Cerebroside-A provides potent neuroprotection after cerebral ischaemia through reducing glutamate release and Ca\(^{2+}\) influx of NMDA receptors

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Abstract

Excessive presynaptic glutamate release after cerebral ischaemia leads to neuronal death mainly through excessive calcium entry of \(N\)-methyl-D-aspartate receptors (NMDARs). Our recent study reported that cerebroside can open large-conductance Ca\(^{2+}\)-activated K\(^+\) (BKCa) channels. The present study evaluated the effects of cerebroside-A (CS-A), a single molecule isolated from an edible mushroom, on brain injury after focal or global ischaemia in adult male mice and rats. We herein report that treatment with CS-A after 60-min middle cerebral artery occlusion dose-dependently reduced the cerebral infarction with at least a 6-h efficacious time-window, which was partially blocked by the BKCa channel blocker charybdotoxin (CTX). Treatment with CS-A after 20 min global cerebral ischaemia (four-vessel occlusion) significantly attenuated the death of pyramidal cells in hippocampal CA1 area, which was also sensitive to CTX. CS-A, by opening the BKCa channel, could prevent excessive glutamate release after oxygen-glucose deprivation (OGD). In addition, CS-A could inhibit NMDAR Ca\(^{2+}\) influx, which did not require the activation of the BKCa channel. Furthermore, CS-A blocked the OGD-induced NMDAR-dependent long-term potentiation in hippocampal CA1 region. These findings indicate that treatment with CS-A after stroke exerts potent neuroprotection through prevention of excessive glutamate release and reduction of Ca\(^{2+}\) influx through NMDARs.

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Introduction

Stroke is the second cause of death worldwide and a leading cause of serious, long-term disability. Immediately after ischaemia, excessive presynaptic glutamate release results in the accumulation of extracellular glutamate reaching concentrations that induce over-activation of glutamate receptors (Jabaudon et al. 2000; Phillis & O’Regan, 2003). Consequently, an excessive calcium entry mainly through ionotropic glutamatergic receptors of the \(N\)-methyl-D-aspartate (NMDA) subtype initiates neuronal death. The process of neurotoxicity has been demonstrated in several experimental models of cerebral ischaemia (Butcher et al. 1990). In humans, increased glutamate levels in fluid and plasma have been reported to persist in some cases for >6 d after the onset of stroke (Dohmen et al. 2003). Therefore, targeting the neurotoxicity process after a stroke event might be important in stopping the progression of brain damage.

Our recent study reported that pure cerebroside isolated from Baifuzi (\(Typhonium\) giganteum Engl.), a traditional Chinese medicine for treating stroke, can...
activate large-conductance Ca\(^{2+}\)-activated K\(^+\) (BKCa) channels by directly binding with a stress axis hormone-regulated exon (STREX) domain of the channel (Chi et al. 2010). BKCa channels are widely expressed in the brain and are preferentially located at glutamatergic synaptic terminals (Hu et al. 2001). The activation of BKCa channels limits the Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels (VGCCs) (Mikawa et al. 1997). BKCa channel openers can minimize the ischaemia-induced neuronal depolarization, accumulation of intracellular Ca\(^{2+}\) and neurotransmitter release (Gribkoff et al. 2001). Blockade of BKCa channels enhances cell damage after oxygen-glucose deprivation (OGD) (Runden-Pran et al. 2002). Thus, it is of interest to investigate the effects of cerebroside on ischaemia-induced neuronal damage.

The neurons in hippocampal CA1 region are one of the most vulnerable structures of the brain with regard to neuronal damage triggered by ischaemia or energy deprivation. In-vitro studies utilizing OGD have produced a large amount of data on the mechanisms of acute and delayed neuronal damage after energy failure. In particular, electrophysiological recordings have shown that energy deprivation destroys ionic homeostasis leading to irreversible membrane depolarization of neurons, which induces long-term potentiation (LTP) at Schaffer collateral-CA1 synapses, a pathological form of synaptic plasticity (Calabresi et al. 2001, 2002). The induction of OGD-LTP is well known to be a synaptic mechanism leading to ischaemic apoptosis by enhancing presynaptic glutamate release and post-synaptic calcium influx (Calabresi et al. 2003).

