cai, H.-Y. Wang, E. Friedman, Increased dopamine receptor signaling and dopamine receptor–G protein coupling in denervated striatum, J. Pharmacol. Exp. Ther. 302 (2002) 1105–1112.
[5] S.J. Carlisle, F. Marciano-Cabral, A. Staab, C. Ludwick, G.A. Cabral, Differential expression of the CB2 cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation, Int. Immunopharmacol. 2 (2002) 69–82.
[6] C.C. Chao, S. Hu, T.W. Molitor, E.G. Shaskan, P.K. Peterson, Activated microglia mediate neuronal cell injury via a nitric oxide mechanism, J. Immunol. 149 (1992) 2736–2741.
[7] R.G. Coffey, Y. Yamamoto, E. Snella, S. Pross, Tetrahydrocannabinol inhibition of macrophage nitric oxide production, Biochem. Pharmacol. 52 (1996) 743–751.

[8] J. De Vry, K.R. Jentzsch, Discriminative stimulus effects of BAY 38-7271, a novel cannabinoid receptor agonist, Eur. J. Pharm. 457 (2002) 147–152.
[9] U. Dirnagl, C. Iadecola, M.A. Moskowitz, Pathobiology of ischaemic stroke: an integrated view, Trends Neurosci. 22 (1999) 391–397.
[10] H. Fujisawa, W.L. Maxwell, D.L. Graham, G.M. Reasdale, R. Bullock, Focal microvascular occlusion after acute subdural hematoma in the rat—a mechanism for ischemic damage and brain swelling, Acta Neurochir. 60 (1994) 193–196.
[11] Y. Gaoini, R. Mechoulam, Isolation, structure and partial synthesis of an active constituent of hashish, J. Am. Chem. Soc. 86 (1964) 1646–1647.
[12] G. Gerdeman, D.M. Lovinger, CB1 cannabinoid receptor inhibits synaptic release of glutamate in rat dorsolateral striatum, J. Neurophysiol. 85 (2001) 468–471.
[13] D. Giulian, T.J. Baker, L.C. Shih, L.B. Lachman, Interleukin 1 of the central nervous system is produced by ameboid microglia, J. Exp. Med. 164 (1986) 594–604.
[14] R.I. Grundy, The therapeutic potential of the cannabinoids in neuroprotection, Expert Opin. Invest. Drugs 11 (2002) 1365–1374.
[15] R.I. Grundy, M. Rabuffetti, M. Beltramo, Cannabinoids and neuroprotection, Mol. Neurobiol. 24 (2001) 29–51.
[16] S. Holmi, T. Mathiesen, Biphasic edema development after experimental brain contusion in rat, Neurosci. Lett. 194 (1995) 97–100.
[17] M.L. Holtz, S.D. Craddock, L.C. Pettigrew, Rapid expression of neuronal and inducible nitric oxide synthases during post-ischemic reperfusion in rat brain, Brain Res. 898 (2001) 49–60.
[18] E. Horváth, K.H. Augstein, Neuroprotection by the novel 5-HT_{1A} receptor agonist BAY×3702 in the rat model of acute subdural hematoma, J. Neurotrauma 14 (1997) 170.
[19] K.L. Jin, X.O. Mao, P.C. Goldsmith, D.A. Greenberg, CB1 cannabinoid receptor induction in experimental stroke, Ann. Neurol. 48 (2000) 257–261.
[20] D.J. Kim, S.A. Thayer, Activation of CB1 cannabinoid receptors inhibits neurotransmitter release from identified synaptic sites in rat hippocampal cultures, Brain Res. 852 (2000) 398–405.
[21] S.M. Knoblach, L. Fan, A.I. Faden, Early neuronal expression of tumor necrosis factor-alpha after experimental brain injury contributes to neurological impairment, J. Neuroimmunol. 95 (1999) 115–125.
[22] S.K. Leong, E.A. Ling, Amoeboid and ramified microglia: their interrelationship and response to brain injury, Glia 6 (1992) 39–47.
