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RESEARCH PAPER

Sinomenine protects against ischaemic brain injury: involvement of co-inhibition of acid-sensing ion channel 1a and L-type calcium channels

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BACKGROUND AND PURPOSE

Sinomenine (SN), a bioactive alkaloid, has been utilized clinically to treat rheumatoid arthritis in China. Our preliminary experiments indicated that it could protect PC12 cells from oxygen-glucose deprivation-reperfusion (OGD-R), we thus investigated the possible effects of SN on cerebral ischaemia and the related mechanism.

EXPERIMENTAL APPROACH

Middle cerebral artery occlusion in rats was used as an animal model of ischaemic stroke *in vivo*. The mechanisms of the effects of SN were investigated *in vitro* using whole-cell patch-clamp recording, calcium imaging in PC12 cells and rat cortical neurons subjected to OGD-R.

KEY RESULTS

Pretreatment with SN (10 and 30 mg·kg^{-1}, i.p.) significantly decreased brain infarction and the overactivation of calcium-mediated events in rats subjected to 2 h ischaemia followed by 24 h reperfusion. Extracellular application of SN inhibited the currents mediated by acid-sensing ion channel 1a and L-type voltage-gated calcium channels, in the rat cultured neurons, in a concentration-dependent manner. These inhibitory effects contribute to the neuroprotection of SN against OGD-R and extracellular acidosis-induced cytotoxicity. More importantly, administration of SN (30 mg·kg^{-1}, i.p.) at 1 and 2 h after cerebral ischaemia also decreased brain infarction and improved functional recovery.

CONCLUSION AND IMPLICATIONS

SN exerts potent protective effects against ischaemic brain injury when administered before ischaemia or even after the injury. The inhibitory effects of SN on acid-sensing ion channel 1a and L-type calcium channels are involved in this neuroprotection.

Abbreviations

ACSF, artificial cerebrospinal fluid; ASICs, acid-sensing ion channels; MCAO, middle cerebral artery occlusion; OGD-R, oxygen-glucose deprivation-reperfusion; SN, sinomenine; VGCCs, voltage-gated calcium channels

Introduction

Ischaemic brain injury is one of the leading causes of death and adult disability due to its high mortality rate in many countries. Abnormal cellular calcium homeostasis may trigger different signalling pathways for cell death and may play a critical role in ischaemic brain injury (Randall and Thayer, 1992; Berliocchi *et al.*, 2005). The use of blockers of neuronal voltage-gated calcium channels (VGCCs) has been considered as a therapeutic approach for post-stroke neuroprotection in humans (Kobayashi and Mori, 1998; Gribkoff and Winquist, 2005). Calcium-dependent neuronal injury is often thought to be related to ischaemic insults, including extracellular acidosis (Yermolaieva *et al.*, 2004; Bano and Nicotera, 2007; Guo *et al.*, 2009). Recent studies have revealed that Ca^{2+}-permeable acid-sensing ion channels (ASICs), especially ASIC1a, also induce Ca^{2+} entry directly and provide an additional Ca^{2+} entry pathway in ischaemic neurons (Xiong *et al.*, 2004; 2008). This novel therapeutic target for ischaemic brain injury has stimulated an interest in searching for the regulators of ASICs as new neuroprotective agents. Previous studies have shown that nonsteroid anti-inflammatory drugs such as aspirin, diclofenac and flurbiprofen directly inhibit ASICs in primary cultured neurons and heterologously expressed ASICs in COS-7 cells (Voilley *et al.*, 2001). Many bioactive components from medicinal plants exhibit significant anti-inflammation properties and our recent studies have revealed that some of them show neuroprotection against cerebral ischaemia; these include baicalein, tetrahydroxystilbene glucoside and theaflavin (Liu *et al.*, 2010; Wang *et al.*, 2009). However, there are few published studies on the effects of these bioactive components from medicinal plants on ASICs.

Sinomenine (SN), a bioactive alkaloid extracted from the Chinese medicinal plant, *Sinomenium acutum*, has been used for the clinic treatment of rheumatoid arthritis in China (Xu *et al.*, 2008). Previous studies have demonstrated that the pharmacological profiles of SN include immunosuppression, anti-inflammation and cytoprotection (Bao *et al.*, 2005; Feng *et al.*, 2006; Qian *et al.*, 2007). Based on its molecular structure (Figure 1A), SN belongs to the family of dextrorotatory morphinan analogues (Jin *et al.*, 2008), indicating that it may be capable of transporting across the blood–brain barrier (Long *et al.*, 2010). Several studies also demonstrated that SN alleviates acute myocardial ischaemia and reperfusion injury by lowering intracellular calcium accumulation (Xie and Jin, 1993). In our previous study, it was found that isoquinoline alkaloids such as berberine and palmatine exhibited significant inhibition of calcium currents in rat isolated hepatocytes, suggesting that alkaloids may exhibit a modulatory action on VGCCs (Wang *et al.*, 2003; 2004). SN is a clinically used anti-inflammatory drug with an alkaloid structure, but its effects on VGCCs and ASICs have not yet been investigated. In the present study, the neuroprotective effect of SN against ischaemic brain injury and its related mechanism of action were investigated.

Methods

Primary cortical neurons culture

Neonatal Sprague–Dawley (SD) rats (day 0–3) were obtained from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science & Technology. All animal care and experimental procedures complied with local and international guidelines on ethical use of animals and were approved by The University Animal Welfare Committee, Tongji Medical College, Huazhong University of Science & Technology. Primary rat cortical neurons were isolated as described in our previous study (Xiong *et al.*, 2006a; Wang *et al.*, 2009) dissected and rinsed in ice-cold Dulbecco's PBS. The dissected tissues were treated with 0.125% trypsin in Hanks' balanced salt solution for 25 min at 37°C and mechanically dissociated using a fire-polished Pasteur pipettes. Cells were collected by centrifugation and resuspended in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) with 10% fetal bovine serum. For whole-cell patch-clamp recording and calcium imaging, cells (20 000–40 000) were seeded on poly-D-lysine coated coverslips and kept at 37°C in a 5% CO_2 incubator. After 24 h, the culture medium was changed to DMEM medium supplemented with 2% B27 and the cortical neurons were fed with fresh medium twice weekly. Microscopically, glial cells were not apparent with this protocol. The neurons were maintained for 7–10 days in primary culture until used for whole-cell patch-clamp recording. The properties of ASICs were characterized in neurons that were kept in culture for at least 10 days.

CHO cells culture and transfection

CHO cells were obtained from Chinese Type Culture Collection and the cultures of CHO were maintained in 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C and subcultured every 2–3 days. The CHO cells were transfected with plasmid pCMV6-ASIC1a using the liposome transfection reagent (Lipofectamin 2000). This vector contained the cDNA of wide-type rat ASIC1a and was sequenced by Invitrogen. To select stable transfectants, cells were grown in complete medium supplemented with Geneticin (G418) antibiotics (Gibco Invitrogen Corporation) at 600 $\mu g \cdot mL^{-1}$ for 1 week and 400 $\mu g \cdot mL^{-1}$ for 2 weeks. Transfectants were tested by PCR and electrophysiological analysis.

PC12 cells culture

Rat PC12 cells were obtained from Chinese Type Culture Collection and were differentiated into neuron-like by treating with nerve growth factor. PC12 cells were maintained in DMEM medium containing 5% heat-inactivated fetal calf serum, 10% heat-inactivated horse serum, 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells (4×10^4 cells per 200 µL) were seeded into 96- or 24-well plates and then incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C for 2 days.