Our recent study demonstrated that the pure cerebroside from ethanol extract of Baifuzi can activate BKCa channels. The present study first evaluated the effects of cerebroside-A (CS-A), a single molecule cerebroside extracted and isolated from an edible mushroom (Qi et al. 2001), on brain injury after focal or global stroke in adult male mice and rats. In order to explore the mechanisms underlying the neuroprotective effect of CS-A, we further investigated the effects of the opening of BKCa channels by CS-A on presynaptic glutamate release, Ca\(^{2+}\) influx through L-type VGCCs and NMDARs, and induction of hippocampal LTP after OGD.

Materials and methods

Extraction and isolation of CS-A

The dried fruiting bodies of *Termitomyces albuminosus* (Jizongjun in Chinese) were extracted to give a MeOH extract, which was washed with hexane, and then partitioned between H\(_2\)O and BuOH. The BuOH fraction was chromatographed on ODS eluted with MeOH/H\(_2\)O to give four fractions. The third fraction, which was eluted with MeOH/H\(_2\)O (95:5, v/v), was separated by a silica gel open column eluted with CHCl\(_3\)/MeOH to give four fractions. The fraction, which was eluted with CHCl\(_3\)/MeOH (90:10, v/v), was subjected to HPLC eluted by MeOH/H\(_2\)O (98:2, v/v) to yield CS-A. The structure of CS-A (Fig. 1) was identified as the reported cerebroside by comparing its physical chemical properties, \(^1\)H- and \(^{13}\)C-NMR, MS data, and specific rotation. The detailed process has been described in our previous paper (Qi et al. 2001).

Experimental animals

The present studies were approved by Animal Care and Ethical Committee of Nanjing Medical University. All procedures were in accordance with the guidelines of the Institute for Laboratory Animal Research of Nanjing Medical University. Male C57BL/6 mice (weighing 25–30 g) and male Sprague–Dawley rats (weighing 200–250 g, Oriental Bio Service Inc., China) were used throughout the study. Animals were housed in a light-controlled room under a 12-h light/dark cycle (lights on 07:00 hours) maintained at 25 °C, with food and water available ad libitum.

Preparation of the focal cerebral ischaemia model

Focal cerebral ischaemia was induced by middle cerebral artery occlusion (MCAO). Mice were anaesthetized with a mixture of 70% N\(_2\)O and 30% O\(_2\) containing 2.5% isoflurane, and were maintained by the inhalation of 1.5% isoflurane during the operation. Briefly, a heat-blunted black monofilament surgical suture (6/0 G) was inserted into the internal carotid artery to occlude the origin of the MCA. Adequacy of vascular occlusion and reperfusion was monitored in the front parietal cortex of the occluded side by Perimed PF5050 Q4 (Perimed, Sweden) multi-channel laser Doppler flowmetry. Body and head temperatures were controlled at 37 ± 0.5 °C using a water pads. Arterial blood pressure and gases were monitored with a femoral catheter. After 60 min occlusion, the
filament was withdrawn to allow reperfusion. Sham-operated animals were treated identically, except that their MCAs were not occluded.

**Preparation of the global brain ischaemia model**

We used a four-vessel occlusion (4VO) method for transient global ischaemia as described previously (Chi et al. 2010). Rats were anaesthetized with chloral hydrate (400 mg/kg i.p.). Briefly, both common carotid arteries (CCAs) were dissected free, and the incision was closed. Immediately after this procedure, both vertebral arteries (VAs) between the first and second cervical vertebrae were exposed and electrocauterized completely using a bipolar cauterizer under an operating microscope (SZH-ILLB, Japan). After 24 h, the common arteries were occluded with aneurysm clips for 20 min. The clips were then removed, and blood flow though the arteries was confirmed before the wound was sutured. Rectal temperature was continually monitored and maintained at 37±0.5°C. Sham-operation (control) groups had a permanent bilateral occlusion of VAs (2VO rats) that was performed in the same manner as in 4VO rats, except that the CCAs were not occluded. All rats were allowed to survive for 8 d after the onset of cerebral ischaemia.

