Cellular/Molecular

Nuclear Ca^{2+} and the cAMP Response Element-Binding Protein Family Mediate a Late Phase of Activity-Dependent Neuroprotection

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The mechanism by which physiological synaptic NMDA receptor activity promotes neuronal survival is not well understood. Here, we show that that an episode of synaptic activity can promote neuroprotection for a long time after that activity has ceased. This long-lasting or "late phase" of neuroprotection is dependent on nuclear calcium signaling and cAMP response element (CRE)-mediated gene expression. In contrast, neuroprotection evoked acutely by ongoing synaptic activity relies solely on the activation of the phosphatidylinositol 3-kinase/Akt pathway. This "acute phase" does not require nuclear calcium signaling and is independent of activation of the CRE-binding protein (CREB) family of transcription factors. Thus, activity-dependent neuroprotection comprises two mechanistically distinct phases that differ in their spatial requirements for calcium and in their reliance on the CREB family.

Key words: **apoptosis; calcium; [Ca]; neuroprotection; NMDA receptors; CREB; CaM kinase**

Introduction

Survival of a wide variety of neurons is dependent on physiological levels of electrical activity, as demonstrated by the deleterious effects of activity blockade *in vivo* and *in vitro* (Catsicas et al., 1992; Linden, 1994; Mennerick and Zorumski, 2000). This susceptibility is most common during development, but, in several cases, it extends into maturity as well (Mennerick and Zorumski, 2000). There is growing evidence that a large part of the neuroprotection afforded by synaptic activity is mediated by the resulting intracellular Ca^{2+} transients. At glutamatergic synapses, Ca^{2+} influx through the NMDA subtype of ionotropic glutamate receptors is a major source of these transients and of the resulting neuroprotection (Hardingham and Bading, 2003). Normal levels of synaptic NMDA receptor activity are strongly neuroprotective as evidenced by the fact that blockade of NMDA receptors *in vivo* causes widespread apoptosis and exacerbates ongoing neurodegeneration in the developing and adult CNS (Ikonomidou et al., 1999, 2000; Olney et al., 2002; Adams et al., 2004).

This study addresses the mechanism by which synaptic NMDA receptor activity can promote neuronal survival. In particular, we studied the role of activation of transcription promoted by the cAMP response element (CRE), mediated by the CRE-binding protein (CREB) family (Mayr and Montminy, 2001; Lonze and Ginty, 2002), in this process. The potential of CREB family-regulated gene products to promote neuronal survival was first demonstrated in the context of neurotrophin signaling (Bonni et al., 1999; Riccio et al., 1999) and by exogenous overexpression (Walton et al., 1999). In addition, studies of mice in which CREB and/or CREB family members have been deleted also point to a prosurvival role for CREB: CREB null mice exhibit considerable death of dorsal root ganglion sensory neurons *in vivo* and sympathetic neurons *in vitro* (Lonze et al., 2002). Mice lacking CREB [and cAMP response element modulator (CREM), another CREB family member] in the CNS during development show extensive neuronal apoptosis (Mantamadiotis et al., 2002). Postnatal disruption of the CREB gene (in CREM null mice) results in progressive neurodegeneration in the hippocampus and dorsolateral striatum (Mantamadiotis et al., 2002).

Synaptic NMDA receptor activity strongly induces CRE-dependent gene expression (Hardingham et al., 2002), making it an attractive mechanism for synaptic NMDA receptor-dependent neuroprotection. Studies have reported correlations between NMDA receptor-dependent survival and CREB phosphorylation in cerebellar granule neurons (Monti et al., 2002), between NMDA receptor-dependent ischemic preconditioning and CREB phosphorylation in hippocampal neurons (Mabuchi et al., 2001), and between NMDA receptor antagonist-induced apoptosis and CREB dephosphorylation in several brain regions *in vivo* (Hansen et al., 2004). However, a causal link between synaptic NMDA receptor-dependent CRE activation and the prevention of apoptosis has not yet been made.

Another key question surrounding the mechanism of activity-dependent prosurvival signals is one of longevity. Neurons are not consistently active *in vivo*, which suggests that activity-dependent prosurvival signals may be able to exert their effects for a period of time after the activity has ceased. In the context of

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synaptic plasticity, CREB plays a key role in prolonging/consolidating activity-dependent changes to synaptic strength (Mayford et al., 1996; Silva et al., 1998; Ahn et al., 1999; Barco et al., 2002, 2003), raising the possibility that it may play a comparable role in prolonging activity-dependent neuroprotection. This possibility has not hitherto been studied, so we investigated the mechanism of neuroprotection not only in neurons experiencing ongoing synaptic activity but also in neurons that have experienced a previous episode of activity but are subsequently inactive.

Materials and Methods

Hippocampal cultures, stimulation, and the induction of apoptosis. Hippocampal neurons were cultured as described previously (Bading and Greenberg, 1991), except that growth medium was supplemented with B27 (Invitrogen, San Diego, CA). Stimulations were done after a culturing period of 9 d during which hippocampal neurons develop a rich network of processes, express functional NMDA-type and AMPA/kainate-type glutamate receptors, and form synaptic contacts (Hardingham et al., 2001a, 2002). Bursts of action potential firing were induced by treatment of cultured hippocampal neurons with 50 μM bicuculline (BiC), and burst frequency was enhanced by the addition of 5 μM 4-aminopyridine (4-AP) (Hardingham et al., 2001a). (+)-5-Methyl-10,11-dihydro-5*H*-dibenzo [a,d] cyclohepten-5,10-imine maleate (MK-801; 10 μM) was obtained from Tocris Cookson (Ballwin, MO), and TTX (2 μM) and 4-aminopyridine were obtained from Calbiochem (La Jolla, CA). Neurons were subjected to trophic deprivation by transferring them from growth medium to a medium containing 10% MEM (Invitrogen) and 90% salt-glucose-glycine medium (Bading and Greenberg, 1991). Chemical inducers of apoptosis were used as follows: staurosporine (50 nM; Calbiochem), 9-*cis*-retinoic acid (50 μM; Sigma, St. Louis, MO), C-2 ceramide (50 μM; Calbiochem), or okadaic acid (3 nM; Calbiochem). Neurons were fixed and subjected to Hoechst staining, and cell death was quantified by counting (blind) the number of apoptotic nuclei as a percentage of the total.

