Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways

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Published online: 15 April 2002, DOI: 10.1038/nn835

Here we report that synaptic and extrasynaptic NMDA (*N*-methyl-D-aspartate) receptors have opposite effects on CREB (cAMP response element binding protein) function, gene regulation and neuron survival. Calcium entry through synaptic NMDA receptors induced CREB activity and brain-derived neurotrophic factor (BDNF) gene expression as strongly as did stimulation of L-type calcium channels. In contrast, calcium entry through extrasynaptic NMDA receptors, triggered by bath glutamate exposure or hypoxic/ischemic conditions, activated a general and dominant CREB shut-off pathway that blocked induction of BDNF expression. Synaptic NMDA receptors have anti-apoptotic activity, whereas stimulation of extrasynaptic NMDA receptors caused loss of mitochondrial membrane potential (an early marker for glutamate-induced neuronal damage) and cell death. Specific blockade of extrasynaptic NMDA receptors may effectively prevent neuron loss following stroke and other neuropathological conditions associated with glutamate toxicity.

Calcium signals are key mediators of numerous adaptive changes in the nervous system that are initiated by electrical activity[1–3]. To control multiple processes using a single second messenger, neurons exploit the spatial and temporal differences of calcium signals associated with electrical activity[4–12]. The site of calcium entry can determine the biological outcome of calcium signaling, as shown by site-specific differences in the regulation of CREB-mediated transcription. Calcium flux though L-type calcium channels is a potent activator of CREB, which in turn increases the expression of the gene encoding BDNF. In contrast, stimulation of NMDA receptors by glutamate bath application activates CREB only transiently and does not induce BDNF expression[4,8,13]. These findings seem to indicate that L-type calcium channels have a privileged role in CREB-mediated gene expression[4,8,13–15]. However, the stimulation methods used in these studies—KCl-induced membrane depolarization or glutamate bath application—only approximate physiological stimuli and thus provide limited insight into how synaptic activity controls nuclear signaling and transcription.

We therefore set up experimental conditions to analyze gene regulation by glutamatergic synaptic transmission. We found that stimulation of NMDA receptors by synaptic activity robustly activated CREB and CREB target gene expression. Genomic events induced by synaptic activity were initiated by calcium flux through synaptic NMDA receptors rather than through L-type calcium channels. The failure of glutamate to activate CREB function was due to specific coupling of extrasynaptic NMDA receptors to a CREB shut-off pathway that antagonized the CREB-promoting activity of synaptic NMDA receptors. The cellular consequences (survival versus death) of NMDA receptor activation were also specified by the location of the receptors activated rather than by their degree of stimulation. Our results suggest the existence of functionally distinct synaptic and extrasynaptic NMDA receptor signaling complexes with directly opposing effects on CREB function, gene regulation and neuronal fate.

Results

We first investigated the calcium-dependent control of genomic events in response to synaptically evoked bursts of action potentials by blocking GABA (γ-aminobutyric acid)$_A$ receptors with bicuculline in hippocampal cultures. As the hippocampal cultures contained inhibitory interneurons (~10%) that imposed a tonic inhibition on the neuronal network, blocking $GABA_A$ receptor function caused the neurons to fire synchronous bursts of action potentials[9]. The bicuculline-induced bursts of action potentials occurred periodically with low frequencies (0.05–0.15 Hz; **Fig. 1a**) and are associated with global calcium transients caused by calcium flux through synaptic NMDA receptors[9].

We also converted the bicuculline-induced 'oscillation type' calcium signals into elevated 'plateau-type' calcium signals by supplementing the bicuculline treatment with 2.5 mM 4-aminopyridine (4-AP)[9], a weak potassium-channel blocker. This treatment markedly increased the burst frequency (**Fig. 1b**) and resulted in elevated calcium plateaus induced by synaptic NMDA receptors (see **Fig. 3a** for typical calcium plateaus induced by