Whole-cell patch-clamp recording

The procedure for whole-cell patch-clamp recording of VGCCs was carried out as described in our previous reports

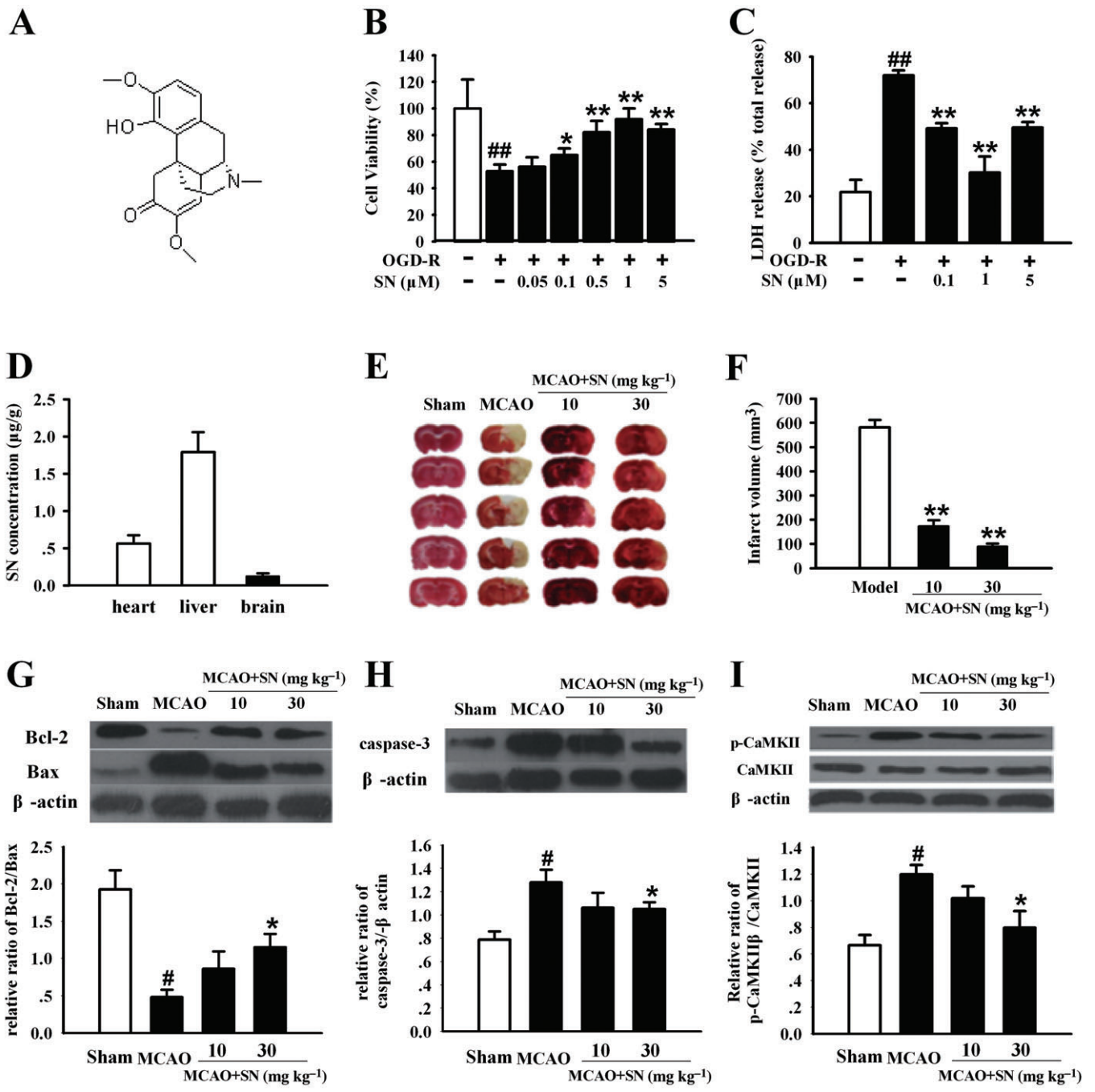

Figure 1

Pretreatment with SN exhibits protective effects against cerebral ischaemia *in vitro* and *in vivo*. (A) The chemical structure of SN. (B) SN reduced OGD-R-induced PC12 cell death in a dose-dependent manner. (C) Different concentrations of SN reduced OGD-R-induced LDH release in PC12 cells. Data are expressed as means ± SEM. $n = 6$, ##$P < 0.01$ vs. control,*$P < 0.05$ and **$P < 0.01$ vs. OGD-R. (D) SN content was measured using LC-MS in heart, liver and brain at 0.5 h after i.p. injection of 10 mg·kg^{-1} SN. $n = 3$. (E) SN attenuated MCAO-induced infarction assessed by TTC staining. The white brain area represents infarcted tissue and the serial sections of each group are from one animal. (F) Infarct volume was measured in the whole brain. Data are expressed as means ± SEM. $n = 6$. **$P < 0.01$ vs. MCAO. (G) Representative immunoreactive bands and histogram showing SN attenuated MCAO-induced decrease in the ratio of Bcl-2/Bax. (H) Representative immunoreactive bands showing SN attenuated MCAO-induced increase in activated caspase-3 and histogram representing the quantitative analysis of activated caspase-3 level normalized to β-actin protein. (I) Representative immunoreactive bands showing SN alleviated MCAO-induced up-regulation of CaMKII auto-phosphorylation and histogram representing the quantitative analysis of autophosphorylated CaMKII level normalized to total protein level. Data are expressed as means ± SEM. $n = 3$, #$P < 0.05$ vs. sham and *$P < 0.05$ vs. MCAO.

with minor modifications (Wang *et al.*, 2008; Ma *et al.*, 2009). The procedure for whole-cell patch-clamp recording of ASICs was also carried out as described in our previous reports with minor modifications (Hu *et al.*, 2010; Huang *et al.*, 2010). The bath solution for recording high-voltage activated calcium current (I_{HVA}) contained (in mM): choline-Cl 110, $MgCl_2$ 2, $CaCl_2$ 10, TEA-Cl 20, HEPES 10, glucose 20, and the pH was adjusted to 7.4 with CsOH. Glass pipettes were used with a resistance of about 2–4 MΩ when filled with the following solution (in mmol·L^{-1}): CsF 64, CsCl 64, $CaCl_2$ 0.1, $MgCl_2$ 2, EGTA 10.0, HEPES 10.0, Tris-ATP 5.0, and the pH was adjusted to 7.2 with CsOH. The bath solution for recording acid-sensing (proton-gated) ion channels currents contained (in mM): NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, glucose 10, and the pH was adjusted to 7.4 with NaOH. Pipettes (2–4 MΩ) were filled with internal solution containing (in mM): KCl 140, NaCl 10, $MgCl_2$ 1, EGTA 5, Mg-ATP 2, HEPES 10, and the pH was adjusted to 7.2 with KOH. The bath solution for recording NMDA-activated currents contained (in mM): NaCl 150, KCl 5, $CaCl_2$ 0.2, HEPES 10, glucose 10; and the pH was adjusted to 7.4 with NaOH. The patch-pipettes (2–4 MΩ) were filled with internal solution containing (in mM): KCl 140, NaCl 5, $MgCl_2$ 1, EGTA 10, Mg-ATP 2, HEPES 10, and the pH was adjusted to 7.2 with KOH. After establishment of a whole-cell configuration, the capacitance compensation and series resistance compensation were adjusted before recording. The current signals were acquired at a sampling rate of 10 kHz and filtered at 3 kHz. Whole-cell patch-clamp recordings were carried out using an EPC-10 amplifier (HEKA, Lambrecht, Germany) driven by Pulse/PulseFit software (HEKA, Southboro, Germany). Drug actions were measured only after steady-state conditions were reached, which were judged by the amplitudes and time when currents remained constant. All the recordings were recorded at room temperature (20–22°C). All experiments were repeated three times using different batches of cells and at least 3–4 dishes of cells were used for recording in each batch of cells.

Calcium imaging

Digital calcium imaging was performed as described by Ming *et al.* (Ming *et al.*, 2006; Lin *et al.*, 2007). The cells were washed three times with artificial cerebrospinal fluid (ACSF) containing (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 10 HEPES (pH 7.3), then loaded with 1 μM Fura-2/AM in ACSF for 30 min at 37°C to remove the excess extracellular Fura-2/AM. Coverslips were then mounted on a chamber positioned on the movable stage of an inverted microscope (TE2000, Japan), which was equipped with a calcium imaging system (PTI, USA). The cells were superfused by ACSF at a rate of 2 mL·min^{-1} for 10 min. Fluorescence was excited at wavelengths of 340 nm for 150 ms and 380 nm for 50 ms at 1 s interval by a monochromator (PTI K-178-S) and the emission was imaged at 510 nm with a video camera (CoolSNAP HQ2, ROPPER, USA) through fluor oil-immersion lens (Nikon) and a wide band emission filter. F340/F380 fluorescence ratio was recorded and analysed by MetaFluor version 6.3 software. All experiments were repeated at least three times using different batches of cells to ensure reproducibility.

Oxygen-glucose deprivation

The procedure for oxygen-glucose deprivation-reperfusion (OGD-R) was performed as described in our previous reports (Wang *et al.*, 2009; Zhang *et al.*, 2009). To initiate the OGD challenge, PC12 cells or cortical neurons at day 7 *in vitro* were rinsed twice and incubated in Earle's solution without glucose containing (in mM): 116 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1.0 NaH_2PO_4, 1.8 $CaCl_2$ and 26 $NaHCO_3$ (pH 7.3). Cells were then incubated at 37°C in an oxygen-free incubator (95% N_2 and 5% CO_2) for 4 h (PC12 cells) or 2 h (cortical neurons). Control cells were incubated in Earle's solution containing 5.6 mM glucose and maintained in an incubator with 5% CO_2 at 37°C. After the deprivation period, cultures were returned back to the normal culture medium under normoxic conditions for 24 h, which was the recovery period. The different concentrations of SN were added to the culture medium 24 h before OGD until the end of recovery.

