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$K_{Ca}2$ channels activation prevents $[Ca^{2+}]_i$ deregulation and reduces neuronal death following glutamate toxicity and cerebral ischemia

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Exacerbated activation of glutamate receptor-coupled calcium channels and subsequent increase in intracellular calcium ($[Ca^{2+}]_i$) are established hallmarks of neuronal cell death in acute and chronic neurological diseases. Here we show that pathological $[Ca^{2+}]_i$ deregulation occurring after glutamate receptor stimulation is effectively modulated by small conductance calcium-activated potassium ($K_{Ca}2$) channels. We found that neuronal excitotoxicity was associated with a rapid downregulation of $K_{Ca}2.2$ channels within 3 h after the onset of glutamate exposure. Activation of $K_{Ca}2$ channels preserved $K_{Ca}2$ expression and significantly reduced pathological increases in $[Ca^{2+}]_i$ providing robust neuroprotection *in vitro* and *in vivo*. These data suggest a critical role for $K_{Ca}2$ channels in excitotoxic neuronal cell death and propose their activation as potential therapeutic strategy for the treatment of acute and chronic neurodegenerative disorders.
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Excessive release of glutamate and uncontrolled neuronal excitations lead to progressive neuronal death following cerebral ischemia. In particular, glutamate-induced stimulation of α-amino-3-hydroxy-5-methyl-4-isoxazol-propionic acid and *N*-methyl-D-aspartate (NMDA) receptors mediates toxic increases in intracellular calcium concentrations ($[Ca^{2+}]_i$). In neurons, disrupted intracellular calcium homeostasis disturbs multiple metabolic processes thereby promoting progressive cell death.[1–3] Consequently, inhibition of NMDA receptors has been extensively investigated in experimental and clinical stroke studies. For example, studies on NMDA receptor antagonists such as dizocilpine (MK-801) or memantine revealed neuroprotective effects against ischemia-induced neuronal death *in vitro* and ischemic brain damage *in vivo*,[4,5] promoting disturbed $[Ca^{2+}]_i$ as a promising therapeutic target. However, inhibitors of NMDA receptors largely failed in clinical studies[6] and, therefore, novel strategies controlling $[Ca^{2+}]_i$ homeostasis are warranted for the development of effective therapies of neurological disorders associated with excessive NMDA receptor stimulation.

It has been suggested that small-conductance calcium-activated potassium (KCNN/SK/$K_{Ca}2$) channels may control membrane excitability through attenuated NMDA receptor activity.[7,8] In neurons, synaptic NMDA receptors are closely associated with $K_{Ca}2$ channels in dendritic spines. Small increases in $[Ca^{2+}]_i$ lead to the activation of $K_{Ca}2$ channels, which induce afterhyperpolarization thereby providing a feedback control that prevents toxic increases in $[Ca^{2+}]_i$ under physiological conditions.[9]

How $K_{Ca}2$ channel-dependent $[Ca^{2+}]_i$ regulation is altered under pathological conditions, in which glutamate toxicity has an essential role, is so far unknown. Thus, we address the question whether pharmacological modulation of $K_{Ca}2$ channels attenuates glutamate-induced $[Ca^{2+}]_i$ deregulation and cell death in primary cultured neurons *in vitro* and reduces infarct size following cerebral ischemia *in vivo*.

Results

Activation of $K_{Ca}2$ channels prevents the increases in $[Ca^{2+}]_i$ after glutamate exposure in neurons. Glutamate immediately induced increases in fluorescence intensities of the calcium-sensitive dye FURA-2 and these increases in $[Ca^{2+}]_i$ over control levels sustained until termination of the measurements (Figure 1a). These glutamate-induced alterations in $[Ca^{2+}]_i$ signals can be subdivided into three phases: an early phase was detected as a very fast and

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Abbreviations: NMDA, *N*-methyl-D-aspartate; $[Ca^{2+}]_i$, intracellular cytosolic calcium concentration; $K_{Ca}2$, small-conductance calcium-activated potassium channels; I_{AHP}, afterhyperpolarization current; DCD, delayed calcium deregulation; TTX, tetrodotoxi; MTT, (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; EGTA, ethylene glycol tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; BHQ, 2,5-di-*t*-butyl-1, 4-benzohydroquinone; ER, endoplasmatic reticulum; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; MCAo, middle cerebral artery occlusion; NF-κB, nuclear factor-κ B; CAF, cytosine arabinoside
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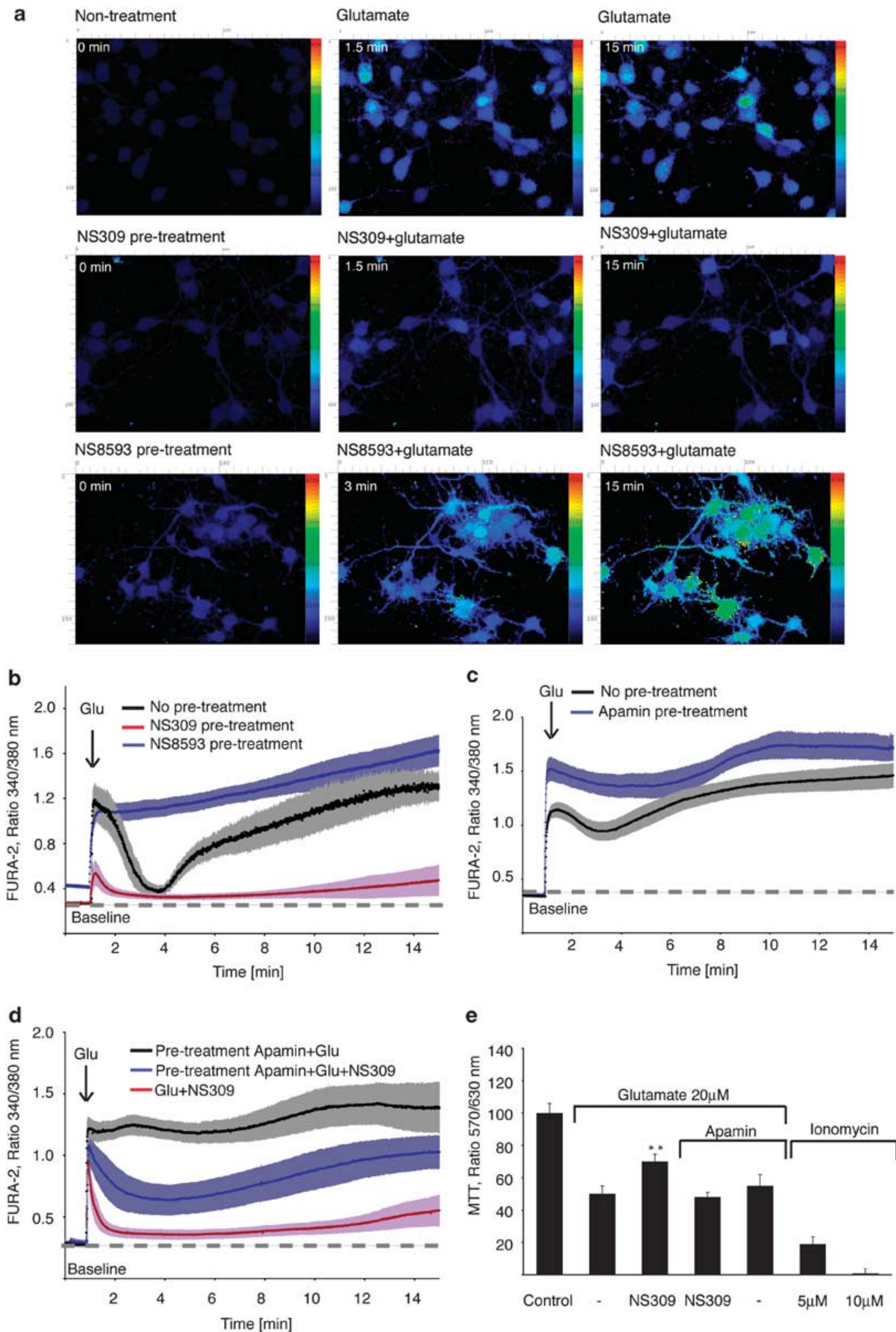

