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NMDA receptors and the differential ischemic vulnerability of hippocampal neurons

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Abstract

Transient cerebral ischemia causes an inhomogeneous pattern of cell death in the brain. We investigated mechanisms, which may underlie the greater susceptibility of hippocampal CA1 vs. CA3 pyramidal cells to ischemic insult. Using an *in vitro* oxygen–glucose deprivation (OGD) model of ischemia, we found that *N*-methyl-D-aspartate (NMDA) responses were enhanced in the more susceptible CA1 pyramidal cells and transiently depressed in the resistant CA3 pyramidal cells. The long-lasting potentiation of NMDA responses in CA1 cells was associated with delayed cell death and was prevented by blocking tyrosine kinase-dependent up-regulation of NMDA receptor function. In CA3 cells, the energy deprivation-induced transient depression of NMDA responses was converted to potentiation by blocking protein phosphatase signalling. These results suggest that energy deprivation differentially shifts the intracellular equilibrium between the tyrosine kinase and phosphatase activities that modulate NMDA responses in CA1 and CA3 pyramidal cells. Therapeutic modulation of tyrosine phosphorylation may thus prove beneficial in mitigating ischemia-induced neuronal death in vulnerable brain areas.

Introduction

A major goal in stroke research is the development of treatments to rescue brain tissue within the ischemic penumbra. Of the numerous potentially neuroprotective drugs tested in recent years, none have passed successfully beyond clinical trials (Gladstone *et al*., 2002). A particular challenge in drug discovery lies in selecting promising targets from among the multitude of proteins involved in cell death pathways. Useful clues might be obtained by characterizing endogenous neuroprotective mechanisms. Examination of ischemic brain tissue reveals an inhomogeneous pattern of neuronal death, indicating a differential vulnerability of neurons to energy deprivation and excitotoxicity. We propose that investigating the intracellular pathways underlying this differential vulnerability will reveal endogenous neuroprotective processes and facilitate the identification of target molecules for therapeutic intervention.

An ideal tissue to study differential neuronal responses to excitotoxic stress is the hippocampus, in which neighbouring populations of neurons with similar morphologies and properties, the CA1 and CA3 pyramidal cells, exhibit markedly distinct reactions after stroke. In patients with brain ischemia, CA1 pyramidal cells are among the most vulnerable in the brain (Zola-Morgan *et al*., 1986; Petito *et al*., 1987), a finding also observed in animal models of stroke (Schmidt-Kastner & Freund, 1991). In comparison, CA3 pyramidal cells remain remarkably resistant to ischemia. Initially, the cells in both areas of the ischemic hippocampus are exposed to similar triggers. The time course of and the rise in glutamate levels are equivalent in the CA1 and CA3 areas (Mitani *et al*., 1992). Energy deprivation reduces intracellular ATP thereby shifting ionic gradients and reversing glutamate transport both in CA1 (Rossi *et al*., 2000) and in CA3 pyramidal cells (Jabaudon *et al*., 2000). The expression of *N*-methyl-D-aspartate (NMDA) receptors, a Ca^{2+}-permeable subset of glutamate receptor that initiates excitotoxic cell death when over-activated (Lipton, 1999), is also similar in CA1 and CA3 pyramidal cells (Monyer *et al*., 1994).

A further unresolved problem with respect to glutamate excitotoxicity is that transient global ischemia induces a brief reversible rise in extracellular glutamate concentration, whereas neuronal death is observed with a delay of two or more days (Petito *et al*., 1987; Kirino, 2000). Thus, although the role of the NMDA receptors in triggering excitotoxic cell death is established, additional mechanisms downstream of NMDA receptors must be responsible for the differential susceptibility to excitotoxic death in CA1 and CA3 pyramidal cells.

Utilizing an *in vitro* hippocampal preparation that replicates the defining functional and morphological characteristics of ischemic neurons *in vivo*, we identified cell-specific differences in the modulation of NMDA receptor currents by tyrosine kinases and phosphatases that may contribute to the preferential cell death of CA1 cells.

Materials and methods

Slice cultures and electrophysiology

Hippocampal slice cultures were prepared from 6-day-old Wistar rats, calbindin knockout mice (Airaksinen *et al*., 1997) or control littermates as described (Gähwiler *et al*., 1998) using the roller-tube technique. Animals were killed by decapitation. After 3–4 weeks *in vitro*, slice

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cultures were transferred to a recording chamber mounted on an upright microscope (Axioscope FS, Zeiss Germany) or processed for propidium iodide (PI) labelling (see below). Slices were superfused at 1–2 mL per min with artificial cerebrospinal fluid (ACSF, in mM) 124 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.26 NaH_2PO_4, 2 $MgCl_2$, 3 $CaCl_2$, 10 D-glucose, and 10 mg/L Phenol Red (pH 7.4, ~310 mOsm, 29 °C, saturated with 95% O_2, 5% CO_2). Whole-cell voltage-clamp recordings at −50 mV were obtained using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Recording pipettes (2–5 MΩ) contained (in mM) 140 K-gluconate, 10 NaCl, 1 $MgCl_2$, 10 HEPES, 1 EGTA (pH 7.2–7.4, ~300 mOsm). Series (7–15 MΩ) and input resistances were monitored regularly. Currents were filtered at 2 kHz and analysed offline (pClamp 7, Axon Instruments). Where indicated 30 mM BAPTA replaced the EGTA and K-gluconate was removed to maintain the osmolarity, BAPTA was infused through the patch pipette for 15 min before recording NMDA currents. Intracellular recordings were made with sharp electrodes filled with 1 M K-acetate (40–90 MΩ) and an Axoclamp 2B amplifier. Bridge balance was maintained throughout the experiments. NMDA potentials were recorded at the resting membrane potential except occasionally when current was injected to ensure the post-oxygen–glucose deprivation (OGD) NMDA potentials were recorded at the same membrane potential as the control NMDA potentials.