Assessments of cell viability

After various treatments, cell viability was measured by using the MTT assay, which was based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases. Cell cultures were incubated with MTT solution (5 mg·mL^{-1}) for 4 h at 37°C. Then, the medium was discarded and DMSO was added to solubilize the reaction product formazan by shaking for 15 min. Absorbance at 492 nm was measured with a microplate reader (ELx800, Bio-Tek, Winooski, VT, USA). Cell viability of vehicle group that was not exposed to OGD was defined as 100%. Cell viability was expressed as a percentage of the value in the control group. To confirm cell death, the amount of LDH released in the medium and total LDH were determined after 18 h of OGD. The assay for LDH activity was performed according to the protocols of the LDH kit. Briefly, an aliquot of the culture supernatants (extracellular) or cell dissociation solution (intracellular) was mixed with nicotinamide adenine dinucleotide and lactate solution. Colorimetric absorbance was measured at 490 nm with a microplate reader. Total LDH activity was calculated by adding the values for extracellular and intracellular LDH that were measured in live cells treated with 1% Triton X-100. The ratio of released LDH (extracellular) versus total LDH (extracellular + intracellular) was calculated and expressed as a percentage of total LDH.

Analysis of drug tissue distribution

Adult male SD rats (3 months), weighing 220–250 g, were used to assess the levels of SN in different tissues. To avoid possible breakdown of SN by intestinal mucosa, SN (10 mg·kg^{-1} body weight) was dissolved in a total volume of 1 mL solvent and administered i.p.; 0.5 h later, the animals were anaesthetized with chloral hydrate (350 mg·kg^{-1}, i.p.) and intracardially perfused with 0.9% saline. Then, the heart, liver and brain samples were collected. Each sample (about 0.5 g wet weight) was rinsed with 0.9% NaCl, weighed, and immediately frozen at –80°C. Ice-cold PBS (0.5 mL) was added to the tissue samples, which were homogenized for 10 min in an ice-water bath. The homogenized mixtures were then centrifuged at 12 000× *g* for 10 min. The supernatant was collected and 1 mL of methanol was added. The mixture was again centrifuged at 12 000× *g* at 4°C for 15 min. The supernatant was collected for analysis and the recovery was

estimated by spiking control samples with known amounts of SN. Twenty microlitres of sample were loaded into the HPLC system coupled with a Thermo Finnigan LC-MS system (ThermoFinnigan Corporation, San Jose, CA, USA), including a Surveyor MS pump, LCQ DECA XPPlus MS detector, an auto sampler and Xcalibur TM 1.3 software. The mass spectra were acquired using electrospray ionization in positive ion mode and MRM. Data were acquired with Xcalibur 1.3. Peak areas were plotted against the concentration of SN and calibration lines were analysed by linear regression analysis. Calibration equation was $y = 158656x + 2E + 06$ with a correlation coefficient (r^2) of 0.9998. This calibration curve was used for the SN quantification.

Focal cerebral ischaemia and drug administration

Adult male rats (3 months, weighing 230–280 g) were housed under a 12 h light : 12 h dark cycle with food and water available *ad libitum* and were subjected to middle cerebral artery occlusion (MCAO) as described previously (Cai *et al.*, 2006; Liu *et al.*, 2010; Wang *et al.*, 2009). To avoid false-positive results of neuroprotection, the rats without deficits after MCAO were therefore excluded. After exclusion of these animals and those that did not survive the surgical procedure, the success rate of the procedure was more than 75%. The MCAO model and analysis was performed by a person who was unaware of the treatments to the different animals.

Rats were briefly anaesthetized with chloral hydrate (350 mg·kg^{-1}, i.p.). The left common carotid artery was exposed at its bifurcation and the external carotid artery was ligated. A 5–0 monofilament long nylon suture (22.0–25.0 mm) with rounded tip was introduced into the MCA through the left internal carotid artery up to the level of the anterior cerebral artery. The suture was inserted 18–20 mm from the bifurcation of common carotid to occlude the middle cerebral artery. After 120 min of MCAO, the suture was slowly withdrawn to allow reperfusion for 24 h. Body temperature was maintained at the 37°C with a homeothermic blanket. Sham-operated control animals underwent all the surgical procedures except occlusion of the MCA by introducing a short thread. A successful occlusion of the right middle cerebral artery is achieved when the left forelimb is paretic after surgery. In the first study of SN pretreatment on cerebral ischaemia, four groups were randomly assigned: sham group, MCAO group, MCAO + SN (10 mg·kg^{-1}) group, and MCAO + SN (30 mg·kg^{-1}) group. SN (10 and 30 mg·kg^{-1}) was dissolved in distilled water and administered i.p. 12 h before MCAO and again 0.5 h before the reperfusion. In the second group, designed to study the time window of SN for the treatment of cerebral ischaemia, SN was administered i.p. at 1, 2 and 3 h after MCAO at the dosage of 30 mg·kg^{-1}. In the final group, to examine the effect of SN on functional recovery after cerebral ischaemia, SN was administered at the dosage of 30 mg·kg^{-1} i.p. 1 h after MCAO and once daily for a week after MCAO. The sham group and MCAO group of animals received an equal volume of distilled water.

Infarct volume measurement

After 24 h of reperfusion, animals were killed by decapitation under 10% chloral hydrate anaesthesia to investigate the effect of SN pretreatment on cerebral ischaemia and its time window. The brains were quickly removed on ice. Five 2 mm consecutive coronal slices were made starting from the anterior pole and incubated in saline solution containing 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 30 min. The slices were then fixed by immersion in 4% formalin neutral buffer solution (pH 7.4) for 1 h. The infarct area in each slice was evaluated by scanned digital images with an image analysing system (NIH image, public domain software developed at the United States National Institutes of Health). Infarct volume of each slice was obtained by multiplying the infarct area by 2 mm thickness. Total infarct volume was determined by adding infarct volume of the five consecutive slices.

Western blot analysis

Each rat was killed by 10% chloral hydrate anaesthesia after 120 min of MCAO followed by 24 h reperfusion to investigate the effect of SN pretreatment on cerebral ischaemia. The infarct side of the cortex (1–5 mm posterior to the bregma and 1–5 mm beside the sagittal suture) was isolated for the assay of protein expression. The brain tissue was washed twice with ice-cold PBS and then lysed on ice in extraction buffer containing 50 mM Tris-base (pH 7.4), 100 mM NaCl, 1% NP-40, 10 mM EDTA, 20 mM NaF, 1 mM PMSF, 3 mM Na_3VO_4 and protease inhibitors. The homogenates were centrifuged at 12 000× *g* for 15 min at 4°C. Supernatant was separated and stored at –80°C until use. Protein concentration was determined using the BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Protein samples (50 μg) were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes. After being blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature, transferred membranes were incubated overnight at 4°C with different primary antibodies (Bcl-2: 1:500 dilution; Bax, Casepase-3: 1:300 dilution; phospho-CaMKII and CaMKII 1: 400 dilution). Following three washes with TBST, membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:3000) in TBST with 1% nonfat milk for 1 h at room temperature. After repeated washes, membranes were reacted with ECL reagents (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) for 5 min, and visualized with X-ray films (Kodak X-Omat, Rochester, NY, USA). The films were scanned and the optical density of the bands was determined using Optiquant software (Packard Instrument). Normalization of results was ensured by running parallel Western blots with β-actin.