Figure 1 Effect of $K_{Ca}2$ channel modulators on the onset of glutamate-induced delayed calcium deregulation. (**a**) Neuronal cells were loaded with FURA-2 AM and then stimulated with glutamate (20 μM) in the presence or absence of NS309 (50 μM), NS8593 (50 μM) or apamin (1 μM) pre-treatment. Glutamate was applied to neurons after monitoring the cells for 1 min, as shown in the $[Ca^{2+}]_i$ kinetic profile. (**a**) Representative FURA-2 fluorescence intensities from neurons stimulated with glutamate (at 1 min) in the presence of NS309, NS8593 or apamin. (**b–d**) Depicted are representative traces of $[Ca^{2+}]_i$ FURA-ratios values ($\pm$ S.E.M.) stimulated with glutamate in the presence of the indicated $K_{Ca}2$ channel modulators ($n = 15$–25). (**e**) MTT analysis of primary neuronal cultures pre-treated with NS309 (50 μM) or apamin (10 μM) and challenged with glutamate (20 μM) for 24 h (**$P < 0.01$ *versus* glutamate-treated neurons, ANOVA, Scheffé's test)

pronounced increase in $[Ca^{2+}]_i$ followed by a second phase with a short decrease of $[Ca^{2+}]_i$, and a third phase characterized by a sustained $[Ca^{2+}]_i$ increase that showed a higher amplitude than the early phase (Figure 1b). The length of the second phase varied in range from seconds to minutes in different experimental settings. Moreover, individual neurons show this intermediate decrease of $[Ca^{2+}]_i$ at different time intervals ranging from 3 to 4 min up to 10 min, which limits the detection of this proposed second phase in average Ca^{2+} kinetics plotted from up to 25 neurons. The third phase of the Ca^{2+} kinetic is regarded as 'delayed calcium deregulation' (DCD), which contributes to delayed cellular death.[1,10] DCD was detected for periods of up to 1–2 h (Supplementary Figure S1A). Measurements of $[Ca^{2+}]_i$ in cortical neurons did not show any spontaneous $[Ca^{2+}]_i$ spikes in both basal conditions or in glutamate-stimulated neurons. This was further demonstrated by application of tetrodotoxin (TTX), which is known to block the transient spontaneous $[Ca^{2+}]_i$ spikes. TTX failed to influence DCD formation (Supplementary Figure S1B). This finding is consistent with other reports that founds that TTX does not block DCD formation[1] and also does not prevent the delayed cell death.[11]

Single-cell fluorescence imaging further revealed that pre-treatment of neurons with NS309, an activator of $K_{Ca}2$ channels,[12] prevented increases in $[Ca^{2+}]_i$, and significantly reduced DCD (Figure 1). Whole-culture $[Ca^{2+}]_i$ recordings for more than 1 h confirmed that the $K_{Ca}2$ channel activator NS309 attenuated the glutamate-induced elevated levels of $[Ca^{2+}]_i$ similar to the observations obtained by single-cell imaging (Supplementary Figure S1A).

In contrast to the findings with the $K_{Ca}2$ activator NS309, pharmacological inhibition of $K_{Ca}2$ channels with NS8593 further increased the glutamate-induced increases in $[Ca^{2+}]_i$ levels in the DCD phase (Figures 1a and b). As NS8593 is a negative modulator for all $K_{Ca}2$ channels,[10] we used the highly specific $K_{Ca}2.2$ channel blocker apamin. In line with findings obtained with NS8593, apamin also promoted a further increase in $[Ca^{2+}]_i$ compared with the glutamate challenge alone (Figure 1c). Moreover, pre-incubation with apamin reduced the effect of NS309 on $[Ca^{2+}]_i$ (Figure 1d).

Previous studies established NS309 as a potent activator of recombinant $K_{Ca}2$ channels that induces an increase of the I_{AHP} in hippocampal brain slices.[12] Here we tested the effect of NS309 (50 μM) on I_{AHP} in CA1 neurons from 17- to 21-day-old mice. Before NS309 application, the depolarization step induced a characteristic I_{AHP}. NS309 induced an increase of IAHP in all cells tested (six of six cells; Supplementary Figure S2). That was detectable even if the activator was removed from the bath (Supplementary Figure S2).

To further strengthen the direct action of $K_{Ca}2$ channels on calcium signaling, we investigated whether other potassium channels might affect calcium homeostasis. To this end, we applied diazoxide, a specific activator of K_{ATP} channels. Pre-treatment and also post-treatment with diazoxide did not affect glutamate-induced DCD, suggesting that K_{ATP} channels do not have a direct effect on DCD signaling. In addition, inhibition of K_{ATP} channels with glibenclamide did not significantly alter the DCD (Supplementary Figure S3).

As $K_{Ca}2$ channel activation reduced $[Ca^{2+}]_i$ after excessive stimulation of glutamate receptors in neurons, we next investigated whether $K_{Ca}2$ channel activation provided neuroprotective effects against glutamate-induced excitotoxicity. Activation of $K_{Ca}2$ channels by NS309 significantly reduced glutamate toxicity (Figure 1e). In contrast, when primary cortical neurons were subjected to toxic doses of glutamate in the presence of the $K_{Ca}2.2$ channel blocker apamin, neuronal cells were not rescued (Figure 1e). Further, apamin also attenuated NS309-induced neuronal survival indicating that neuroprotection by NS309 was mediated through $K_{Ca}2.2$ channel activation (Figure 1e). Although NS8593 (50 μM) in combination with the glutamate challenge resulted in a further increase in DCD mean values compared with DCD induced by glutamate alone, NS8593 failed to show a further induction of apoptotic markers, such as caspase 3 activation (Supplementary Figure S3). As a positive control for a complete induction of cellular death we used ionomycin at different concentrations (Figure 1e). Notably, NS309 failed to promote cellular survival against glutamate toxicity when $K_{Ca}2.2$ channels were downregulated by siRNA, confirming the particular role of this subtype as previously demonstrated by pharmacological compounds (Supplementary Figure S4). Overall, these findings strongly suggest that $K_{Ca}2$ channel activity was critical for the maintenance of intracellular Ca^{2+} homeostasis and protection against glutamate-induced excitotoxicity in primary neurons.

Modulation of $K_{Ca}2$ channels during deregulated Ca^{2+} homeostasis. To assess the effectiveness of $K_{Ca}2$ channel modulators on regulating glutamate-induced $[Ca^{2+}]_i$ deregulation, we also applied NS309 and NS8593 after the onset of the glutamate challenge. When NS309 was applied to neurons after the onset of the early peak of $[Ca^{2+}]_i$, a significant recovery of $[Ca^{2+}]_i$ was observed (Figures 2a and b). NS309 reduced $[Ca^{2+}]_i$ by $\sim$75% in the first minute and by $\sim$90% after 5 min. In contrast, inhibition of $K_{Ca}2$ channels by NS8593 further elevated $[Ca^{2+}]_i$ by 30% in the first 5 min and by 60% after 10 min (Figure 2b).