**Drug administration**

All animals were randomly divided into vehicle- or drug-treated groups. CS-A was diluted in 0.5% ethanol and injected (i.p.) twice daily with a 12-h interval starting at 1, 4, 6, 8 or 12 h after MCAO for two successive days. The BKCa channel blocker charybdotoxin (CTX; Sigma, USA) was dissolved in 0.9% saline and injected into the cerebroventricle (i.c.v.) once daily for two successive days through an implanted guide cannula (2.2 mm length, 23-gauge) above the left lateral ventricle. The drugs were injected with a stepper-motorized micro-syringe (Stoelting, USA). Control mice were given an equal volume of vehicle. The mice did not show weight loss or abnormal behaviours during or after treatment with CS-A (data not shown).

**Measurement of cerebral infarct**

Using 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, USA) staining, the infarct size was measured at day 3 post-MCAO. All animals were killed under deep halothane anaesthesia, and the brains were quickly frozen and sliced into 2-mm-thick sections. Slices were photographed and studied using image analysis software (MCID; Imaging Research, Canada). The infarct volume was expressed as a percentage of the contralateral hemisphere.

**Electrophysiological recording and analysis**

**Slice preparation**

Mice were decapitated under deep anaesthesia with ethyl ether. Brains were rapidly removed and coronal slices (200-μm-thick) were cut using a vibrating microtome (Micslicer DTK 1500, Dousaka EM Co., Japan). The hippocampal slices were incubated in artificial cerebrospinal fluid (ACSF) at 36±1°C for 60 min. ACSF was composed of (mM): 124 NaCl, 2 CaCl₂, 4.5 KCl, 1 MgCl₂, 26 NaHCO₃, 1.2 NaH₂PO₄, and 10 d-glucose (pH 7.4) and was oxygenated with a gas mixture of 95% O₂/5% CO₂.

**Field potential recording**

The slice was transferred into a recording chamber and perfused continuously with oxygenated ACSF. The experiments were conducted at 30±1°C. A bipolar tungsten electrode was placed in the CA1 radiatum layer to stimulate Schaffer collaterals. Constant current pulses (0.05 Hz) were supplied by a stimulator (SEN-3301, Japan). Excitatory post-synaptic potentials (EPSPs) were recorded in the radiatum layer with a 4–5 MΩ resistance glass microelectrode that was connected to a neutralized, high-input impedance preamplifier with a high-pass filter at 5 kHz. Stimulus intensity was set to 50% of the maximal intensity that evoked a saturated EPSP to avoid possible deprivation of readily releasable transmitters. Signals were amplified using a differential AC amplifier (A-M Systems, model 1700, USA). EPSPs were digitized and saved using the pCLAMP system (Axon Instruments Inc., USA).

**OGD-induced LTP**

To avoid slice preparation resulting in sequential damage after brain ischaemia, we used a slice model of LTP induced by OGD to explore the mechanism of the neuroprotective effect of CS-A as described previously (Calabresi et al. 2001, 2002). Glucose was totally removed from ACSF and sucrose was added to balance the osmolarity. Oxygen deprivation was obtained by bubbling ACSF in a gas mixture containing 95% N₂/5% CO₂. This procedure did not affect the resting membrane potential or the input resistance of the recorded cells. Ischaemic solutions entered the recording chamber no later than 20 s after turning a
three-way tap. Complete replacement of the medium in the chamber took 90 s.

