Calpain-mediated Degradation of Myocyte Enhancer Factor 2D Contributes to Excitotoxicity by Activation of Extrasynaptic N-Methyl-D-aspartate Receptors*

Gengze Wei[‡], Yue Yin[§], Wenming Li[‡], Haruhiko Bito[¶], Hua She[‡], and Zixu Mao[‡1]

From the [‡]Departments of Pharmacology and Neurology, Emory University School of Medicine, Atlanta, Georgia 30322, the [§]Fourth Military Medical University, Xi'an, Shaanxi 710032, China, and the [¶]Department of Neurochemistry, Graduate School of Medicine, University of Tokyo, Tokyo 113-0033, Japan

Background: Myocyte enhancer factor 2D (MEF2D) plays important roles in neuronal survival.
Results: Activation of extrasynaptic NMDAR causes calpain-mediated cleavage of MEF2D.
Conclusion: Extrasynaptic NMDA receptors-induced excitotoxicity is in part mediated by degradation of MEF2D.
Significance: Learning how MEF2D is dysregulated by excessive NMDA-activated calpain may provide a therapeutic strategy by inhibiting MEF2D degradation for excitotoxicity-associated diseases.

Synaptic and extrasynaptic NMDA receptors (NMDARs) appear to play opposite roles in neuronal survival and death. Here we report the new findings on the dysregulation of survival factor, myocyte enhancer factor 2D (MEF2D), by extrasynaptic NMDARs. Excitotoxicity led to the NMDAR-dependent degradation of MEF2D protein and inhibition of its transactivation activity in mature cortical neurons. The activation of extrasynaptic NMDARs alone was sufficient for degradation of MEF2D. Calpain directly cleaved MEF2D *in vitro* and blocking this protease activity greatly attenuated NMDAR signaled degradation of MEF2D in neurons. Consistently, inhibition of calpain protected cortical neurons from NMDA-induced excitotoxicity. Furthermore, knockdown of MEF2D sensitized neurons to NMDA-induced excitotoxicity, which was not protected by calpain inhibition. Collectively, these findings suggest that dysregulation of MEF2D by calpain may mediate excitotoxicity via an extrasynaptic NMDAR-dependent manner.

NMDA receptors (NMDARs)[2] are cationotropic channels gated by glutamate, the main excitatory neurotransmitter in the central nervous system, and play important roles in synaptic transmission and plasticity, modulate learning and memory, and initiate neuronal death response (1). NMDAR activities are dysregulated under conditions that comprise neuronal viability. This is thought to occur when the intensity and duration of an excitatory signal exceed a certain level, causing sustained high levels of Ca^{2+} influx and toxicity. There is strong evidence implicating the involvement of NMDAR signaling in causing neuronal death during brain ischemia, hypoxia, traumatic injury (2), as well as in other neurodegenerative diseases such as Alzheimer and Parkinson disease (3, 4). Overstimulation of NMDARs has been shown to cause mitochondrial dysfunction and bioenergetic failure, generate reactive oxygen species, dysregulate protease calpain, activate p38MAPK and JNK, and disrupt the protein complex involving postsynaptic density protein 95.

Interestingly, maintaining proper NMDAR activities are needed to protect neurons during development or after trauma (3–8). A number of pathways such as Akt and cAMP-response element-binding protein (CREB) are known to confer the neuroprotective effects of NMDARs (1). The molecular basis of this dichotomy of NMDARs signaling to both neuroprotective and neurodestructive events is not fully understood. There is indication that the location of NMDARs can affect the nature of NMDA signals. It has been proposed that synaptic NMDAR activation promotes survival, whereas activation of extrasynaptic NMDARs signals opposes synaptic NMDARs activity and causes stress and death (9).

