Potassium Channel Blockers Attenuate Hypoxia- and Ischemia-Induced Neuronal Death In Vitro and In Vivo

Ling Wei, MD; Shan Ping Yu, MD, PhD; Frank Gottron, PhD; B. Joy Snider, MD, PhD; Gregory J. Zipfel, MD; Dennis W. Choi, MD, PhD

Background and Purpose—In light of recent evidence suggesting that an upregulation of K⁺ efflux mediated by outward delayed rectifier (Iₖ) channels promotes central neuronal apoptosis, we sought to test the possibility that blockers of Iₖ channels might be neuroprotective against hypoxia/ischemia-induced neuronal death.

Methods—Membrane currents were recorded with the use of patch clamp recordings in cultured murine cortical neurons. Protective effects of K⁺ channel blockers were examined in rats subjected to transient middle cerebral artery occlusion followed by 14-day reperfusion.

Results—The K⁺ channel blocker tetraethylammonium (TEA) (5 mmol/L) selectively blocked Iₖ without affecting N-methyl-D-aspartate receptor–mediated current or voltage-gated Ca²⁺ currents. Both TEA and a lipophilic K⁺ channel blocker, clofilium, attenuated neuronal apoptosis induced by hypoxia in vitro and infarct volume induced by ischemia in vivo.

Conclusions—These data are consistent with the idea that K⁺ channel–mediated K⁺ efflux may contribute to ischemia-triggered apoptosis and suggest that preventing excessive K⁺ efflux through K⁺ channels may constitute a therapeutic approach for the treatment of stroke. (Stroke. 2003;34:1281-1286.)

Key Words: hypoxia ■ ischemia ■ middle cerebral artery occlusion ■ tetraethylammonium

Brain ischemia-induced neuronal death has been linked in part to excess Ca²⁺ influx through ionotropic glutamate receptors and voltage-gated Ca²⁺ channels.¹ ² As a result, considerable effort has been directed toward reducing neuronal circuit excitation as a strategy for neuroprotective intervention. Besides direct blockade of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors, additional strategies, including enhancing synaptic inhibition, reducing glutamate release, and reducing neuronal firing by blocking Na⁺ channels or opening K⁺ channels, have been tested.³ ⁴ The latter strategy, opening K⁺ channels, is supported by the observation that drugs that enhance opening of ATP-sensitive K⁺ channels (KₐTP channels) can reduce ischemia-induced necrotic cell death in the heart⁶ and brain.⁷ ⁸ Furthermore, the novel Ca²⁺-activated maxi-K channel opener, BMS-204352, was neuroprotective in rodent focal stroke models,⁹ although clinical trial results have been disappointing.¹⁰

Excessive K⁺ efflux and intracellular K⁺ depletion have been hypothesized to be key steps in the apoptotic cascade of many cells, including central neurons.¹¹ ⁻¹³ One possible factor limiting the benefit of enhancing K⁺ channel activity in the ischemic brain might be enhancement of an apoptotic component of focal ischemic neuronal death,¹⁴ ⁻¹⁶ a component that might be especially prominent when infarction is delayed after mild transient insults.¹⁶ ¹⁷ We observed that several apoptotic insults induced enhancements of the outward delayed rectifier (Iₖ) currents in cultured cortical neurons. This enhancement occurred well before cells committed to die, consistent with a potential role as an early mechanism in apoptosis.¹¹ ⁻¹⁸ ¹⁹ A close relationship between enhanced K⁺ channel activity and apoptosis is also supported by studies in other types of neurons and peripheral cells.²⁰ The potassium channel blockers tetraethylammonium (TEA) and clofilium or elevated extracellular K⁺ attenuated neuronal apoptosis in neurons¹¹ ⁻¹⁸ ⁻²⁰ ; the neuroprotection persisted even when the concurrent Ca²⁺ influx and intracellular Ca²⁺ increase were prevented.¹¹ ⁻¹⁸ ¹⁹ Recently, TEA was found to be neuroprotective against CA1 hippocampal injury after global ischemia in rats.²¹