Plasmids, antibodies, and Western blot analysis. CRE-Luc, thymidine kinase promoter-driven renilla luciferase (pTK-RL), and pEGFPN1 [a plasmid encoding enhanced green fluorescent protein (eGFP)] were obtained from Promega (Madison, WI). The following plasmids have been described previously: pSVICERI [a plasmid encoding inducible cAMP early repressor (ICE1) under an RSV promoter] (a gift from Dr. Paulo Sassone-Corsi, Centre National de la Recherche Scientifique–Institut National de la Santé et de la Recherche Médicale, Strasbourg, France), CaMKIV313 (constitutively active truncated mutant of CaM kinase IV) active (Sun et al., 1994), 4X nuclear localized calmodulin-binding peptide (CAMBP4nuc) (Wang et al., 1995) (exclusive nuclear localization of CAMBP4nuc was confirmed by immunolocalization of the FLAG tag on CAMBP4nuc) (data not shown), GAG-AKT (Burgering and Coffer, 1995), and p110αCAAX (Didichenko et al., 1996). Anti-phospho (Ser473) Akt, anti-Akt, and anti-phospho-extracellular signal-regulated kinase 1/2 (ERK1/2) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-phospho-CREB and CREB antibodies were obtained from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Gel electrophoresis and Western blotting of protein samples were performed using an Xcell Surelock system (Invitrogen) using precast gradient gels (4–20%). For visualization of Western blots, HRP-based secondary antibodies were used followed by chemiluminescent detection on Kodak (Rochester, NY) X-Omat film.

Transfections and gene expression analysis. Neurons were transfected at 8 d *in vitro* using Lipofectamine 2000 (Invitrogen) and stimulated 24 h after transfection. For CRE reporter assays, to demonstrate the efficacy of the inhibitors of CRE activation (ICER and CAMBP4nuc), the ratio of reporter plasmid to inhibitor plasmid was 1:2 (plus 0.25 parts of pTK-RL transfection efficiency control). Stimulation (for 8 h) was 24 h after transfection, which was sufficient time for the plasmids encoding CREB inhibitors to be expressed. Luciferase assays were performed using the Dual Glo assay kit (Promega) with Firefly luciferase-based reporter gene activity normalized to the renilla control (pTK-RL plasmid) in all cases. Mean ± SEM of four experiments is shown. For the neuronal viability assays, peGFP was used to track the fate of transfected neurons expressing the plasmid of interest. To ensure that GFP-positive neurons were also expressing the plasmid of interest, an even more favorable ratio was used than that shown to be sufficient for the gene expression studies (peGFP, plasmid of interest; 1:3). Coexpression at this ratio was confirmed in the case of pRFP (plasmid encoding red fluorescent protein) and also pCAMBP4nuc (which is FLAG tagged, and expression was analyzed immunocytochemically). As with the reporter assays, BiC/4-AP stimulations were performed at 24 h after transfection, and either neurons were allowed to experience continuous activity (for studying protection afforded by ongoing activity) or activity was terminated after 12 h by addition of TTX plus MK-801 to eliminate all NMDA receptor activation (for studying long-lasting neuroprotection).

Fluorescence image capture and monitoring the fate of transfected neurons. All GFP fluorescent images were taken on a Zeiss (Thornwood, NY) Axioplan2 microscope using a laser-scanning confocal microscope driven by LSM510 software. For each transfected dish, images of 50–200 GFP-expressing neurons were taken across a marked area of ~7 mm^2. Images were first taken at 48 h after transfection, when little cell death has taken place under any conditions. Images of the same cells were then taken at 72 h and then at 96 h, after which the neurons were fixed and stained with Hoechst. Photographs of Hoechst-stained nuclei were taken with a CCD camera driven by Openlab (Improvision, Lexington, MA) software. Pictures of the GFP-expressing neurons at 48, 72, and 96 h were then compared to ascertain whether the cell survived through the course of the experiment or died by the time the 72 h image was taken or by the time the 96 h image was taken. In the vast majority of cases, death was easily spotted as an absence of a GFP-expressing cell where one once was, frequently with fragments of fluorescent cellular debris in its place. In other cases, neurons were at an earlier stage of death, characterized by broken, fragmented neurites and rounded cell bodies. For each treatment, the fate of 200–1000 neurons was monitored over four to nine independent experiments.

Results

Synaptic NMDA receptor activity is strongly neuroprotective

To analyze the molecular mechanisms involved in synaptic NMDA receptor-dependent neuroprotection, we recapitulated activity-dependent neuroprotection *in vitro* using established models for neuronal apoptosis. The simplest model used was trophic deprivation: when hippocampal neurons were transferred from growth medium to basal medium, they died over 72–96 h, exhibiting shrunken nuclei and large round clumps of chromatin resembling apoptosis. To assess the neuroprotective effects of synaptic NMDA receptor activation, bath application of glutamate or NMDA is inappropriate because these unphysiological stimuli also activate extrasynaptic NMDA receptors, which can antagonize synaptic NMDA receptor signaling and promote neuronal death (Hardingham et al., 2002). To enhance synaptic NMDA receptor activity, tonic network inhibition was blocked with the $GABA_A$ receptor antagonist bicuculline, inducing bursts of action potentials (Fig. 1*A*, inset), which are associated with global synaptic NMDA receptor-dependent Ca^{2+} transients (Hardingham et al., 2001a). Cell death after trophic deprivation was inhibited >90% by triggering burst activity with bicuculline (Fig. 1*A*). This protection was abolished by the NMDA receptor antagonist MK-801. Although enhancing synaptic NMDA receptor activity is neuroprotective, inhibiting basal levels of activity exacerbates death: treatment with the $GABA_A$ agonist muscimol abolished spontaneous firing as measured by multielectrode array (data not shown) and exacerbated death to a similar degree as tetrodotoxin (Fig. 1*A*). Treatment of neurons with MK-801 alone also exacerbated neuronal death, underlining the importance of synaptic NMDA receptors in activity-dependent protection (Fig. 1*A*). Resistance to chemical inducers of apoptosis also varied with synaptic NMDA receptor activity: application of