Optical recording

The slices were stained with a voltage-sensitive dye RH155 (0.2 mg/ml, Nippon Kanko Shikiso Kenkyuyo, Japan) for 15 min and were transferred into a recording chamber on the stage of an Olympus inverted microscope (IMT-2, Japan). A bipolar tungsten electrode was placed in the CA1 radiatum layer to stimulate Schaffer collaterals. Light from a tungsten halogen lamp (type JC-24V/200W, Japan) was collimated and rendered with an interference filter at 700±10 nm (Olympus Optical). Changes in light absorption associated with membrane potentiation were detected by metal oxide (MOS) image sensors with the aid of a 690-nm interference filter (bandwidth ±30 nm) and were measured by a high-speed optical recording system (HR Deltaron-1700 Fujix; Fuji Photo Film, Japan). In each trial, a background image recorded for 16 ms before electrical stimulation was stored as a reference image. To make more quantitative analyses of the amplitude–time relationship (time-course) of optical signals, data from each pixel were stored and retrieved, and the amplitude (indicated as a percentage change in optical absorbency) was plotted as a function of time.

Calcium imaging

The Ca\(^{2+}\) indicator, Indo-1 AM, was loaded into hippocampal granule cells in the slice using a local ester loading method (Chen et al. 2007). The slice labelled with Indo-1 was mounted in a recording chamber and observed with an upright microscope (Olympus BX51WI) through a water immersion lens (×20 Olympus XLMPlanFL). Indo-1 fluorescence was observed by a multi-photon laser scanning microscope (BioRad Radiance 2000 MP) with a direct detector system. Indo-1 was excited at 710–730 nm, and the resulting fluorescence was measured at 390 nm and 495 nm every 5 s. When the hippocampal slices were exposed to NMDA (5 μM) or high K\(^+\) (20 mM) extracellular solution ([K\(^+\)]\(_{e}\)) for 1 min, the changes in fluorescence intensities (F390 and F495) were calculated as an indicator of [Ca\(^{2+}\)]\(_i\). Ca\(^{2+}\) signals were averaged for 20–30 cells under the same microscopic field.

Data analysis/statistics

Data were retrieved and processed with Microcal Origin 6.1 software (USA). The group data were expressed as mean ± standard error (s.e.). Experimental results were compared among treatment groups by ANOVA followed by Bonferroni’s post-hoc test. Statistical analysis was performed using Stata version 7 software (Stata Corporation, USA). Differences at p < 0.05 were considered statistically significant.

Results

CS-A reduces MCAO-induced cerebral infarction

At day 3 post-MCAO, the ischaemic hemisphere showed approximately 31.6% cerebral infarction (n=8, Fig. 2a). The twice daily treatment (i.p.) with CS-A (0.05, 0.5, 1.0, 5.0 mg/kg) at 1 h post-MCAO for 2 d could dose-dependently reduce the size of infarction compared to vehicle-treated MCAO mice (n=8, Fig. 2b). Furthermore, CS-A (1 mg/kg) when administered from 1 h to 6 h post-MCAO could perfectly reduce the size of infarction (n=8, Fig. 2c), but it had no effect at 8 h and 12 h after MCAO (n=8). These results suggest that CS-A dose-dependently reduces the MCAO-induced cerebral injury with an efficacious time-window of at least 6 h. The treatment (i.c.v.) with the BKCa channel blocker CTX at 30 min prior to CS-A administration attenuated the neuroprotective effect of CS-A (1 mg/kg) at 1 h after MCAO (p<0.01 vs. CS-A-treated MCAO mice, n=8; Fig. 2d), while the treatment with CTX alone had no effect on the MCAO-induced cerebral infarction (p>0.05 vs. CS-A-treated MCAO mice, n=8). Pre-treatment with CTX could not completely prevent the neuroprotective effect of CS-A (p<0.05 vs. CTX-treated MCAO mice), indicating that this effect of CS-A against ischaemic brain injury partially depends on opening the BKCa channel. At day 8 after 4VO, the number of pyramidal cells was reduced by approximately 40% (p<0.01 vs. 2VO rats, n=8; Fig. 2e, f). Treatment with CS-A (1 mg/kg) at 1 h post-4VO significantly attenuated the loss of pyramidal cells (p<0.01 vs. 4VO rats, n=8), which were also sensitive to pre-treatment with CTX (p<0.05 vs. CS-A-treated 4VO rats, n=8).