Assay for functional recovery after cerebral ischaemia

In the final study, the effect of SN on functional recovery after cerebral ischaemia was examined. After 120 min of MCAO, the neurological scores were recorded on a scale of 0 to 4 as described previously (Murakami *et al.*, 1998) every day for a week to evaluate the effect of SN on functionary recovery after cerebral ischaemia. Neurological scores were graded as follows: score 0 means no neurological deficit; 1 means failure to fully extend the right forepaw; 2 means circling to

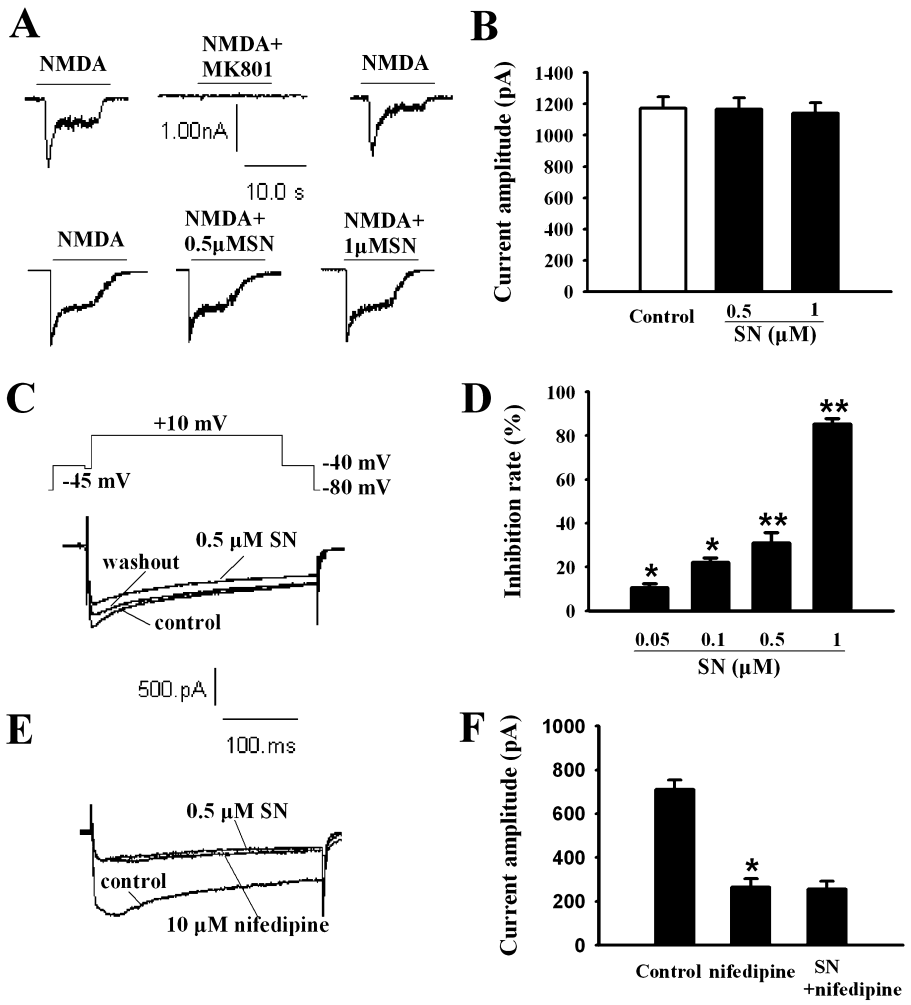

Figure 2

SN inhibits VGCCs, but not NMDA currents in rat cultured cortical neurons. SN failed to affect NMDA-activated currents in rat cultured cortical neurons. Representative traces (A) and histogram (B) showing that 0.5 and 1 μM SN failed to inhibit NMDA-activated currents in rat cultured cortical neurons. Data are expressed as means ± SEM and were analysed by Student's paired *t*-test. $n = 4$. (C) Representative traces and (D) histogram showing that 0.5 μM SN inhibited I_{HVA} in cortical neurons. (E) and (F) Representative traces and histogram showing that 0.5 μM SN failed to inhibit I_{HVA} in the presence of nifedipine (10 μM) in cultured cortical neurons. Data are expressed as means ± SEM and were analysed by Student's paired *t*-test. $n = 6$, $^*P < 0.05$ and $^{**}P < 0.01$ vs. control.

the right; 3 means falling to the right; and 4 means unable to walk spontaneously. Body weights were also monitored every day for a week.

Statistical analysis

Data are expressed as means ± SEM. Student's paired *t*-test was used for the statistical analysis of electrophysiological data in Figures 2 and 3 using SPSS 11.0 software. Student's *t*-test was used to analyse the data of neurological scores and body weight in Figure 6 for each test day respectively between drug- and vehicle-treated groups. Other data were analysed by ANOVA followed by Student-Newman–Keuls test using SPSS 11.0 software. Differences were considered significant at $P < 0.05$.

Materials

Sinomenine (dissolved in distilled water, molecular weight: 329.39, purity above 98%) was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). DMEM, fetal bovine serum, horse serum and G418 were purchased from Gibco Invitrogen Corporation (Carlsbad, CA, USA). pCMV6-ASIC1a plasmid was purchased from Bioyong Technologies Inc (Beijing, China). Lipofectamin 2000 was obtained from Invitrogen. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and trypsin were obtained from Sigma-Aldrich (St. Louis, MO, USA). The reagent kit for determining LDH was purchased from Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China). Fura-2/AM was obtained from Biotium (Hayward, CA, USA). Psalmotoxin 1 was purchased

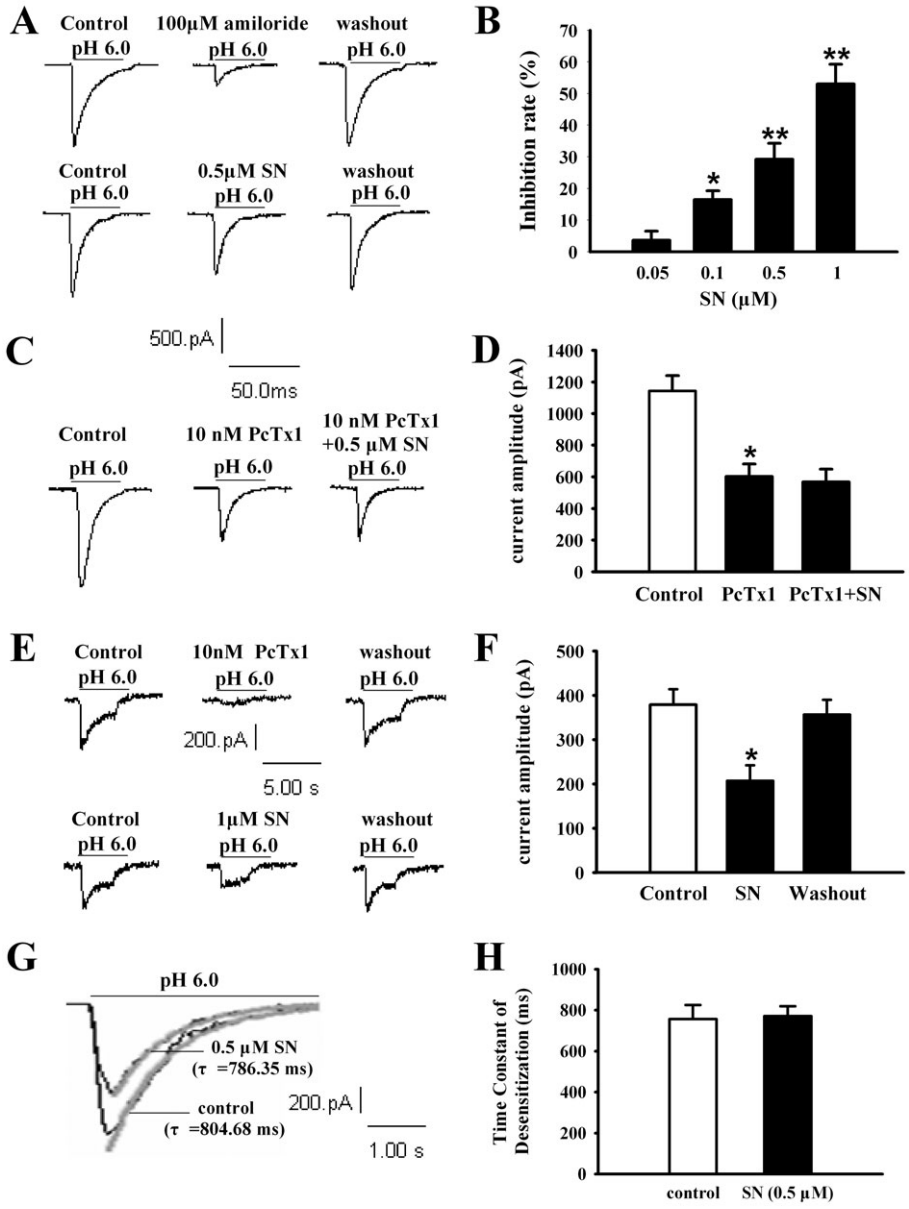

Figure 3

SN inhibits ASICs in rat cultured cortical neurons and transfected CHO cells. (A) and (B) Representative traces and histogram of results showing that different concentrations of SN inhibited ASICs currents in cortical neurons ($n = 6$). (C) and (D) Representative traces and histogram of results showing that 0.5 μM SN failed to inhibit ASICs currents in the presence of an ASIC1a blocker in cortical neurons ($n = 4$). (E) and (F) Representative traces and histogram showing that 10 nM PcTx1 and 1 μM SN inhibited the ASIC1a current expressed in CHO cells ($n = 6$). Data are expressed as means ± SEM and were analysed by Student's paired *t*-test, $^*P < 0.05$ and $^{**}P < 0.01$ vs. control. (G) and (H) Representative traces and summary data showing SN had no effect on the desensitization of ASICs currents in cortical neurons. $n = 6$.

from Alomone labs (Jerusalem, Israel). Primary antibodies of Bax and Bcl-2 were purchased from Cell Signaling Technology Inc. (San Francisco, CA, USA). Primary antibodies of TNF-α, activated-caspase-3, phospho-CaMKII and CaMKII were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other general agents were commercially available.