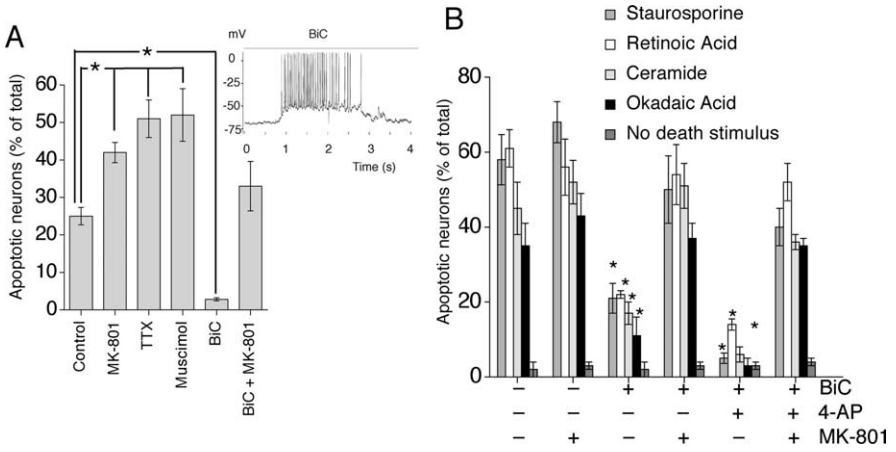

Figure 1. Neuroprotection afforded by ongoing synaptic NMDA receptor activity. ***A***, Apoptosis induced by trophic deprivation is prevented by synaptic NMDA receptor activity. Trophic deprivation for 72 h in the presence or absence of BiC (50 μM), MK-801 (10 μM), TTX (2 μM), or muscimol (10 μM). Mean ± SEM is shown in this and all subsequent figures containing error bars (*$p <$ 0.001; paired two-tailed *t* test; $n = 8$). Inset, Whole-cell current-clamp recording showing an example of a BiC-induced burst of action potentials. ***B***, Synaptic NMDA receptor activity protects neurons against inducers of apoptosis. Neurons treated in the presence or absence of the indicated drugs, plus the indicated inducers of apoptosis for 36 h (*$p < 0.05$; paired two-tailed *t* test; $n = 3–4$). No death stimulus refers to neurons placed in standard transfection medium containing the insulin-transferrin-selenite supplement (Bading et al., 1993).

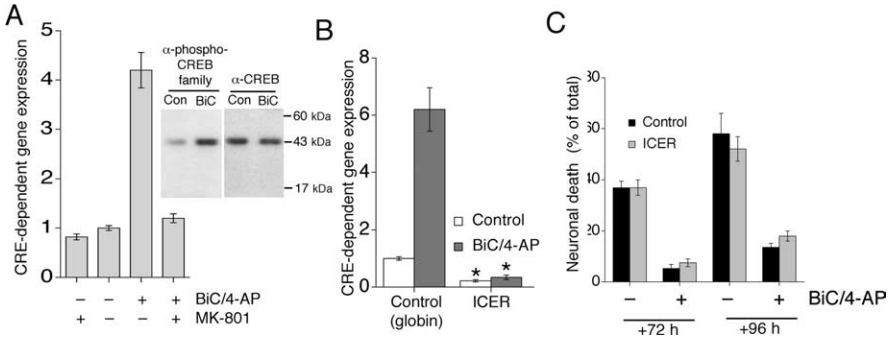

Figure 2. Neuroprotection afforded by ongoing synaptic NMDA receptor activity does not require activation of CRE-mediated gene expression. ***A***, Synaptic NMDA receptor activity regulates CRE reporter expression. MK-801 was used at 5 μM. Inset, Activated (phospho-) CREB is the only activated CREB family member detectable in hippocampal neurons. Western blot analysis shows neuronal extracts ±BiC, probed with antibodies against the phospho-forms of CREB family members and against CREB. Con, Control. ***B***, Expression of ICER inhibits the activation of a CRE reporter. CRE-firefly luciferase expression (normalized to renilla transfection control) in control (globin) or ICER-transfected neurons is shown ±BiC/4-AP (8 h) (*$p < 0.05$; paired two-tailed *t* test; $n = 5$). ***C***, CREB family-independent neuroprotection afforded by ongoing synaptic activity. Neurons transfected with the indicated plasmids, plus an eGFP marker, are shown. The fate of the neurons after trophic deprivation was monitored as described in the supplemental material (available at www.jneurosci.org). For each treatment, the fate of 300–600 cells was monitored over five (+72 h) or three (+96 h) independent experiments.

staurosporine, C-2 ceramide, retinoic acid, or okadaic acid all caused widespread cell death, which was reduced by bicuculline-induced activity (Fig. 1*B*). Moreover, increasing burst frequency with a low concentration (5 μM) of the K^+ channel blocker 4-aminopyridine (BiC/4-AP) (Hardingham et al., 2001b) rendered the neurons even more resistant to the apoptosis inducers (Fig. 1*B*). All bicuculline and BiC/4-AP-induced protection was reversed by MK-801 (Fig. 1*B*).

Activation of CRE-mediated gene expression is not needed for neuroprotection evoked by ongoing synaptic activity

The NMDA receptor-dependent activation status of CREB correlates well with neuronal survival (Mabuchi et al., 2001; Monti et al., 2002; Hansen et al., 2004). We also confirm this in our experimental system: survival-inducing bicuculline treatment (Fig. 1*A*,*B*) also triggers the strong NMDA receptor-dependent induction of a CRE reporter gene (Hardingham et al., 2002) (Fig. 2*A*). Of the CREB family members, CREB itself is likely to be the major mediator of CRE-dependent transcription in hippocampal neurons. Western blot analysis of cell extracts (obtained from unstimulated or BiC-stimulated hippocampal neurons) with an antibody that recognizes activated phospho-(serine 133) CREB and equivalent residues on CREM and ATF1 (activating transcription factor 1) reveals only a single very tight doublet at 43 kDa (Fig. 2*A*, inset), which corresponds to the size of CREB (the doublet is consistent with the two major isoforms of CREB-α and -δ). When the blot is stripped and reprobed with an antibody specific for CREB, this doublet is picked up once again, confirming it as CREB. Even on overexposed films, we observed no inducible phosphorylation apart from at CREB (data not shown). Thus, in hippocampal neurons, CREB is the only activated CREB family member that we can detect (in contrast to AtT20 cells) (Chawla et al., 1998) and thus is likely to be the major mediator of CRE-dependent gene expression. However, we cannot rule out minor contributions from other CREB family members.

To investigate a causal role of CRE-dependent gene expression in activity-dependent neuroprotection, we specifically inhibited activation of CRE-dependent gene expression and asked whether synaptic NMDA receptor activity was still effective in preventing apoptosis. To inhibit CRE activation, we expressed an endogenous inhibitory member of the CREB family, ICER1 (De Cesare and Sassone-Corsi, 2000), which strongly inhibited activation of a CRE reporter (Fig. 2*B*). ICER had no effect on reporter genes driven by either a constitutively active (TK) promoter, a minimal TATA-containing promoter, or on the basal or activity-induced levels of reporter activity driven by the serum response element (data not shown). Thus, ICER is specific in its effects on CRE-dependent gene expression.

To monitor the fate of individual neurons expressing ICER, we coexpressed an eGFP marker. After trophic deprivation, neurons expressing the control plasmid exhibited considerable cell death at 72 h, which was increased at 96 h (Fig. 2*C*). However, bicuculline-induced synaptic NMDA receptor activity dramatically reduced the amount of neuronal death at 72 and 96 h of trophic deprivation (Fig. 2*C*). Surprisingly, we found that expression of ICER had no effect on neuronal survival: its expression neither exacerbated neuronal death in the electrically silent neurons nor reduced activity-dependent neuroprotection in the bicuculline-treated neurons (Fig. 2*C*). Thus, using this model for neuronal death, neuroprotection induced by ongoing synaptic activity does not require CRE activation, raising the question as to what is required.