To investigate a possible role of K⁺ channels in ischemia-induced apoptosis and potential therapeutic implications of the K⁺ channel hypothesis for apoptosis in ischemic brain injury, the present study explored protective effects of K⁺ channel blockers against neuronal death induced by hypoxia in vitro and by mild transient focal ischemia in the rat. This work was partially reported in an abstract.²²
Materials and Methods

Mouse Cortical Cultures
Mixed cortical cultures, containing both neurons and glia, were prepared as described previously.25 Swiss Webster mice of 15 to 17 days’ gestation (Taconic Farms, Germantown, NY) were anesthetized with inhaled halothane in accordance with institutional guidelines. After brain removal, dissociated neocortices were plated onto a previously established glial monolayer, at a density of 4 hemispheres per 10 mL, in 35-mm culture dishes for whole cell recordings and in 24-well plates for toxicity experiments, in Eagle’s minimal essential medium (Earle’s salts) containing 25 mmol/L glucose, 5% fetal bovine serum, and 5% horse serum. Cultures were kept in a 37°C, humidified incubator (Heraeus, Kendro) in a 5% CO2 atmosphere. All experiments were performed after 10 to 14 days in vitro. Glial cultures were prepared from dissociated neocortices of 1- to 3-day postnatal mice.

Electrophysiology
Whole cell voltage clamp and current clamp recordings were performed on the stage of an inverted microscope (Nikon) with an EPC-7 amplifier (List-Electronic, Germany); patch electrodes had tip resistance of 7 to 10 MΩ (fire-polished). Current and voltage tracings were collected with the use of a data acquisition/analysis program package, PULSE (HEKA Electoronik). Different solutions were used for recording K+, Ca²⁺, and NMDA receptor currents.11,18,19 In Ca²⁺ current recordings, TEA (10 mmol/L) was included in the external solution to block K⁺ channels. The membrane potential was measured in current clamp mode. For oxygen-glucose deprivation (OGD), recordings were performed 5 to 20 minutes after cultures were removed from the hypoxia chamber. All external solutions contained 0.1 μmol/L tetrodotoxin to prevent activation of Na⁺ channels. Solution pH was adjusted to 7.3; experiments were performed at room temperature (21°C to 22°C).

Oxygen-Glucose Deprivation
Mixed cortical cultures were placed into an anaerobic chamber (ThermoForma) containing a gas mixture of 5% CO₂, 10% H₂, and 85% N₂. The culture medium was exchanged with deoxygenated glucose-free Earle’s balanced salt solution (BSS) (in mmol/L: NaCl 116, KCl 5.4, MgSO₄ 0.8, NaH₂PO₄ 1.0, CaCl₂ 1.8, NaHCO₃ 26.2, glucose 5.6, and KCl 20 mL/L). Oxygen was removed from BSS, by bubbling solution with the anaerobic gas mixture. Cultures were deprived of oxygen and glucose for 45 to 55 minutes, then washed back into glucose-containing media and returned to the normoxic incubator for 24 hours. In some experiments, glutamate antagonists (1 μmol/L MK-801 and 100 μmol/L 6-cyano-7-nitroquinolin-xaline-2,3-dione [CNQX]) were included in exposure media, and the OGD period was extended to 110 minutes (“blocked OGD”). Cultures were washed back into glucose-containing media and returned to the incubator for 48 hours before the assessment of neuronal death.24

Cell Death Assays
Neuronal cell death was estimated by phase contrast microscopy and quantified by lactate dehydrogenase (LDH) release.25 For each culture plate the total amount of neuronal LDH was determined by incubating the cells with 300 μmol/L LDH release. For each culture plate the total amount of neuronal LDH was determined by incubating the cells with 300 μmol/L NMDA for 24 hours. Neuronal loss was assessed by LDH activity released to the bathing medium, minus the background level found in sham-treated wells, expressed relative to total neuronal LDH. There was no significant glial death detected by trypan blue staining.