Results

SN exerts protective effects against OGD-R-induced neurotoxicity in PC12 cell and cerebral ischaemia in rats

Firstly, the cytoprotection of SN was evaluated using PC12 cells. As shown in Figure 1B, the cell viability decreased by 47.21 ± 5.12% after exposed to OGD for 4 h followed by 24 h

re-oxygenation by MTT assay (n = 6, P < 0.01 vs. control). To examine the cytoprotective effects of SN against OGD-R-induced cytotoxicity, different concentrations of SN were added to the culture medium 24 h before OGD until the end of recovery. As shown in Figure 1B, pretreatment with SN significantly attenuated OGD-R-induced PC12 cell death in a concentration-dependent manner. The cell viability was significantly increased to 64.78 ± 5.17% (n = 6, P < 0.05 vs. OGD-R), 82 ± 8.55%, 91.81 ± 8.21% and 84.11 ± 4.11% (n = 6, P < 0.01 vs. OGD-R) by administration of 0.1, 0.5, 1 and 5 μM SN, respectively. In order to confirm the cytoprotective effect of SN, another cell viability assay that measured LDH release was performed after OGD treatment in PC12 cells. It has been previously established that LDH release correlates linearly with the number of damaged cells after toxic insult. In this study, SN at 0.1, 1 and 5 μM significantly reduced OGD-induced LDH release by 22.73 ± 1.96%, 41.72 ± 1.79% and 22.44 ± 2.09%, respectively (n = 6, P < 0.01 vs. OGD-R, Figure 1C).

Secondly, the protective effects of SN on cerebral ischaemia in rats were investigated. As shown in Figure 1D, SN concentration in the brain was 0.11 ± 0.04 μg·g^{-1} (0.340 ± 0.134 μM) (n = 3) at 0.5 h after i.p. injection of 10 mg·kg^{-1} SN, which offered cytoprotection to cells, suggesting that SN can cross the blood–brain barrier and act as a protective agent in the CNS. Next, a model of transient focal ischaemia produced by MCAO was employed and SN was administered i.p. at doses of 10 mg·kg^{-1} and 30 mg·kg^{-1} 12 h before MCAO and again 0.5 h before the onset of reperfusion following 120 min of MCAO in rats. TTC staining showed both doses of SN significantly reduced the infarct area in all five consecutive coronal sections compared with corresponding ischaemic sections (Figure 1E) and decreased the total infarct volume from 581.16 ± 30.70 mm^3 to 172.62 ± 24.98 mm^3 and 94.10 ± 15.75 mm^3, respectively (n = 6, P < 0.01 vs. MCAO, Figure 1F). SN (30 mg·kg^{-1}) seemed to have no effect on TTC staining in the sham rats (Figure S1). Furthermore, the expression levels of Bcl-2, Bax and activated caspase-3 proenzyme in cerebral cortical tissues were analysed by Western blotting. After 24 h reperfusion, the expression of Bax and activated caspase-3 increased and the ratio of Bcl-2/Bax expression was decreased markedly in the ipsilateral hemisphere compared with the levels obtained in the corresponding areas of the sham group. However, pretreatment with SN (30 mg·kg^{-1}) significantly inhibited the elevation of active caspase-3 from 1.25 ± 0.16 to 0.9 ± 0.08 and attenuated the reduction of the Bcl-2/Bax ratio induced by ischaemic cerebral injury from 0.50 ± 0.12 to 1.13 ± 0.22, respectively (n = 3, P < 0.05 vs. MCAO, Figure 1G, H), suggesting that SN exhibited neuroprotection *in vivo* via inhibition of the increase in apoptosis-related protein induced by brain ischaemia. CaMKII regulates many functions related to calcium signal and a massive increase in the intracellular concentration of free calcium following brain ischaemia leads to an over-autophosphorylation of CaMKII. We chose phosphorylation of CaMKII as an indicator to reflect the effect of SN on the calcium-mediated events *in vivo*. As shown in Figure 1I, after 2 h of MCAO treatment and 24 h reperfusion, the autophosphorylation level of CaMKII was up-regulated from 0.66 ± 0.08 to 1.20 ± 0.07 (n = 3, P < 0.05 vs. sham). However, the up-regulation was significantly attenuated by SN (30 mg·kg^{-1}) pretreatment from 1.20 ± 0.07 to 0.80 ± 0.12 (n = 3, P < 0.05 vs. MCAO), which was consistent with our observation with calcium imaging *in vitro*. Considering that SN had no direct effect on the autophosphorylation of CaMKII in sham rats (Figure S2), the inhibition of calcium signals in pathological conditions may be involved in the neuroprotective effect of SN.

SN inhibits L-type calcium currents, but not NMDA current in rat cultured cortical neurons

Abnormal entrance of calcium into neurons during ischaemia is recognized as the main factor to trigger cascade pathways associated with cell injury. The NMDA receptor is one of the most important targets for the treatment of cerebral ischaemia, due to its permeability to calcium during this procedure. In order to determine weather the effect of SN is produced by regulation of NMDA receptor current, the NMDA-activated current was investigated in rat cultured cortical neurons. As shown in Figure 2A, B, at a holding potential of –60 mV, application of 100 μM NMDA to rat cultured cortical neurons evoked an inward current, which was abolished by a selective NMDA receptor blocker MK801 (30 μM). However, SN (0.5 and 1 μM) had no significant effect on NMDA-activated currents (n = 4, P < 0.05 vs. control), suggesting that other targets may be involved in the effect of SN on cellular calcium signals.

Next, we observed the effect of SN on VGCCs, another main pathway for calcium entrance. The high voltage-activated calcium current (I_{HVA}) was investigated in the rat cultured cortical neurons. The cells were stepped from the holding potential of –80 mV to –40 mV (50 ms), and then depolarized to +10 mV (200 ms) after the brief hyperpolarization to –45 mV for 10 ms. The I_{HVA} was activated by the second depolarization. The protocol was applied every 5 s. Extracellular application SN inhibited VGCCs currents in a concentration-dependent manner. The inhibition rate of 0.05, 0.1, 0.5 and 1 μM SN on I_{HVA} was 10.63 ± 1.73%, 22.28 ± 1.88%, 30.99 ± 4.81% and 85.33 ± 2.52%, respectively (n = 6, P < 0.05 vs. control, Figure 2C, D). The inhibitory effect was partially reversible by application of bath solution and the IC_{50} was 0.56 ± 0.13 μM. In order to determine the selectivity of SN on different VGCC subtypes, we performed additional experiments by blocking the L-type calcium channel. In the presence of the L-type calcium channel blocker nifedipine (10 μM), SN did not inhibit VGCCs any more (n = 6, P < 0.05 vs. control, Figure 2E, F), suggesting that inhibition of the L-type calcium channel mediates the main effect of SN on VGCCs.