The phosphatidylinositol 3-kinase-Akt pathway mediates neuroprotection evoked by ongoing synaptic activity

The phosphatidylinositol 3-kinase (PI3K)-Akt pathway (Brazil et al., 2004) is activated by NMDA receptor activity and has neuroprotective capabilities (Brunet et al., 2001; Lafon-Cazal et al., 2002). Moreover, bicuculline treatment activates Akt in an NMDA receptor-dependent manner (Fig. 3*A*, inset). Treatment of neurons with the PI3K inhibitor 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) (100 μM) eliminated the protective effect of bicuculline in trophically deprived conditions but also caused increased cell death in control neurons (Fig. 3*B*), showing that basal levels of Akt are important in neuronal survival. Treatment of neurons with a submaximal concentration of LY294002 (30 μM) blocked bicuculline-induced protection (Fig. 3*B*), indicating that the enhanced Akt activity triggered by synaptic NMDA receptor activity is necessary for neuroprotection. We obtained similar results using the structurally distinct PI3K inhibitor wortmannin as well as with the recently developed 3′-modified phosphatidyl inositol analog 1L-6-hydroxymethyl-chiro-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate (HIMO), which inhibits Akt (Hu et al., 2000) (data not shown).

As well as being necessary for neuronal survival, activation of the PI3K-Akt pathway is also sufficient; expression of plasmids encoding active forms of PI3K (p110CAAX) and Akt (Akt-GAG) in electrically silent neurons was sufficient to confer a level of neuroprotection observed in electrically active (BiC/4-AP-stimulated) neurons (Fig. 3*C*). Figure 3*D* shows some example pictures; note how the p110CAAX-transfected and Akt-GAG-transfected neurons survive in contrast to many of their untransfected neighbors. For comparison, control-transfected neurons ±BiC/4-AP stimulation are also shown. Thus, the data from Figure 3*A–C* support the conclusion that the PI3K-Akt pathway is an important mediator of neuroprotection afforded by ongoing synaptic NMDA receptor activity, although contributions from other signal pathways cannot be ruled out.

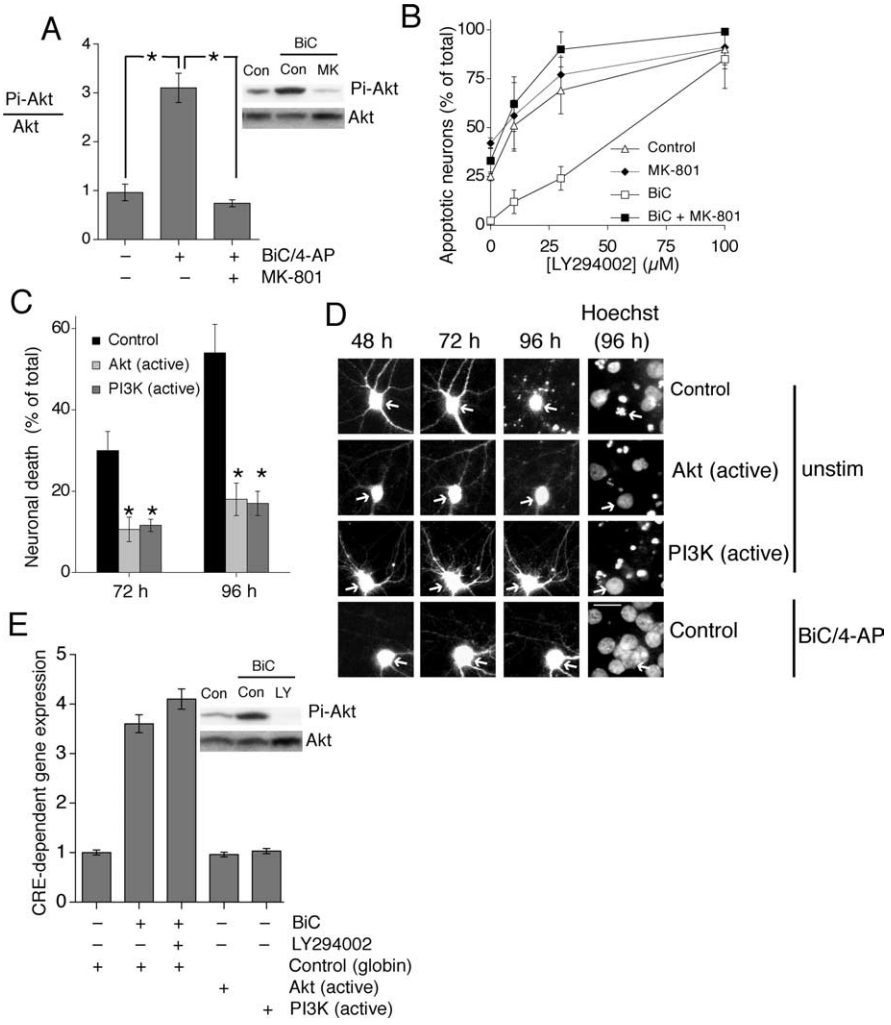

Figure 3. The PI3K-Akt pathway is necessary and sufficient for neuroprotection afforded by ongoing synaptic activity. ***A***, Synaptic NMDA receptor activity induces Akt activation. Western blot analysis of phospho- (Ser-473) Akt levels in neurons treated for 30 min with BiC ± MK-801 (MK). Phospho-Akt (Pi-Akt) Western band intensity normalized to levels of Akt (*$p < 0.05$; paired two-tailed *t* test; $n = 3$) is shown. Inset, Example of one of the Western blots analyzed. ***B***, Submaximal levels of the PI3K inhibitor LY294002 block synaptic NMDA receptor-dependent neuroprotection. Neurons placed in trophically deprived medium and treated with LY294002 1 h before treatment with BiC and/or MK-801 are shown. Death was assessed after 72 h. ***C***, ***D***, Constitutive activation of Akt or PI3K is sufficient to confer neuroprotection. Neurons were transfected with the indicated plasmids plus an eGFP marker, and their fate under trophic deprivation was monitored at +72 and +96 h (examples in ***D***). Scale bar, 40 μm. Analysis of the data shown in ***C*** (*$p < 0.05$; paired two-tailed *t* test; $n = 3$) is shown. Arrows indicate the nucleus of the transfected cell monitored over time. ***E***, The PI3K-Akt pathway does not influence CRE-mediated gene expression in hippocampal neurons. BiC-induced CRE reporter activation was assessed ±100 μM LY294002 (LY). Also, levels of CRE reporter activity in neurons expressing constitutively active Akt or PI3K were measured, compared with the control (globin). $n = 3$. Inset, LY294002 inhibits BiC-induced Akt activation. Con, Control; unstim, unstimulated. Error bars represent SEM.

