Molecular and Cellular Neuroscience 46 (2011) 720–730

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Excitotoxic neuroprotection and vulnerability with CaMKII inhibition

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ARTICLE INFO

Article history:
Received 20 September 2010
Revised 2 February 2011
Accepted 3 February 2011
Available online 21 February 2011

Keywords:
Excitotoxicity
Aberrant calcium signaling
Ischemia
Kinase inhibitors
Cortical neurons
CaMKII inactivation

ABSTRACT

Aberrant calcium signaling is a common feature of ischemia and multiple neurodegenerative diseases. While activation of calcium–calmodulin (CaM)-dependent protein kinase II (CaMKII) is a key event in calcium signaling, its role in excitotoxicity is controversial. Our findings demonstrate neuroprotection in neuronal cultures treated with the small molecule (KN-93) and peptide (tat-AIP and tat-CN21) inhibitors of CaMKII immediately prior to excitotoxic glutamate/glycine insult. Unlike KN-93 which blocks CaMKII activation, but not constitutively active forms of CaMKII, tat-CN21 and tat-AIP significantly reduced excitotoxicity in cultured neurons when applied post-insult. We observed that the neuroprotective effects of tat-CN21 are greatest when applied before the toxic glutamate challenge and diminish with time, with the neuroprotection associated with CaMKII inhibition diminishing back to control 3 h post glutamate insult. Mechanistically, tat-CN21 inhibition of CaMKII resulted in an increase in CaMKII activity and the percentage of soluble αCaMKII observed in neuronal lysates 24 h following glutamate stimulation. To address the impact of prolonged CaMKII inhibition prior to excitotoxic insult, neuronal cultures were treated with CaMKII inhibitors overnight and then subjected to a sub-maximal excitotoxic insult. In this model, CaMKII inhibition prior to insult exacerbated neuronal death, suggesting that a loss of CaMKII enhances neuronal vulnerability to glutamate. Although changes in αCaMKII or NR2B protein levels are not responsible for this enhanced glutamate vulnerability, this process is blocked by the protein translation inhibitor cycloheximide. In total, the neuroprotection afforded by CaMKII inhibition can be seen as neuroprotective immediately surrounding the excitotoxic insult, whereas sustained CaMKII inhibition produced by excitotoxicity leads to neuronal death by enhancing neuronal vulnerability to glutamate.

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Introduction

Finely-tuned transient increases in intracellular calcium are essential for neuronal development, communication and plasticity. However, dysregulated calcium signaling can produce neuronal death *via* necrotic and programmed cell death mechanisms (Portera-Cailliau et al., 1997; Dirnagl et al., 1999; Snider et al., 1999). Excitotoxicity is a hallmark of most neurodegenerative diseases; a process that leads to excessive accumulation of intracellular calcium *via* the over-activation of excitatory glutamate receptors. Because of its prominent role in neuronal calcium signaling, calcium/calmodulin-dependent protein kinase II (CaMKII) may contribute to excitotoxic neurodegeneration for the following reasons, 1) CaMKII is activated and autophosphorylated in stroke, brain trauma and epilepsy (Perlin et al., 1992; Churn et al., 1995; Zalewska and Domanska-Janik, 1996), 2) the calmodulin inhibitor calmidazolium is a neuroprotective agent in ischemia (Pohorecki et al., 1990), and 3) ischemia induces CaMKII translocation (Aronowski et al., 1992; Morioka et al., 1995; Onodera et al., 1995 Aronowski and Grotta, 1996; Hu et al., 1998; Dosemeci et al., 2001) and phosphorylation of key post-synaptic substrates (i.e. post-synaptic density) (Meng and Zhang, 2002; Takagi et al., 2003; Fu et al., 2004; Hao et al., 2005). Previous studies using small molecule and peptide inhibitors of CaMKII such as KN-93 and autocamtide-2 inhibitory protein (AIP) have been shown to be neuroprotective when applied before an excitotoxic insult *in vitro* (Hajimohammadreza et al., 1995; Laabich and Cooper, 2000; Takano et al., 2003; Fan et al., 2006; Goebel, 2009; Vest et al., 2010). However, because CaMKII also regulates substrates involved in neuronal survival (e.g. L-type calcium channels, CREB and BCL-2 etc.), it is also possible that inhibiting CaMKII may exacerbate excitotoxic neuronal death (Dash et al., 1991; Bok et al., 2007; Wheeler et al., 2008). In support of this, an ischemic insult in αCaMKII knock-out mice leads to much greater neuronal death than in wild-type litter mates, suggesting that CaMKII activity is important for neuronal survival to excitotoxicity (Waxham et al., 1996).