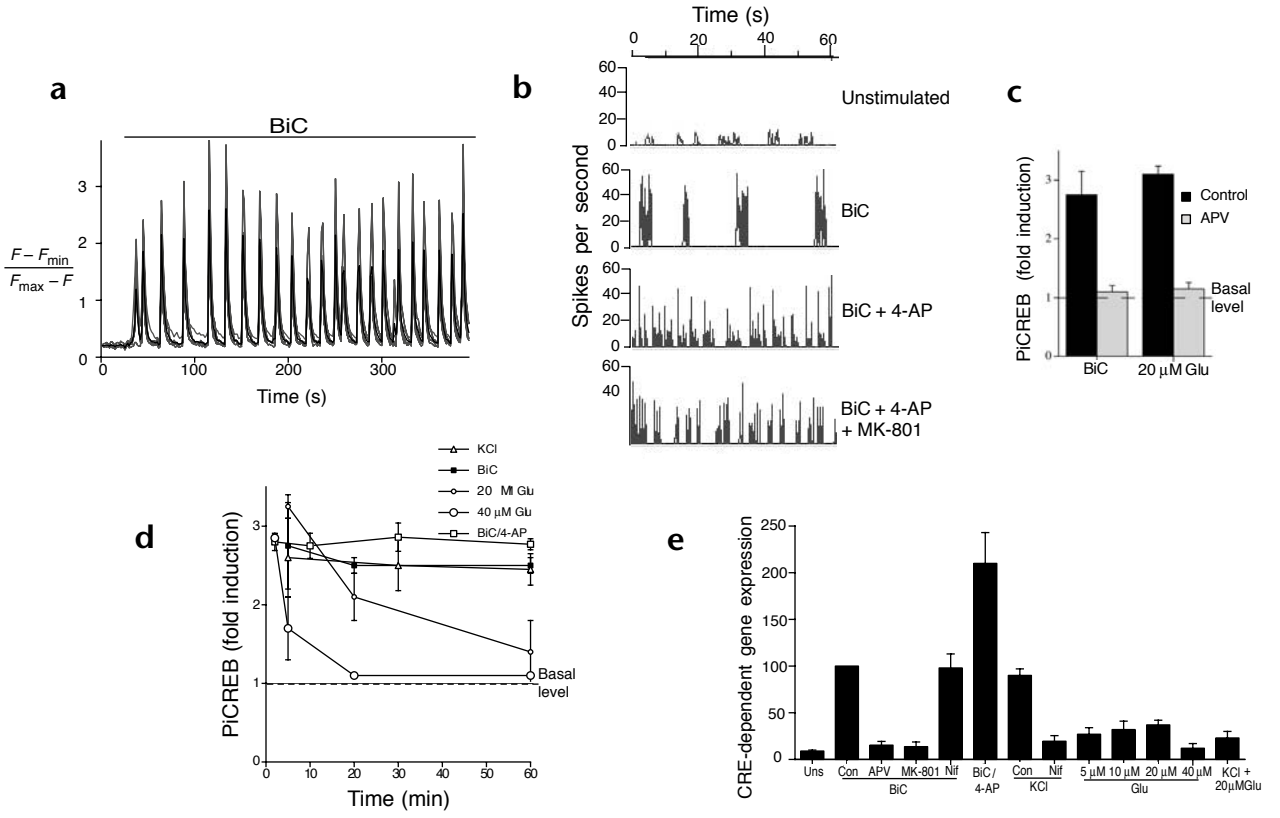

Fig. 1. Calcium entry through NMDA receptors rather than through L-type calcium channels is responsible for nuclear signaling to CREB and induction of CRE-dependent transcription in synaptically activated hippocampal neurons. (**a**) Imaging of global calcium transients in hippocampal neurons during a 400-s exposure to bicuculline (BiC, 50 μM); a typical example is shown. Calcium concentrations expressed as $[(F-F_{min})/(F_{max}-F)]$ (see Methods). (**b**) Example of a typical multi-electrode array recording of bicuculline-induced action potential bursting in the presence or absence of the indicated compounds (4-AP, 2.5 mM; MK-801, 10 μM). Treatments with 4-AP also contained 5 μM nifedipine to block calcium entry through L-type calcium channels. The global calcium transients evoked by the bursting (in the presence or absence of nifedipine) are NMDA receptor–dependent[9] (see also **Figs. 3b** and **7e**). (**c**) Immunocytochemical analysis of CREB phosphorylation on Ser 133 in hippocampal neurons exposed for 5 min to 50 μM bicuculline (BiC) or 20 μM glutamate (Glu) in the presence or absence of 250 μM APV. (**d**) Immunocytochemical analysis of the phosphorylation and dephosphorylation of CREB on serine 133 in hippocampal neurons after the indicated treatments (BiC, 50 μM; 4-AP, 2.5 mM). (**e**) Analysis of CRE reporter gene expression in unstimulated (Uns) hippocampal neurons and in hippocampal neurons after the indicated treatments (BiC, 50 μM; 4-AP, 2.5 mM; MK-801, 10 μM; APV, 500 μM; Nif, 5 μM). Reporter gene expression is presented relative to the expression of EGFP from a co-transfected plasmid to normalize for transfection efficiency. Con, control.

bicuculline/4-AP treatment). Bath application of glutamate also induces plateau-type calcium signals that are mediated by calcium flux through NMDA receptors[8,16] (**Fig. 3a**), whereas exposing hippocampal neurons to elevated levels of extracellular KCl causes membrane depolarization and calcium influx through L-type calcium channels[4,8,14,15].

Synaptic NMDA receptors stimulate CREB function

We analyzed two nuclear events triggered by the entry of calcium into hippocampal neurons: phosphorylation of CREB at the activator-site residue Ser 133 (ref. 17) and induction of CRE (cAMP response element)–dependent reporter gene expression[4,7,8]. These events were induced either by synaptically evoked bursts of action potentials or by glutamate bath application. All were blocked by the NMDA receptor antagonists D-(–)-2-amino-5-phosphonovalerate (APV) or MK-801 and thus were initiated by calcium flux through NMDA receptors (**Fig. 1c** and **e**). Nuclear events triggered by KCl-induced membrane depolarization were activated by calcium flux through nifedipine-sensitive L-type calcium channels (**Fig. 1e** and data not shown).

As compared to L-type calcium channels, NMDA receptors (activated by bicuculline-induced action potentials with or without 4-AP) were equally potent in causing both sustained CREB phosphorylation at Ser 133 and CRE-mediated gene expression (**Fig. 1d** and **e**). In addition, we found that bursts of action potentials, stimuli that are more physiologically relevant than KCl-induced membrane depolarization, activate CRE-dependent gene expression independently of L-type calcium channels. Blocking L-type calcium channels with nifedipine did not reduce CRE-reporter gene expression after action potential bursts, though it did inhibit the response triggered by KCl-induced membrane depolarization (**Fig. 1e**).

Synaptic NMDA receptors induce BDNF gene expression

The potent activation of CREB after synaptically evoked entry of calcium through NMDA receptors was also reflected by strong BDNF expression. The BDNF gene is controlled by several transcription factors, with CREB playing a central role[18–20]. We found that synaptic NMDA receptors induced BDNF expression to about the same extent as did the opening of L-

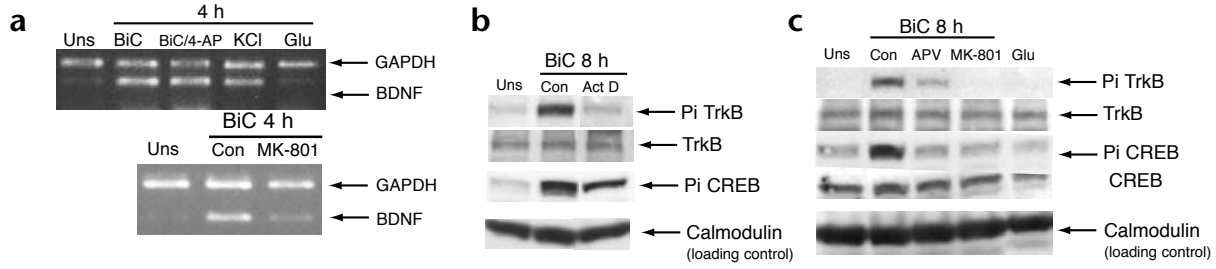

Fig. 2. NMDA receptors activated by synaptic activity robustly induced BDNF expression. (**a**) Expression of BDNF mRNA and GAPDH mRNA (control) was measured by RT-PCR in unstimulated hippocampal neurons (Uns) and in hippocampal neurons stimulated for 4 h with: 50 μM bicuculline (BiC) in the presence or absence of 10 μM MK-801; 20 μM glutamate (Glu); 50 mM KCl. (**b, c**) Immunoblot analysis of TrkB phosphorylation on tyrosine 515 (Pi TrkB), TrkB expression, CREB phosphorylation on serine 133 (Pi CREB), and calmodulin (loading control) in unstimulated hippocampal neurons (Uns) or in hippocampal neurons treated for 8 hours with either 50 μM bicuculline (BiC) in the absence (Con) or presence of 500 μM APV, 10 μM MK-801, 10 μg/ml actinomycin D (Act D), or with 20 μM glutamate (Glu). The levels of CREB phosphorylation on serine 133 obtained 8 hours after bicuculline treatment were similar to the levels obtained after 5 minutes of bicuculline stimulation; stimulations with 50 μM bicuculline for 2–4 h only slightly increased TrkB phosphorylation (data not shown).

type calcium channels, previously thought to be the only calcium channels capable of increasing BDNF expression[13] (**Fig. 2a**). This increase in BDNF messenger RNA (mRNA) led to an increase in biologically active BDNF, which binds to its receptor, TrkB. This binding causes phosphorylation of TrkB on Tyr 515, which was measured using an antibody to phospho-TrkB. We found that bicuculline increased TrkB phosphorylation, and that this increase was blocked both by the NMDA receptor blocker MK-801 and by actinomycin D (an inhibitor of gene transcription), indicating that it was due to an induction of BDNF transcription triggered by calcium flux through synaptic NMDA receptors (**Fig. 2b** and **c**).