Activation of CRE-mediated gene expression by intracellular Ca^{2+} transients in neurons is primarily mediated by a combination of the Ras-ERK1/2 pathway and nuclear CaM kinases, particularly CaM kinase IV, (Impey and Goodman, 2001; West et al., 2001; Wu et al., 2001; Hardingham and Bading, 2003). However, because the PI3K-Akt pathway has been reported to be able to influence CRE-mediated gene expression in some experimental systems (Du and Montminy, 1998), we decided to clarify the role (if any) of this pathway in the induction of the CRE by synaptic activity. We found no role for the PI3K-Akt pathway in activity-dependent signaling to the CRE; LY294002 blocks the induction of Akt by bicuculline (Fig. 3*E*, inset) but has no effect on the induction of a CRE reporter by bicuculline (Fig. 3*E*). In addition, neither wortmannin nor the Akt inhibitor HIMO affects the induction of a CRE reporter by bicuculline (data not shown). Moreover, expression of constitutively active forms of Akt or PI3K has no effect on CRE reporter activity (Fig. 3*E*). Therefore, the ability of the PI3K-Akt pathway to confer neuroprotection is very unlikely to involve CRE regulation, consistent with the fact that CRE-mediated gene expression is not required for neuroprotection promoted by ongoing synaptic NMDA receptor activity.

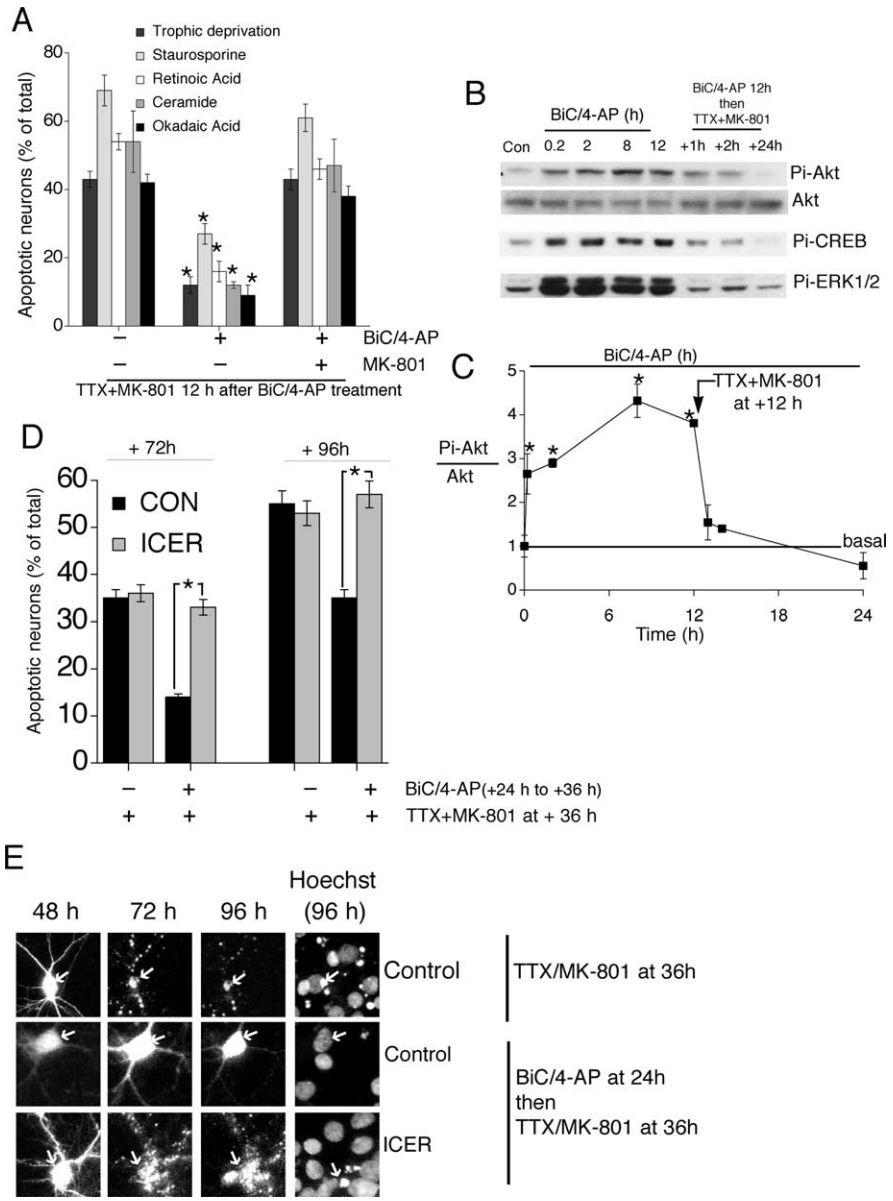

Figure 4. The CREB family mediates long-lasting activity-dependent neuroprotection. ***A***, Synaptic NMDA receptor activity is neuroprotective long after activity has ceased. BiC/4-AP-induced activity (±MK-801) was permitted for 12 h and terminated by TTX plus MK-801 treatment. Neurons were then left in trophically deprived medium for 60 h or exposed to the indicated apoptosis inducers for 36 h (*$p < 0.05$; paired two-tailed *t* test; 12 h BiC/4-AP stimulation compared with control conditions; $n = 3–4$). ***B***, ***C***, Ser-473 Akt phosphorylation decays after cessation of synaptic activity. BiC/4-AP stimulation was followed, where indicated, by TTX plus MK-801. Pi-Akt, Phospho-Akt. ***B***, An example of a Western blot, with the decay of phospho-CREB (Pi-CREB) and phospho-ERK1/2 (Pi-ERK1/2) also included for comparison. Con, Control. ***C***, Analysis of the decay in Akt phosphorylation normalized to Akt levels (*$p < 0.05$; paired two-tailed *t* test; the asterisk indicates significantly higher Pi-Akt levels than basal; $n = 3$). ***D***, ***E***, Long-lasting neuroprotection is dependent on the previous activation of CRE-dependent gene expression. Neurons were transfected either with control (CON) (globin) plasmid or the CRE inhibitor ICER before experiencing a 12 h episode of BiC/4-AP-induced activity (terminated by TTX plus MK-801). Images monitoring the subsequent survival of the neurons in trophically deprived medium were then taken at the indicated times (***E***, examples). Scale bar, 40 μm. Cell death is expressed as an increase above the low levels of death that is observed if BiC/4-AP activity is ongoing. For each treatment, 400–1000 neurons were analyzed. *$p < 0.005$; paired two-tailed *t* test; $n = 6$. Error bars represent SEM.

death-inducing stimulus (Fig. 4*A*). We found that the neuroprotection afforded by an episode of synaptic activity lasts far beyond the point at which activity is terminated; 12 h of bicuculline-induced activity at the beginning of trophic deprivation (terminated by TTX plus MK-801) has a significant positive effect on cell fate when analyzed 60 h later (Fig. 4*A*). Similarly, a previous episode of BiC/4-AP-induced activity (12 h) also confers neuroprotection on electrically silent neurons in the face of apoptosis-inducing agents applied for 36 h after the cessation of activity (Fig. 4*A*). Thus, neuronal fate in these scenarios is governed not by the current level of synaptic activity but by the "activity history" of the neuron. This long-lasting or "late phase" of activity-dependent neuroprotection is NMDA receptor dependent (blocked by MK-801) (Fig. 4*A*) and can be induced, albeit with diminishing effectiveness, by stimulation times as low as 30 min (data not shown).