As demonstrated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, NS309 unequivocally protected the neurons against glutamate toxicity when applied before and up to 3 h after the onset of a glutamate challenge, in line with the restoration of calcium homeostasis (Figure 2c).

$K_{Ca}2$ channels reduce the Ca^{2+} influx from the extracellular space. Although NS309 completely restored $[Ca^{2+}]_i$ when applied in the first minutes after onset of glutamate exposure (Figures 2b and 3a), NS309 only partially attenuated the DCD when applied during the late phase of increased $[Ca^{2+}]_i$ (Figure 3b). The remaining question of why $K_{Ca}2$ channel activation failed to completely recover $[Ca^{2+}]_i$ applied after the onset of DCD prompted us to investigate the sources of $[Ca^{2+}]_i$ increases. To this end, ethylene glycol tetraacetic acid (EGTA) or ethylenediaminetetraacetic acid (EDTA; 4 mM) were applied to complex extracellular $[Ca^{2+}]$. Under these conditions, glutamate neither increased $[Ca^{2+}]_i$ nor induced DCD (Figure 3c), suggesting that extracellular Ca^{2+} was

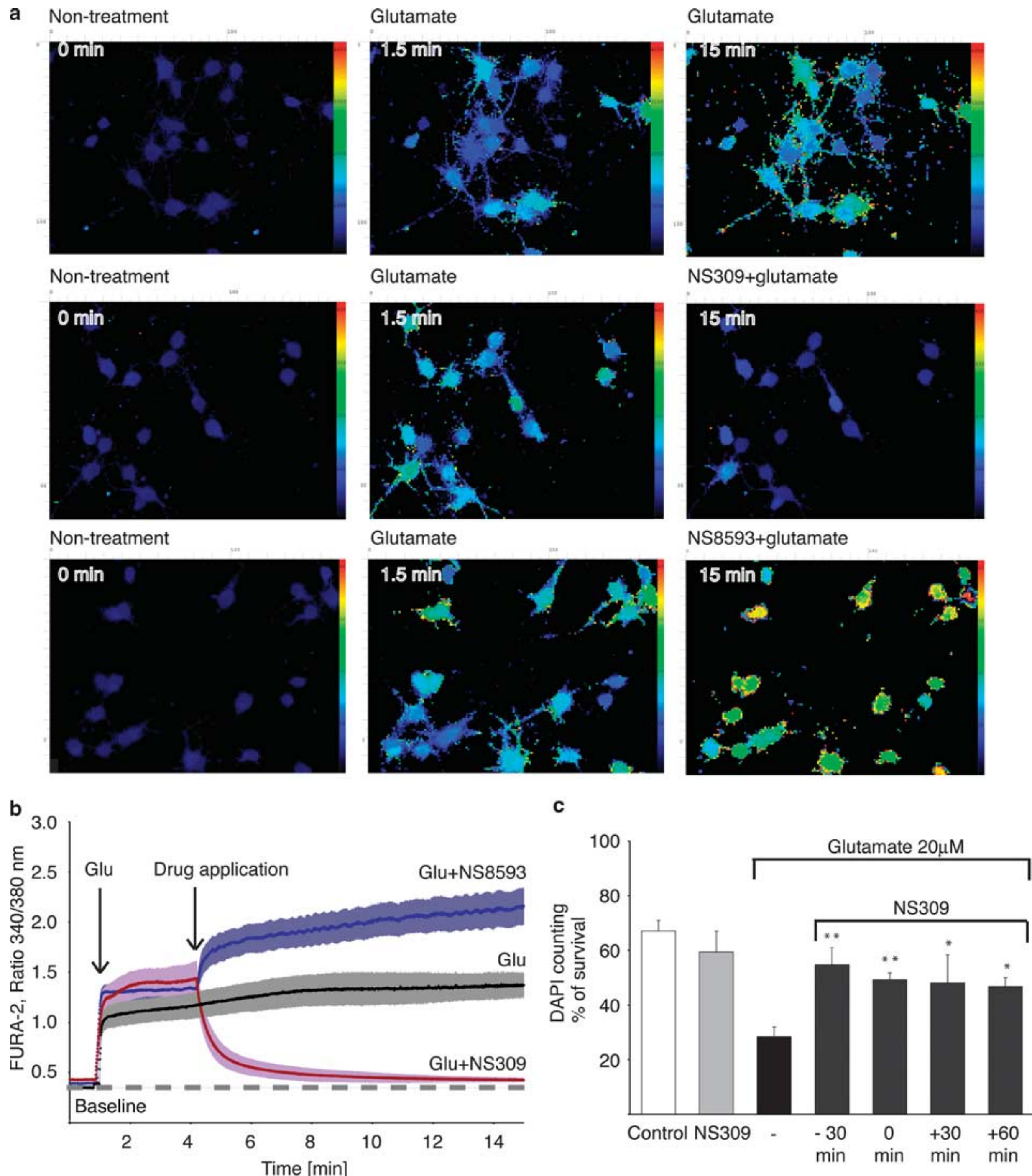

Figure 2 Effect of $K_{Ca}2$ channel modulators after the onset of glutamate-induced delayed calcium deregulation. (**a**) Representative FURA-2 fluorescence intensities from single cells stimulated with 20 μM glutamate (at 1 min). NS309 (50 μM) or NS8593 (50 μM) were applied at 4 min after onset of the glutamate challenge. (**b**) Kinetic profiles of $[Ca^{2+}]_i$ values (± S.E.M.) stimulated with glutamate (20 μM) and then treated with NS309 (50 μM), NS8593 (50 μM) at the indicated time ($n=15$–20). (**c**) DAPI counting of neurons challenged with glutamate (20 μM) in the presence or absence of NS309 (50 μM) applied as indicated (*$P<0.05$, **$P<0.01$ *versus* glutamate-treated neurons, ANOVA Scheffé's test)

required for $[Ca^{2+}]_i$ deregulation. Complete removal of external Ca^{2+} during the early glutamate-induced $[Ca^{2+}]_i$ peak led to a fast $[Ca^{2+}]_i$ recovery (Figures 3d and f). However, when extracellular Ca^{2+} was depleted with EDTA after the onset of DCD, $[Ca^{2+}]_i$ recovery was delayed and incomplete (Figure 3g). The low-sensitivity of DCD to extracellular Ca^{2+} removal was similar to the previously observed failure of NS309 to completely restore the $[Ca^{2+}]_i$ at late time points. The remaining $[Ca^{2+}]_i$ could be a result of delayed Ca^{2+} release from intracellular stores from the