SN inhibits ASIC1a currents in rat cultured cortical neurons and transfected CHO cells

Because ASICs, especially ASIC1a, also induce Ca^{2+} entry directly and constitute an additional Ca^{2+} entry pathway in ischaemic neurons, the effect of SN on ASICs currents in rat cultured cortical neurons was investigated using the whole-cell patch-clamp techniques. At a holding potential of –80 mV, a rapid reduction of extracellular pH to 6.0 evoked large transient inward currents, ASICs currents, which was blocked by 100 μM amiloride (Figure 3A). Extracellular application SN inhibited ASICs currents in a concentration-

dependent manner. SN 0.1, 0.5 and 1 μM significantly inhibited ASICs currents and the inhibition rate was 16.43 ± 2.82%, 29.20 ± 5.09% and 53.04 ± 6.22%, respectively. However, 0.05 μM SN exhibited little effect on ASICs currents ($n = 6$, $P < 0.05$ vs. control, Figure 3B). The inhibitory effect of SN was partially reversible by application of bath solution and the IC_{50} was 0.27 ± 0.09 μM. In order to determine the selectivity of SN on different ASIC subtypes, we performed additional experiments to investigate the effect of SN on ASICs in the presence of a selective ASIC1a blocker PcTx1. It was found that SN had no effect on ASICs in the presence of PcTx1 (10 nM), suggesting that the inhibition of ASICs by SN is ASIC1a-dependent ($n = 4$, $P < 0.05$ vs. control, Figure 3C, D). To determine whether inhibition of ASICs currents by SN in cortical neurons was a direct pharmacological action on ASIC1a, the effect of SN on ASIC1a currents in transfected CHO cells was examined. As shown in Figure 3E, ASIC1a currents expressed in CHO cells, which can be completely abolished by 10 nM PcTx1, were significantly reduced by 1 μM SN from 378.81 ± 35.36 to 207.16 ± 35.43 pA, with an inhibitory rate of 47.32 ± 4.73% ($n = 8$, $P < 0.05$ vs. control, Figure 3F). These results suggest that SN directly inhibits ASIC1a currents in cultured cortical neurons and ASIC1a expressed CHO cells. As the kinetics of channel opening may affect channel activity and increase calcium influx, the time constant (τ) of desensitization of ASICs currents was fitted to a mono-exponential function. It was found that the τ value of ASICs currents in the control and 0.5 μM group was 756.89 ± 68.66 and 771.87 ± 48.22 ms, respectively, indicating that SN has no affect on ASICs currents desensitization in cortical neurons ($n = 6$, Figure 3G, H).

SN inhibits KCl and acidification-mediated increase in $[Ca^{2+}]_i$ in rat cultured cortical neurons

Overactivation of calcium-mediated events plays an essential pathological role in the irreversible neuronal injury caused by cerebral ischaemia. The resultant opening of VGCCs after depolarization of cell membrane potential caused by inactivation of Na^+, K^+-ATPase and activation of ASIC1a after extracellular acidulation contribute to calcium influx under ischaemic conditions. With regard to the simultaneous regulation of ASICs and VGCCs by SN, we further investigated the effect of SN on $[Ca^{2+}]_i$ increase caused by depolarization of cell membrane potential (KCl) and extracellular acidosis. It was found that 30 mM KCl initiated a significant increase in $[Ca^{2+}]_i$ by 105.88 ± 9.68% in cultured cortical neurons (Figure 4A), and pretreatment with SN (1 μM) attenuated this increase to 35.29 ± 10.05% (Figure 4B). Extracellular acidosis also initiated a significant increase in $[Ca^{2+}]_i$ by 48.08 ± 7.04% (Figure 4C), and pretreatment with SN (1 μM) attenuated this increase to 9.62 ± 6.86% (Figure 4D). These results suggest that SN antagonizes the increase in $[Ca^{2+}]_i$ after cerebral ischaemia.

Antagonism of L-type calcium channel and ASIC1a contribute to the neuroprotection of SN against ischaemic insults in cultured cortical neurons

In order to study whether the inhibition of L-type calcium channels and ASIC1a by SN is really involved in the neuroprotective effect, the neuroprotection of SN was investigated using cultured cortical neurons. After OGD-R treatment, the cell viability was significantly decreased by 43.89 ± 5.29%. Pretreatment with 0.1, 0.5 and 1 μM SN significantly increased cell viability to 72.09 ± 4.54%, 82.35 ± 7.12% and 88.29 ± 3.90%. The L-type calcium channel blocker nifedipine (10 μM) also produced significant protection, while the ASIC1a blocker PcTX1 (10 nM) seemed to have no effect ($n = 8$, $P < 0.05$ vs. OGD-R, Figure 5A). As the *in vitro* model of OGD-R cannot mimic the extracellular acidosis directly that occurs during cerebral ischaemia, direct extracellular acidosis was employed to induce neurotoxicity. After being exposed to extracellular acidosis (pH = 6.0) for 2 h, the cell viability was decreased by 32.98 ± 6.81%. SN (0.5, 1 μM) did not change the pH of the extracellular solution. However, PcTX1 10 nM and SN (0.5, 1 μM) increased cell viability to 95.44 ± 4.44%, 79.14 ± 5.28% and 92.55 ± 5.58%, respectively ($n = 8$, $P < 0.05$ vs. pH 6.0, Figure 5B). Interestingly, 10 μM nifedipine had no effect. As both of OGD and extracellular acidosis are pathological factors involved in cerebral ischaemia, the antagonism of SN to ASICs and VGCCs contribute, at least partially, to its neuroprotection against ischaemic brain injury *in vivo*.

Administration of SN after cerebral ischaemia protects against brain injury and improves functional recovery

We further investigated the time window for SN treatment after cerebral ischaemia, because a suitable time window for treatment of ischaemic brain injury is very important for the therapeutic applicability of SN in the clinic. SN was infused i.p. at 1, 2 and 3 h after MCAO and the volume of the ipsilateral hemisphere infarct was evaluated respectively. Compared with the MCAO group, i.p. administration of SN (30 $mg \cdot kg^{-1}$) at 1 and 2 h after MCAO significantly reduced the infarct volume from 573.35 ± 28.69 mm^3 to 142.50 ± 48.72 mm^3 and 403.44 ± 29.62 mm^3, respectively. However, SN administration (30 $mg \cdot kg^{-1}$) at 3 h after MCAO did not reduce the infarct volume ($n = 5$, Figure 6A, B). To evaluate the effects of SN administration on functional recovery and survival after cerebral ischaemia, SN (30 $mg \cdot kg^{-1}$) or saline was administered i.p. 1 h after MCAO and once daily for a week after surgery. As shown in Figure 6, compared to the MCAO group, SN administration significantly improved behaviour scores after stroke and recovered body weight ($n = 5$, $^*P < 0.05$ and $^{**}P < 0.01$ vs. MCAO, Figure 6C, D). These results suggest that administration of SN after cerebral ischaemia protects brain injury and improves functional recovery.

Discussion

In the present study, it was first demonstrated that SN, a plant-derived bioactive alkaloid with clinical anti-inflammatory effect (Feng *et al.*, 2006), has a significant inhibitory effect on ASICs and VGCCs in rat cultured cortical neurons. As a dual blocker of ASICs and VGCCs, SN attenuated the increase in $[Ca^{2+}]_i$ caused by depolarization of cell membrane potential and extracellular acidification in cortical