NMDA currents were isolated by adding the AMPA/kainate antagonist 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulphonamide (NBQX, 10 μM), the $GABA_A$ antagonist picrotoxin (100 μM) and 0.5 μM tetrodotoxin. NMDA (200 μM) was pressure-ejected (0.5 bar, 200 ms) from a micropipette at 40 s intervals. Individual responses to NMDA were measured from the holding current to the peak. For each cell, 3–5 responses were averaged in each condition. NMDA-evoked responses were blocked completely by the specific antagonist 3-((*R*)-2-carboxypiperazin-4yl)-propyl-1-phosphonic acid (CPP, 40 μM) at the end of the recordings. (S)-α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) currents (100 μM) were recorded and analysed as for NMDA currents in the presence of picrotoxin (100 μM) and tetrodotoxin (0.5 μM). Whole-cell synaptic currents (at +40 mV) were evoked with ACSF-filled electrodes (10–20 μA, 200 μs pulses) placed in the CA3 pyramidal layer or dentate gyrus.

Oxygen-glucose deprivation (OGD)

Glucose in the superfusate was replaced with 8 mM sucrose and 3 mM 2-deoxyglucose, saturated with 95% N_2, 5% CO_2 and 3 mM NaN_3 was added.

Propidium iodide (PI) labelling

Slice cultures, in culture tubes, were washed 3× and incubated in a water bath for 4 min with either ACSF (control) or with the OGD solution at 35 ± 1 °C. Cultures were washed 3× with ACSF and returned to culture medium containing 2.5 μg/mL PI and 1 : 500 penicillin/streptomycin. Genistein and PP2 were added immediately and CPP 2 h after OGD. SU6656 was added to the culture medium the previous day. Experiments were performed at least twice on different batches of cultures. After 48 h cultures were photographed with constant settings using an Axiocam and Axiovision software mounted on an Axiophot microscope (excitation 546 nm, emission > 590 nm, Zeiss). Images were photographed and analysed blind to the treatment group. A circle (6000 pixels) was positioned over the brightest region of the pyramidal layers CA1 and CA3 and the average intensity was determined using Image J (download from http://rsb.info.nih.gov/ij/).

Intracellular Ca^{2+} imaging

Twenty micromolar Oregon Green 488 BAPTA-2 (Molecular Probes, Leiden, Netherlands) was added to the intracellular solution (K_d ~580 nM) and allowed to diffuse for 20 min. Excitation illumination was applied at 488 nm using a TILL Photonics Polychrome I monochromator (Planegg, Germany). Emitted images were collected with a cooled CCD camera (Princeton Instruments, Trenton, NJ) after passing them through a TILL FITC filter set, stored and analysed using Axon Imaging Workbench (Axon Instruments). Exposures were made at 15 s intervals for 400–1000 ms. Average fluorescence was determined for regions of interest in proximal dendrites and background fluorescence was subtracted. $\Delta F/F$ was calculated for each image ($\Delta F/F$ = (fluorescence-average baseline fluorescence)/average baseline fluorescence).

Immunostaining

Mouse and rat slice cultures were double-immunostained for calbindin expression and the neuronal marker NeuN. Slices were fixed overnight at 4 °C in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Free-floating cultures were washed in 0.1 M PB then blocked and permeabilized in 0.1 M PB, 0.4% Triton X-100 and 5% heat-inactivated horse serum (HS) for 24 h at 4 °C. Primary rabbit α-calbindin antibody (rabbit α-CB, 1 : 5000, Swant, CH) was applied for 24 h at 4 °C in 0.1 M PBS, 0.4% Triton X-100 and 2.5% HS. To visualize the anatomy of the cultures, a primary antibody that recognizes mature neurons (neuronal nuclear antigen, mouse α-NeuN, 1 : 400, Chemicon, CA, USA) was applied together with the α-CB antibody. Cultures were washed with 0.1 M PB 0.4% Triton X-100 (4 × 30 min) and incubated at 4 °C overnight with the secondary antibodies donkey α-mouse Alexa 546 and goat α-rabbit Alexa 488 (1 : 350 each in 0.1 M PB, 0.4% Triton X-100 and 2.5% HS, Molecular Probes). After extensive washing in 0.1 M PB, cultures were mounted using the ProLong® Antifade Kit (Molecular Probes) and stored in the dark at 4 °C. Fluorescent images were acquired with a Hamamatsu C4742-95 digital camera (Hamamatsu, Ichino, Japan) mounted on a Leitz DMRB fluorescent microscope with a 20 × 0.50 PH2 lens (Leica, Wetzlar, Germany) and analysed using Openlab 3.5.2 (Improvision, Coventry, UK).

Drugs

Stock solutions were prepared in distilled H_2O or dimethylsulfoxide as appropriate and diluted in ACSF to the working concentration immediately before use. AMPA, NMDA, picrotoxin and nifedipine were purchased from Sigma. 4′,5,7-trihydroxyisoflavone (genistein) was from Sigma or Tocris-Cookson (Avonmouth, UK). 3-(4-chlorophenyl)1-(1,1-dimethylethyl)-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (PP2) was from Tocris-Cookson. 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine 1 (PP1) was from Alexis (San Diego, CA). NBQX was from AG Scientific (San Diego, CA), SU6656 from Biaffin (Kassel, Germany) or Sigma and tetrodotoxin from Latoxan (Valence, France). CPP was kindly provided by Novartis (Basel, Switzerland). Dimethylsulfoxide never exceeded 0.02% and did not affect NMDA currents. Pervanadate was prepared by heating freshly dissolved 100 mM Na orthovanadate (Sigma) in H_2O to 95 °C then cooling to 25 °C on ice and adjusting pH to 10. Just before use one part 500 mM H_2O_2 was combined with 50 parts of the 100 mM Na orthovanadate, left to stand for 10 min then diluted 1 : 1000 in ACSF and applied.

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Statistical analysis

Data are presented as mean percentage control ± SEM. Paired Student's *t*-tests were used to compare non-normalized NMDA current amplitudes (average of 3–5 successive traces) before and 20 min after OGD. PI experiments were analysed independently then pooled as the results were not different. For the PI stainings, two-way ANOVA followed by multiple comparisons using the least significant differences was performed. Comparisons were made with SPSS software (SPSS Chicago IL) with significance at $P = 0.05$.