CS-A opening of BKCa channels reduces presynaptic glutamate release

To investigate the mechanisms underlying the neuroprotective effect of CS-A in opening BKCa channels, the effects of CS-A on presynaptic glutamate release was first examined in control mice. The results in Fig. 3a clearly show that perfusion of CS-A (10 μM) in hippocampal slices reversibly attenuates the slope of EPSP at Schaffer collateral-CA1 synaptic transmission (p<0.01, n=10 slices/6 mice), which was sensitive to the BKCa channel blocker CTX (0.1 μM, p<0.01).
Fig. 2. Cerebroside-A (CS-A) reduces brain damage after focal and global cerebral ischaemia. (a) Representative photographs of cerebral infarction in middle cerebral artery occlusion (MCAO) mice treated with CS-A. Note that treatment with CS-A after stroke significantly reduces the MCAO-induced cerebral infarction. (b) Dose-dependency of the neuroprotective effect of CS-A. Bar graphs show the mean infarct volume percentage in MCAO mice that were treated with various concentrations of CS-A (0.05, 0.5, 1.0, 5.0 mg/kg) at 1 h after MCAO (** p < 0.01 vs. sham-operated (sham-op) mice; ## p < 0.01 and # p < 0.05 vs. MCAO mice). (c) Time-window of neuroprotective effect of CS-A. Bar graphs show the mean infarct volume percentage in MCAO mice that were treated with CS-A (1.0 mg/kg) starting 1, 4, 6, 8 or 12 h after MCAO (** p < 0.01 and * p < 0.05 vs. MCAO mice). (d) Effects of BKCa channel blocker charybdotoxin (CTX) on the neuroprotective effect of CS-A (** p < 0.01 vs. MCAO mice; p < 0.01 vs. CS-A-treated MCAO mice; + p < 0.05). (e) Representative sections of hippocampal CA1 area in 2VO rats, 4VO rats, and 4VO rats treated with CS-A (1 mg/kg) or CS-A/CTX 1 h post-4VO. Scale bar, 100 μm. (f) Bar graphs represent the number of surviving pyramidal cells in hippocampal CA1 of 2VO (□) and 4VO rats (■) treated with CS-A or CTX (** p < 0.01 vs. 2VO rats; # p < 0.01 vs. 4VO rats; + p < 0.05 vs. CS-A treated 4VO rats).
The inhibitory effect of CS-A exhibited a sigmoidal shape with an apparent IC$_{50}$ of 5.83 μM (Fig. 3b). In addition, the values of paired-pulse ratio (PPR) with 25–75 ms interpulse intervals (IPIs) in the presence of CS-A were markedly larger than the values in the absence of CS-A (p < 0.05, n = 10 slices/6 mice; Fig. 3c), suggesting that opening of BKCa channels by CS-A reduces presynaptic glutamate release.

**CS-A’s opening of BKCa channels prevents excessive glutamate release after OGD**

In hippocampal slices stained with RH155 dye, release of presynaptic glutamate by stimulating Schaffer collaterals elicited an optical signal in the CA1 radiatum layer containing two components: an initial spike-like signal representing a presynaptic fibre volley and a large signal reflecting EPSP (first trace; Fig. 4a). Because the large depolarizing component was suppressed by the addition of 10 μM CNQX/50 μM AP5 (second trace), the spike-like signal (third trace) was abolished by 1 μM TTX (fourth trace). Notably, in the presence of CNQX/AP5, a delayed depolarizing signal remained (second trace), which was abolished by the glial glutamate transporter GLT-1 blocker dihydromakaiainate (DHK, 1 μM), and was thus identified as synaptically induced glial depolarization (SIGD). This signal has been demonstrated to reflect the probability of glutamate release (Chen & Sokabe, 2005). As shown in Fig. 4b, the application of CS-A (10 μM) significantly reduced the SIGD area (vs. basal SIGD area, p < 0.01, n = 11 slices/6 mice), which was abolished by CTX (0.1 μM, p < 0.01), indicating that opening of BKCa channels by CS-A can reduce presynaptic glutamate release. In hippocampal slices subjected to 5 min OGD,
the SIGD area increased at 10 min after replacement of ACSF (vs. basal SIGD area, \( p < 0.01, n = 10 \) slices/6 mice; Fig. 4c), showing an increase in glutamate release after delivering OGD. Importantly, application of CS-A was able to prevent the OGD-induced increase of SIGD area (\( p < 0.01, n = 10 \) slices/6 mice), which was sensitive to pre-treatment with CTX (\( p < 0.01, n = 10 \) slices/6 mice). These findings indicate that the opening of BKCa channels by CS-A might prevent excessive glutamate release after stroke.