To date, the discrepancies found between the *in vivo* knock-out model and *in situ* small molecule and peptide inhibitor experiments are not understood. While it is possible that differences could be due to model systems (genetic knock-out *versus* pharmacological manipulation), the impact that both short-term and sustained CaMKII

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doi:10.1016/j.mcn.2011.02.003

inhibition prior to excitotoxic insult have on neuronal survival has not been explored in one system concurrently. Furthermore, the small molecule and peptide inhibitors of CaMKII used in previous experiments have been shown to have a variety of off-target effectors. The family of KN-drugs has been shown to inhibit a variety of CaM-kinase family members as well as voltage-gated potassium and calcium channels (Enslen et al., 1994; Ledoux et al., 1999; Gao et al., 2006). AIP, which mimics the autoregulatory domain of CaMKII, has also been shown to inhibit other CaM-kinase family members (Smith et al., 1990).

In order to determine whether CaMKII inhibition is neuroprotective, a battery of CaMKII inhibitors including those presented in earlier studies (KN-93 and AIP) as well as the highly-specific CaMKIINtide peptide inhibitor, CN21 (Vest et al., 2007), were applied either immediately before or after the onset of excitotoxic insult. KN-93, AIP, and CN21 all afforded neuroprotection when applied prior to the onset of an excitotoxic insult. Interestingly, only AIP and CN21, which inhibit the autonomous form of CaMKII (Ishida et al., 1995; Chang et al., 1998; Rose and Hargreaves, 2003), afforded neuroprotection when applied after insult. The translocation and loss of CaMKII activity observed 24 h after glutamate excitotoxicity were prevented in a time-dependent manner by CaMKII inhibition. To examine the effect that prolonged loss of CaMKII activity has on neuronal sensitivity to excitotoxicity, neuronal cultures were treated with CaMKII inhibitors and were then subjected to a sub-maximal glutamate/glycine insult. Prolonged CaMKII inhibition (>8 h) exacerbates neuronal death following an excitotoxic challenge; a process that requires protein synthesis. Taken together, these data indicate that acute inhibition of CaMKII is neuroprotective when applied immediately surrounding an excitotoxic insult, whereas prolonged inhibition enhances neuronal death to an excitotoxic challenge.

Results

Characterization of neuronal cultures and their death following glutamate–glycine application in cortical neurons

After 7–8 days *in vitro* (DIV), 97% of the cultured cortical cells are MAP2 positive. Less than 2% of the remaining cells stained with the astrocyte marker GFAP, indicating that these cultures are predominantly neurons (Figs. 1A–B). Over 90% of the cultured cells were also CaMKII positive, as detected by both a monoclonal αCaMKII and polyclonal pan-CaMKII antibody (Fig. 1B). Neuronal viability was assessed using a differential calcein-AM (FITC green) to label vital cells and ethidium homodimer to label cytotoxic cells (Texas Red) (Figs. 1C–D). Dose-dependent applications of glutamate/glycine