Transient Brain Focal Ischemia in the Rat
Focal ischemia, confined to the cerebral cortex in the right middle cerebral artery (MCA) territory of Long-Evans male rats (weight, 300 to 350 g; Charles River, Wilmington, Del) was induced by temporary ligations of the right MCA and both common carotid arteries (CCAs).26 The rats were anesthetized by injection of chloral hydrate 400 mg/kg IP. Anesthesia and surgical procedures were performed in accordance with institutional guidelines. Local blood flow was measured by [14C]iodoamphetamine (IAP) quantitative autoradiography as described previously27 or monitored by laser-Doppler flowmetry (TF5000, Perimed). At the end of the ischemic period (30 or 45 minutes), the right MCA ligation and both CCA clips were released. Rectal temperature was closely monitored and maintained at 37±0.3°C during MCA occlusion (MCAS) and for up to 2 hours after ischemia via an electronic temperature controller (Cole-Parmer) linked to a heating pad and a heating lamp. Free access to food and water was allowed after recovery from anesthesia. All rats were kept in air-conditioned cages with room temperature maintained at 24±0.5°C.

The right femoral artery in some rats was cannulated for monitoring arterial blood pressure and heart rate and for obtaining blood samples for glucose and gas analysis before, during, and after ischemia. Sham-operated and drug-treated rats were treated by intracerebroventricular injection (total volume=2 μL) or by intraperitoneal injection (total volume=0.5 mL) according to the experimental plan.

Fourteen days after onset of MCAO, animals were killed with an overdose of pentobarbital (100 mg/kg) followed by intracardiac perfusion of 200 mL 0.9% NaCl. The brains were then sliced into 2-mm-cortical sections. Cortical infarct volume was morphometrically measured after staining with 2% triphenyltetrazolium chloride (TTC) in phosphate-buffered saline (pH 7.4) at 37°C for 20 minutes and then stored in 10% neutral-buffered formalin. The cross-sectional area of the TTC-unstained region was determined with the use of an image analyzer (MCID Imaging System) and by the indirect method.28,29 Hemisphere cortical volume was calculated by summation of infarct volumes measured in component brain slices. Infarct volumes defined by the TTC method in this model correlated well with those defined by histological examination after hematoxylin and eosin staining.29 Surgery and infarct measurements were performed under double-blind conditions.

Materials
Clofilitum, NMDA, MK-801, and CNQX were from Research Biochemicals International (RBI); other compounds were from Sigma.

Statistical Analysis
We used Student’s 2-tailed t test for comparison of 2 experimental groups; we used 1-way ANOVA test followed by Tukey test for multiple pairwise tests. Changes were identified as significant if probability value was <0.05. Mean±SEM values were reported.

Results

Effects of TEA and Clofilitum on K⁺ Current, NMDA Receptor Current, and Ca²⁺ Current
Previous studies in cultured cortical neurons showed that TEA and clofilitum attenuated apoptosis by inhibiting the delayed rectifier IK current.11,19 At 5 mmol/L, TEA blocked 39±5% of the IK blocked by voltage steps from −70 to +40 mV (n=10 cells) (Figure 1A). Current clamp recordings revealed that bath-applied 5 mmol/L TEA-induced transient mild depolarization: the membrane potential shifted from the control −56±3 mV to −51±3 mV after 10 minutes of continuous application of TEA (P=0.04; n=18). However, membrane potential returned to baseline after 20 to 30 minutes in TEA (−57±3 mV; P=0.65 compared with controls; n=9). As an open channel blocker, clofilitum preferably blocked the IK steady state current; 0.1 μmol/L clofilitum suppressed 36±7% of IK (n=4) (Figure 1B) without affecting the fast inactivating IK current.19 Clofilitum (0.1 μmol/L) did not alter the resting membrane potential (−58±3 mV and −60±2 mV before and during 30 minutes of exposure, respectively; n=6).

High concentrations of TEA may block NMDA receptor channels, but this blockade becomes progressively smaller at
TEA and Clofilium Effect on NMDA and Ca2+ Currents

The TEA and clofilium effect was reversible (data not shown). C, NMDA receptor current was evoked by local application of 200 μmol/L NMDA and 10 μmol/L glycine at a holding potential of −70 mV (n = 7) as well as more depolarized potentials (data not shown). High voltage-activated (HVA) Ca2+ currents were triggered by voltage steps from −70 to 0 mV (n = 4); the L-type Ca2+ current was activated by voltage steps from −40 to +10 mV (n = 3). For the NMDA-triggered current, peak and steady state currents were measured; for voltage-gated Ca2+ current, steady state current was measured. TEA (5 mmol/L) showed no effect on NMDA current or voltage-gated Ca2+ currents.