Results

Validation of the ischemia model

We verified that simulating transient ischemia by oxygen-glucose deprivation (OGD) in the hippocampus *in vitro* induces delayed neuronal death as occurs *in vivo* (Schmidt-Kastner & Freund, 1991; Lipton, 1999). Cell death was assayed by adding PI to hippocampal slice cultures. PI rapidly penetrates cells with damaged membranes and fluoresces upon binding to nucleic acids (Macklis & Madison, 1990). OGD for 4 min at 35 °C did not result in immediately detectable PI staining (not shown). However, after re-incubation in normal culture medium for 48 h, PI staining was evident ($P < 0.001$ cntl vs. OGD, two-way ANOVA, Fig. 1) and was significantly greater in the CA1 area than in the CA3 area ($P = 0.029$, LSD). The delayed neuronal death was prevented by the NMDA receptor antagonist CPP (40 μM) applied 2 h after OGD ($P = 0.70$ vs. cntl, two-way ANOVA, Fig. 1). When OGD was maintained for 20–30 min, anoxic depolarization was induced and the entire hippocampus was labelled rapidly with PI (not shown). These results confirm that transient OGD induces NMDA receptor-dependent delayed neuronal death preferentially in the CA1 area of hippocampal slice cultures as reported (Vornov *et al*., 1994; Abdel-Hamid & Tymianski, 1997).

Comparison of functional responses in CA1 and CA3 pyramidal cells

Ischemia rapidly depresses synaptic transmission by blocking stimulus-evoked transmitter release (Hershkowitz *et al*., 1993). If ischemic depression of excitatory transmission were more pronounced in the CA3 than in the CA1 area, CA3 pyramidal cells would be less exposed to excitotoxic neurotransmitter. There was no difference, however, in the depression of synaptic responses ($P = 0.89$ CA1 vs. CA3 at maximum; Supplementary material Fig. S1, A).

A critical step in NMDA receptor-dependent excitotoxicity is intracellular Ca^{2+} overload, which kills cells through activation of proteases, production of free-radicals, and mitochondrial dysfunction (Lipton, 1999). The OGD-induced somatic Ca^{2+} signal was transient and recovered rapidly in both CA1 and CA3 pyramidal cells ($P = 0.6$ CA1 vs. CA3 at peak; Supplementary material Fig. S1, B). Thus, the greater vulnerability of CA1 cells cannot be explained by a sustained Ca^{2+} signal. Furthermore, the transient nature of the Ca^{2+} response indicates that the immediate OGD-induced rise in Ca^{2+} is perhaps a trigger rather than the executor of delayed neuronal death.

As NMDA receptors are fundamental in initiating cell death, we compared the effect of OGD on responses to pressure application of NMDA in the two cell types. In CA1 pyramidal cells, NMDA-induced currents slightly decreased in amplitude during the 4 min OGD but immediately thereafter currents progressively increased reaching a plateau after 20 min (143 ± 12% $P < 0.001$; Fig. 2A and B). This potentiation of the NMDA response did not diminish for the duration of the recordings (up to ∼1 h) as described previously (Hammond *et al*., 1994). In striking contrast, the same protocol depressed NMDA currents for approximately 5 min in CA3 pyramidal cells ($P = 0.042$; Fig. 2A and B). Recovery but no potentiation was observed after 20 min ($P = 0.16$; Fig. 2A and B). The same effects were observed when we recorded synaptic NMDA responses. Pharmacologically isolated synaptic NMDA currents recorded at +40 mV were transiently depressed by OGD to the same extent in CA1 and CA3 pyramidal cells (Fig. 2C left bars). After 20 min, however, synaptic NMDA currents were significantly potentiated in CA1 pyramidal cells ($P = 0.04$) but not in CA3 pyramidal cells ($P = 0.17$; Fig. 2C right bars).

The observed modulation of NMDA responses could reflect an action at the receptor level, or an indirect effect due to differential changes in the electrical membrane properties. We found that differences in membrane properties could not account for the differences in NMDA currents as OGD caused similar changes in input resistance or holding current in CA1 and CA3 pyramidal cells ($P = 0.7$, $P = 0.67$, respectively, Supplementary material, Fig. S2). These findings suggest that divergent modulation of NMDA receptors accounts for the greater susceptibility of CA1 vs. CA3 pyramidal cells to ischemic neuronal death.

Previous work demonstrated that the regulation of NMDA receptors by metabotropic receptor activation is bi-directional depending on the contribution of various Ca^{2+}-dependent processes (Grishin *et al*.,

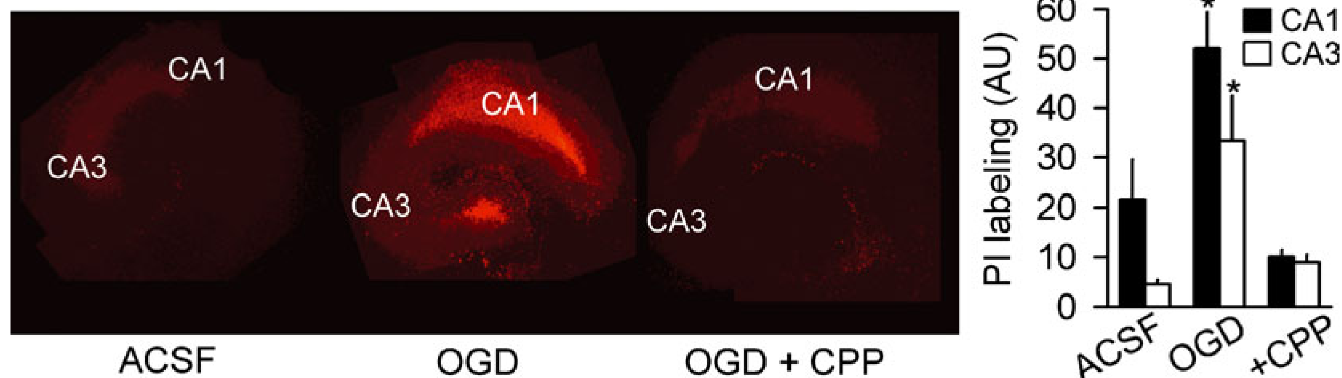

Fig. 1. Oxygen-glucose deprivation (OGD) induces NMDA receptor-dependent delayed neuronal death in organotypic hippocampal slice cultures. 48 h following 4′ OGD, propidium iodide labelling of hippocampal slice cultures ($n = 8$) increased relative to cultures treated for 4′ with ACSF ($n = 8$). Labelling intensity was significantly greater in the CA1 pyramidal cell layer. Adding the NMDA receptor antagonist CPP 2 h after OGD significantly reduced propidium iodide labelling ($n = 10$). At right, quantification of labelling intensity. Asterisks $P < 0.05$.