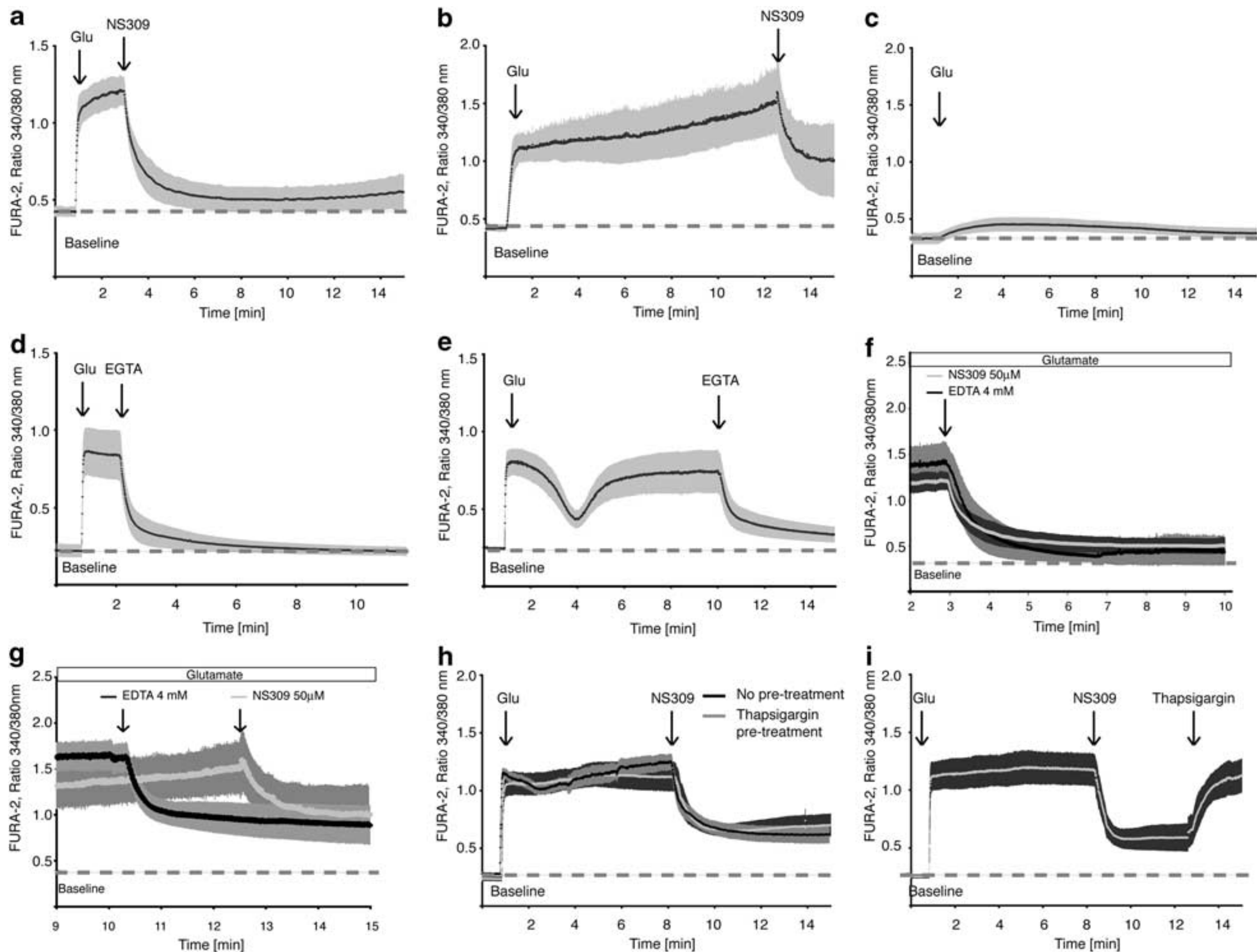

Figure 3 Depletion of extracellular Ca^{2+} prevents the formation of delayed calcium deregulation (DCD). Neuronal cells were loaded with FURA-2 and then stimulated with glutamate (20 μM). The $K_{Ca}2$ channel activator (50 μM NS309), or EGTA (4 mM) were applied to neurons 2 min (**a**) or 9–12 min (**c**) after exposure to glutamate. The influences of NS309 (**a**, **b**, **f**–**i**), EDTA (**c**, **f** and **g**) and EGTA (**d** and **e**) on the kinetic profile of $[Ca^{2+}]_i$ were evaluated prior and after the onset of DCD. Depicted are the kinetic profiles of $[Ca^{2+}]_i$ ($\pm$ S.E.M.; $n = 15$–20). (**h** and **i**) The depletion of ER intracellular Ca^{2+} pool was achieved with 100 μM thapsigargin prior to or after the onset of DCD

endoplasmic reticulum (ER). To deplete Ca^{2+} from ER stores and to block sarco/ER Ca^{2+}-ATPase (SERCA) pumps, we applied thapsigargin and 2,5-di-t-butyl-1,4-benzohydroquinone (BHQ). High concentrations of thapsigargin (100 μM) mediated very small $[Ca^{2+}]_i$ increases followed by complete $[Ca^{2+}]_i$ recovery within 10 min (Supplementary Figure S5A). Pre-treatment with thapsigargin did not influence the glutamate-induced $[Ca^{2+}]_i$ deregulation (Figure 3h However, when $K_{Ca}2$ channels were activated after the onset of DCD, 100 μM thapsigargin completely blocked the protective effect of NS309 on $[Ca^{2+}]_i$ (Figure 3i). Thapsigargin and BHQ at lower concentrations (10 and 25 μM, respectively) only partially blocked the $K_{Ca}2$ channel-mediated rescue of $[Ca^{2+}]_i$ (Supplementary Figure S5B). Altogether, these findings suggest that the remaining delayed increases in $[Ca^{2+}]_i$ were attributed to extracellular and intracellular sources.

Activation of $K_{Ca}2$ channels attenuates NMDA-induced $[Ca^{2+}]_i$ deregulation. Glutamate-induced increases in $[Ca^{2+}]_i$ through the NMDA receptor has a dominant role in $[Ca^{2+}]_i$ deregulation and in neuronal death.[10,13] In primary neurons, NMDA increased $[Ca^{2+}]_i$ and shaped the Ca^{2+} kinetics in a similar manner as glutamate (Supplementary Figure S6A). Pre-treatment with the NMDA antagonist MK801 attenuated the magnitude of the early glutamate-induced $[Ca^{2+}]_i$ peak and prevented the onset of DCD (Supplementary Figure S6B). In addition, MK801 partially restored $[Ca^{2+}]_i$ levels when added after the onset of NMDA-induced DCD (Supplementary Figure S6C). These data supported the conclusion that the initial increase in $[Ca^{2+}]_i$ was mediated by NMDAR stimulation, whereas the following DCD was mediated by additional sources of Ca^{2+}. Activation of $K_{Ca}2$ channels by NS309 resulted in a complete recovery of $[Ca^{2+}]_i$ when applied before NMDA-induced $[Ca^{2+}]_i$ deregulation (Supplementary Figure S6A). However, NS309 only partially restored $[Ca^{2+}]_i$ when applied after the onset of NMDA-induced DCD (Supplementary Figure S6D). Moreover, MTT analysis revealed that pre-treatment with NS309 rescued primary neurons from NMDA-induced neuronal death, suggesting that $K_{Ca}2$ channels affected NMDA receptor activity and attenuated NMDA-mediated excitotoxicity (Supplementary Figure S6E).