TEA and Clofilium Attenuate Apoptotic Death Induced by OGD in Cortical Culture

OGD induces NMDA receptor–mediated excitotoxic death of cultured cortical neurons.6 In this necrosis in vitro model, TEA (5 mmol/L) or clofilium (0.1 μmol/L) (both during and 24 hours after OGD) did not attenuate neuronal injury caused by 50 minutes of OGD (data not shown). Addition of NMDA and AMPA antagonists during the OGD period along with extension of insult duration to 110 minutes (blocked OGD) results in neuronal death with morphological and biochemical features of apoptosis.24,32 After this blocked OGD treatment, the membrane potential was depolarized by >20 mV, from −65±2 mV in sham control cells (n = 9) to 44±3 mV in post-OGD cells (n = 11; P < 0.05), favorable for activation of voltage-gated channels. When tested in this model of ischemic apoptosis, TEA (5 mmol/L) or clofilium (0.1 μmol/L) attenuated neuronal death by 30±6% and 44±9%, respectively (P < 0.01 for each blocker compared with sham controls; n = 26 and n = 18 for TEA and clofilium groups, respectively; Figure 2). Since drugs were added in the presence of NMDA and non-NMDA receptor blockers, the observed neuroprotective effects were not likely to be mediated by glutamate receptor antagonism. TEA at 5 mmol/L was mildly neuroprotective even when added immediately after the blocked OGD (Figure 2).

TEA and Clofilium Reduce Cerebral Infarct Volume Induced by Transient Focal Ischemia in the Rat

Transient focal ischemia was induced by 30-minute ligations of the right MCA and both CCAs.17 Local blood flow in the ischemic core area during MCA and CCA occlusions decreased from the normal 125±10 mL/100 g per minute to <10 mL/100 g per minute (>90% reduction; n = 6 rats) as measured by [14C]IAP quantitative autoradiography; complete reperfusion was observed soon after ligation release (131±15 mL/100 g per minute). To ensure that any delayed infarction was complete and to assess long-term effects of tested drugs, we measured cerebral infarct volume 14 days after the ischemic insult (infarct volume in sham controls = 159±11 mm3; n = 31). In rats given

Figure 2. K+ channel blockers protect against OGD-induced neuronal apoptosis. Mixed cortical cultures were exposed to OGD for 110 minutes in the presence of 1 μmol/L MK-801 and 100 μmol/L CNQX (blocked OGD). Resulting neuronal apoptosis, assessed by LDH release 24 hours after the onset of blocked OGD and expressed as the percentage of cell death induced by blocked OGD alone, was attenuated by cycloheximide (CHX) (1 μg/mL), TEA (5 mmol/L), or clofilium (0.1 μmol/L) applied during and after the insult or TEA applied only after the insult (TEA Post) (n = 8 cultures per condition). *Significant difference from blocked OGD alone (P < 0.05).
TEA (5 μg/kg ICV) or clofilium (0.3 ng/kg ICV) (equivalent to ±5 mmol/L TEA and ±0.1 μmol/L clofilium, respectively) 1 to 3 minutes before MCAO, infarct volume 14 days later was 112±30 mm³ (30±20% reduction; P<0.05; n=29) and 119±22 mm³ (25±12% reduction; P<0.05; n=5), respectively (Figure 3A, 3B., and 3C). The neuroprotective effect of TEA diminished when its dosage was reduced to 1 μg/kg ICV (Figure 3B).

The neuroprotective effect of TEA and clofilium persisted when tested against prolonged MCAO of 45 minutes. Cerebral infarction volume assessed 14 days later was 134±28 mm³ after MCAO alone (n=22) but was 94±6 mm³ (30±8% reduction; n=10; P<0.05) and 95±15 mm³ (29±11% reduction; n=10; P<0.05) in animals given 5 μg/kg TEA ICV or 0.3 ng/kg clofilium ICV, respectively, immediately before MCAO.