Bath glutamate can suppress CREB function

In striking contrast to the activation of NMDA receptors by synaptic activity, stimulation of NMDA receptors by glutamate bath application poorly induced expression of the CRE-dependent reporter gene and only transiently increased CREB phosphorylation on Ser 133 (**Fig. 1d** and **e**), confirming previous reports[4,8]. Moreover, glutamate suppressed the induction of the CRE reporter gene after stimulation of L-type calcium channels (**Fig. 1e**). The fact that glutamate treatment did not promote CREB function was not due to a dosage problem, as a full range of glutamate concentrations were tested (**Fig. 1d** and **e**). Glutamate treatment also did not induce BDNF expression or TrkB phosphorylation (**Fig. 2a** and **c**).

Next we investigated the molecular basis of this pronounced stimulation-dependent difference in NMDA receptor signaling. The inability of glutamate to stimulate CREB function was not due to activated $GABA_B$ or metabotropic glutamate receptors, as their respective antagonists, CGP 54626 (1 μM) and α-methyl-4-carboxyphenylglycine (MCPG; 0.5 mM), did not increase the efficacy of nuclear signaling to CREB after glutamate treatment (data not shown). Neither was it due to differences in calcium transients induced by the stimulation method used, bicuculline/4-AP versus bath glutamate. This was shown by calcium imaging (**Fig. 3a**). Hippocampal neurons stimulated with bicuculline/4-AP generated elevated, global (cytoplasmic and nuclear) calcium plateaus (**Fig. 3a**). After removal of bicuculline/4-AP and blockade of electrical activity using tetrodotoxin (TTX), which caused calcium concentrations to return to basal levels, the neurons were exposed to either 20 μM or 40 μM glutamate. Glutamate treatment also induced plateau-type global calcium transients (**Fig. 3a**); these calcium plateaus were due to NMDA receptor activation (ref. 16 and data not shown) and resembled those after bicuculline/4-AP stimulation. This similarity suggests that calcium profile differences do not underlie the marked signaling differences between the two stimulation methods.

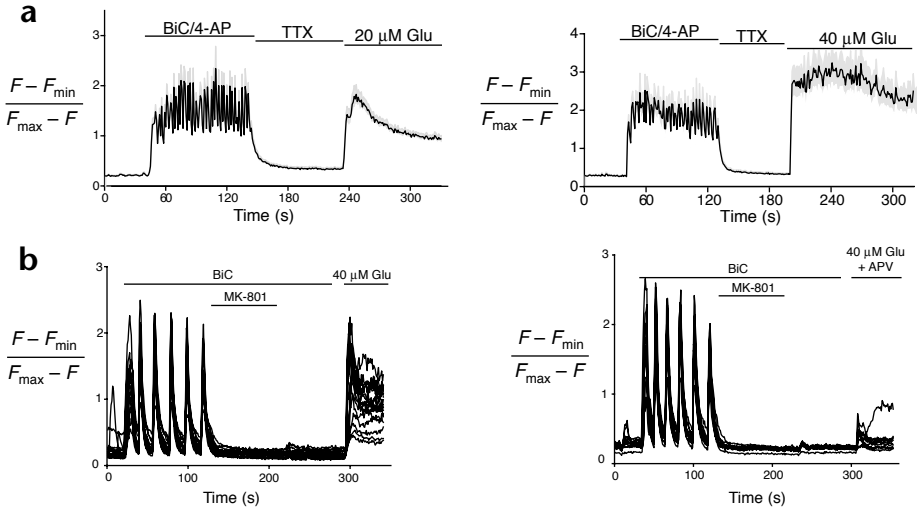

Fig. 3. Comparison of NMDA receptor–mediated calcium signals triggered by bursts of action potentials or by glutamate bath application. Calcium concentrations expressed as $[(F - F_{min})/(F_{max} - F)]$ (see Methods). (**a, b**) Imaging of global calcium transients in hippocampal neurons after the indicated treatments. Bicuculline (BiC), 50 μM; 4-AP, 2.5 mM; TTX, 2 μM; MK-801, 10 μM; APV, 500 μM. (**a**) Mean calcium levels of 13 hippocampal neurons ± s.e.m. (shaded area). (**b**) Examples of individual neurons (thin lines); thick line represents the mean. Similar results were obtained in other experiments.

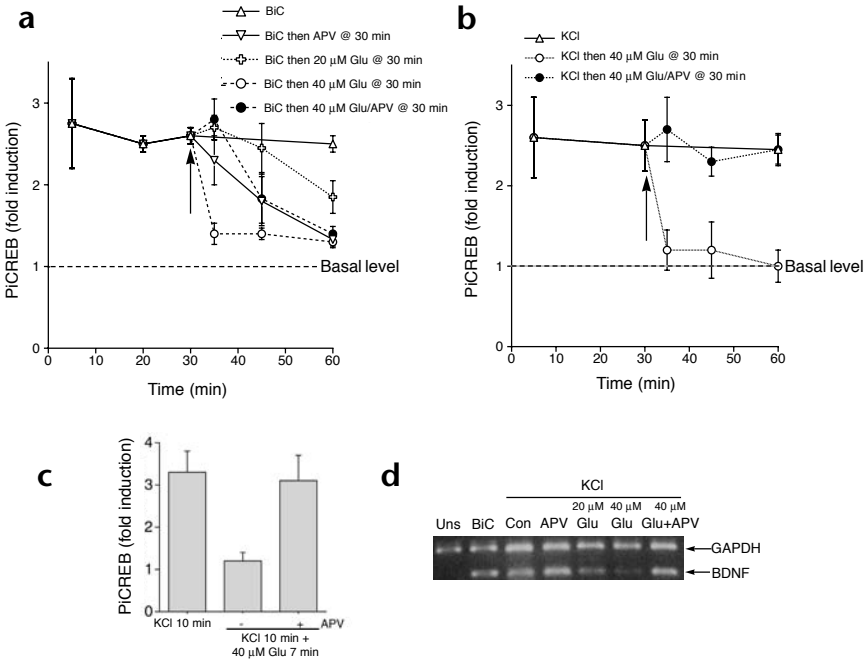

Fig. 4. Calcium flux through extrasynaptic NMDA receptors initiates a CREB shut-off signal and suppresses induction of BDNF expression. (**a, b**) Immunocytochemical analysis of the rapid shut-off of CREB phosphorylation on serine 133 in hippocampal neurons after stimulation (arrow) with 20 or 40 μM glutamate in the presence or absence of 100 μM APV. CREB phosphorylation was induced by stimulating for 30 min either calcium flux through synaptic NMDA receptors with 50 μM bicuculline (BiC) or calcium flux through L-type calcium channels with 50 mM KCl. (**c**) Hippocampal neurons were first subjected to bicuculline/ MK-801 pre-treatment to inactivate synaptic NMDA receptors. CREB phosphorylation was subsequently induced by activating for 10 min calcium flux through L-type calcium channels with 50 mM KCl. CREB phosphorylation was assessed immunocytochemically after the indicated treatments. Glutamate, 40 μM; APV, 250 μM. (**d**) Expression of BDNF mRNA and of GAPDH mRNA, as control (Con), were measured by RT-PCR in unstimulated hippocampal neurons (Uns) and in hippocampal neurons 4 h after the indicated treatments. Bicuculline, 50 μM; KCl, 50 mM; APV, 500 μM.