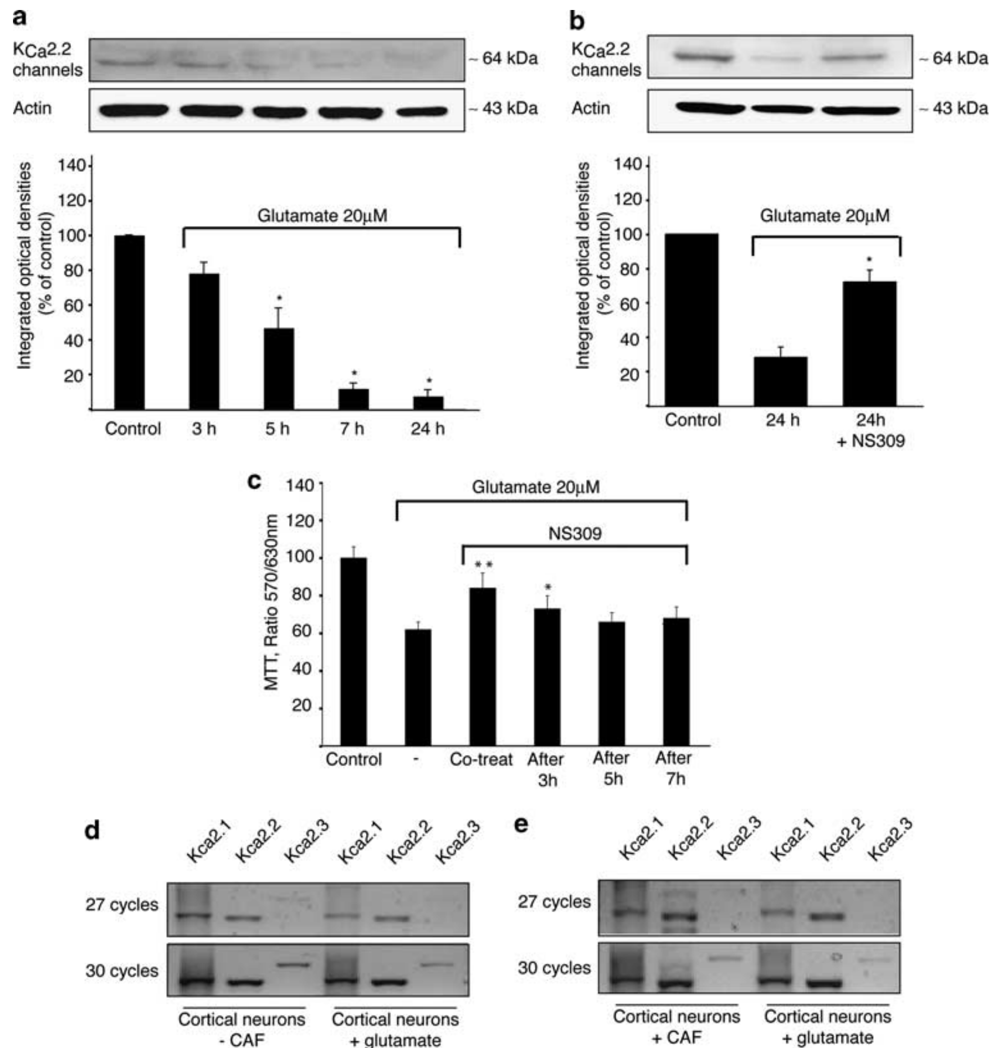

Figure 4 Glutamate attenuates the $K_{Ca}2.2$ channel protein expression. (**a**) Western blot analysis of $K_{Ca}2.2$ channel expression at the indicated time points after glutamate exposure (20 μM). Representative immunoblots are shown at the upper part of the quantified data of $K_{Ca}2.2$ channels analyzed from different experiments ($n=3$, $^{*}P<0.05$ *versus* non-treated PCN). (**b**) Neuronal cells were challenged with glutamate for 24 h with or without 30 min pretreatment with NS309 (50 μM). Representative immunoblots are shown at the upper part of the quantified data of $K_{Ca}2.2$ channels ($n=3$, $^{*}P<0.05$ *versus* non-treated PCN). (**c**) MTT analysis of neurons treated with NS309 (50 μM) 0 h, 3 h, 5 h and 7 h after the application of glutamate (20 μM). $^{*}P<0.05$, $^{**}P<0.01$ *versus* glutamate-treated neurons were considered to be significant, ANOVA Scheffé's test. (**d** and **e**) mRNA analysis of $K_{Ca}2$ channel subtypes in the presence or absence of glutamate in **(d)** astrocyte–neuron co-culture and in **(e)** pure cortical neuronal cultures

Glutamate neurotoxicity is associated with reduced $K_{Ca}2$ channel protein levels. To gain further insight into the relation between glutamate toxicity and $K_{Ca}2$ channel-mediated $[Ca^{2+}]_i$ regulation, we next investigated the expression levels of $K_{Ca}2$ channels after the glutamate challenge. Glutamate reduced the protein levels of $K_{Ca}2.2$ channels in a time-dependent manner (Figure 4a). These results suggested that the observed DCD after glutamate receptor stimulation and the failure of NS309 to rescue $[Ca^{2+}]_i$ levels when applied with delay was at least in part attributed to $K_{Ca}2$ channel degradation. In fact, the $K_{Ca}2$ channel activator NS309 prevented the glutamate-induced decrease in $K_{Ca}2.2$ channel expression (Figure 4b) and NS309 protected neurons against glutamate toxicity when applied up to 3 h after the onset of glutamate challenge, which was perfectly in line with the observed protein expression levels of $K_{Ca}2.2$ channels (Figure 4c). Further, EDTA application was able to block glutamate-induced $K_{Ca}2.2$ channel downregulation, suggesting that increased $[Ca^{2+}]_i$ mediated this effect (Supplementary Figure S7A). On the other hand, increase in $[Ca^{2+}]_i$ can activate Ca^{2+}-dependent genes, which could control the expression of $K_{Ca}2.2$ channels. Interestingly, short application of the selective glutamate receptor agonist NMDA induced an upregulation of $K_{Ca}2.2$ channel protein levels in the long term (Supplementary Figure S7B). This was potentiated in cells in which calpain activity was blocked, suggesting that Ca^{2+}-dependent activation of calpains negatively controls $K_{Ca}2.2$ channel protein levels.

Analysis of mRNA levels of $K_{Ca}2$ channel subtypes revealed that cortical neurons express all $K_{Ca}2$ subtypes: $K_{Ca}2.1$, $K_{Ca}2.2$ and $K_{Ca}2.3$ (Figures 4d and e). The $K_{Ca}2.3$

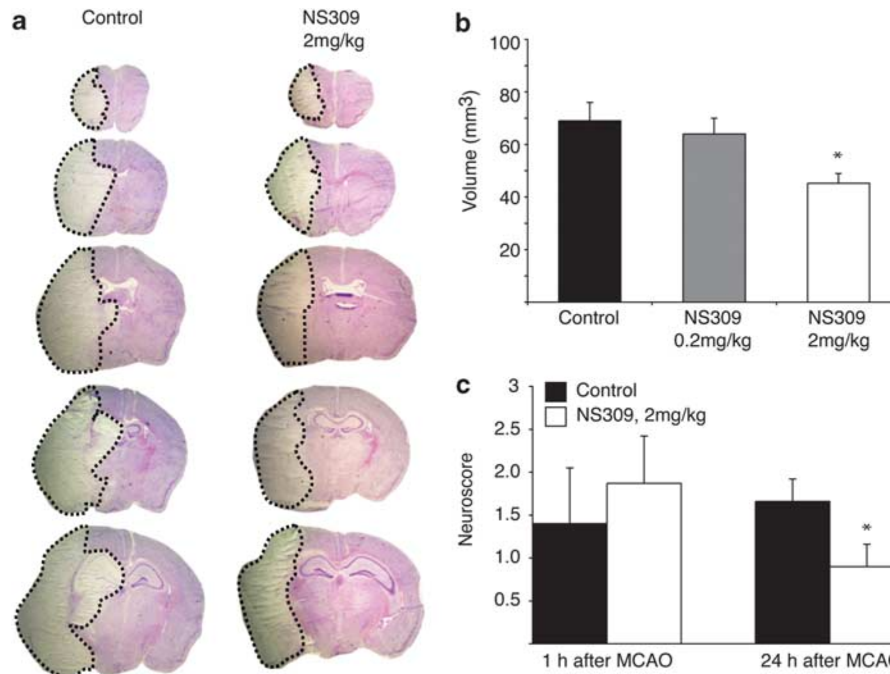

Figure 5 $K_{Ca}2$ channel activator NS309 reduces infarct volume after transient focal cerebral ischemia. (**a**) Representative coronal brain slices obtained at 24 h after focal cerebral ischemia from vehicle control animals (left) and mice treated with 2 mg/kg NS309 right. The slices were stained with cresyl violet to identify unstained infarct areas as marked by the dashed line. (**b**) Quantification of total infarct volume determined 24 h after MCAo in mice treated with 0.2 or 2 mg/kg NS309 compared with vehicle-treated controls. (**c**) Neuroscore measured at indicated time points after middle cerebral artery occlusion ($n = 6$, $^*P < 0.05$ *versus* non-treated mice. Mann–Whitney *U*-test)