Myocyte enhancer factor 2 (MEF2) A–D are transcription factors known to play important roles in neuronal development, synaptic plasticity, and survival (10, 11). Indeed, MEF2 proteins promote the survival of several types of neurons under different conditions (12–15). Nuclear MEF2D is regulated by chaperone-mediated autophagy (16). Interestingly, MEF2D has been shown to be present and function in mitochondria (17). Dysregulation of the chaperone-mediated autophagy-MEF2D pathway or mitochondrial MEF2D occurs in Parkinson disease. Importantly, enhancing MEF2 activity broadly or in mitochondria attenuates the loss of dopaminergic neurons in substantia nigra pars compacta (SNpc) in a 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine mouse model of Parkinson disease (17, 18). Many stress signals inhibit MEF2 proteins. It is reported that caspases are involved in the degradation of MEF2 proteins under the stress and neuronal death conditions (19–21). In the current study, we identify that extrasynaptic NMDARs as the primary channel mediate excitotoxicity and cause calpain-de-
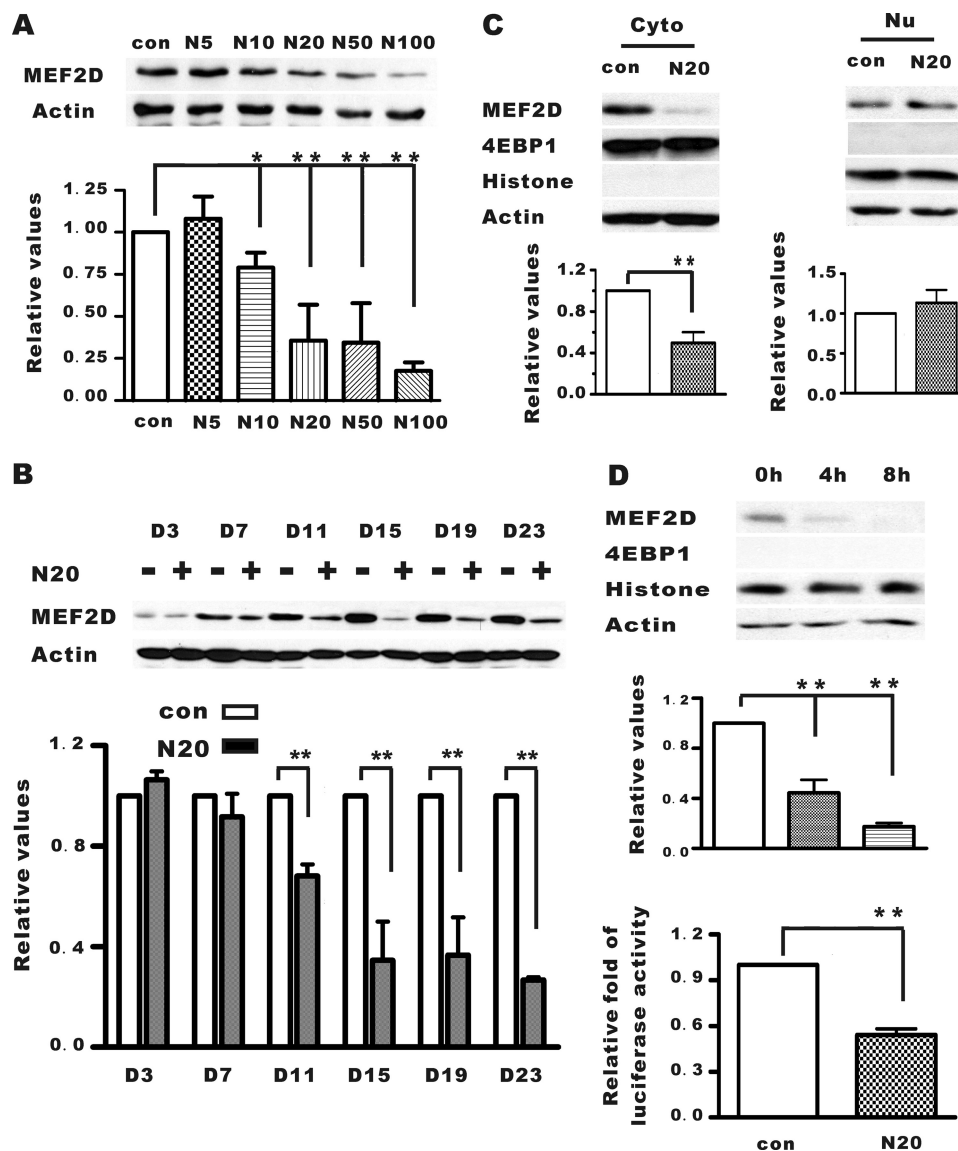
FIGURE 1. **NMDA regulation of MEF2D in cortical neurons.** *A*, NMDA concentration-dependent decrease of MEF2D expression following 1 h of treatment in DIV 15 cortical neurons (*N5–N100* indicate 5–100 μM NMDA). *B*, NMDA inhibited MEF2D expression in mature neurons. Cortical neurons cultured at different DIVs were treated with 20 μM NMDA for 1 h; total proteins were collected for Western blotting. *, $p < 0.05$ and **, $p < 0.01$ compared with control (*con*) groups ($n = 4$). *C*, MEF2D expression decreased in the cytoplasm after 1 h of treatment of 20 μM NMDA. *D*, NMDA reduced nuclear MEF2D level after 4 h and transcription activity (luciferase) at 8 h following NMDA treatment (1-h treatment + 3-h or 7-h culture); 4EBP1, cytoplasmic (*Cyto*) marker and histone, nuclear (*Nu*) marker.