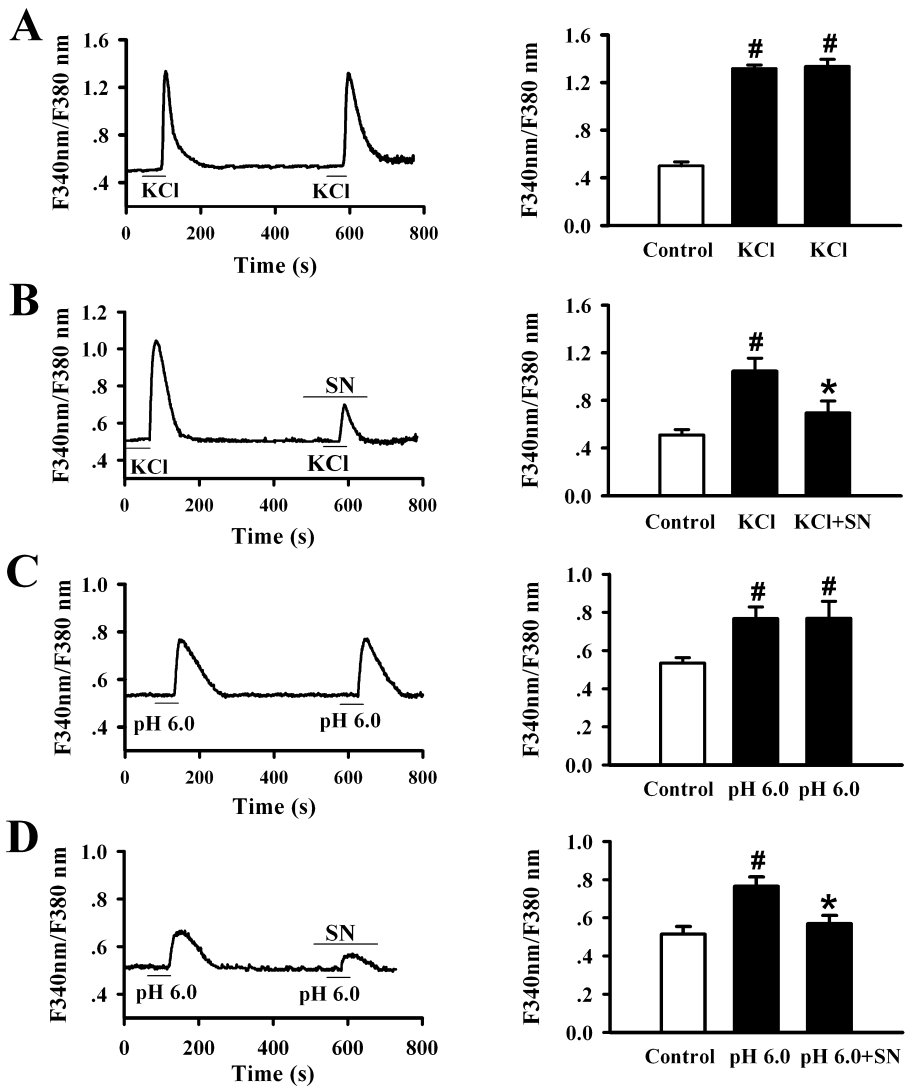

Figure 4

SN inhibits $[Ca^{2+}]_i$ increase induced by KCl and extracellular acidosis in rat cultured cortical neurons. (A) Representative traces and histogram of results showing that $[Ca^{2+}]_i$ responds to repeated KCl stimulation under control conditions with a time interval of 5 min between stimuli. Repeated stimulation with KCl (30 mM) produced an equal transient $[Ca^{2+}]_i$ increase. $n = 7$. (B) Representative traces and histogram of results showing that SN inhibited $[Ca^{2+}]_i$ increase induced by KCl. $n = 10$. (C) Representative traces and histogram of results showing that $[Ca^{2+}]_i$ responds to repeated acid stimulation (extracellular acid solution, pH = 6.0) under control conditions with a time interval of 5 min between stimuli. $n = 8$. (D) Representative traces and histogram of results showing that SN inhibited $[Ca^{2+}]_i$ increase induced by extracellular acid solution. $n = 9$. Data are expressed as means ± SEM and were analysed by Student's paired *t*-test, #$P < 0.05$ vs. control, *$P < 0.05$ vs. KCl or pH 6.0.

neurons. SN conferred marked cytoprotection against OGD-R and extracellular acidosis-induced cell injury. Pretreatment with SN (10 and 30 mg·kg^{-1}, i.p.) significantly decreased brain infarction in rats subjected to 120 min ischaemia followed by 24 h reperfusion. Down-regulation of calcium-mediated events after cerebral ischaemia was at least partly involved in the neuroprotection of SN. More importantly, administration of SN (30 mg·kg^{-1}, i.p.) after cerebral ischaemia also protected brain injury and improved functional recovery. Our results suggest that co-inhibition of ASIC1a and L-type calcium channels contribut to the neuroprotection of SN. Therefore, SN appears to fall into the class of multifunctional compounds, which may be a potential new drug for the treatment of stroke.

Ischaemic brain injury is one of the leading causes of death and adult disability worldwide, and identifying new therapeutic agents is a major health imperative. Although the preclinical studies are encouraging, the trials for acute stroke therapy are disappointing. Therefore, recent efforts have been focused on drugs with multifaceted mechanisms of action, such as PAN-811 (Jiang *et al.*, 2006a), which possesses at least two definite and independent action targets and shows a much wider spectrum of action and more potent protection.

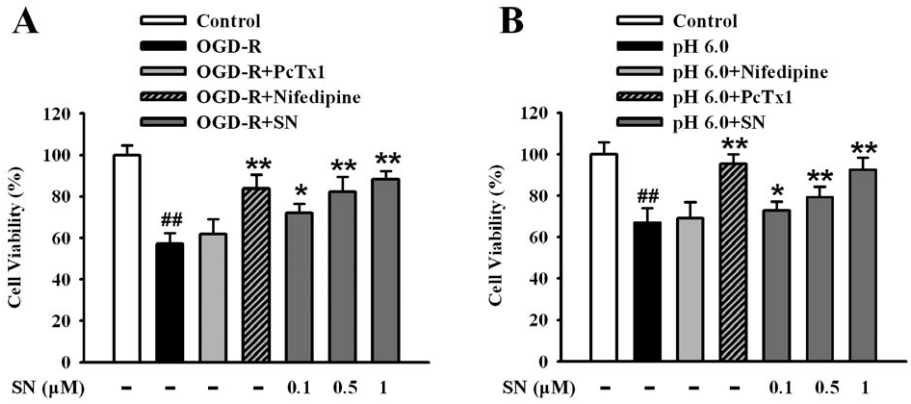

Figure 5

Antagonism of L-type channels and ASIC1a contribute to the neuroprotection of SN against ischaemic insults in cortical neurons. (A) Nifedipine (10 μM) and different concentrations of SN reduced OGD-R-induced cell death in cultured cortical neurons. (B) PcTx1 and different concentrations of SN reduced extracellular acidosis-induced cell death in cultured cortical neurons. Data are expressed as means ± SEM. $n = 8$, ##$P < 0.01$ vs. control, *$P < 0.05$ and **$P < 0.01$ vs. OGD-R or pH 6.0.

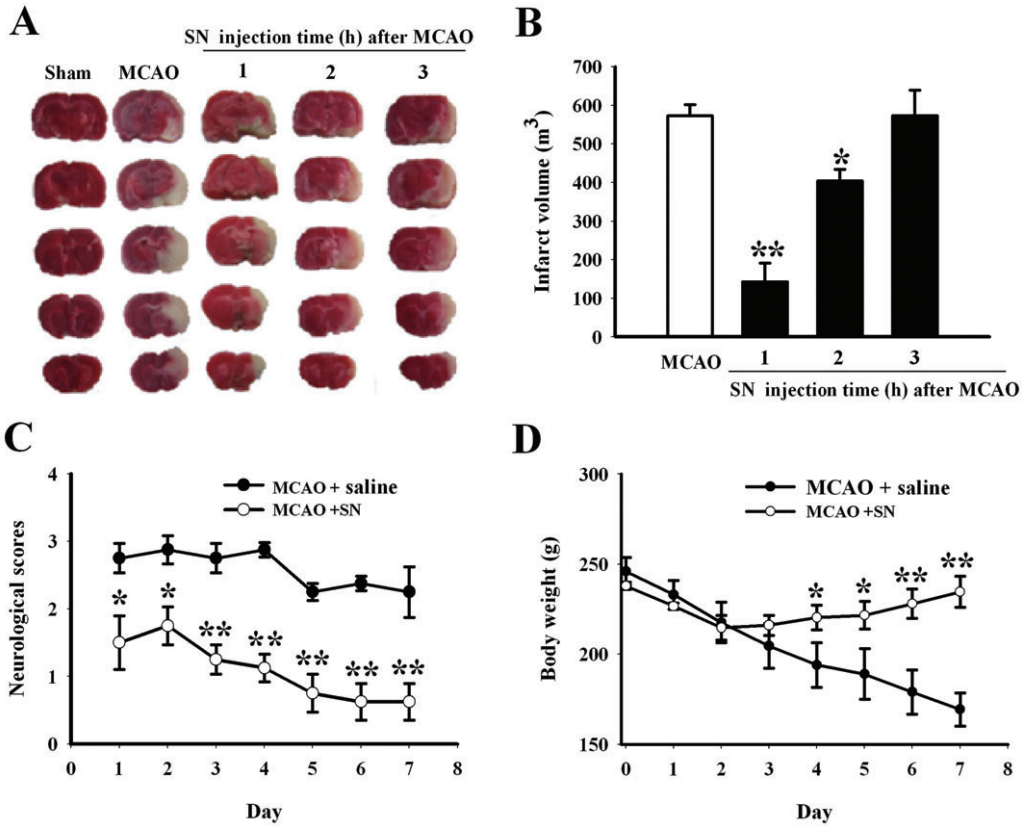

Figure 6

Administration of SN after cerebral ischaemia protects against brain injury and improves functional recovery. (A) Effect of SN (30 mg·kg^{-1}, i.p) administration, at 1, 2 and 3 h after cerebral ischaemia, on infarct volume induced by 120 min of MCAO, evaluated 24 h after induction of ischaemia. The white brain area represents infarcted tissue. (B) Infarct volume was measured in total brain. Data are expressed as means ± SEM, $n = 5$. *$P < 0.05$ and **$P < 0.01$ vs. MCAO. (C) Neurological scores were graded. SN (30 mg·kg^{-1}) or saline (control) was injected 1 h after cerebral ischaemia and once daily for a week. Data are expressed as means ± SEM. $n = 4$, *$P < 0.05$ and **$P < 0.01$ vs. saline at the same day. (D) Body weights were monitored after administration of saline or SN (30 mg·kg^{-1}) at 1 h after cerebral ischaemia and once daily for a week. Data are expressed as means ± SEM. $n = 5$, *$P < 0.05$ and **$P < 0.01$ vs. saline at the same day.