Despite being a quaternary ammonium compound, clofilium is relatively lipophilic because of the large hydrophobic alkyl side chains linked to the nitrogen and thus may pass immediately before MCAO. C, TEA (5 μg/kg ICV 1 to 2 minutes before MCAO; n=11) or clofilium (0.6 μg/kg IP before MCAO and 2 more doses at 24 and 48 hours later; n=15) reduced the infarction areas at each stereotaxic level when assessed 14 days later. D, Systemic administration of clofilium (0.3 to 60 μg/kg per day ×3 days) dose-dependently attenuated ischemic infarction assessed 14 days later (n=9). A 30-minute delay of the first administration of clofilium still antagonized infarction formation (n=9); the protective effect was diminished when clofilium was given 1 hour after the onset of MCAO (n=10). *Significant difference from sham control (P<0.05).

**Figure 3.** K⁺ channel blockers attenuated focal ischemia-induced cerebral infarction. A, Cerebral infarction in brain sections 14 days after 30 minutes of transient focal ischemia, stained with TTC. Infarction and cavitation were reduced by TEA (5 μg/kg ICV) given 1 to 3 minutes before ischemia. B, Protective effects against 30-minute ischemia-induced cerebral infarction by MK-801 (intraperitoneal; n=5), TEA (intracerebroventricular; n=9), and clofilium (intracerebroventricular; n=5). All 3 drugs were administered 1 to 3 minutes before MCAO. C, TEA (5 μg/kg ICV 1 to 2 minutes before MCAO; n=11) or clofilium (0.6 μg/kg IP before MCAO and 2 more doses at 24 and 48 hours later; n=15) reduced the infarction areas at each stereotaxic level when assessed 14 days later. D, Systemic administration of clofilium (0.3 to 60 μg/kg per day ×3 days) dose-dependently attenuated ischemic infarction assessed 14 days later (n=9). A 30-minute delay of the first administration of clofilium still antagonized infarction formation (n=9); the protective effect was diminished when clofilium was given 1 hour after the onset of MCAO (n=10). *Significant difference from sham control (P<0.05).

**Physiological Parameters Before, During, and After MCA Occlusion**

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<th>TEA</th>
<th>Clofilium</th>
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<tr>
<td></td>
<td>Control</td>
<td>During MCAO</td>
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<tr>
<td><strong>Hematological</strong></td>
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<tr>
<td>PO₂, mm Hg</td>
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<td>80.3±3.2</td>
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<tr>
<td>PCO₂, mm Hg</td>
<td>52.7±1.2</td>
<td>56.0±8.8</td>
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<tr>
<td>pH</td>
<td>7.3±0.02</td>
<td>7.2±0.17</td>
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<tr>
<td>Heart rate, beats/min</td>
<td>320±44</td>
<td>370±34*</td>
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<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>76.3±7.4</td>
<td>78.7±6.1</td>
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<tr>
<td>Plasma glucose, mg/dL</td>
<td>269.6±11.4</td>
<td>318.6±32.6</td>
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TEA, 5 μg/kg ICV; clofilium, 6 μg/kg IP, n=3 to 15 rats for each measurement. The heart rate increased during MCAO with or without a K⁺ channel blocker. In control animals, TEA or clofilium did not change the heart rate.

*Significant difference from controls (P<0.05).
volume; Figure 3D). When the first dose of clofilium (6 μg/kg IP) was delayed until the end of 30 minutes of MCAO, infarct volume was still reduced by 21±5% (P<0.05). Delaying clofilium administration until 1 hour after the onset of ischemia diminished the protective effect (Figure 3D).

Rectal temperature, blood pressure, blood O₂, blood CO₂, and blood glucose levels remained constant during 45 minutes of MCAO and 60 minutes of reperfusion in the presence of TEA 5 μg/kg ICV or clofilium 6 μg/kg IP (Table); no clinical seizures were observed. Rectal temperature was maintained at 37.0±0.3°C during MCAO and recovery from anesthesia; it was additionally monitored for up to 19 hours after ischemia. No significant changes in these physiological parameters were seen in animals given TEA or clofilium.