channel is less abundant than $K_{Ca}2.1$ and $K_{Ca}2.2$ channel subtypes in both pure neuronal cultures (treated with cytosine arabinoside (CAF)) and in neuron–astrocyte co-cultures (without CAF treatment). In the pure cortical neuronal cultures, $K_{Ca}2.3$ channels seemed to be expressed at lower levels compared with neuron–astrocyte co-cultures, suggesting that $K_{Ca}2.1$ and $K_{Ca}2.2$ channels mediated the effects of NS309 on glutamate-induced calcium deregulation. Glutamate did not drastically affect the $K_{Ca}2.1$ and $K_{Ca}2.2$ mRNA expression (Figures 4d and e).

$K_{Ca}2$ channel activator NS309 reduces ischemic brain damage after experimental middle cerebral artery occlusion (MCAo) in mice. To translate our results from *in vitro* studies in which NS309 promoted neuroprotection against glutamate and NMDA toxicity, we next evaluated whether NS309 could provide neuroprotective effects in a model of ischemic brain damage *in vivo*. NS309 (0.2 and 2 mg/kg) was applied intraperitoneally 30 min before transient focal cerebral ischemia. Controls received vehicle, 2% DMSO in 0.9% saline. At 24 h after reperfusion, infarct volume was $69 \pm 7\,mm^2$ (mean ± S.E.M.) in the control group (Figures 5a and b). Non-treated animals showed extensive ischemic injury, whereas significant protection was observed in NS309-treated mice (Figure 5a). Histo-morphometrical analysis demonstrated that a single dose of NS309 (2 mg/kg) reduced the infarct volume to $45 \pm 4\,mm^3$ compared with the vehicle-treated controls ($69 \pm 7\,mm^2$; mean ± S.E.M.; Figure 5b). The lower dose of NS309 (0.2 mg/kg) did not reduce the infarct volume compared to the controls. NS309-treated mice (2 mg/kg) exhibited fast and extensive recovery with improved neurological function score[14] compared with controls as determined 24 h after MCAo (Figure 5c).

Discussion

In this study, we identified $K_{Ca}2$ channels as a promising target to preserve $[Ca^{2+}]_i$ homeostasis by counteracting glutamate- and NMDA-induced $[Ca^{2+}]_i$ deregulation and neurotoxicity *in vitro* and reducing infarct development after cerebral ischemia *in vivo*. In neurons, glutamate triggers immediate increases in $[Ca^{2+}]_i$ through activation of NMDA-receptors followed by sustained disturbances in the intracellular Ca^{2+} homeostasis.[10,15] However, key regulators of intracellular Ca^{2+} homeostasis that may serve as targets to prevent such sustained glutamate toxicity in neurological diseases have not been identified.

In this study, the $K_{Ca}2$ activator NS309 significantly attenuated $[Ca^{2+}]_i$ increase when applied before the onset of DCD, and partially contributed to $[Ca^{2+}]_i$ recovery when applied after onset of glutamate-induced DCD. Further, we observed a close correlation of the neuroprotective potential of $K_{Ca}2$ channel activation and their expression levels. Notably, activation of the $K_{Ca}2$ channels preserved their expression and their protective potential after exposure to glutamate or NMDA. Previous reports suggested that reduced expression and functional loss of $K_{Ca}2$ channels were connected to hyperexcitability in neurodegenerative processes.[16,17] The exact mechanism of such $K_{Ca}2$ channel inactivation associated with neurodegenerative processes has not been clarified so far. Our results now indicate that

activation of NMDAR was involved in glutamate-induced $[Ca^{2+}]_i$ deregulation and subsequent delayed neuronal death. Both, NMDA inhibitors and pharmacological activation of $K_{Ca}2$ channels attenuated NMDA- and glutamate-induced $[Ca^{2+}]_i$ deregulation. In the soma of CA1 hippocampal regions, single-channel measurements showed that L-type calcium channel and $K_{Ca}2$ channels are co-localised.[9] The contribution of voltage-dependent channels to the formation and maintenance of DCD is unlikely, as previous experiments using the L-type calcium channel blocker nitrendipine had little effect on the delayed Ca^{2+} overload once DCD had been established.[1] However, a more recent study showed the involvement of transient receptor potential and store-operated Ca^{2+} channels, as the respective inhibitors 2-aminoethoxydiphenyl borate and La^{3+} attenuated glutamate-induced DCD.[18] In bullfrog sympathetic neurons, Ca^{2+}-induced Ca^{2+} release activation prolongs Ca^{2+} transients and activates BK channels that are closely associated with N-type Ca^{2+} channels, ryanodine and $K_{Ca}2$ channels.[9] Ryanodine and IP3 receptors might also be involved in DCD regulatory processes, as application of ryanodine or caffeine was shown to inhibit glutamate-induced DCD.[18] Removing the extracellular Ca^{2+} by adding the extracellular Ca^{2+} chelators EDTA or EGTA resulted in maintenance of $[Ca^{2+}]_i$. Thus, activation of $K_{Ca}2$ appears to be highly efficient to block pathological Ca^{2+} influx from the extracellular space in the context of glutamate excitotoxicity.

Pharmacological blockage of SERCA pumps with thapsigargin or BHQ partially blocked the protective effect of NS309 on DCD. However, thapsigargin alone did not alter $[Ca^{2+}]_i$ and did not further exacerbate DCD, arguing against a major contribution of intracellular store depletion for the observed glutamate-induced DCD. Our observation is in agreement with a report describing that thapsigargin did not increase mean onset time or incidence of DCD.[18] Overall, our findings propose that NS309-mediated $K_{Ca}2$ activation restores Ca^{2+} homeostasis by diminishing the Ca^{2+} entry from the extracellular pool but still requires functional intracellular Ca^{2+} stores for full effectiveness.

Another important finding was that NS309 preserved the expression of $K_{Ca}2$ channels in neurons exposed to glutamate. This suggests that glutamate-induced excitotoxicity is mediated by activation of NMDAR and concomitant disruption of counteractive mechanisms such as $K_{Ca}2$ channels that disappear after the excitotoxic stimulus. This observation may explain both, the lack of adaptation to glutamate receptor overstimulation, and the therapeutic time window observed for NS309. As shown in this study, NS309 mediated neuroprotection only when applied up to 3 h after the onset of $[Ca^{2+}]_i$ deregulation. This is in agreement with the time window of the progressive decline in $K_{Ca}2.2$ channel expression levels upon glutamate damage. After exposure to glutamate for 5 h or more, neurons expressed less $K_{Ca}2.2$ channels and around this time point NS309 lost the neuroprotective potential. Apparently, such reduced $K_{Ca}2.2$ protein levels resulted in a failure to regulate neuronal excitability despite increased Ca^{2+} concentrations that usually enhance the activity of the channel. Thus, neurons undergoing delayed neuronal death after the glutamate challenge were continuously deprived of this important feedback mechanism of $[Ca^{2+}]_i$, therefore showing increased excitability and deregulated calcium homeostasis. These findings are in agreement with the direct correlation between the prevention of DCD and reduced $K_{Ca}2.2$ channel degradation. The mechanism of $K_{Ca}2$ channel regulation is still unclear but may result from Ca^{2+}-dependent protein degradation or enhanced internalization from the membrane, which might be dependent on calpain activation.