pendent cleavage of MEF2D, which may contribute to excitotoxicity. Thus, our studies provide a mechanism that controls a key neuronal survival factor by coordinated signaling via NMDARs in mediating excitotoxicity in neurons.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—MEF2D C-terminal antibody was purchased from BD Transduction Laboratories; MEF2 N-terminal antibody from Santa Cruz Biotechnology; histone, 4EBP1, Fodrin, and GST antibodies from Cell Signaling; and actin antibody from Sigma-Aldrich. NMDA, DL-2-amino-5-phosphonopentanoic acid, bicuculline, 4-aminopyridine, MK801, Ro 25-6981, ifenprodil, and ZnCl$_2$ were purchased from Sigma-Aldrich; tetrodotoxin, memantine, MDL28170, and calpeptin from Tocris; and Suc-LLVY-AMC from Calbi-

ochem. AK295 was gifted by Dr. Jonathan Glass (Emory University). NVP-AAM077 was kindly provided by Dr. Yves Auberson (Novartis).

Primary Neuron Cultures—Culture of primary cortical neurons from Long Evans rats at embryonic day 18 was carried out as described previously (22). Briefly, Cortical neurons were digested with trypsin and plated on poly-L-lysine-coated plates with neurobasal medium (Invitrogen) containing 2% B27 (Invitrogen) and 0.5 mM glutamine (Cellgro). Cortical neurons were treated at 15 days *in vitro* (DIV), except that culture time was mentioned specially.

NMDA Treatment—Neurons were preincubated in medium with 1 μM tetrodotoxin for 3 h before stimulation. For synaptic stimulation, 50 μM bicuculline and 2.5 mM 4-aminopyridine were added in medium, and neurons were incubated for 1 h. For

extrasynaptic stimulation, 1 μM MK801 was used to irreversibly inhibit synaptic NMDA receptors for 5 min under the condition of synaptic stimulation. After being washed three times with PBS, neurons were incubated in medium with or without containing 20 μM NMDA for 1 h. For whole cell NMDA stimulation, neurons were incubated in medium containing various concentrations of NMDA for 1 h.

Luciferase Reporter Assay—MEF2 reporter assay was carried out as described previously (21).

Calpain Activity Assay—Cleavage of Suc-LLVY-AMC by calpain to fluorescent product AMC was used to measure calpain activity. Neuron lysates following various treatments were incubated in assay buffer (50 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$, and 2 mM DTT) containing Suc-LLVY-AMC for 30 min at room temperature. Fluorescence was recorded by a BioTek Synergy HT Microplate Reader at 360 nm excitation and 440 nm emission.

MEF2D RNAi Lentivirus Infection—The siRNA against rat MEF2D targeted the sequence 5′-GTAGCTCTCTGGT-CACTCC-3′ as described by Flavell *et al.* (23). To produce a nonsilencing scrambled siRNA, the sequence of the MEF2D siRNA was altered such that it targeted the sequence, 5′-GTA**T**CTCTCTGGTC**G**CTCC-3′ (mismatches in boldface type) (17, 23, 24). For the MEF2D RNAi lentivirus construct, the RNAi cassettes were cloned into lentiviral vector pFUGW (24). Lentiviruses were produced in HEK293T cells at the Emory Viral Core facility by co-transfection of pFUGW-MEF2D RNAi, packaging plasmid p8.91, and vesicular stomatitis virus envelope expression plasmid (pMD.G). Lentiviruses were concentrated and titrated on HEK293T cells. Lentiviruses were applied to cortical neurons at DIV 12 and experiments were performed 72 h after virus infection.