Glutamate stimulates extrasynaptic NMDA receptors

Although bicuculline-induced bursting and bath application of glutamate both activate synaptic NMDA receptors, extrasynaptic receptors can be activated only by glutamate application[21]. Perhaps the observed difference in nuclear signaling was due to differential stimulation of extrasynaptic NMDA receptors. As glutamate causes dephosphorylation of CREB under certain conditions[22], we considered the possibility that extrasynaptic NMDA receptors could initiate a CREB-inhibiting pathway that is dominant over the CREB-promoting activity of synaptic NMDA receptors.

We first investigated whether glutamate bath application induces substantial calcium flux through extrasynaptic NMDA receptors. To test this, we selectively blocked synaptic NMDA receptors to assess whether glutamate bath application generated an additional calcium transient by stimulating extrasynaptic NMDA receptors. Synaptic NMDA receptors were selectively inactivated using MK-801 in conjunction with bicuculline. MK-801 blocked synaptic NMDA receptor–induced calcium transients (**Fig. 3b**) without compromising bicuculline-induced firing bursts of action potentials (**Fig. 1b**). Owing to the irreversible inhibition by MK-801, calcium concentrations remained low even after MK-801 washout despite the continuous presence of bicuculline (**Fig. 3b**). Because MK-801 is an open channel blocker and binds only activated NMDA receptors, the population of NMDA receptors not stimulated by bicuculline-induced firing (those not located at synapses) was left unblocked. This population of extrasynaptic NMDA receptors was then activated by adding glutamate to the medium. Indeed, glutamate exposure gave rise to calcium transients that were blocked (>90% inhibition) by APV (**Fig. 3b**).

Extrasynaptic receptors shut off CREB

To investigate whether extrasynaptic NMDA receptors antagonize nuclear signaling to CREB, we analyzed the effects of calcium flux through extrasynaptic NMDA receptors on the phosphorylation of CREB at serine 133. Calcium flux through synaptic NMDA receptors, triggered by bicuculline exposure, induced robust and sustained phosphorylation of Ser 133 of CREB (**Fig. 4a**; also **Fig. 1d**) and also potently activated CREB-mediated transcription (**Fig. 1e**). The increase in CREB phosphorylation was due to activation of NMDA receptors, as it was blocked by APV (**Fig. 1c**). In contrast, activation of the extrasynaptic population of NMDA receptors by glutamate initiated a CREB shut-off signal: CREB phosphorylation (previously induced by bicuculline) decayed rapidly and in a dose-dependent matter after glutamate treatment (**Fig. 4a**).

Activation of NMDA receptors with 40 μM NMDA (in the presence of 2 mM glycine) was as potent as glutamate in inducing the CREB shut-off pathway (data not shown). Given that bath application of glutamate or NMDA activates both extrasynaptic and synaptic NMDA receptors, these findings show that extrasynaptic NMDA receptors can override the CREB-promoting effects of synaptic NMDA receptors. CREB immunoreactivity was not affected by glutamate treatment (data not shown), indicating that this was a specific dephosphorylation signal, not a signal that resulted in the degradation of CREB. Treatment with 40 μM glutamate triggered CREB dephosphorylation more rapidly than merely blocking synaptic NMDA receptors with APV (**Fig. 4a**), indicating that the CREB dephosphorylation signal is an active one, rather than one that interferes with the phosphorylation-promoting signal. The CREB shut-off signal was NMDA receptor dependent: adding 40μM glutamate in the presence of APV caused the decay in CREB phosphorylation to slow to a rate similar to that in neurons treated with APV alone (**Fig. 4a**).

The NMDA-receptor dependency of the shut-off mechanism was also shown in experiments in which CREB phosphorylation was first induced using NMDA receptor–independent stimulations. We found that, consistent with a previous report[22], glutamate treatment shuts off, in an APV-sensitive manner, CREB phosphorylation initiated by KCl-induced calcium flux through

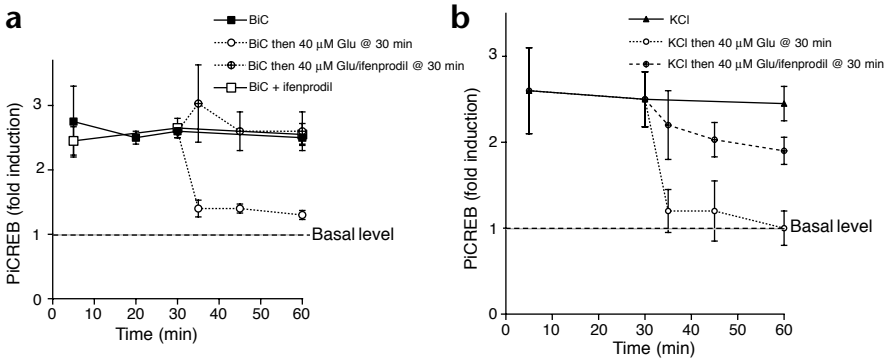

Fig. 5. Inhibition of the predominantly extrasynaptically localized NMDA receptor subunit NR2B with ifenprodil blocks the CREB shut-off process. Immunocytochemical analysis was done as described in Fig. 4. CREB shut-off was induced using 40 μM glutamate in the presence or absence of 10 μM ifenprodil.

L-type calcium channels (**Fig. 4b**) or by increasing intracellular levels of cAMP (data not shown). Thus, whereas synaptic NMDA receptors are linked to a CREB activity-inducing pathway, extrasynaptic NMDA receptors initiate a general CREB shut-off mechanism that is dominant over many CREB phosphorylation pathways.

To determine conclusively whether stimulation of extrasynaptic NMDA receptors is sufficient to trigger the CREB shut-off, we used a method similar to the one described in Fig. 3b. Hippocampal neurons were stimulated with bicuculline followed by the addition of MK-801 to block synaptic NMDA receptors. After washout of bicuculline/MK-801, CREB phosphorylation was induced by stimulating calcium flux through L-type calcium channels using KCl-mediated membrane depolarization. Extrasynaptic NMDA receptors were then activated by glutamate. We found that this stimulation of extrasynaptic NMDA receptors caused rapid dephosphorylation of CREB that was blocked by APV (**Fig. 4c**). Given that synaptic NMDA receptors remain inactive for at least 30 minutes after the pre-blocking procedure with bicuculline/MK-801 (data not shown), these results indicate that calcium flux through extrasynaptic NMDA receptors is sufficient to trigger the CREB shut-off pathway.

If extrasynaptic NMDA receptors couple to a dominant CREB shut-off pathway, do they also suppress expression of the CREB target gene BDNF? Indeed, we found that stimulation of extrasynaptic NMDA receptors with glutamate overrides, in an APV-dependent manner, the BDNF gene transcription–inducing activity of L-type calcium channels (**Fig. 4d**).