[23] E.A. Ling, W.C. Wong, The origin and nature of ramified and amoeboid microglia: a historical review and current concepts, Glia 7 (1993) 9–18.
[24] P. Lipton, Ischemic cell death in brain neurons, Physiol. Rev. 79 (1999) 1431–1568.
[25] L.A. Matsuda, S.J. Lolait, M.J. Brownstein, A.C. Young, T.I. Bonner, Structure of a cannabinoid receptor and functional expression of the cloned cDNA, Nature 346 (1990) 561–564.
[26] F. Mauler, T. Fahrig, E. Horváth, R. Jork, Inhibition of evoked glutamate release by the neuroprotective 5-HT_{1A} receptor agonist BAY×3702 in vitro and in vivo, Brain Res. 888 (2001) 150–157.
[27] F. Mauler, J. Mittendorf, E. Horváth, J. de Vry, Characterization of the diarylether sulfonylester (−)-(*R*)-3-(2-hydroxymethylindanyl-4-oxy)phenyl-4,4,4-trifluoro-1-sulfonate (BAY 38-7271) as a potent cannabinoid receptor agonist with neuroprotective properties, J. Pharmacol. Exp. Ther. 302 (2002) 359–368.
[28] J.D. Miller, R. Bullock, D.I. Graham, M.H. Chen, G.M. Teasdale, Ischemic brain damage in a model of acute subdural hematoma, Neurosurgery 27 (1990) 433–439.
[29] F. Molina-Holgado, E. Molina-Holgado, C. Guaza, N.J. Rothwell, Role of CB1 and CB2 receptors in the inhibitory effects of cannabinoids on lipopolysaccharide-induced nitric oxide release in astrocyte cultures, J. Neurosci. Res. 67 (2002) 829–836.
[30] J. Mu, S.Y. Zhuang, M.T. Kirby, R.E. Hampson, S.A. Deadwyler, Cannabinoid receptors differentially modulate potassium A and D currents in hippocampal neurons in culture, J. Pharmacol. Exp. Ther. 291 (1999) 893–902.
[31] S. Munro, K.L. Thomas, M. Abu-Shaar, Molecular characterization of a peripheral receptor for cannabinoids, Nature 365 (1993) 61–65.
[32] K. Murakami, T. Kondo, G. Yang, S.F. Chen, Y. Morita-Fujimura, P.H. Chan, Cold injury in mice: a model to study mechanisms of brain edema and neuronal apoptosis, Prog. Neurobiol. 57 (1999) 289–299.
[33] D. Panikashvili, C. Simeonidou, S. Ben-Shabat, L. Hanus, A. Breuer, R. Mechoulam, E. Shohami, An endogenous cannabinoid (2-AG) is neuroprotective after brain injury, Nature 413 (2001) 527–531.
[34] S. Parmentier-Batteur, G.A. Bohme, D. Lerouet, L. Zhou-Ding, V. Beray, I. Margaill, M. Plotkine, Antisense oligodeoxynucleotide to inducible nitric oxide synthase protects against transient focal cerebral ischemia-induced brain injury, J. Cereb. Blood Flow Metab. 21 (2001) 15–21.
[35] G. Paxinos, C. Watson, The Rat Brain in Stereotaxic Coordinates, 3rd Edition, Academic Press, New York, 1996.
[36] R.G. Pertwee, Pharmacology of cannabinoid CB1 and CB2 receptors, Pharmacol. Ther. 74 (1997) 129–180.
[37] T. Petrov, A.B. Page, C.R. Owen, J.A. Rafols, Expression of the inducible nitric oxide synthase in distinct cellular types after traumatic brain injury: an in situ hybridization and immunocytochemical study, Acta Neuropathol. (Berl.) 100 (2000) 196–204.
[38] D. Piomelli, A. Giuffrida, A. Calignano, F. Rodríguez de Fonseca, The endocannabinoid system as a target for therapeutic drugs, Trends Pharmacol. Sci. 21 (2000) 218–224.