Assessment of Neuronal Viability—Neurons were treated as indicated for 8 h. The morphology of neurons was observed and recorded under microscope. Culture media were collected and analyzed for lactate dehydrogenase (LDH) activity using the LDH cytotoxicity kit (Cayman Chemical) following the manufacturer's protocol. We calculated the percentage of dead cells by the following equation. Cell death (%) = (treatment group LDH release − control group LDH release) × 100/maximum LDH release. For maximum LDH release, the cells were lysed along with the culture medium by freezing/thawing and then assayed for the LDH activity.

Calpain Cleavage of MEF2D and Protein Sequencing—Purified recombinant GST-MEF2D(1–86) and GST-MEF2D(87–507) were co-incubated with calpain 2 (Calbiochem) in reaction buffer (50 mM Tris-HCl, pH 7.4, 5 mM $CaCl_2$, and 1 mM DTT) for 15 min at room temperature. Samples were then subjected to SDS-PAGE for Coomassie Brilliant Blue Staining and Western blotting. The cleavage band was excised for protein sequencing via mass spectrometry.

Oxygen and Glucose Deprivation (OGD)—At DIV 15, cortical neurons were washed twice with and placed in glucose-free DMEM (OGD medium, Invitrogen) in a hypoxia chamber containing 1% O_2, 5% CO_2, and N_2 balance at 37 °C. Neurons were harvested after 1 h of OGD. For LDH assay, culture media were collected from neurons 4 h after treatment.
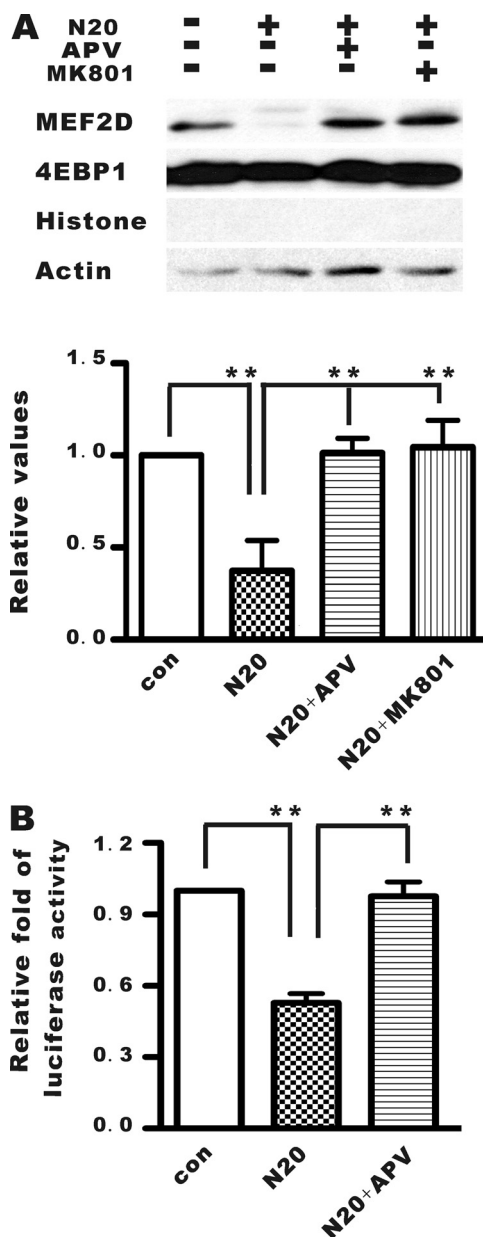

FIGURE 2. **Involvement of NMDA receptors in NMDA-mediated degradation of MEF2D.** *A*, NMDA receptor inhibitors (DL-2-amino-5-phosphonopentanoic acid (*APV*) and MK801) blocked 20 μM NMDA-induced degradation of MEF2D in cortical neurons (DIV 15). *B*, NMDAR inhibitors blocked NMDA induced-decrease of MEF2D transcription activity. **, $p < 0.01$ compared with indicated groups ($n = 5$). *N20*, 20 μM NMDA; *4EBP1*, cytoplasmic marker; *con*, control; and *histone*, nuclear marker.