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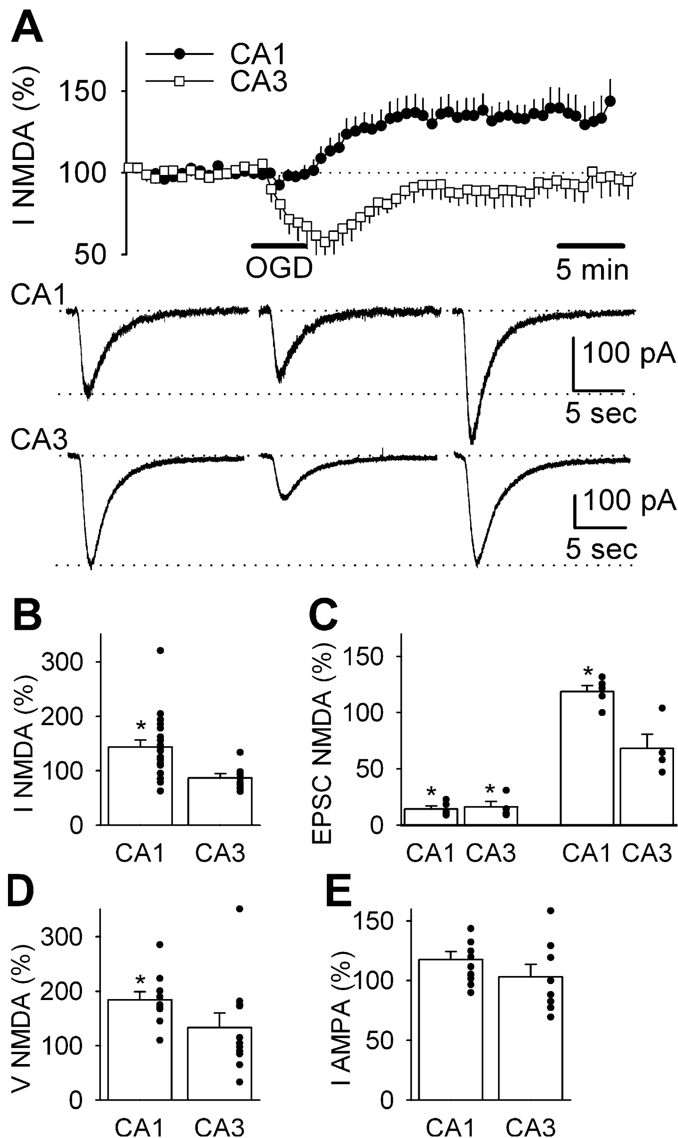

FIG. 2. Synaptic and NMDA-induced NMDA responses undergo OGD-induced potentiation in CA1 but not in CA3 pyramidal neurons. (A and B) Whole-cell currents induced by puffing NMDA are significantly enhanced following 4′ OGD in CA1 pyramidal neurons ($n = 20$) but not in CA3 pyramidal neurons ($n = 8$). Traces show individual responses from one CA1 and one CA3 pyramidal neuron before (left), during (middle) and 20′ after (right) OGD. (C) Synaptically evoked whole-cell NMDA currents are first equally depressed in CA1 ($n = 5$) and CA3 pyramidal neurons ($n = 4$, first set of bars) and 20′ later significantly enhanced in CA1 but not in CA3 pyramidal neurons (second set of bars). (D) Intracellularly recorded NMDA-induced potentials are significantly enhanced after OGD in CA1 ($n = 10$) but not in CA3 pyramidal neurons ($n = 11$). (E) AMPA-induced responses were not significantly changed following OGD in CA1 ($n = 11$) or CA3 ($n = 8$) pyramidal neurons. Asterisks indicate significantly different from baseline ($P < 0.05$).

2004). To ensure that the difference between CA1 and CA3 pyramidal cells was not due to dialysis of Ca^{2+} buffers or ATP/GTP during patch pipette recordings, we obtained intracellular recordings of NMDA-evoked depolarizations with sharp electrodes. Again, NMDA responses in CA1 pyramidal cells were significantly increased 20 min after OGD ($P < 0.001$) whereas no change occurred in CA3 pyramidal cells ($P = 0.34$; Fig. 2D). AMPA responses were not, however, potentiated in either CA1 or CA3 pyramidal neurons following OGD ($P = 0.06$, $P = 0.98$, respectively, CA1 vs. CA3 $P = 0.27$ Fig. 2E) as was reported previously for CA1 cells (Crepel *et al*., 1993; but see Quintana *et al*., 2006). Thus, OGD selectively potentiated NMDA responses in CA1 pyramidal cells an observation that was seen with whole-cell voltage-clamp recordings, intracellular current-clamp recordings and whole-cell recordings of synaptically evoked NMDA responses demonstrating that the effect is robust, not dependent on membrane potential and not particular to a single recording condition.

Inhibition of tyrosine kinase prevents the OGD-induced potentiation of NMDA current and confers neuroprotection. A common motif in the up-regulation of NMDA receptors by diverse pathways is the activation of the tyrosine kinase Src, which phosphorylates many proteins including the NR2A and NR2B subunits of the NMDA receptor (Salter & Kalia, 2004). Moreover, Ca^{2+} is necessary for the activation of signalling pathways that target Src in CA1 pyramidal cells (Kotecha & MacDonald, 2003). When the Ca^{2+} chelator BAPTA (30 mM) was included in the patch pipette, the OGD-induced potentiation of NMDA currents was no longer observed in CA1 pyramidal cells ($P = 0.18$; Fig. 3A). In the continuous presence of the broad-spectrum tyrosine kinase inhibitor genistein (30 μM), OGD transiently depressed NMDA currents ($P = 0.05$; Fig. 3B), which recovered within 5 min ($P = 0.17$). Likewise, the OGD-induced potentiation of NMDA currents was blocked by PP1 (10 μM), a specific inhibitor of Src family tyrosine kinases ($P = 0.52$, Fig. 3C). A second, unrelated Src family tyrosine kinase inhibitor SU6656 (20 μM; Blake *et al*., 2000) also prevented the OGD-induced potentiation of NMDA receptors when preincubated for 1–2 h ($P = 0.18$; Fig. 3C). Furthermore, as with genistein, PP1 unmasked a transient depression of NMDA current during OGD ($P = 0.04$). Treatment with genistein also reduced the delayed neuronal death 48 h after OGD ($P = 0.052$ vs. ACSF, $P = 0.0006$ vs. ischemia Fig. 3D) as did the more specific Src tyrosine kinase inhibitor PP2 (10 μM $P = 0.85$ vs. ACSF, $P < 0.0001$ vs. ischemia) suggesting that tyrosine phosphorylation leading to a potentiation of NMDA receptors may play a role in ischemic neuronal death. SU6656, however, did not confer protection and resulted in a greater amount of fluorescence even in ACSF treated cultures (data not shown).