It has been demonstrated that abnormal cellular calcium load plays a critical role in ischaemic brain injury. Inactivation of Na^+, K^+-ATPase caused by ATP depletion results in depolarization of cell membrane potential in neurons. This may consequently initiate cation influx via voltage-gated cation channels. In previous studies, much attention has been paid to VGCCs and blockers of these calcium channels, which are used as neuroprotective agents in clinical therapy (Miyazaki *et al.*, 1999; Hicks *et al.*, 2000; Taya *et al.*, 2000). Our present study has demonstrated that SN strongly inhibits I_{HVA} and significantly attenuates KCl-induced calcium overload in rat cultured cortical neurons, suggesting that SN can be used as a novel blocker of VGCCs and can reduce calcium-related pathobiochemical processes. In the presence of the L-type calcium channel blocker nifedipine, SN had no effect on VGCCs, suggesting that the regulation of VGCCs by SN is mediated by L-type calcium channels. CaMKII is a ubiquitously expressed protein kinase and regulates many functions related to calcium signals. Elevated intracellular free calcium triggers autophosphorylation of CaMKII at a threonine residue in the inhibitory domain (Thr286 in CaMKIIα), thereby converting the kinase to a persistently active state (Lisman *et al.*, 2002). However, the increase in intracellular free calcium following brain ischaemia leads to a massive and sustained autophosphorylation of CaMKII and contributes to ischaemic brain injury (Waxham *et al.*, 1996; Matsumoto *et al.*, 2002). In our study, the over-autophosphorylation of CaMKII after 120 min ischaemia and 24 h reperfusion was significantly reduced by SN treatment, suggesting SN inhibits the calcium overload induced by ischaemia in these pathological conditions. The increase in $[Ca^{2+}]_i$ can initiate neuronal death by promoting apoptosis. Pretreatment with SN (30 $mg \cdot kg^{-1}$) significantly inhibited the elevation of active caspase-3 and attenuated the reduction of the Bcl-2/Bax ratio induced by cerebral ischaemia. This anti-apoptosis effect may be involved in the protective effect of SN.

In addition to VGCCs, Ca^{2+}-permeable acid-sensing ion channels ASIC1a constitute an important Ca^{2+} entry pathway in ischaemic neurons. Therefore, a redirection of attention to other cation channels, including ASICs, has emerged in recent years as a novel therapeutic target of stroke. Acidosis is a common feature in acute ischaemic brain injury; previous studies have demonstrated that activation of calcium-permeable ASIC1a is largely responsible for acidosis-mediated neuronal injury (Yermolaieva *et al.*, 2004; Xiong *et al.*, 2006b). Activation of ASICs can directly or indirectly induce an increase in the concentration of intracellular calcium ($[Ca^{2+}]_i$) and cause further neuronal injury. Intracerebroventricular administration of ASIC1a blockers or knockout of the ASIC1a gene protects brain from injury (Xiong *et al.*, 2004). Hence, ASICs have been proved to be a novel neuroprotective target for stroke patients and the search for ASIC inhibitors has attracted much interest and pharmacological research. Previous studies revealed that NSAIDs such as aspirin, diclofenac and flurbiprofen directly inhibit ASIC currents in neurons and heterologously expressed COS-7 cells (Voilley *et al.*, 2001). In the present study, we demonstrated for the first time that SN has an inhibitory effect on ASIC currents in cortical neurons and ASIC1a expressed in CHO cells. After application of PcTx1, an ASIC1a inhibitor, the inhibitory effect of SN was attenuated, indicating that the effect of SN on ASICs is mediated by ASIC1a. Previous studies have demonstrated that PcTx1 inhibits the acid response by 47, 53.2 and 46% in cortical neurons, medium spiny neurons and hippocampal neurons (Baron *et al.*, 2002; Xiong *et al.*, 2004; Jiang *et al.*, 2009). Consistent with previous findings, our results show that 10 nM PcTx1 inhibits the acid response by about 52.7%. PcTx1 is regarded as a 'gating modifier', as it affects the opening and closing properties of ASIC1a. Moreover, PcTx1 does not inhibit homomeric ASIC1b, ASIC2a or ASIC3, nor heteromeric ASIC1a-containing channels (Escoubas *et al.*, 2000; Diochot *et al.*, 2007). In our experimental settings, whole-cell recording data indicated that SN is selective for homomeric ASIC1a. However, further investigations, using CHO cells transfected with heteromeric channels containing ASIC1a, are needed to confirm this.

The development of novel pharmacological approaches for ischaemic brain injury with multifunctional neuroprotection has attracted much attention in recent years (Youdim and Buccafusco, 2005; Jiang *et al.*, 2006b; Minnerup and Schabitz, 2009). Multiple pathological mechanisms are involved in brain ischaemia, therefore, new multifunctional neuroprotective agents theoretically are more effective clinically than classical drugs. SN is a bioactive alkaloid derived from the medicinal plant, *Sinomenium acutum*, and has been used clinically as an anti-inflammatory agent for many years with low toxicity and few side effects. Many alkaloids contained in medicinal herbs or health foods, such as berberine, oxymatrine and rutaecarpine, exhibit neuroprotection against several diseases involving the CNS (Dai *et al.*, 2008; Zhou *et al.*, 2008; Cui *et al.*, 2009; Liu *et al.*, 2009). Based on its molecular structure, SN belongs to the family of dextrorotatory morphinan analogues, and has been observed to afford neuroprotection in a variety of experimental models of CNS injury (Steinberg *et al.*, 1988; Werling *et al.*, 2007; Keller *et al.*, 2008). A recent study indicated that SN protects against the death of dopaminergic neurons by inhibiting microglial activation and this may become a new strategy to treat inflammation-mediated neurodegenerative diseases (Qian *et al.*, 2007). In the present experiments, the neuroprotective effects of SN against ischaemic brain injury both *in vivo* and *in vitro* were investigated. SN was found to inhibit ASICs currents and VGCCs currents, even at a relatively low concentration, 0.1 μM (ASICs) and 0.05 μM (VGCCs), close to the concentration obtained in the brain after administration of 10 $mg \cdot kg^{-1}$ SN. This indicates that SN is able to reach the concentrations required to bring blockade of these relevant targets *in vivo*. Blockade of L-type calcium channels and the ASIC1a channel were found to attenuate cell injury in cortical neurons induced by OGD-R and extracellular acidosis, respectively. SN did not affect NMDA receptor-induced currents, but the results do show that the blockade of cation channels by SN *in vitro* is associated with its neuroprotective effect *in vivo*. It is likely that its effects on VGCCs and ASICs are involved in its protective effects against brain injury caused by the overactivation of calcium-mediated events after cerebral ischaemia *in vivo*. The anti-inflammatory effect of SN may also be involved in its neuroprotection, as inflammation is one of the key mechanisms associated with the pathology of cerebral stroke. Hence, the effect of SN on brain ischaemia may involve multiple targets and mechanisms. However, it

has been demonstrated that the anti-inflammatory properties of SN, such as inhibition of TNF-α activity and blockade of NF-κB activation require a relatively high concentration (0.25–5 mM) (Huang *et al.*, 2007; Zhao *et al.*, 2007; Ou *et al.*, 2009), therefore, the inhibition of cation channels by low concentrations of SN is more likely to be responsible for its effect in the CNS.

A suitable therapeutic time window of novel agents is very important for them to have clinical value in the treatment of cerebral ischaemia. Our study further demonstrated that administration of SN (30 mg·kg^{-1}, i.p.) at 1 and 2 h after cerebral ischaemia significantly attenuated brain injury. In addition, administration of SN (30 mg·kg^{-1}, i.p.) 1 h after cerebral ischaemia and once daily for a week also improved functional recovery. It should be noted that SN needed to be applied within 2 h of ischaemic brain injury to produce a significant improvement.

In conclusion, SN exerts a potent protective effect against ischaemic brain injury when administered before ischaemia or even after the injury. The pharmacological profile of SN involved in the neuroprotection may include co-inhibition of ASIC1a and L-type calcium channel. Considering its diverse pharmacological profile, long history of clinical application, relatively low cost of preparation and appropriate physical properties, SN has the potential to be a suitable treatment for stroke.

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Conflict of interest

No conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Representative section showing SN administration had no influence on TTC staining in sham rats. (n = 4).
Figure S2 Representative immunoreactive bands showing SN had no influence on the activation of apoptosis-related protein and calcium signal in sham rats.

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