Statistical Analysis—Analysis of variance followed by SNK test was employed for tests of significance between three or more groups. The comparison between two groups was performed by Student's *t* test. Results were expressed as means ± S.E., and statistical significance was accepted at $p < 0.05$.

RESULTS

Toxic NMDA Decreases MEF2D Level and Activity—We tested first whether excitotoxicity regulated survival factor MEF2D. Primary cortical neurons at DIV 15 were treated with different concentrations of NMDA for 1 h, a time point before overt neuronal loss was evident, and measured for MEF2D lev-

els. NMDA significantly reduced the levels of MEF2D protein in a concentration-dependent manner (Fig. 1A). Because MEF2D levels change as neurons mature in culture, we treated primary cortical neurons cultured for time periods from 3 to 23 days. Our quantification analysis showed that 20 μM NMDA treatment reduced MEF2D in neurons cultured for 11 days or older (Fig. 1B). Based on these, we chose 20 μM of NMDA and neurons cultured at DIV 15 for most of the subsequent experiments. Previous studies indicate that MEF2D is present in both nuclear and cytoplasmic compartments (16). We determined which pool of MEF2D was affected by NMDA. Subcellular fractionation showed that NMDA caused a rapid decrease in MEF2D in the cytoplasm but not in the nucleus of cortical neurons within 1 h (Fig. 1C). However, nuclear MEF2D was reduced if cortical neurons were returned to normal culture medium and incubated for an additional 3 h following the initial NMDA treatment (Fig. 1D, top and middle panels). Consistent with this, MEF2 transcription activity was inhibited at 8 h after NMDA treatment (Fig. 1D, bottom). These results suggest that toxic NMDA induces a sequential decrease of MEF2D levels early in the cytoplasm and then in the nucleus in mature neurons.

NMDARs Are Involved in MEF2D Degradation—To explore the mechanisms by which NMDA reduced MEF2D, we tested whether NMDARs were involved in mediating the suppressive effect on MEF2D level and activity. We employed two widely used NMDAR antagonists, DL-2-amino-5-phosphonopentanoic acid (a competitive antagonist) and MK801 (a non-competitive antagonist), to block NMDA neurotoxicity. These two antagonists completely protected MEF2D from the NMDA-mediated excitotoxicity (Fig. 2A). Consistent with this, NMDAR antagonist DL-2-amino-5-phosphonopentanoic acid restored MEF2D transcription function in the presence of NMDA (Fig. 2B). These results indicate that NMDARs play a dominant role in mediating the toxic signal and degradation of MEF2D.