CA3 pyramidal cells are protected from ischemic NMDA receptor potentiation through tyrosine phosphatase activity. The above findings raise the question why OGD fails to potentiate NMDA responses in CA3 pyramidal cells, even though Src-dependent potentiation of NMDA currents in these cells is observed after activation of metabotropic glutamate receptors (Xiong *et al*., 1999; Benquet *et al*., 2002). We hypothesized that in CA3 pyramidal cells, phosphorylation by Src tyrosine kinase may be counteracted by Ca^{2+}-dependent tyrosine phosphatase activity, as an increase in Ca^{2+} prevents NMDA receptor up-regulation in these cells (Grishin *et al*., 2004). Intracellular Ca^{2+} chelation with BAPTA (30 mM), combined with nifedipine (10 μM) to block Ca^{2+} channels, resulted in a shift from a transient depression to a significant OGD-induced potentiation of NMDA currents in CA3 pyramidal cells ($P = 0.048$; Fig. 4A). This OGD-induced potentiation of NMDA currents was blocked by genistein (30 μM; $P = 0.29$; Fig. 4B) and PP2 ($P = 0.44$, $n = 4$, data not shown) demonstrating that OGD does activate tyrosine kinases in CA3 cells but a Ca^{2+}-dependent process counteracts the phosphorylation and prevents the potentiation. Likewise, when tyrosine phosphatases were blocked with pervanadate (100 μM), OGD still transiently depressed NMDA currents ($P = 0.044$; Fig. 4C), but this depression was followed by a strong potentiation of NMDA current ($P = 0.038$). As expected, the OGD-induced potentiation of NMDA currents revealed by coapplication of pervanadate was

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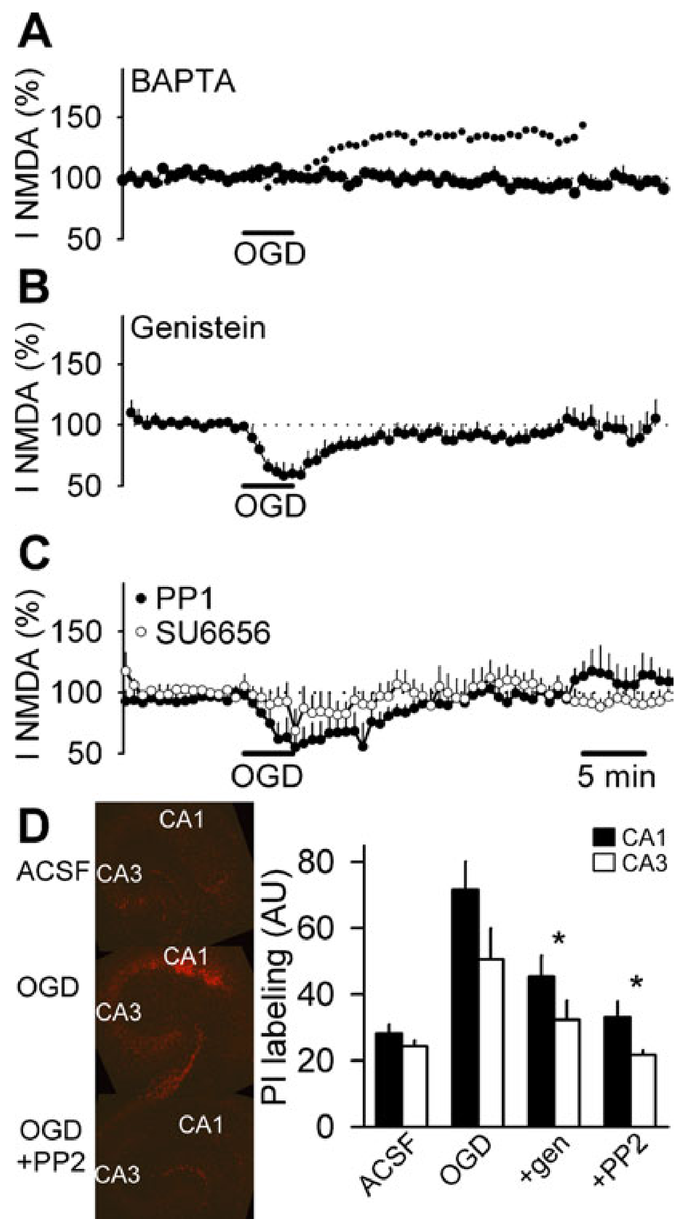

FIG. 3. An increase in intracellular Ca^{2+} and Src tyrosine kinase activation are required for potentiation of NMDA responses and delayed neuronal death in CA1 pyramidal neurons. (A) Intracellular dialysis of 30 mM BAPTA prevents OGD potentiation of NMDA responses in CA1 pyramidal neurons ($n = 4$). For comparison, the mean NMDA responses without Ca^{2+} buffering are reproduced here from Fig. 3A (dots). (B) The general tyrosine kinase inhibitor genistein prevents OGD potentiation of NMDA responses in CA1 pyramidal neurons ($n = 5$). (C) The Src family tyrosine kinase inhibitors PP1 ($n = 5$) and SU6656 ($n = 8$) also prevented potentiation of NMDA responses in CA1 pyramidal neurons. (D) Genistein and PP2 reduced OGD-induced delayed neuronal death as indicated by propidium iodide uptake ($n = 17, 18, 17, 16$). *Indicates PI labelling in both CA1 and CA3 is significantly reduced relative to OGD alone (*posthoc* pairwise comparison $P < 0.05$).