**CS-A reduces \( \text{Ca}^{2+} \) influx through NMDARs**

Excessive calcium entry mainly through NMDARs and L-VGCCs after cerebral ischaemia leads to neuronal death (Seeburg, 1993). We further evaluated the influence of CS-A on the \( \text{Ca}^{2+} \) influx through NMDARs or L-type VGCCs in hippocampal CA1 pyramidal cells. A pair of typical images for NMDA (5 \( \mu \)M) increased \([\text{Ca}^{2+}]_i\) in the absence and presence of CS-A is shown in Fig. 5a. The addition of CS-A (10 \( \mu \)M) for 5 min did not affect the basal level of \([\text{Ca}^{2+}]_i\) (lane 2, \( n = 8 \) slices/5 mice), while significantly attenuating the NMDA-induced increase in \([\text{Ca}^{2+}]_i\)

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**Fig. 4.** Cerebroside-A (CS-A)’s opening of BKCa channels reduces presynaptic glutamate release after stroke. (a) Sample traces of optical signal were obtained from the same site sequentially from left to right in ACSF, +CNQX/AP5, +DHK, and +TTX. (b) Effects of CS-A on glutamate release. Bar graph shows mean value of the synaptically induced glial depolarization (SIGD) area expressed as percentage of control value in ACSF (\( ** p < 0.01 \) vs. control group; \( # # p < 0.01 \) vs. CS-A-alone-treated group). (c) Effects of CS-A on glutamate release after delivering oxygen-glucose deprivation (OGD) (■). Bar graph shows mean value of the SIGD area expressed as percentage of control value (\( ** p < 0.01 \) vs. control group; \( # # p < 0.01 \) vs. OGD group; \( ++ p < 0.01 \) vs. OGD group treated with CS-A).

**Fig. 5.** Cerebroside-A (CS-A) reduces NMDAR Ca\(^{2+}\) influx. (a, b) Effects of CS-A on NMDA-induced \([\text{Ca}^{2+}]_i\) increase in hippocampal CA1 region. Typical images were obtained from a same slice sequentially from left to right in artificial cerebrospinal fluid (ACSF), NMDA, CS-A and CS-A/NMDA. Colour changes indicate level of \([\text{Ca}^{2+}]_i\) (red > yellow > green). Scale bar, 100 \( \mu \)M. Bar graph shows mean level of \([\text{Ca}^{2+}]_i\) (\( ** p < 0.01 \); \( # # p < 0.01 \) vs. NMDA-alone group). (c) Effects of CS-A on \( \text{Ca}^{2+} \) influx by voltage-gated \( \text{Ca}^{2+} \) channels (VGCCs) (\( ** p < 0.01 \); \( # # p < 0.01 \) vs. high \( K^+ \) group).
Fig. 6. Cerebroside-A (CS-A) blocks the induction of NMDAR-dependent oxygen-glucose deprivation–long-term potentiation (OGD-LTP) by reducing NMDAR-Ca\(^{2+}\) influx. (a) Effects of NMDAR antagonist MK801 on OGD-LTP induction. Each point represents group mean value (± S.E.M.) of excitatory post-synaptic potential (EPSP) slope expressed as the percentage of baseline before OGD are plotted against the recording time. Sample traces were obtained at 5 min pre-OGD (left) and 60 min post-OGD.
(lane 5, \( p < 0.01 \), \( n = 8 \) slices/5 mice; Fig. 5b). Interestingly, pre-treatment with CTX (0.1 \( \mu M \)) hardly affected the effect of CS-A (lane 6). In contrast, high \( [K^+]_o \) (20 mM) could elevate \( [Ca^{2+}]_i \) (lane 4, vs. basal level lane 1, \( p < 0.01 \), \( n = 8 \) slices/5 mice; Fig. 5c), which was sensitive to the application of L-type VGCC blocker nifedipine (lane 5, \( p < 0.01 \), \( n = 8 \) slices/5 mice) but not CS-A (lane 6, \( p > 0.05 \), \( n = 8 \) slices/5 mice). The findings indicate that CS-A can inhibit \( Ca^{2+} \) influx through NMDARs in opening BKCa channels in an independent manner.