Brief application of the glutamate receptor agonist NMDA, on the contrary, increased $K_{Ca}2.2$ channel protein levels, which was further augmented by calpain inhibition. This suggests that activation of calcium-dependent proteases and/or Ca^{2+}-dependent gene transcription may regulate $K_{Ca}2.2$ channel expression physiologically. Although Ca^{2+}-activated calpains may enhance $K_{Ca}2.2$ degradation, nuclear factor-κ B (NF-κB) activity may enhance neuroprotective $K_{Ca}2.2$ expression.[19,20] It has been shown that preserved transcriptional activity of NF-κB in neurons provides cerebroprotective effects in a variety of degenerative conditions after acute brain damage.[21,22] Thus, in addition to direct pharmacological activation as achieved here with NS309, induction of $K_{Ca}2$ channel expression through enhanced NF-κB transcriptional activity may be an additional strategy to activate this system of intracellular Ca^{2+} regulation.

The promising effects of $K_{Ca}2$ channel activation demonstrated *in vitro* are also relevant for ischemic neuronal death *in vivo*. Our data strongly suggest a therapeutic potential for $K_{Ca}2$ channel activators in paradigms of excitotoxic neuronal damage that contributes to infarct development after cerebral ischemia. In line with our findings, overexpression of $K_{Ca}2$ channels in the dentate gyrus attenuated kainic acid-induced hippocampal CA3 lesion, suggesting that $K_{Ca}2$ channels are a common motif of Ca^{2+} autoregulation at different glutamate receptors.[23] *In vivo*, the protective function of NS309 may be extended to non-neuronal cells, as NS309 can enhance ATP-evoked membrane hyperpolarization, along with acute endothelial NO synthesis in isolated endothelial cells. In addition, NS309 was shown to augment the acetylcholine-induced vasodilatation in small-resistance arterioles that might improve the recovery of damaged tissue.[24] It is interesting to note that two FDA-approved drugs, namely, riluzole (used in the treatment of amyotrophic lateral sclerosis) and chlorzoxazone (used as central myorelaxant)[25] have been reported to enhance the activity of $K_{Ca}2$ channels,[26] suggesting a clinical relevance of the present findings on neuroprotection mediated by $K_{Ca}2$ channel activation *in vitro* and *in vivo*. For example, $K_{Ca}2$ channel activation may significantly contribute to the reported effects of the neuroprotectant riluzole, that is, that it inhibits the release of glutamate from nerve terminals, modulates NMDA receptors and reduces neuronal excitability.[27]

In conclusion, our data suggest that activation of $K_{Ca}2$ channels promotes neuroprotection *in vitro* and *in vivo* by reducing glutamate- and NMDA-induced $[Ca^{2+}]_i$ deregulation. $K_{Ca}2$ channels may, therefore, have a major role in the regulatory feedback loop that interrupts the glutamate-triggered neuronal hyperexcitability, progressive disturbance of Ca^{2+} homeostasis and excitotoxic neuronal death. Accordingly, $K_{Ca}2$ channels may serve as novel therapeutic targets to prevent intracellular Ca^{2+} overload under

pathological conditions associated with glutamate-induced excitotoxicity.

Materials and Methods

Primary cortical neuron culture. Primary cortical neurons were plated at a density of 16×10^3 cells/well (96 well plates) and 3×10^5 cells/well (6 well plates) on polyethyleneimine pre-coated plates. Neurobasal medium supplemented with 5 mM HEPES, 1.2 mM glutamine, 2% (v/v) B27 supplement (20 ml/l) and gentamicin (0.1 mg/ml) was used as a culture medium. Neurons were treated with the $K_{Ca}2$ channel activator NS309, the $K_{Ca}2$ channel blocker NS8593 or apamin for the indicated time periods. On the basis of previous kinetic studies, NS309 was used at a concentration of 50 μM.[19]

Evaluation of cell viability. Neuronal viability was determined by the colorimetric MTT assay. The absorbance of each well was determined with an automated FLUOstar Optima reader (BMG Labtech, Offenburg, Germany) at 570 nm with a reference filter at 630 nm.

Calcium measurements in single neurons using calcium imaging. Primary cortical cells were incubated with 2 μM FURA-2 AM for 30 min at 37°C in HEPES-ringer buffer. Drugs were diluted in HEPES-ringer buffer (20 μM glutamate, 500 μM NMDA, 50 μM NS309, 50 μM NS8593, 1 μM apamin, 25 μM MK801, 4 mM EDTA and 4 mM EGTA). Fluorescence intensities from single cells excited at the two wavelengths (F340 and F380) were recorded separately and combined (fluorescence ratio: $r = F340/F380$) after background subtraction (fluorescence of a cell-free area).

Protein analysis. Primary cortical neurons were lysed in 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% TritonX, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, complete mini protease inhibitor cocktail tablet and phosphatase inhibitor cocktail 1 and 2. The membranes were incubated overnight with primary antibodies (1 : 3000; rabbit anti-$K_{Ca}2.2$[28] channel at 4°C and afterwards with peroxidase-conjugated secondary antibodies (1 : 2500).

RT-PCR. Total RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). RT reactions were conducted in a thermo cycler with setting at 42°C for 30 min. Amplifications using specific primers[29] by PCR were carried out for 27 or 30 cycles at various steps: (1) denaturing at 95°C for 4 min; (2) 94°C for 30 s; (3) annealing temperature (T_m) for 30 s, depending on the $K_{Ca}2$ isoform of interest; and (4) extension at 72°C for 30 s. The final extension step was set to 72°C for 5 min. The T_m for $K_{Ca}2.1$ was 63°C, 57,3°C for $K_{Ca}2.2$ and 61°C for $K_{Ca}2.3$.

Transient focal cerebral ischemia. All animal experiments were conducted according to the guidelines of Government of Upper Bavaria. Male C57BL/6 mice were subjected to 60 min transient MCAo by an intraluminal filament as previously described.[14] Mice were killed and brains were removed and frozen in powdered dry ice 24 h after reperfusion. Infarct volume was calculated by multiplying the infarct areas with the distance between sections. NS309 was administered 30 min before MCAo by intraperitoneal injection (100 μl per 20 g) at a concentration of 0.2 mg/kg and 2 mg/kg. Saline (0.9%) with 2% DMSO was used as vehicle.

Statistical analysis. All data are given as means ± S.D. For statistical comparisons between two groups, Student's *t*-test was used; multiple comparisons were performed by ANOVA followed by Scheffé's *post hoc* test. Calculations were performed with the Winstat standard statistical software package. For the MCAo experiments we have used the Mann–Whitney *U*-test for the analysis of differences between groups and also by ANOVA followed by Scheffé's or Tukey's as *post hoc* analysis. A statistically significant difference was assumed at $P < 0.05$.

Other methods, including hippocampal slice preparation, patch-clamp recordings and data sanalysis were carried out as described in Andres *et al.*[29], Li *et al.*[30], Pedarzani *et al.*[31] and Landshamer *et al.*[32] Further details are provided in Supplementary Materials and Methods.

Conflict of interest

The authors declare no conflict of interest.