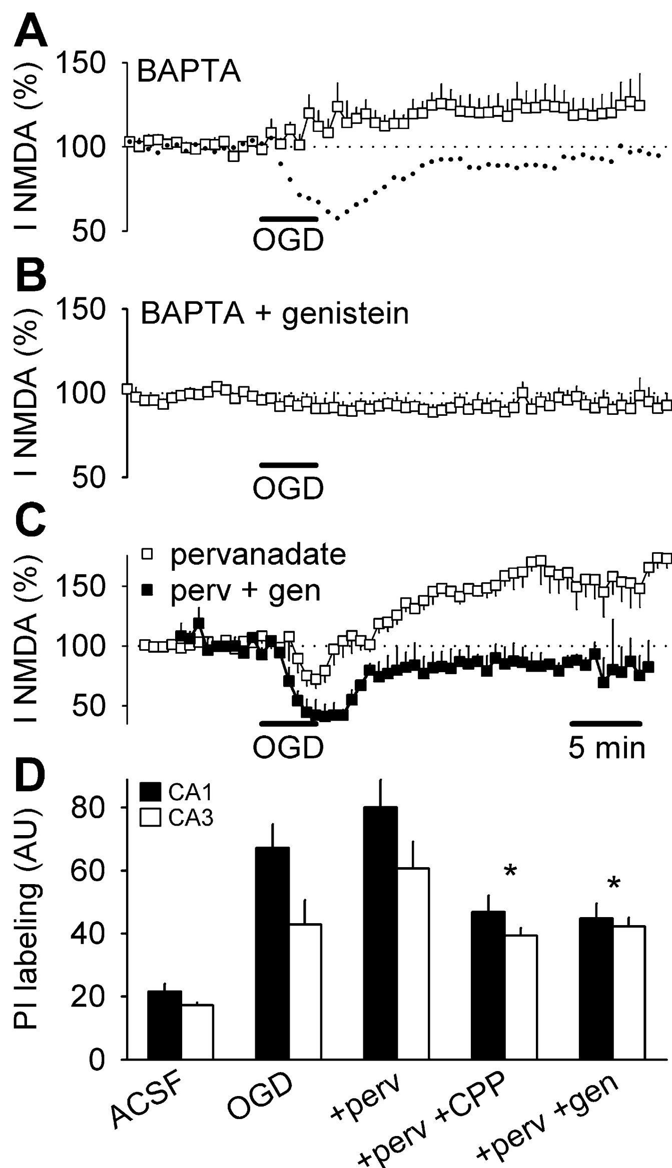

FIG. 4. In CA3 pyramidal cells, Ca^{2+}-dependent activation of tyrosine phosphatase suppresses Src-dependent potentiation of NMDA responses. (A) Intracellular dialysis of 30 mM BAPTA converts the OGD-induced depression of NMDA receptors into potentiation ($n = 8$). (B) This potentiation of NMDA responses revealed by chelating Ca^{2+} is blocked by the tyrosine kinase inhibitor genistein ($n = 5$). (C) Blocking tyrosine phosphatases with pervanadate reveals OGD-induced potentiation of NMDA receptors in CA3 pyramidal neurons ($n = 4$), which is blocked by the addition of genistein ($n = 4$). (D) Pervanadate enhanced the OGD-induced propidium iodide labelling. Blocking NMDA receptors with CPP or tyrosine kinases with genistein reduced the OGD-induced neuronal death in the presence of pervanadate ($n = 18, 18, 18, 18, 17$). *Indicates significant reduction of PI labelling relative to OGD plus pervanadate (*posthoc* pairwise comparison $P < 0.05$).

blocked by the tyrosine kinase inhibitor genistein ($P = 0.233$; Fig. 4C). Thus, in CA3 pyramidal neurons, Ca^{2+}-dependent phosphatase activity counteracts and masks the OGD-induced phosphorylation that leads to potentiation of NMDA receptors.

Application of pervanadate during and after OGD significantly enhanced the amount of propidium iodide labelling 48 h later ($P = 0.023$; Fig. 4D). The NMDA receptor antagonist CPP significantly reduced the propidium iodide labelling induced by OGD in the presence of pervanadate ($P < 0.0001$; Fig. 4D), as did the tyrosine kinase inhibitor genistein ($P = 0.0001$; Fig. 4D).

We next examined whether the apparent balance between phosphorylation and dephosphorylation in CA1 and CA3 pyramidal cells differs under resting conditions. Genistein had no effect on NMDA

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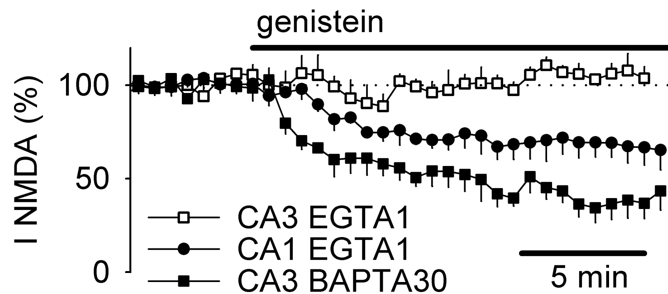

FIG. 5. Differential sensitivity to tyrosine kinase inhibition in CA1 vs. CA3 pyramidal neurons. Under nonischemic conditions genistein reduced NMDA responses in CA1 cells ($n = 5$) but not in CA3 cells recorded with 1 mM EGTA ($n = 4$), indicating that NMDA responses in CA1 cells are tonically enhanced by tyrosine phosphorylation. NMDA currents in CA3 pyramidal neurons became sensitive to genistein when intracellular Ca^{2+} was chelated with 30 mM BAPTA ($n = 4$).