CREB shut-off is blocked by NR2B antagonist

The subunit composition of extrasynaptic and synaptic NMDA receptors is not identical. In hippocampal neurons, extrasynaptic NMDA receptors are composed predominantly of NMDA receptor 1 (NR1) and NMDA receptor 2B (NR2B) subunits, whereas synaptic NMDA receptors also contain NR2A subunits[21]. Ifenprodil, a selective but incomplete inhibitor of the NR2B subunit[23], may therefore preferentially inhibit extrasynaptic NMDA receptors. Calcium imaging revealed that ifenprodil was more effective in blocking calcium transients induced by extrasynaptic NMDA receptors (71 ± 5% inhibition) than those induced by synaptic NMDA receptors (53 ± 4% inhibition). Given these pharmacological properties, we expected ifenprodil to be an effective inhibitor of extrasynaptic NMDA receptor–induced CREB shut-off. To test this, we induced CREB phosphorylation by activating synaptic NMDA receptors using the bicuculline treatment, then triggered the decay of CREB phosphorylation by stimulating extrasynaptic NMDA receptors with glutamate. We found that ifenprodil completely blocked this decay (**Fig. 5a**). Indeed, CREB phosphorylation remained high during the course of the experiment, indicating that the inhibitory action of ifenprodil on synaptic NMDA receptors was not strong enough to compromise their CREB phosphorylation–promoting activity. Ifenprodil also inhibited glutamate-induced CREB shut-off in experiments in which CREB phosphorylation was induced by opening L-type calcium channels using KCl (**Fig. 5b**).

Hypoxic/ischemic conditions trigger CREB shut-off

Stimulation of extrasynaptic NMDA receptors may occur during hypoxic/ischemic insults. These pathological conditions are characterized by glutamate transporters operating in reverse (pumping glutamate out of the cells)[24], leading to stimulation of all NMDA receptors, including extrasynaptic ones. We found that, similar to glutamate bath application, depriving primary hippocampal neurons of oxygen and glucose caused a decay of CREB phosphorylation (**Fig. 6**). This CREB dephosphorylation was inhibited by APV and ifenprodil (**Fig. 6**), indicating that hypoxic/ischemic conditions initiate the CREB shut-off pathway through the activation of extrasynaptic NMDA receptors.

Extrasynaptic NMDA receptors trigger cell death

Given the dramatic differences in signaling from synaptic and extrasynaptic NMDA receptors (**Figs. 1–6**), we tested the hypothesis that cell survival after NMDA receptor activation is also specified by the location, not the degree, of NMDA receptor activation. We compared stimuli that evoke calcium entry through synaptic NMDA receptors, through synaptic and extrasynaptic NMDA receptors or through L-type calcium channels (**Fig. 7a**). These

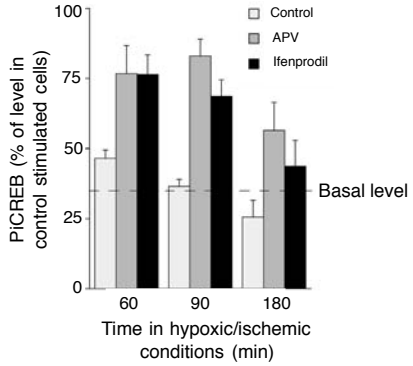

Fig. 6. Extrasynaptic NMDA receptors mediate CREB shut-off induced by hypoxia/ischemia. Immunocytochemical analysis of the dephosphorylation of phospho-serine 133-CREB in hippocampal neurons subjected for the indicated times to hypoxic/ischemic conditions in the presence or absence of 500 μM APV or 10 μM ifenprodil. Neurons were treated with 10 μM forskolin/0.5 mM IBMX for 30 min before exposure to hypoxic/ischemic conditions; this pre-treatment stimulates CREB phosphorylation on serine through activating cAMP-dependent protein kinase.