[39] L. Puebla, E. Arilla-Ferreiro, Modulation of somatostatin receptors, somatostatin content and G_i proteins by substance P in the rat frontoparietal cortex and hippocampus, J. Neurochem. 84 (2003) 145–156.
[40] M. Sawada, N. Kondo, A. Suzumura, T. Marunouchi, Production of tumor necrosis factor-alpha by microglia and astrocytes in culture, Brain Res. 491 (1989) 394–397.
[41] Y. Shapira, D. Setton, A.A. Artru, E. Shohami, Blood–brain barrier permeability, cerebral edema, and neurologic function after closed head injury in rats, Anesth. Analg. 77 (1993) 141–148.
[42] M. Shimizu-Sasamata, P. Bosque-Hamilton, P.L. Huang, M.A. Moskowitz, E.H. Lo, Attenuated neurotransmitter release and spreading depression-like depolarizations after focal ischemia in mutant mice with disrupted type I nitric oxide synthase gene, J. Neurosci. 18 (1998) 9564–9571.
[43] L.J. Sim-Selleya, L.J. Vogt, B.A. Vogt, S.R. Childers, Cellular localization of cannabinoid receptors and activated G-proteins in rat anterior cingulate cortex, Life Sci. 71 (2002) 2217–2226.
[44] V. Taupin, S. Toulmond, A. Serrano, J. Benavides, F. Zavala, Increase in IL-6, IL-1 and TNF levels in rat brain following traumatic lesion. Influence of pre- and post-traumatic treatment with Ro 54864, a peripheral-type (p site) benzodiazepine ligand, J. Neuroimmunol. 42 (1993) 177–185.
[45] E. Tsuchida, R. Bullock, The effect of the glycine site-specific ACEA1021 on ischemic brain-damage caused by acute subdural-hematoma in the rat, J. Neurotrauma 12 (1995) 279–288.
[46] Y. Waksman, J.M. Olson, S.J. Carlisle, G.A. Cabral, The central cannabinoid receptor (CB1) mediates inhibition of nitric oxide production by rat microglial cells, J. Pharmacol. Exp. Ther. 288 (1999) 1357–1366.
[47] L. Walter, A. Franklin, A. Witting, C. Wade, Y. Xie, G. Kunos, K. Mackie, N. Stella, Nonpsychotropic cannabinoid receptors regulate microglial cell migration, J. Neurosci. 23 (2003) 1398–1405.
[48] C.X. Wang, A. Shuaib, Involvement of inflammatory cytokines in central nervous system injury, Prog. Neurobiol. 67 (2002) 161–172.
[49] L. Wang, J. Liu, J. Harvey-White, A. Zimmer, G. Kunos, Endocannabinoid signaling via cannabinoid receptor 1 is involved in

ethanol preference and its age-dependent decline in mice, Proc. Natl. Acad. Sci. USA 100 (2003) 1393–1398.
[50] M.N. Woodroofe, G.S. Sarna, M. Wadhwa, G.M. Hayes, A.J. Loughlin, A. Tinker, M.L. Cuzner, Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by in vivo microdialysis: evidence of a role for microglia in cytokine production, J. Neuroimmunol. 33 (1991) 227–236.
[51] G.H. Xi, Y. Hua, R.F. Keep, J.G. Younger, J.T. Hoff, Systemic complement depletion diminishes perihematomal brain edema in rats, Stroke 32 (2001) 162–167.
[52] Q. Zhao, Transient middle cerebral artery occlusion in rats, Doctoral dissertation (LUMEDW/(MEXB-1013) 1-62), Reprocentralen, Byggnadsbyran, Lund University, Lund, Sweden, 1995.
[53] M. Zwienenberg, Q.Z. Gong, L.L. Lee, R.F. Berman, B.G. Lyeth, ICP monitoring in the rat: comparison of monitoring in the ventricle, brain parenchyma, and cisterna magna, J. Neurotrauma 16 (1999) 1095–1102.