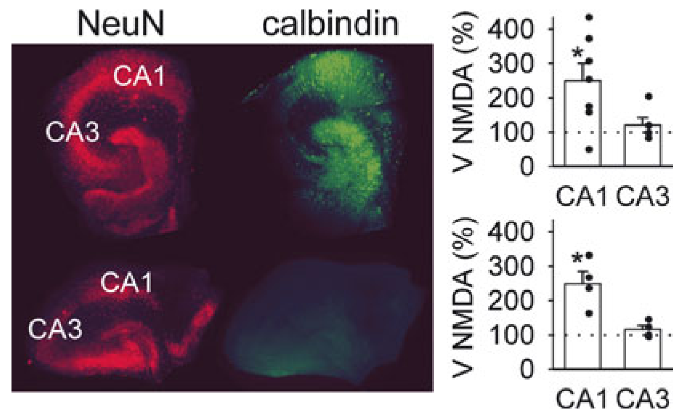

FIG. 6. The difference between OGD-induced NMDA receptor potentiation in pyramidal cells is not due to the selective presence of the Ca^{2+} binding protein calbindin as intracellularly recorded NMDA potentials are selectively potentiated in CA1 pyramidal neurons of both control (upper) and calbindin knock-out mice (lower). The left panel shows immunofluorescence labelling for the neuronal marker NeuN and the right panel for calbindin. Note the absence of calbindin immunoreactivity in the CA3 pyramidal cell layer in control (upper right) and the complete absence in the knockout (lower right). *Indicates significantly different from baseline ($P < 0.05$).

currents in CA3 pyramidal cells indicating that the NMDA currents were not significantly enhanced by tyrosine phosphorylation ($P = 0.57$, Fig. 5). In CA1 pyramidal cells however, NMDA currents were substantially reduced by genistein ($P = 0.049$, Fig. 5) indicating a tonic regulation by ongoing kinase activity. Interestingly, when intracellular Ca^{2+} was chelated with BAPTA, genistein significantly reduced NMDA currents in CA3 pyramidal cells ($P = 0.043$, Fig. 5), again suggesting that tyrosine kinase activity in CA3 pyramidal cells is counteracted by Ca^{2+}-dependent tyrosine phosphatase activity. As increasing the Ca^{2+} buffering of CA3 cells led to CA1-like responses to OGD and genistein, we hypothesized that the presence of a high affinity Ca^{2+} binding protein expressed predominantly in CA1 cells could account for the differences between CA1 and CA3 pyramidal cells (Grishin *et al*., 2004). Calbindin is an endogenous Ca^{2+} buffer, which is present in CA1 but not CA3 pyramidal cells (Sloviter, 1989). Moreover, CA1 pyramidal cells in calbindin knockout mice are more resistant to ischemia (Klapstein *et al*., 1998). Intracellular recordings from control mouse slice cultures revealed that, as in rat hippocampus, OGD induced potentiation of NMDA currents in CA1 but not in CA3 pyramidal cells ($P = 0.04$, $P = 0.35$, respectively, Fig. 6). However, we observed a similar pattern of OGD-induced potentiation of NMDA responses in calbindin knock-out mice (in CA1 $P = 0.05$, in CA3 $P = 0.23$ Fig. 6) suggesting that calbindin is not involved in the differential modulation of NMDA responses in CA1 vs. CA3 pyramidal cells.

Discussion

After years of concerted research efforts, the development of neuroprotective drugs for the treatment of stroke remains unsuccessful. Complicating issues include the multifactorial aetiology of stroke in human patients as compared with simplified animal models, and an inadequate understanding of the cellular processes culminating in ischemic cell death. Our study shows that in CA1 pyramidal cells, which are particularly vulnerable to stroke, a brief episode of energy deprivation leads to long-term potentiation of NMDA receptors. As a result, even normal synaptic activity during the postischemic period would lead to enhanced NMDA receptor activity. Although the phenomenon of NMDA receptor potentiation after anoxia has been widely observed (Urban *et al*., 1990; Hori *et al*., 1991; Hammond *et al*., 1994; Mitani *et al*., 1998; Huang & Hsu, 1999), the presence of an overriding mechanism that suppresses NMDA receptor potentiation in neurons resistant to ischemia was not known. Furthermore, our data in support of a central role for tyrosine phosphorylation in the potentiation of NMDA responses identifies a novel prospective target for stroke therapy. This finding is important as blocking NMDA receptors is not a viable treatment option (Kemp & McKernan, 2002). Interestingly, systemic injection of the Src inhibitors PP1 (Paul *et al*., 2001) and PP2 (Lennmyr *et al*., 2004), also used in our experiments, is tolerated in rodents, and significantly reduced infarct size following permanent and transient middle cerebral artery occlusion, respectively. This neuroprotective effect of Src inhibition was attributed to decreased oedema and improved cerebral perfusion. We predict that Src inhibitors permeating the blood–brain barrier will also exert protective effects by reducing NMDA receptor potentiation in vulnerable types of neurons. Ultimately, a multipronged approach will likely be required for clinical applications, as a whole series of processes that can lead to neuronal death are implicated in stroke including mitochondrial dysfunction (Chan, 2004; Starkov *et al*., 2004), TRP channels (Aarts *et al*., 2003), acid sensitive ion channels (Xiong *et al*., 2004; Gao *et al*., 2005), and JNK (Borsello *et al*., 2003) to name a few.

Several observations implicate a potentiation of NMDA responses in the phenomenon of delayed neuronal death. First, antagonists of NMDA receptors block both the ischemia-induced potentiation of the receptors (Hammond *et al*., 1994) and delayed neuronal death (Lipton, 1999) (Fig. 1B). Second, buffering intracellular Ca^{2+} prevents ischemia-induced NMDA receptor potentiation (Crepel & Ben Ari, 1996) (Fig. 4A) and delayed neuronal death (Abdel-Hamid & Tymianski, 1997). Third, inhibitors of tyrosine kinase block ischemia-induced NMDA receptor potentiation (Fig. 3A) and delayed neuronal death (Kindy, 1993; Ohtsuki *et al*., 1996; Paul *et al*., 2001; Lennmyr *et al*., 2004) (Fig. 3D). and fourth, in CA3 pyramidal cells OGD did not lead to NMDA receptor potentiation (Fig. 2A) and these cells are more resistant to delayed neuronal death and excitotoxicity (Schmidt-Kastner & Freund, 1991) (Fig. 1). The observation that SU6656 attenuated NMDA receptor potentiation but not propidium iodide labelling suggests either that it is possible to dissociate OGD induced NMDA receptor potentiation from the neuronal death. Alternatively this is a neurotoxic effect of this compound that is independent of its ability to block Src family tyrosine kinases. As far as we are aware no other studies have

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assessed whether SU6656 is effective at preventing neuronal death in a model of ischemia.