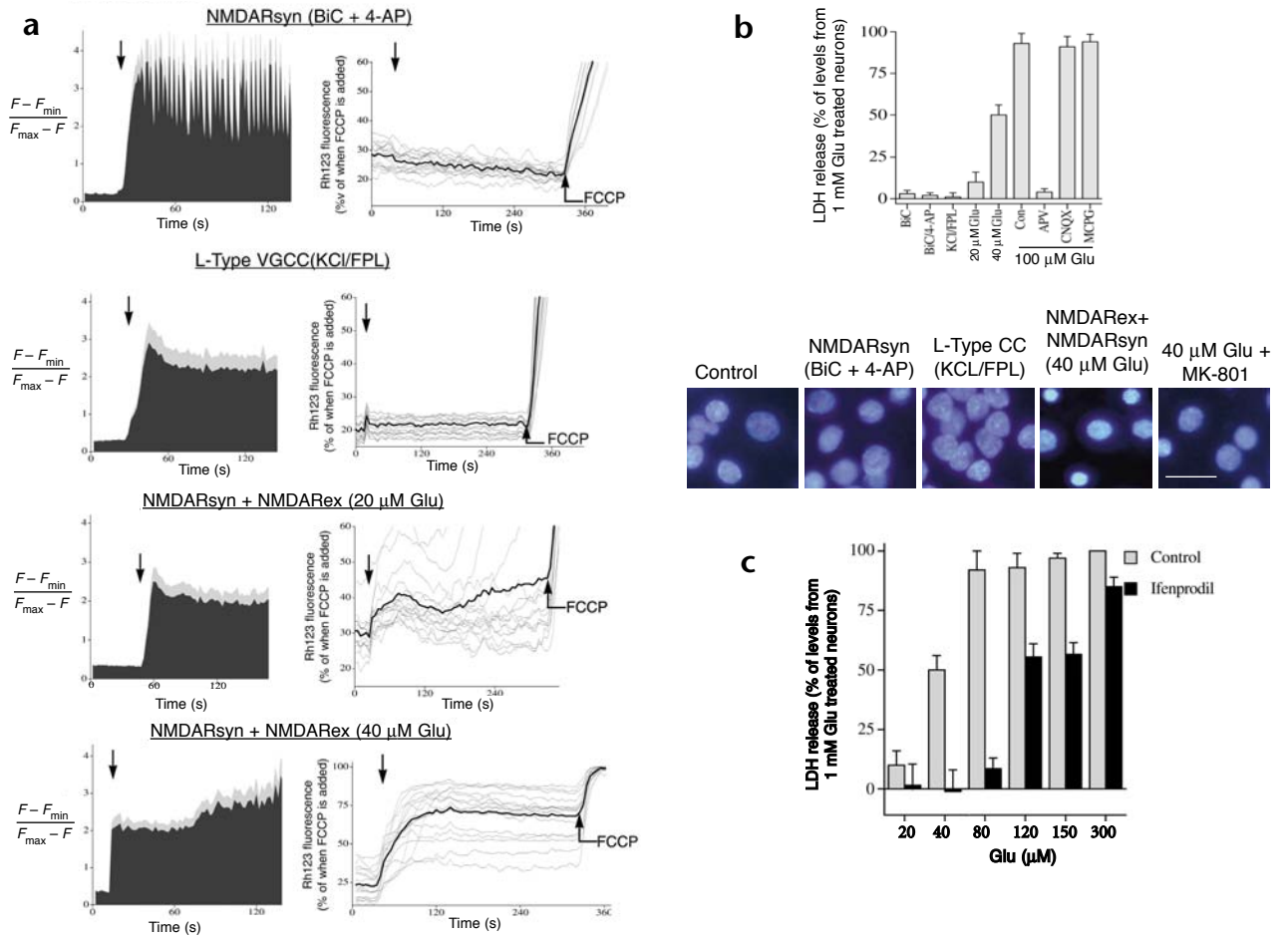

Fig. 7. Extrasynaptic NMDA receptors are linked to mitochondrial dysfunction and neuronal death. (**a**) Analysis of calcium transients (left) and mitochondrial membrane potential (right) in hippocampal neurons after activation of either synaptic NMDA receptors (50 μM bicuculline/2.5 mM 4-AP/ 5 μM nifedipine), or synaptic *and* extrasynaptic NMDA receptors (20 μM and 40 μM glutamate), or L-type calcium channels induced by increasing the extracellular KCl concentration by 50 mM in the presence of the L-type calcium channel agonist FPL 64176 (5 μM) (KCl/FPL). Arrows indicate start of stimulation. The area underneath the calcium traces is dark shaded to emphasize the calcium load; the lightly shaded area represents the s.e.m.. Mitochondrial membrane potential of individual neurons (thin lines) are shown; thick line represents the mean. (**b**) Analysis of excitotoxic cell death of hippocampal neurons stimulated as in (**a**). APV, 500 μM; MK-801, 10 μM; MCPG, 500 μM; CNQX, 400 μM. Examples of Hoechst-stained hippocampal neurons are shown to illustrate the distinct chromatin fragmentation observed after necrotic cell death. Similar excitotoxic cell death was observed when NMDA was used instead of glutamate (data not shown); both glutamate and NMDA-induced death was blocked by NMDA receptor antagonists. The presence of the L-type calcium channel blocker nifedipine did not alter glutamate-induced toxicity (data not shown). (**c**) Effect of ifenprodil (10 μM) on excitotoxic cell death of hippocampal neurons induced with the indicated concentrations of glutamate. (**d**) Imaging of calcium (left) and mitochondrial membrane potential (right) in hippocampal neurons first exposed to a stimulus activating synaptic NMDA receptor (50 μM bicuculline and increasing amounts of 4-AP) and then to a stimulus activating synaptic *and* extrasynaptic NMDA receptor (glutamate). Between stimuli, neurons were treated with 2 μM TTX to block all electrical activity. (**e**) Calcium profile (left) and breakdown of mitochondrial membrane potential (right) specifically evoked by extrasynaptic NMDA receptors. Hippocampal neurons were sequentially exposed to stimuli activating synaptic NMDA receptors (50 μM bicuculline, 2.5 mM 4-AP, 5 μM nifedipine) and, after blocking synaptic NMDA receptors with 10 μM MK-801, to stimuli activating extrasynaptic NMDA receptors (60–100 μM glutamate).

stimuli were, respectively, (i) induction of near-continuous action potential bursts, (ii) exposure of the neurons to glutamate and (iii) an increase in the extracellular KCl concentration by 50 mM in the presence of the L-type calcium channel agonist FPL 64176 (KCl/FPL stimulation). Calcium imaging showed that near-continuous bursting and glutamate exposure gave rise to NMDA receptor–dependent elevated calcium plateaus with similar amplitudes; KCl/FPL-induced calcium plateaus were L-type calcium channel dependent[8,9,16] (**Fig. 7a**; also **Fig. 7e**).

To investigate which stimulus links to cell death, we monitored the mitochondrial membrane potential in living hippocampal neurons. A breakdown of the mitochondrial membrane potential is an early marker of excitotoxic cell death[25–28]. We also measured lactate dehydrogenase (LDH) release and stained hippocampal neurons with Hoechst 33342 to determine the percentage of hippocampal nuclei with small, irregular chromatin clumps, a characteristic of necrotic cell death[29]. We found that hippocampal neurons remained healthy, with intact mitochondrial membrane potential, after stimulation of calcium entry through synaptic NMDA receptors and through L-type calcium channels (**Fig. 7a** and **b**). In contrast, stimulation with 40 μM glutamate (or, to a lesser extent, with 20 μM glutamate) caused a rapid breakdown of the mitochondrial membrane potential leading to cell death (**Fig. 7a** and **b**). Glutamate-induced excitotoxic cell death was blocked by APV but not by MCPG or by the AMPA/kainate receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; **Fig. 7b**). Ifenprodil efficiently inhibited glutamate-induced death; however, because it is an incomplete blocker of NR2B, the protective potential of ifenprodil broke down at very high glutamate concentrations (**Fig. 7c**). The neuroprotective properties of ifenprodil suggest the involvement of extrasynaptic NMDA receptors in glutamate-induced neuronal damage.

To address the possibility that the observed difference in cell death was due to differences in calcium load, we measured calcium concentration and mitochondrial membrane potential in hippocampal neurons that were exposed first to near-continuous bursting (activating synaptic NMDA receptors), then to glutamate (activating both synaptic and extrasynaptic NMDA receptors). The results show that only glutamate exposure caused a breakdown of the mitochondrial membrane potential, although both stimuli induced calcium plateaus of similar amplitude (**Fig. 7d**). Thus, stimulation-induced calcium signals can be similar in profile yet strikingly different in their cellular consequences (in agreement with other reports[30,31]). This indicates that it is not the profile of the NMDA receptor–induced calcium signals but possibly differences in the location of the receptors activated that is functionally important.

As stimulation of calcium entry through synaptic NMDA receptors (or through L-type calcium channels) did not compromise the health of hippocampal neurons, we reasoned that extrasynaptic NMDA receptors may specifically link to a cell death pathway. We used a stimulation protocol similar to that illustrated in Fig. 3b to selectively induce calcium flux through extrasynaptic NMDA receptors. The neurons were first exposed to bicuculline plus 4-AP (which leads to calcium flux through synaptic NMDA receptors), then MK-801 (**Fig. 7e**). MK-801 did not block bicuculline-induced bursting of action potentials (**Fig. 1b**), but inactivated all synaptic NMDA receptors stimulated during bicuculline/4-AP treatment, causing calcium concentrations to return to near basal levels (**Fig. 7e**). MK-801 spared NMDA receptors not stimulated during bicuculline/4-AP exposure (namely extrasynaptic NMDA receptors); this population of receptors was then activated by glutamate treatment (**Fig. 7e**). We found that extrasynaptic NMDA receptors do indeed link to a cell death pathway and cause a breakdown of the mitochondrial membrane potential (**Fig. 7e**). The extrasynaptic NMDA receptor–induced loss of mitochondrial membrane potential was blocked (more than 95 % inhibition) by APV or MK-801. Intense stimulation of synaptic NMDA receptors, in contrast, had no effect on mitochondrial membrane potential (**Fig. 7a, d,** and **e**).