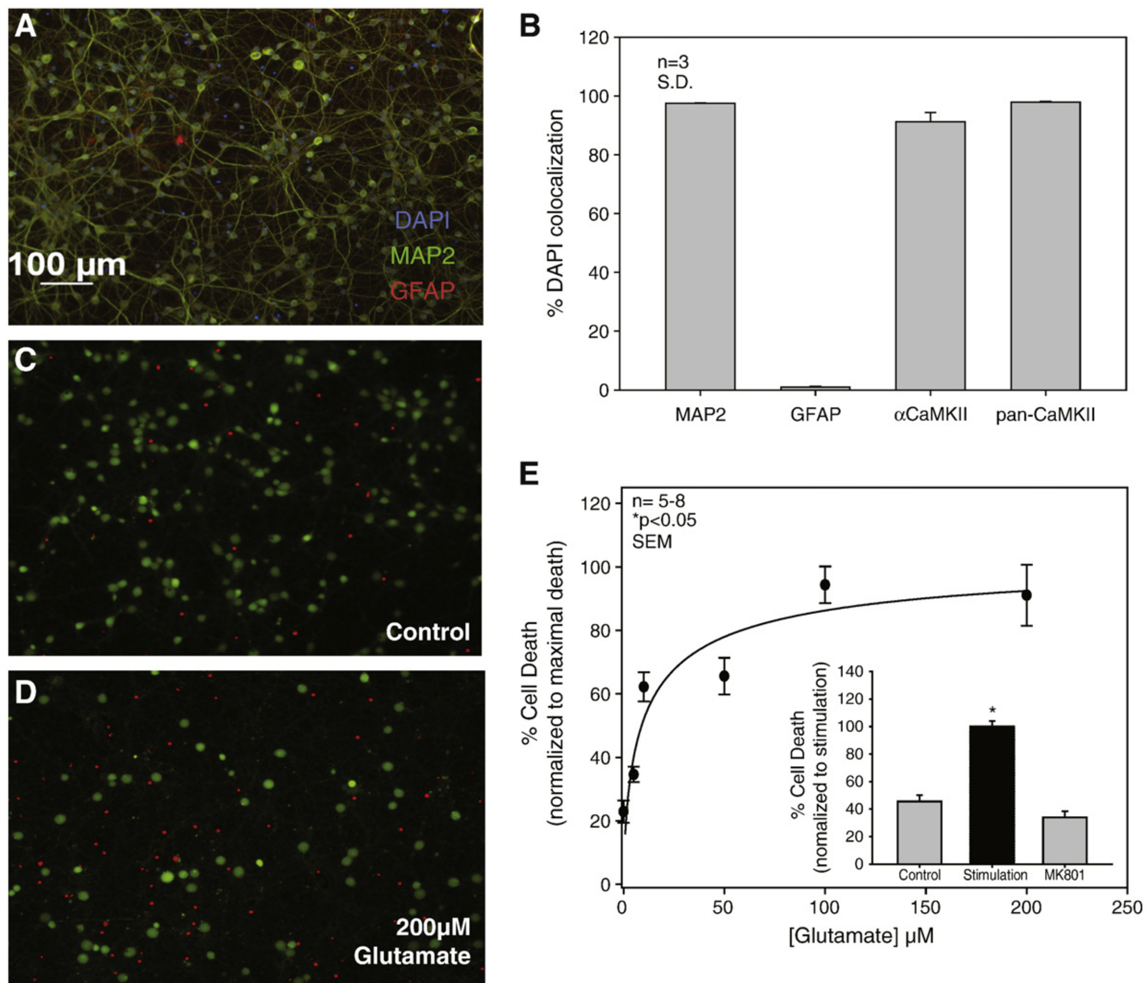

Fig. 1. Glutamate–glycine induced excitotoxicity in cortical neuron cultures. A, Primary cortical neurons 7 DIV, stained with MAP-2 (green), GFAP (red), DAPI (blue). B, Average number of cells (±SD, n=3) stained for the neuronal marker (MAP-2), astrocyte marker (GFAP), αCaMKII, and pan-CaMKII at 7 DIV. C–D, Representative images of control cultures, C, and cultures treated with 200 μM glutamate/20 μM glycine for 1 h, D, stained with LIVE/DEAD Viability/Cytotoxicity kit (Molecular probes) 24 h following treatment. This differentially stains viable cells (green) and cytotoxic cells (red). E, Average cell death (normalized to maximal death, ±SEM, n=5–8) in control cultures and cultures treated with varying concentrations of glutamate. Inset, average cell death (±SEM, n=5–8) in control cultures, cultures stimulated with 200 μM glutamate/20 μM glycine, and cultures pretreated with 20 μM MK-801 followed by glutamate stimulation (*p<0.05; One-way ANOVA, post-hoc Dunnett's test).

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