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1. Randall RD, Thayer SA. Glutamate-induced calcium transient triggers delayed calcium overload and neurotoxicity in rat hippocampal neurons. *J Neurosci* 1992; **12**: 1882–1895.
2. Bano D, Nicotera P. Ca2+ signals and neuronal death in brain ischemia. *Stroke* 2007; **38**: 674–676.
3. Lipton SA, Nicotera P. Calcium, free radicals and excitotoxins in neuronal apoptosis. *Cell Calcium* 1998; **23**: 165–171.
4. Zhang L, Mitani A, Yanase H, Kataoka K. Countinous monitoring and regulating of brain temperature in the conscious and freely moving ischemic gerbil: effect of MK801 on delayed neuronal death in hippocampal CA1. *J Neurosci Res* 1997; **47**: 440–448.
5. Olsson T, Wieloch T, Smith ML. Brain damage in a mouse model of global cerebral ischemia. Effect of NMDA receptor blockade. *Brain Res* 2003; **982**: 260–269.
6. Parsons CG, Stoffler A, Danysz W. Memantine: a NMDA receptor antagonist that improves memory by restoration of homeostasis in the glutamatergic system – too little activation is bad, too much is even worse. *Neuropharmacology* 2007; **53**: 699–723.
7. Ngo-Anh TJ, Bloodgood BL, Lin M, Sabatini BL, Maylie J, Adelman JP. SK channels and NMDA receptors form a Ca^{2+}-mediated feedback loop in dendritic spines. *Nat Neurosci* 2005; **8**: 642–649.
8. Faber ES, Delaney AJ, Sah P. SK channels regulate excitatory synaptic transmission and plasticity in the lateral amygdala. *Nat Neurosci* 2005; **8**: 635–641.
9. Stocker M. Ca^{2+}-activated K^+ channels: molecular determinants and function of the SK family. *Nat Rev Neurosci* 2004; **5**: 758–770.
10. Tymianski M, Charlton MP, Carlen PL, Tator CH. Source specificity of early calcium neurotoxicity in cultured embryonic spinal neurons. *J Neurosci* 1993; **13**: 2085–2104.
11. Peterson C, Neal JH, Cotman CW. Development of *N*-methyl-D-asparate excitotoxicity in cultured hippocampal neurons. *Brain Res Dev Brain Res* 1989; **48**: 187–195.
12. Strøbaek D, Teuber L, Jørgensen TD, Ahring PK, Kjaer K, Hansen RS *et al.* Activation of human IK and SK Ca^{2+}-activated K^+ channels by NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime). *Biochim Biophys Acta* 2004; **1665**: 1–5.
13. Khodorov B. Glutamate-induced deregulation of calcium homeostasis and mitochondrial dysfunction in mammalian central neurones. *Prog Biophys Mol Biol* 2004; **86**: 279–351.
14. Plesnila N, Zinkel S, Le DA, Amin-Hanjani S, Wu Y, Qiu J, Chiarugi A *et al.* BID mediates neuronal cell death after oxygen/glucose deprivation and focal cerebral ischemia. *Proc Natl Acad Sci USA* 2001; **98**: 15318–15323.
15. Thayer SA, Miller RJ. Regulation of the intracellular free calcium concentration in single rat dorsal root ganglion neurones *in vitro*. *J Physiol* 1990; **425**: 85–115.
16. Boettger MK, Till S, Chen MX, Anand U, Otto WR, Plumpton C *et al.* Calcium-activated potassium channel SK1- and IK1-like immunoreactivity in injured human sensory neurons and its regulation by neurotrophic factors. *Brain* 2002; **125**: 252–263.
17. Shakkottai VG, Chou CH, Oddo S, Sailer CA, Knaus HG, Gutman GA *et al.* Enhanced neuronal excitability in the absence of neurodegeneration induces cerebellar ataxia. *J Clin Invest* 2004; **113**: 582–590.
18. Chinopoulos C, Gerencser AA, Doczi J, Fiskum G, Adam-Vizi V. Inhibition of glutamate-induced delayed calcium deregulation by 2-APB and La3+ in cultured cortical neurones. *J Neurochem* 2004; **91**: 471–483.
19. Dolga AM, Granic I, Blank T, Knaus HG, Spiess J, Luiten PG *et al.* TNF-alpha-mediates neuroprotection against glutamate-induced excitotoxicity via NF-kappaB-dependent up-regulation of $K_{Ca}2.2$ channels. *J Neurochem* 2008; **107**: 1158–1167.
20. Kye MJ, Spiess J, Blank T. Transcriptional regulation of intronic calcium-activated potassium channel SK2 promoters by nuclear factor-kappa B and glucocorticoids. *Mol Cell Biochem* 2007; **300**: 9–17.
21. Mattson MP, Culmsee C, Yu Z, Camandola S. Roles of nuclear factor kappaB in neuronal survival and plasticity. *J Neurochem* 2000; **74**: 443–456.
22. Culmsee C, Siewe J, Junker V, Retiounskaia M, Schwarz S, Camandola S *et al.* Reciprocal inhibition of p53 and nuclear factor-kappaB transcriptional activities determines cell survival or death in neurons. *J Neurosci* 2003; **23**: 8586–8589.
23. Lee AL, Dumas TC, Tarapore PE, Webster BR, Ho DY, Kaufer D *et al.* Potassium channel gene therapy can prevent neuron death resulting from necrotic and apoptotic insults. *J Neurochem* 2003; **86**: 1079–1088.
24. Sheng JZ, Ella S, Davis MJ, Hill MA, Braun AP. Openers of SKCa and IKCa channels enhance agonist-evoked endothelial nitric oxide synthesis and arteriolar vasodilation. *FASEB J* 2009; **23**: 1138–1145.
25. Pedarzani P, Stocker M. Molecular and cellular basis of small-and intermediate-conductance, calcium-activated potassium channel function in the brain. *Cell Mol Life Sci* 2008; **65**: 3196–3217.
26. Cao Y, Dreixler JC, Roizen JD, Roberts MT, Houamed KM. Modulation of recombinant small-conductance Ca^{2+}-activated K^+ channels by the muscle relaxant chlorzoxazone and structurally related compounds. *J Pharmacol Exp Ther* 2001; **296**: 683–689.

27. Killestein J, Kalkers NF, Polman CH. Glutamate inhibition in MS: the neuroprotective properties of riluzole. *J Neurol Sci* 2005; **233**: 113–115.
28. Sailer CA, Kaufmann WA, Marksteiner J, Knaus HG. Comparative immunohistochemical distribution of three small-conductance Ca^{2+}-activated potassium channel subunits, SK1, SK2, and SK3 in mouse brain. *Mol Cell Neurosci* 2004; **26**: 458–469.
29. Andres MA, Baptista NC, Efird JT, Ogata KK, Bellinger FP, Zeyda T. Depletion of SK1 channel subunit leads to constitutive insulin secretion. *FEBS Lett* 2009; **583**: 369–376.
30. Li W, Halling DB, Hall AW, Aldrich RW. EF hands at the N-lobe of calmodulin are required for both SK channel gating and stable SK-calmodulin interaction. *J Gen Physiol* 2009; **134**: 281–293.
31. Pedarzani P, McCutcheon JE, Rogge G, Jensen BS, Christophersen P, Hougaard C *et al.* Specific enhancement of SK channel activity selectively potentiates the afterhyperpolarizing current I (AHP) and modulates the firing properties of hippocampal pyramidal neurons. *J Biol Chem* 2005; **280**: 41404–41411.
32. Landshamer S, Hoehn M, Barth N, Duvezin-Caubet S, Schwake G, Tobaben S, Kazhdan I *et al.* Bid-induced release of AIF from mitochondria causes immediate neuronal cell death. *Cell Death Differ* 2008; **15**: 1553–15563.

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