Synaptic NMDA receptor activation is anti-apoptotic

Synaptic NMDA receptor activation was found to be neuroprotective. This was demonstrated using a standard apoptosis-inducing protocol that uses the general protein kinase inhibitor staurosporine. Hippocampal neurons exposed to staurosporine for 36 hours showed typical signs of apoptosis (shrunken cell body and large, round chromatin clumps; **Fig. 8**). Stimulating synaptic activity with bicuculline for 16 hours before staurosporine treatment reduced the number of apoptotic neurons (**Fig. 8**). This bicuculline-induced neuroprotective effect was blocked with MK-801 (**Fig. 8**), indicating that it resulted from stimulation of synaptic NMDA receptors. The most complete protection was observed when bicuculline-induced calcium entry through synaptic NMDA receptors was further enhanced by 2.5 mM 4-AP, which increases the burst frequency as shown in Fig. 1b (**Fig. 8**). The bicuculline/4-AP-induced anti-apoptotic activity was blocked by MK-801 (**Fig. 8**). Conversely, staurosporine-induced death is exacerbated by blocking spontaneous synaptic NMDA receptor activity (either by using

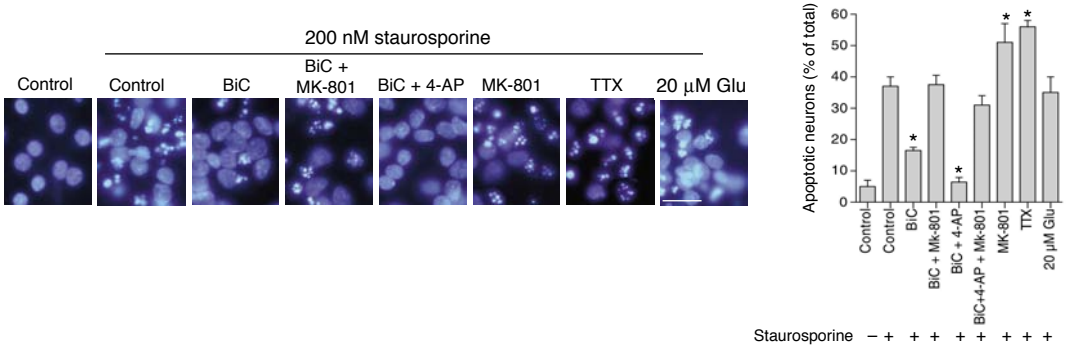

Fig. 8. Stimulation of synaptic NMDA receptors is anti-apoptotic. Staurosporine-induced apoptosis in hippocampal neurons with or without pretreatment for 16 h with the indicated compounds (BiC, 50 μM; 4-AP, 2.5 mM; MK-801, 10 μM; TTX, 2 μM). Examples of Hoechst-stained hippocampal neurons illustrate the characteristic chromatin fragmentation of apoptotic neurons. Statistically significant differences ($P < 0.05$, two-tailed, unpaired *t*-test; $P < 0.05$, Mann-Whitney *U* test) compared with control condition (treatment with staurosporine alone) are indicated with an asterisk.

MK-801 or by blocking all electrical activity with TTX; **Fig. 8**). This further underscores the importance of synaptic NMDA receptors in promoting neuron survival. As we observed no neuroprotective effect after stimulation of the neurons with a low dose (20 μM) of glutamate (**Fig. 8**), this pro-survival activity could not simply have been due to electrical activation of the neurons or to tolerance after mild excitotoxic insults.

Discussion

We have shown that synaptic and extrasynaptic NMDA receptors have directly opposing effects on CREB function and neuronal fate. Our results indicate that hippocampal neurons have at least two functionally distinct NMDA receptor signaling complexes. Synaptic complexes promote nuclear signaling to CREB, induce BDNF gene expression, and activate an anti-apoptotic pathway; extrasynaptic complexes antagonize nuclear signaling to CREB, block induction of BDNF expression, and are involved in mitochondrial dysfunction and cell death. Thus, the biological consequences of NMDA receptor activation in hippocampal neurons are specified by the location of the NMDA receptor signaling complex activated. This location-specific difference in NMDA receptor signaling provides a long-sought molecular basis for why activation of NMDA receptors by glutamate is a poor inducer of CREB-mediated gene expression.

It has been suggested that L-type calcium channels have a privileged role in calcium regulation of CREB. The results presented here alter this 'source specificity' model for CREB-mediated gene expression. Calcium flux through L-type calcium channels and through synaptic NMDA receptors are equally potent in stimulating CREB-mediated gene expression, and synaptic NMDA receptors (rather than L-type calcium channels) mediate genomic responses following bursts of action potential firing. Calcium flux through extrasynaptic NMDA receptors, unlike through synaptic NMDA receptors, actively suppresses CREB activity by initiating a general CREB shut-off mechanism. Antagonistic signaling of calcium-permeable ion channels has also been reported in ciliary ganglion neurons[32]. However, in those cells, calcium-entry through L-type calcium channels can suppress CREB phosphorylation on Ser 133 (ref. 32).

The exact nature of the CREB shut-off pathway and the mechanism by which extrasynaptic NMDA receptors activate it remain to be investigated. It may involve inactivation of CREB phosphorylation mechanisms including several CREB kinases such as calcium/calmodulin-dependent protein kinases and cAMP-dependent protein kinase. Alternatively, it is conceivable that extrasynaptic NMDA receptors control the action of the CREB-phospho-serine 133 phosphatase. Inhibition of calcineurin, a calcium/calmodulin-dependent protein phosphatase that may have a function in CREB regulation[5,32], has little or no effect on the rapid decay of CREB phosphorylation (ref. 22 and unpublished data). Inhibition of protein phosphatase 1 (PP1), the principal CREB phospho-serine 133 phosphatase[33], does prevent the dephosphorylation of CREB at Ser 133 (refs. 5,22 and unpublished data); however, the interpretation of these results is complicated by the observation that inhibiting PP1 with okadaic acid compromises the health of the hippocampal neurons and can lead to cell death (unpublished results). There is currently little evidence that PP1 activity is regulated by calcium signaling pathways (except via calcineurin and inhibitor 1; refs. 5,32). One potential mechanism of regulation could involve one of the many different regulatory subunits of PP1 that can modulate PP1 activity and target the enzyme to specific substrates[34]. Thus, extrasynaptic NMDA receptors may induce specific targeting of PP1 to CREB, which is consistent with previous observations that glutamate bath application does not compromise transcription mediated by CBP (CREB binding protein), c-Jun, and the serum response element[4,8,35].

The marked difference in cellular responses induced by synaptic versus extrasynaptic NMDA receptors is likely due to differences in the receptor signaling complexes. Many proteins are known to associate with NMDA receptors[36], but which ones are specific to synaptic or extrasynaptic NMDA receptors is less clear. Molecules involved in CREB shut-off or in mitochondrial depolarization may interact directly with extrasynaptic NMDA receptors but not with synaptic NMDA receptors because of shielding by the post-synaptic density or differences in the receptor subunit composition. One possible physiological model that would lead to stimulation of extrasynaptic NMDA receptors is glia-to-neuron communication. Glial cells are known to release glutamate under certain conditions[37]. Glial cell-derived glutamate could act on extrasynaptic NMDA receptors and thereby negatively regulate neuronal signaling pathways to CREB.