We hypothesized that long-lasting neuroprotection was attributable to Akt activity persisting long after activity had been terminated. However, whereas phospho-Akt levels are sustained for as long as bicuculline-induced bursting is allowed, it decays rapidly after the cessation of activity (Fig. 4*B*,*C*). We next hypothesized that long-lasting neuroprotection involved the activation of CRE-dependent gene expression. CREB phosphorylation also decays rapidly after the cessation of synaptic activity (Hardingham et al., 2002) (Fig. 4*B*), because ERK1/2 activity, the key pathway that determines the duration of activity-dependent CREB phosphorylation (Hardingham et al., 2001b; Impey and Goodman, 2001; Wu et al., 2001), also decays quickly (Fig. 4*B*). However, we reasoned that the expression of CREB-regulated prosurvival gene products, enhanced by the episode of synaptic activity, would persist long after activity had ceased and CREB itself had become inactivated.

To test the role of CRE activation in the late phase of activity-dependent neuroprotection, the CRE inhibitor ICER was expressed in neurons for 24 h before the 12 h episode of BiC/4-AP-induced activity (terminated by TTX plus MK-801). Survival of the neurons in trophically deprived medium was then monitored over time (Fig. 4*D*,*E*). We found that, whereas a previous episode of synaptic activity conferred considerable neuroprotection in control (globin) transfected neurons (Fig. 4*D*), expression of ICER completely blocked this long-lasting protection; levels of neuronal death were as high as if the episode of activity had never taken place (Fig. 4*D*,*E*, example pictures). Thus, activation of CRE-dependent gene expression, while completely redundant in sce-

Long-lasting activity-dependent neuroprotection requires CREB family activation

Because neurons are not continuously bursting *in vivo*, we determined whether an episode of synaptic activity could confer neuroprotection during a subsequent silent period. To test this, we induced strong synaptic NMDA receptor activity (by BiC/4-AP treatment) and then terminated it and subsequently applied a

narios in which activity is continuous, is absolutely required for the process whereby a period of activity confers neuroprotection during times when a neuron is subsequently silent. It remains a possibility that this late phase of neuroprotection also contains a contribution from long-lasting consequences of PI3K-Akt activation.

The role of nuclear Ca^{2+}/calmodulin signaling in activity-dependent neuroprotection

Although the activation of the PI3K-Akt pathway is widely regarded to take place in the cytoplasm, the activation of CREB by intracellular Ca^{2+} transients requires nuclear Ca^{2+}/calmodulin signaling (Hardingham et al., 1997, 2001a; Deisseroth et al., 1998; Limback-Stokin et al., 2004). Given this, we predicted that neuroprotection triggered by ongoing activity would not require an elevation in nuclear Ca^{2+} or nuclear Ca^{2+}/calmodulin signaling, whereas the induction of long-lasting neuroprotection would require it. To test this, we expressed a nuclear-localized inhibitor of Ca^{2+}/calmodulin signaling (CAMBP4nuc) (Wang et al., 1995; Deisseroth et al., 1998) and assessed its impact on activity-dependent CRE reporter activation and neuroprotection. Expression of CAMBP4nuc significantly impaired the activation of CRE-dependent gene expression by BiC/4-AP treatment, while leaving basal CRE reporter activity unaffected (Fig. 5*A*). Just as we found with ICER expression, CAMBP4nuc expression had no effect on the ability of ongoing synaptic activity to evoke neuroprotection (Fig. 5*B*) but completely blocked long-lasting neuroprotection triggered by a previous episode of synaptic activity (Fig. 5*C*). Thus, nuclear Ca^{2+}/calmodulin signaling is an essential requirement for triggering the late phase of activity-dependent neuroprotection.

Discussion

This investigation into synaptic NMDA receptor-dependent neuroprotection has unearthed two mechanistically distinct phases. Neuroprotection evoked acutely while activity is ongoing is independent of CRE activation; activation of the PI3K-Akt pathway is both necessary and sufficient for this phase of neuroprotection. However, we also show that activity-dependent neuroprotection lasts long after activity has ceased; the vulnerability of electrically silent neurons to apoptosis-inducing trauma is critically dependent on their previous activity history. This long-lasting phase of neuroprotection relies on the nuclear Ca^{2+}-dependent activation of CRE-dependent gene expression.

The PI3K-Akt pathway in activity-dependent neuroprotection

Activation of Akt by PI3K involves membrane recruitment of Akt (by binding 3′ phosphoinositide products of PI3K catalysis), which facilitates its phosphorylation on Thr308 by phosphoinositide-dependent protein kinase 1 (PDK1) and on Ser473 by the unidentified PDK2 (Brazil et al., 2004). Activation of the PI3K-Akt pathway by growth factors and cytokines typically takes place when PI3K (via its Src homology 2 domain) binds to specific phospho-tyrosine residues on activated receptor tyrosine kinases. However, the mechanism by which Ca^{2+} influx can activate this pathway is less clear. Although PI3K-independent activation of Akt by CaM kinase kinase has been reported previously (Yano et al., 1998), in our study, the PI3K inhibitor completely blocked activity-dependent Akt activation (Fig. 3*E*, inset), in agreement with other previous studies (Lafon-Cazal et al., 2002; Sutton and Chandler, 2002). Both CaM kinase activity (Sutton and Chandler, 2002) and ERK1/2 activity (Lafon-Cazal et al., 2002) have been reported to contribute to the Ca^{2+}-dependent activation of PI3K, as has the direct activation of PI3K by Ca^{2+}/calmodulin (Joyal et al., 1997). We also observe a partial inhibitory effect of inhibitors of CaM kinases (KN-62) and of ERK1/2 activation (the MEK1 [microtubule-associated protein (MAP) kinase kinase 1 inhibitor PD98059 [2-(2-amino-3-