**CS-A blocks OGD-induced LTP**

To explore the effects of CS-A-inhibiting NMDAR \( Ca^{2+} \) influx on ischaemia-induced excitotoxicity, we observed the induction of ischaemia-induced LTP (Bagetta et al. 2008; Calabresi et al. 2003). In hippocampal slices subjected to 5 min OGD, the EPSP slope at the Schaffer collateral-CA1 synapses slightly reduced within 5 min following replacement of ACSF, then rapidly increased by approximately 1.5-fold of baseline throughout the observation period for over 60 min (\( n = 10 \) slices/6 mice; Fig. 6a), indicative of OGD-LTP. The treatment with the NMDAR blocker MK801 (10 \( \mu M \)) blocked the induction of OGD-LTP leaving a transient increase of EPSP slope \( [n=10 \text{ slices/6 mice; Fig. 6b(i)}] \) with the reduction of PPR \( [\text{lane 2 in 10 min post-OGD vs. lane 1 in 5 min pre-OGD, } p < 0.05; \text{Fig. 6b(ii)}] \), reflecting an OGD-induced increase in presynaptic glutamate release. The treatment with CS-A (10 \( \mu M \)) not only blocked OGD-LTP but also abolished OGD-induced early transient increase of the EPSP slope \( [n=10 \text{ slices/6 mice; Fig. 6c(i)}] \) and the reduction of PPR \( [\text{lane 2 vs. lane 1, } p > 0.05; \text{Fig. 6c(ii)}] \), indicating that CS-A prevents excessive glutamate release after OGD. Furthermore, co-application of CTX and CS-A could also block OGD-LTP \( [n=8 \text{ slices/6 mice; Fig. 6d(i)}] \), although it could not abolish the OGD-induced early transient increase of the EPSP slope and the reduction of PPR \( [\text{lane 2 vs. lane 1, } p < 0.05; \text{Fig. 6d(ii)}] \). The results indicate that CS-A blocks the induction of OGD-LTP by reducing NMDAR \( Ca^{2+} \) influx.

**Discussion**

Excessive glutamate release and subsequent NMDAR \( Ca^{2+} \) influx are major pathological factors leading to degeneration and death of neuronal cells after stroke (Jabaudon et al. 2001; Phillis & O’Regan, 2003). The present study provides clear evidence that CS-A exerts a potent neuroprotective effect with at least a 6-h efficacious time-window after stroke by reducing glutamate release and NMDAR \( Ca^{2+} \) influx.

Prominent features of cerebral ischaemia are the strong depolarization of neuronal membranes resulting in intracellular accumulation of both calcium and sodium. The final outcome of these ion movements is an excessive release of neurotransmitters such as glutamate, which subsequently trigger various processes including modulation of synaptic transmission, excitotoxic cell death. The neuroprotective effect of CS-A against ischaemia-induced cerebral injury depends on the activation of the BKCa channel. Consistent with previous studies (Ghatta et al. 2006; Hu et al. 2001), we observed that CS-A, by opening BKCa channels, diminished presynaptic glutamate release. More importantly, CS-A’s opening of BKCa channels could prevent the excessive presynaptic glutamate release after OGD. Chi et al. (2010) have demonstrated that cerebroside increases BKCa channel currents through interaction with the channel’s STREX domain located at the cytoplasmic side. The STREX domain is important for the activation of BKCa channels induced by lipid-soluble chemicals (Chi & Qi, 2006). Inclusion of STREX exons can facilitate voltage- and \( Ca^{2+} \)-dependent activation of BKCa channels (Erxleben et al. 2002). Cerebroside can activate BKCa channels in excised membrane (Chi et al. 2010), suggesting that its action is not likely to be through intracellular diffusible signals. Because cerebroside does not elevate \( [Ca^{2+}]_i \) levels in hippocampal neurons (Chi et al. 2010), the activation of BKCa channels is not likely to be via \( Ca^{2+} \) triggering.