The opposite effects of synaptic and extrasynaptic NMDA receptors on neuronal fate help explain the paradox that both NMDA receptor stimulants and NMDA receptor antagonists can cause neuronal degeneration. The increase in neuronal apoptosis after injection of MK-801 in the developing rat brain, and the enhancement of apoptosis by MK-801 after traumatic injury or metabolic inhibition in adult rats[38,39], are explained by our finding that synaptic NMDA receptors have pro-survival activity that would be blocked by MK-801. This could also explain why environmental enrichment, stimulating synaptic activity, reduces spontaneous apoptotic cell death in the hippocampus and protects against neurotoxic injuries[40].

The link between extrasynaptic NMDA receptors and mitochondrial dysfunction and cell death may be particularly relevant to the pathophysiology of hypoxic/ischemic cell death and epileptic brain damage[41]. During intense seizure activity in the brain, synaptically released glutamate may spill out of the synaptic cleft. Given the small volume of extracellular space in brain tissue, increased extracellular glutamate concentrations could lead to stimulation of extrasynaptic NMDA receptors and eventually cell injury or death[42]. The reverse operation of glutamate transporters during hypoxic/ischemic episodes[24] may increase extracellular glutamate concentrations, stimulating extrasynaptic NMDA receptors. Severe hypoxia/ischemia may lead to a rapid and complete loss of mitochondrial membrane potential causing cellular ATP depletion and acute necrotic cell death. This could explain necrotic cell death observed in the center of brain areas damaged by hypoxia/ischemia[43,44]. Neurons experiencing shorter, less severe hypoxic/ischemic episodes or neurons in the periphery of the hypoxic/ischemic core (the so-called penumbra[45]) may suffer only a transient and incomplete depolarization of the mitochondria. Although these cells may not undergo acute necrotic cell death, their health could be compromised because stimulation of extrasynaptic NMDA receptors (even with non-toxic glutamate concentrations) triggers the CREB shut-off pathway, blocking the pro-survival activity of CREB[46,47].

Consistent with this hypothesis are the observations of CREB shut-off in *in vivo* stroke conditions (ref. 47 and references therein) and of apoptotic cell death in the penumbra[43,44]. Stroke-surviving neurons (such as those in the peri-infarct area) have sustained concentrations of phospho-CREB and elevated concentrations of BDNF[47,48]. This could be due to increased firing (induced by extracellular glutamate) in areas around the penumbra; this firing may induce calcium influx through synap-

tic NMDA receptors, stimulating CREB function, BDNF expression and other pro-survival events. Within the infarct area, the CREB- and survival-promoting activity of synaptic NMDA receptors may be occluded by the dominant action of extrasynaptic NMDA receptors. Our results support a new pharmacological/genetic approach to minimizing cell death after hypoxic/ischemic insults, head injury or certain chronic neurodegenerative diseases in which glutamate excitotoxicity has been implicated: selectively blocking extrasynaptic NMDA receptors while permitting or enhancing the activity of synaptic NMDA receptors.

METHODS

Hippocampal cultures and stimulations. Hippocampal neurons were cultured as described[49] and stimulated after a culturing period of 10–12 days. Hypoxic/ischemic conditions were induced by exposing hippocampal cultures to glucose-free SGG (ref. 4) in a humidified atmosphere containing 95% nitrogen and 5% CO_2. APV, forskolin, CNQX, 3-isobutyl-1-methylxanthine (IBMX) and FPL 64176 were from Sigma (St. Louis, Missouri); MK-801, ifenprodil, MCPG, and CGP 54626 were from Tocris Cookson (Bristol, UK); TTX and NMDA were from CN Bioscience (Darmstadt, Germany).

Imaging and multi-electrode array recordings. Fluo-3 calcium imaging and confocal laser scanning microscopy was done as described[8]. Calcium concentrations were expressed as a function of the Fluo-3 fluorescence $[(F - F_{min})/(F_{max} - F)]$. Mitochondrial membrane potential was analyzed as described[50] using Rh123 (Molecular Probes, Eugene, Oregon). Hippocampal neurons were loaded with Rh123 (10 μg/ml) in SGG (ref. 4) for 15 min followed by extensive washing with SGG. Maximum Rh123 signal was obtained by completely eliminating the mitochondrial potential by exposing the neurons to the mitochondrial uncoupler FCCP (2.5 μM; Sigma, St. Louis, Missouri). Multi-electrode array recordings were done as described[9].

Signaling and gene expression. Immunocytochemistry and immunoblotting were done as described[8]. Phospho-CREB immunofluorescence was quantified on a MRC 600 confocal laser scanning microscope (excitation 488 nm, emission >515 nm). Within each individual experiment, the black level was set to eliminate background fluorescence, and the gain was set so that the brightest cells gave a high signal, without saturating (pixel intensity <255 in all cases). At least three fields (each with about 20–30 cells) were measured; the mean values were expressed as fold induction over control. The antibody to calmodulin and to phospho-CREB were from Upstate Biotechnology (Lake Placid, New York); the phospho-TrkA antibody that also recognizes TrkB phosphorylated on tyrosine 515 was from New England Biolabs (Beverly, Massachusetts). The antibody to TrkB was from Santa Cruz Biotechnology (Santa Cruz, California). Neurons were transfected after 10 days of culturing using Lipofectamine 2000 (Promega UK, Southampton, UK). The *c-fos*-based, *myc*-tagged CRE reporter pF222myc[7,8] was transfected alongside a plasmid encoding enhanced green fluorescent protein (EGFP) under the control of a cytomegalovirus (CMV) promoter. Reporter-gene expression was analyzed immunocytochemically using the 9E10 monoclonal antibody and quantified using confocal laser scanning microscopy as described[7,8]. Gene expression responses were normalized for transfection efficiency to the EGFP signal and expressed as a myc-tag/GFP ratio. RT-PCR was done using a kit from Stratagene (La Jolla, California). The sequences of the primers were 5′-TCATCCAGTTCCACCAGG-3′, 5′-CCCTCATAGACATGTTTGC-3′ for BDNF, and 5′-TGTCGTGGAGTCTACTGG-3′, 5′-CAGCATCAAAGGTGGAGG-3′ for GAPDH.

Assessment of cell death. Glutamate-induced acute cell death was assessed 4 h after stimulation by staining hippocampal neurons with Hoechst and determining the percentage of hippocampal nuclei with small, irregular chromatin clumps characteristic of acute excitotoxically damaged neurons[29]. Cell death was also quantified by measurements of LDH release using a kit (Promega UK, Southampton, UK) 8 h after stimulation; the difference between released LDH in stimulated and control cultures was expressed as a percentage of the total releasable LDH obtained with 1 mM glutamate, which rapidly kills all neurons. Staurosporine-induced apoptosis was assessed, 36 h after staurosporine (200 nM) exposure, by determining the percentage of hippocampal neurons with shrunken cell body and large round chromatin clumps[29].

Acknowledgments

We thank F. Arnold for help with the multi-electrode array recordings, and P. Vanhoutte and B. Wisden for discussion. This work was supported by the Medical Research Council, Clare College, Cambridge, and the Alexander von Humboldt Foundation.

Competing interests statement

The authors declare that they have no competing financial interests.

RECEIVED 26 NOVEMBER 2001; ACCEPTED 13 MARCH 2002

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