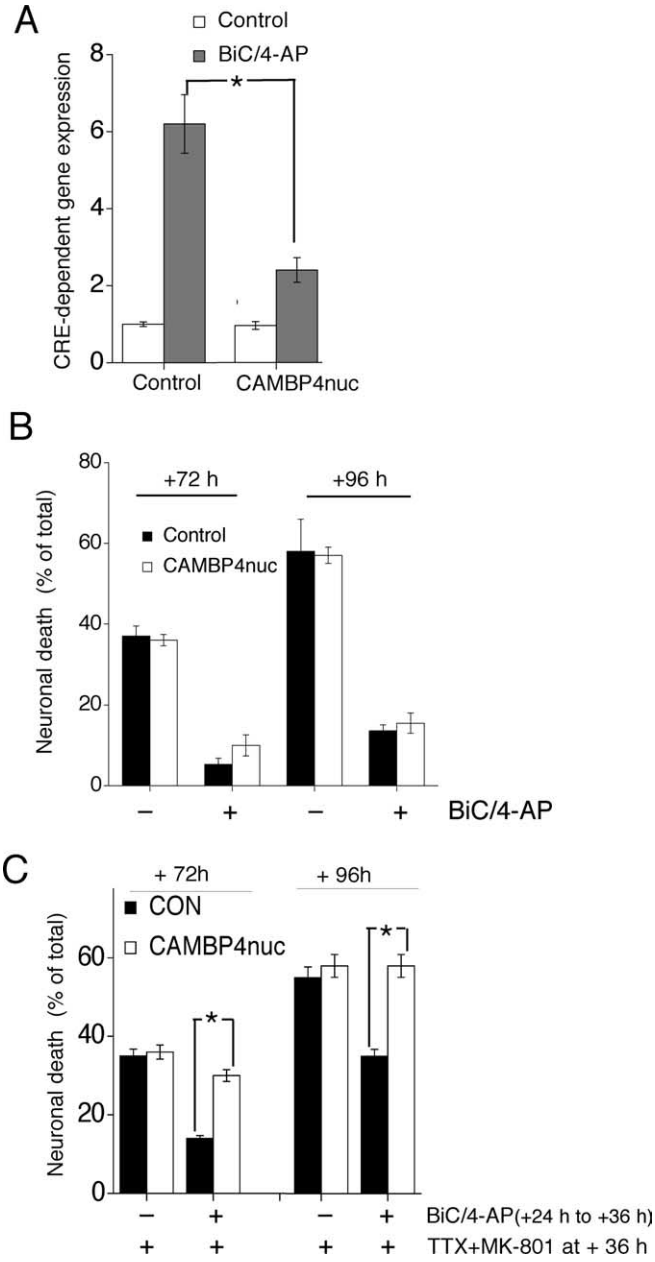

Figure 5. Nuclear Ca^{2+}/calmodulin signaling is required for long-lasting activity-dependent neuroprotection but is not needed for protection afforded by ongoing activity. ***A***, Expression of CAMBP4nuc inhibits stimulated, but not basal, CRE reporter activity. CRE-firefly luciferase expression in control (globin) or CAMBP4nuc-transfected neurons ± BiC/4-AP (8 h) is shown (*$p < 0.05$; paired two-tailed *t* test; $n = 5$). ***B***, Nuclear Ca^{2+}/calmodulin signaling is not needed for neuroprotection afforded by ongoing synaptic activity. Neurons transfected with the indicated plasmids, plus an eGFP marker, are shown. For each treatment, the fate of 300–600 cells under trophic deprivation was monitored over five (+72 h) or three (+96 h) independent experiments. ***C***, Long-lasting neuroprotection is dependent on the previous activation of nuclear Ca^{2+}/calmodulin signaling. Neurons were transfected with either control (CON) (globin) plasmid or CAMBP4nuc before experiencing a 12 h episode of BiC/4-AP-induced activity (terminated by TTX plus MK-801). Images monitoring the subsequent survival of the neurons in trophically deprived medium were then taken at the indicated times. For each treatment, 400–1000 neurons were analyzed. *$p < 0.005$; paired two-tailed *t* test; $n = 5$.

methyoxyphenyl)-4*H*-1-benzopyran-4-one]) on Akt activation (data not shown).

There are a number of ways in which the PI3K-Akt pathway can evoke neuroprotection (Brunet et al., 2001). Akt can promote survival posttranslationally by phosphorylating and inactivating both glycogen synthase kinase-3β (Crowder and Freeman, 2000; Hetman et al., 2000) and the proapoptotic bcl-2 family member BAD (Bcl-2-associated death protein) (Datta et al., 1997, 2002). Akt also phosphorylates and triggers nuclear export of the FOXO (forkhead box *O*) subfamily of forkhead transcription factors, which control the expression of prodeath genes such as Fas ligand and possibly also BIM-1 (Bc1-2-interacting mediator of cell death) and p27KIP1 (Kops et al., 1999; Brunet et al., 2001; Arden and Biggs, 2002). Akt can also promote survival via the inactivation of the transcription factor p53 (Yamaguchi et al., 2001), which controls the expression of prodeath genes such as BAX (Bc1-2-associated X protein). Akt has also been reported to be able to phosphorylate CREB (Du and Montminy, 1998). However, we found no role for Akt in the regulation of CRE-mediated gene expression in our system (Fig. 3*E*), indicating that Akt is unlikely to promote survival via this route.

The finding that synaptic NMDA receptor activity can provide resistance to ceramide (an endogenous inducer of apoptosis) (Goswami and Dawson, 2000; Pettus et al., 2002) underlines another potential Akt-dependent prosurvival mechanism. Ceramide and Akt are mutually antagonistic; Akt prevents ceramide-induced apoptosis and stress-induced ceramide synthesis (Goswami et al., 1999), whereas ceramide itself promotes cell death by inactivating Akt (Zhou et al., 1998). Indeed, exogenous C-2 ceramide causes Akt dephosphorylation, which is antagonized by bicuculline-induced Akt phosphorylation (S. Papadia, P. Stevenson, and G. E. Hardingham, unpublished observations). Because many stressful stimuli, including trophic deprivation, staurosporine, and retinoic acid, can induce intracellular ceramide accumulation (Wiesner and Dawson, 1996; Herget et al., 2000; Toman et al., 2002), activity-dependent Akt activation may promote protection in these cases by antagonizing ceramide-mediated apoptosis.

The CREB family mediates long-lasting neuroprotection, mirroring its role in long-lasting synaptic plasticity

This study demonstrates a causal link between CREB family activation and activity-dependent neuroprotection. We showed that neuronal survival and resistance to trauma is not just dependent on the degree of ongoing synaptic activity, it can also depend on the activity history of the neuron. A previous episode of synaptic activity confers significant neuroprotection during the subsequent period when the neuron is electrically silent. CREB family activation is crucial for this long lasting or late phase of activity-dependent neuroprotection. After the cessation of activity, phospho-Akt levels quickly decay, but so do phospho-CREB levels. However, one can envisage that the changes in gene expression that took place in response to CREB activation will remain long after CREB has become inactivated. These changes are demonstrably needed for long-lasting neuroprotection (possibly in conjunction with Akt-mediated changes to gene expression).