After transient cerebral ischaemia, the two main sites for calcium entry are NMDARs and L-type VGCCs (Seeburg, 1993). An earlier study (Mikawa et al. 1997) reported that activation of BKCa channels
limited the Ca\(^{2+}\) influx of VGCCs. Our results showed that activation of BKCa channels by CS-A did not affect the Ca\(^{2+}\) influx through L-type VGCCs. Importantly, CS-A could suppress the Ca\(^{2+}\) influx of NMDARs, which did not require the activation of BKCa channels. Some lines of evidence indicate that, in hippocampal neuronal cells, NMDARs are located in specific domains of post-synaptic membrane (Abulrob et al. 2005) enriched in sphingolipids and cholesterol. These domains, the so called 'lipids rafts', are plasma membrane structures (Besshoh et al. 2005). The analysis of glutamate-evoked calcium flux found that cholesterol depletion of rat hippocampal pyramidal cells reduces NMDAR function (Frank et al. 2008). Depleted membrane cholesterol level can protect neurons from death induced by excessive stimulation of NMDARs (Bosel et al. 2005). Simvastatin reduces the association of NMDARs with lipid rafts to exert a neuroprotective effect after stroke (Ponce et al. 2008). In addition, cholesterol depletion of rat hippocampal pyramidal cells leads to inhibition of post-tetanic LTP by preventing the NMDAR response (Frank et al. 2008). We observed also that pre-incubation with CS-A to rat hippocampal slices could block the induction of NMDAR-dependent OGD-LTP and frequency-dependent LTP in the hippocampal CA1 region (Supplementary Fig. S2). Taking into account our data, as well as other studies, we propose that CS-A reduces the Ca\(^{2+}\) influx of NMDARs to block the NMDAR-mediated excitotoxicity after brain ischaemia probably through manipulation of lipid content.

Many BKCa channel openers have been reported to prevent neuronal degeneration after ischaemia in preclinical studies; however, only activating BKCa channels has failed to improve the clinical outcome in patients affected by stroke (Nardi & Olesen, 2008). Thus, there is no approval of BKCa channel openers for clinical use so far. Treatment with CS-A not only diminished presynaptic glutamate release through opening BKCa channels, but could also suppress the Ca\(^{2+}\) influx of NMDARs and the induction of NMDAR-dependent OGD-LTP in a BKCa channel-independent manner. The findings give an indication that treatment with CS-A after stroke might exert a stronger neuroprotective effect than BKCa channel openers. On the other hand, CS-A inhibiting NMDAR Ca\(^{2+}\) influx and presynaptic glutamate release could block the induction of frequency-dependent LTP, a cellular model of spatial memory. However, we observed that the inhibitory effects of CS-A on LTP induction were reversible (Supplementary Fig. S3). Moreover, treatment with CS-A for 2 d did not affect the synaptic plasticity (Supplementary Fig. S4) or the spatial cognitive function (Supplementary Fig. S5). In addition, mushrooms have an established history of use in traditional oriental medicine and food, where most medicinal mushroom preparations are regarded as a tonic, i.e. they have beneficial health effects without known negative side-effects and can be moderately used on a regular basis without harm. Mushrooms comprise a vast and yet largely untapped source of powerful new pharmaceutical products. This study provides evidence that treatment with the single molecule CS-A after stroke exerts a potent neuroprotective effect with at least a 6-h efficacious time-window. Thus, CS-A might be a promising new compound for treating stroke.

Note
Supplementary material accompanies this paper on the Journal’s website (http://journals.cambridge.org/pnp).

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Statement of Interest
None.

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