Our conclusion that ischemia potentiates NMDA receptors via tyrosine kinase phosphorylation is supported by several biochemical studies (Shamloo & Wieloch, 1999; Cheung *et al*., 2000; Liu *et al*., 2001; Takagi *et al*., 2003). In gerbil hippocampus, 5 min of global ischemia followed by reperfusion increases tyrosine phosphorylation of NR2A and NR2B subunits of NMDA receptors and the association with FAK, Pyk2, Src and PSD-95 (Zalewska *et al*., 2005). Tyrosine phosphorylation of NR2A and NR2B containing NMDA receptors increases in rat hippocampus after 15 min of global ischemia (Takagi *et al*., 2003). However, the NR2A subunit, which is implicated in current gating and LTP (Kohr & Seeburg, 1996; Liu *et al*., 2004), exhibited greater tyrosine phosphorylation in CA1 than in CA3 tissue only 48 h after ischemia, whereas immediately after global ischemia the increase in NR2A phosphorylation was greater in CA3 than in CA1 (Takagi *et al*., 2003). These results are not entirely consistent with our functional data perhaps reflecting that biochemical assays measure the combined signal from principal cells, interneurons and glia whereas we assayed only pyramidal cells. Furthermore, the specific tyrosine residue(s) whose phosphorylation leads to potentiation of NMDA responses in neurons has yet to be identified and tyrosine phosphorylation of an accessory protein, rather than the direct phosphorylation of NMDA receptors may be responsible for increasing NMDA currents (Salter & Kalia, 2004). Thus it remains unknown whether there is specific regulation of phosphorylation of the relevant tyrosine residues. Additional mechanisms that contribute to the modulation of NMDA currents following ischemic-like insults are redox regulation (Gozlan *et al*., 1994) and changes in pH (Cronberg *et al*., 2005).

In addition to our evidence for an immediate modulation of NMDA receptor function following energy deprivation, a delayed serine phosphorylation-dependent potentiation of NMDA responses in CA1 pyramidal cells occurs 12 hours after cerebral ischemia. Calpain-induced cleavage of p35 to p25 leads to activation of cyclin-dependent kinase 5 (CDK5) and the serine phosphorylation (Wang *et al*., 2003). It would appear that Src-dependent phosphorylation of NMDA receptors is a prerequisite for the CDK5-dependent mechanism as calpain efficiently cleaves NMDA receptors that are not tyrosine phosphorylated by Src (Rong *et al*., 2001).

In CA3 pyramidal cells, energy deprivation resulted in transient depression of NMDA responses due to a Ca^{2+}-dependent (Fig. 4A) and phosphatase-independent (Fig. 4C) process. This response is followed by a return to baseline levels, even though these cells contain the molecular machinery for Src activation and can exhibit Src-dependent potentiation of NMDA responses after metabotropic receptor activation (Xiong *et al*., 1999; Benquet *et al*., 2002). Our data indicate that, in contrast to the response in CA1 pyramidal cells, energy deprivation in CA3 pyramidal cells shifts the kinase/phosphatase balance in favour of tyrosine phosphatase activity. Under nonischemic conditions, Ca^{2+} entry through NMDA receptors activates the Ca^{2+}-dependent phosphatase calcineurin, which in turn activates the tyrosine phosphatase STEP (striatal-enriched phosphatase) (Paul *et al*., 2003). In hippocampus, STEP is associated with the NMDA receptor complex and in its activated form depresses NMDA receptor currents by acting as a functional opponent of Src (Pelkey *et al*., 2002). Whether STEP counteracts Src activity in CA3 pyramidal cells during ischemia is unknown. During ischemia a lower molecular weight isoform of STEP is released into the cytoplasm, the functional implications of which remain to be determined (Gurd *et al*., 1999).

Why should an increase in intracellular Ca^{2+} favour tyrosine kinase activity in CA1 pyramidal cells and tyrosine phosphatase activity in CA3 pyramidal cells? Our data suggest this is the case although it remains unclear why under conditions of high Ca^{2+}-buffering tyrosine kinase activity would be maintained in CA3 pyramidal cells and blocked in CA1 pyramidal cells. In CA3 pyramidal neurons, both genistein and PP2 were able to block the OGD-induced potentiation of NMDA receptors revealed with high BAPTA suggesting that it was not only a special, nonspecific genistein effect that was responsible for this result. The activity of Src family tyrosine kinases is not known to be Ca^{2+}-dependent. However, activation of Src can occur downstream of both phosphatase and kinase activity and many of these enzymes are Ca^{2+}-dependent. The upstream effectors may be different in CA1 and CA3 pyramidal cells and may therefore have different dependencies on Ca^{2+}. Other studies have also addressed whether NMDA responses are tonically regulated by tyrosine kinases. The tyrosine kinase inhibitors genistein and lavendustin A rapidly decrease NMDA responses in CA1 and dorsal horn neurons (Wang & Salter, 1994; Lu *et al*., 1999), whereas loading cells with an inhibitory Src peptide did not decrease CA1 NMDA responses (Lu *et al*., 1998) despite the fact it rapidly depresses single channel activity in inside-out patches under conditions of high Ca^{2+} buffering (Yu *et al*., 1997). Perhaps differences in recording conditions or preparations account for these discrepancies. How a rise in Ca^{2+} leads to apparently opposite effects in CA1 and CA3 pyramidal neurons cannot be answered satisfactorily at present, however, possible explanations include differences in the Ca^{2+} sensitivity of the tyrosine kinase and tyrosine phosphatase pathways, differences in endogenous Ca^{2+} buffering and differences in compartmentalization of Ca^{2+} signalling complexes between CA1 and CA3 pyramidal cells.

An emerging view in stroke research is that targeting signalling components downstream from the NMDA receptors holds greater promise for neuroprotection than attempts to manipulate the receptors themselves (Aarts *et al*., 2002; Borsello *et al*., 2003). Drugs that modulate NMDA receptors or disrupt distal events in the neurotoxic cascade are more likely to be effective during an extended time window. Thus the development of therapies that mimic the brain's own neuroprotective strategy of down-regulating neuronal Src signalling may prove useful in the treatment of stroke.

Supplementary material

The following supplementary material may be found on http://www.blackwell-synergy.com

Fig. S1. OGD similarly affected synaptic currents and Ca^{2+} rises in CA1 and CA3 pyramidal cells.

Fig. S2. OGD induced similar changes of input resistance and holding current in CA1 and CA3 pyramidal cells.

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Abbreviations

ACSF, artificial cerebrospinal fluid; NMDA, *N*-methyl-D-aspartate; OGD, oxygen–glucose deprivation; PI, propidium iodide.

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