**Discussion**

This investigation was driven by the hypothesis that excessive K⁺ efflux via activated Iₑ channels and consequent intracellular K⁺ depletion promote neuronal apoptosis. Extracellular K⁺ increases during and after cerebral ischemia or hypoxia/anoxia because of excessive K⁺ efflux and failure of membrane Na⁺/K⁺-ATPase.34–36 We observed a 20-mV membrane depolarization shortly after conclusion of blocked OGD, likely reflecting partial recovery from more severe depolarization.34,37 Ischemia/hypoxia-induced membrane depolarization is sure to lead to activation of voltage-gated K⁺ channels and consequent loss of intracellular K⁺,36,38 but the effects of this loss on cell survival after ischemia have received little attention. We present here evidence that K⁺ channel blockers can attenuate hypoxic/ischemic apoptotic death either in cortical cultures or in vivo; furthermore, the protective effects of TEA and clofilium were maintained 14 days after in vivo ischemia.

TEA and clofilium did not reduce neuronal death induced by hypoxia in vitro in the absence of glutamate antagonists. In this model, neuronal death has morphological features of necrosis and is insensitive to apoptotic maneuvers such as inhibition of protein synthesis or caspases.32 This system does not accurately model the more indolent, potentially apoptotic neuronal death that can occur in the ischemic penumbra. TEA and clofilium reduced neuronal death after OGD in the presence of glutamate receptor antagonists (blocked OGD). This observation confirmed that the injury-reducing effects of TEA and clofilium were not due to antagonism at NMDA/AMPA receptors. The blocked OGD model is an artificial system designed to model the slowly evolving apoptotic component of ischemic neuronal death; in vivo, TEA and clofilium may have complex neuroprotective effects that would not be detected in this model. For example, membrane hyperpolarization or localized high concentrations of TEA or clofilium might reduce glutamate receptor activation when MK-801 and CNQX are not present. K⁺ channel blockers may also help to preserve the K⁺ electrochemical gradient that is a driving force for neuronal and astroglial high-affinity glutamate transporters,39 thus reducing extracellular glutamate concentrations and consequent excitotoxicity. Clofilium is a class III antiarrhythmic agent, recognized as an open channel blocker of voltage-gated K⁺ channels.40 Clofilium has been reported to have inhibitory effects on voltage-gated Na⁺ and Ca²⁺ currents in guinea pig ventricular myocytes;41 however, these effects were not replicated in a study of acutely isolated ventricular cells.32 Clofilium at high concentrations (eg, 10 μmol/L) may induce apoptosis by itself,43 perhaps as a result of nonspecific inhibition of Na⁺,K⁺-ATPase (S.P. Yu, MD, unpublished data, 2003).

Potassium channel openers are neuroprotective against ischemic cell death in rodents, presumably as a result of membrane hyperpolarization and reduced membrane excitability.6–9 K⁺ channel openers may interfere with Ca²⁺ mobilization from intracellular stores;44 in addition, these openers may mimic preconditioning45 or alter K ATP channels on mitochondrial membranes.6,46 One can anticipate these benefits being lost, or even reversed, after pharmacological blockade of K⁺ channels; therefore, the present demonstration of a net neuroprotective effect with K⁺ channel blockers is all the more surprising.

K⁺ channel blockers can cause seizures, but no clinical seizures or other side effects were observed at dosages showing protective effects, probably because neuroprotective K⁺ channel blockers suppress only a minority fraction of Iₑ current.11 This is consistent with the observation that neuronal membrane potential was not much changed by 5 mmol/L TEA or 0.1 μmol/L clofilium.

Apoptosis likely occurs even after more severe ischemia;47 this is consistent with the observation that TEA and clofilium reduced infarct volume after a longer ischemic insult (45 minutes). The protective effect was modest and diminished with delayed administration. Taken together, these observations support the idea that strategies aimed at maintaining cellular K⁺ homeostasis may be of therapeutic benefit in the treatment of cerebral ischemia.

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