The identity of the CRE-regulated gene(s) responsible for long-lasting protection against apoptosis is a matter of ongoing investigation, initially by expression analysis of neurons experiencing varying levels of synaptic NMDA receptor activity *in vivo* and *in vitro*. One CRE-regulated, NMDA receptor-activated gene known to promote neuronal survival is BDNF (Shieh et al., 1998; Tao et al., 1998). BDNF mRNA expression is reduced after NMDA receptor blockade *in vivo*, and supplementation of neurons with BDNF *in vitro* can rescue neuronal death caused by NMDA receptor antagonists (Hansen et al., 2004). Synaptic NMDA receptor activity strongly activates BDNF expression and tyrosine receptor kinase B (TrkB) activation (Hardingham et al., 2002), and BDNF supplementation can inhibit neuronal death triggered by trophic deprivation in the presence of NMDA receptor antagonists (data not shown). However, any contribution from BDNF is likely to be only partial; TrkB activation decays quickly after the cessation of activity (data not shown), and the cell-autonomous requirement for CRE activation to evoke long-lasting neuroprotection argues for intracellular gene products being important, rather than extracellular ligands. The upregulation of CREB targets involved in metabolism/respiration (e.g., cytochrome *c*), responses to oxidative stress (e.g., superoxide dismutase 2) or in inhibiting apoptosis (e.g., bcl-2) (Riccio et al., 1999) all provide potential ways in which CREB can promote survival and resistance to trauma. The very large number of CRE-containing genes (Mayr and Montminy, 2001; Impey et al., 2004) makes it unlikely that a single gene is responsible for inducing long-lasting neuroprotection. Interestingly, a recent study indicated that activity-dependent CRE activation in developing CNS neurons may protect against excitotoxic trauma (Lee et al., 2005), which would otherwise result in rapid cell death resembling necrosis. Given the differences between acute excitotoxicity/necrosis and apoptosis, it is conceivable that different CRE-regulated genes are responsible for neuroprotection against these different forms of cell death. Nuclear Ca^{2+} signaling may be a common mediator of both neuroprotective programs, although so far a role has only been established for apoptosis (our study). Also, whereas the anti-apoptotic effects of synaptic NMDA receptor activity *in vivo* are well established (Ikonomidou et al., 1999, 2000; Olney et al., 2002; Adams et al., 2004), it is not yet clear under what physiological scenarios activity-dependent protection against excitotoxicity in the developing CNS manifests itself.

The characterization of two phases of activity-dependent neuroprotection raises the question as to which may be the most important *in vivo*. Because neurons are not continuously active under normal physiological conditions, the CRE-dependent late phase of activity-dependent neuroprotection may contribute significantly to survival promotion *in vivo*. The role of the CREB family in long-lasting neuroprotection afforded by a previous episode of synaptic activity is conceptually similar to its role in stabilizing long-lasting changes to synaptic strength induced by a previous episode of activity [late long-term potentiation (LTP) and long-term depression (LTD)] (Ahn et al., 1999; Barco et al., 2002; Korzus, 2003). Short-term plasticity does not rely on CREB-regulated gene products; however, activity-dependent CREB induction plays a central role in prolonging/consolidating these changes to synaptic strength. Thus, the production of CRE-driven gene products appears to be fundamental to the way neurons cement the physiological changes induced by synaptic activity.

Nuclear Ca^{2+} signaling is essential for long-lasting activity-dependent neuroprotection

Nuclear Ca^{2+}/calmodulin signaling is important for the Ca^{2+}-dependent activation of CREB resulting from its requirement for the activation of nuclear CaM kinase IV (Hardingham et al., 1997, 2001b; Deisseroth et al., 1998). We found that inhibiting nuclear Ca^{2+}/calmodulin signaling by expression of the nuclear-localized Ca^{2+}/calmodulin inhibitor, CAMBP4nuc (Wang et al., 1995), inhibited both activity-dependent induction of CRE-dependent gene expression and the late phase of activity-dependent neuroprotection. CAMBP4nuc expression had no ef-

fect on neuroprotection induced by ongoing activity, consistent with activation of the PI3K-Akt pathway requiring cytoplasmic and not nuclear Ca^{2+} elevation. Thus, global activity-dependent Ca^{2+} transients that invade the nucleus are likely to induce neuroprotection that lasts longer than that induced by spatially restricted calcium transients that do not invade the nucleus.

It is possible that the role of nuclear Ca^{2+}/calmodulin signaling in promoting neuroprotection extends beyond its role in activating CREB, because the effect of CAMBP4nuc expression on long-lasting neuroprotection is just as severe as ICER expression, despite being a less-effective inhibitor of CRE reporter activation (compare Figs. 2*C*, 5*A*). Nuclear Ca^{2+} signaling is able to modify transcription in ways other than through the activation of CREB. For example, nuclear Ca^{2+} elevation induces the activity of the broad specificity coactivator CREB-binding protein (CBP) (Chawla et al., 1998; Hardingham et al., 1999) and thus has the ability to modify the transactivating potential of other transcription factors with which CBP interacts (Goldman et al., 1997). Furthermore, nuclear Ca^{2+}/calmodulin signaling mediates the activity-dependent nuclear export of broad specificity transcriptional corepressors SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) and the class II histone deacetylases (HDACs) (Chawla et al., 2003; Mckenzie et al., 2005), which can result in the sensitization or derepression of transcription factor targets of these corepressors (Mckenzie et al., 2005). The transcription factor myocyte-enhancing factor 2 (MEF2) is implicated in mediating activity-dependent neuroprotection (Mao et al., 1999; Okamoto et al., 2000; Linseman et al., 2003) and is repressed by the class II histone deacetylases. Although MEF2 can be activated by p38 MAP kinase-dependent phosphorylation, it can also be activated via nuclear CaM kinase-dependent export of the class II HDACs (McKinsey et al., 2000; Miska et al., 2001; Linseman et al., 2003). Thus, nuclear Ca^{2+}/calmodulin signaling may also promote survival by triggering the export of class II HDACs, causing the derepression of MEF2.

The importance of nuclear Ca^{2+}/calmodulin signaling has also been demonstrated in the consolidation/prolonging of memory and synaptic plasticity (Ahn et al., 1999; Limback-Stokin et al., 2004). Transgenic mice that inducibly express CAMBP4nuc in the forebrain exhibit diminished activity-dependent signaling to CREB and impaired long-term but not short-term memory (although maximal LTP induction was also reduced). Moreover, expression of CAMBP4nuc blocks the late, CREB-dependent phase of cerebellar LTD but not the early induction phase (Ahn et al., 1999). Thus, the importance of direct signaling to CREB by nuclear Ca^{2+}/calmodulin for the capture of long-term memory and plasticity bears striking resemblance to its role in prolonging the neuroprotective effects of an episode of synaptic activity.

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