Activation of Extrasynaptic NMDARs alone Is Sufficient for MEF2D Degradation—Our above data showed that bath application of NMDA reduced MEF2D. Recent reports suggest that the localization of NMDARs determines its signaling outcome with activation of synaptic NMDARs being neuroprotective, whereas with activation of extrasynaptic NMDARs invoking pro-death response (9, 25, 26). We examined the role of synaptic *versus* extrasynaptic NMDARs in regulating MEF2D levels. First, we determined appropriate concentrations of MK801 for sequential experiments. Among concentrations from 0.1 to 10 μM, 1 and 10 μM MK801 completely prevented NMDA-induced neurotoxicity, but 1 μM MK801 could be completely washed out for the subsequent extrasynaptic stimulation. Neurons were treated with 1 μM MK801 for 5 min, washed three times, and then exposed to 20 μM NMDA for 1 h. Seven hours later, the cell viability was measured by LDH assay. Compared with that of NMDA alone group, the viability of MK801-washed out group showed no difference. This suggested that extrasynaptic NMDARs were solely responsible for all the NMDA-induced excitotoxicity. Then, we showed that activation of synaptic but not extrasynaptic NMDARs led to increased phosphorylation of ERK under our experimental conditions
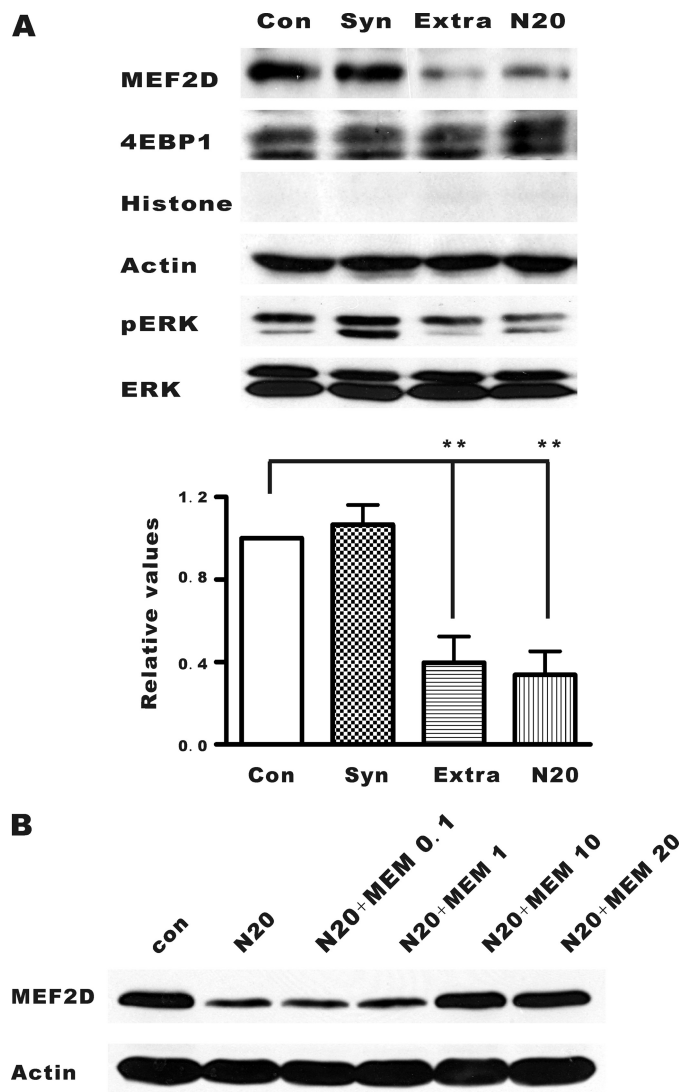

FIGURE 3. **NMDA induced-degradation of MEF2D through extrasynaptic NMDAR activities.** *A*, 50 μM bicuculline and 2.5 mM 4-aminopyridine were used to activate synaptic NMDA receptor. 1 μM MK801 was then added to block all synaptic NMDA receptors. After washing, NMDA was applied to activate only extrasynaptic NMDA receptors. Stimulation of extrasynaptic NMDA receptors, but not synaptic ones, were sufficient for degradation of MEF2D. **, $p < 0.01$ compared with control groups ($n = 4$). *B*, memantine significantly inhibited the degradation of MEF2D by NMDA. Cortical neurons were preincubated with memantine at concentrations from 0.1 to 20 μM and then exposed to 20 μM NMDA for 1 h. Cytoplasmic proteins were collected from the above samples for Western blotting detected with MEF2D antibody. *N20*, 20 μM NMDA; *Syn*, synaptic NMDAR stimulation; *Extra*, extrasynaptic NMDAR stimulation; *MEM*, memantine; *4EBP1*, cytoplasmic marker; and *Histone*, nuclear marker.

(Fig. 3A), recapitulating the reported role of synaptic activity in neuroprotective signaling (27). Stimulation of extrasynaptic NMDARs led to a significant decrease in MEF2D as bath application of NMDA did, but nor stimulation of synaptic NMDARs (Fig. 3A). Treating neurons with memantine, which preferentially blocks extrasynaptic NMDARs (25, 28), totally blocked NMDA-induced degradation of MEF2D (Fig. 3B), further confirming that only extrasynaptic NMDARs are involved in degradation of MEF2D.

Stimulation of Extrasynaptic NMDARs Leads to Activation of Calpain—Many studies have shown that NMDA-mediated excitotoxicity is related to protease calpain activation (26,
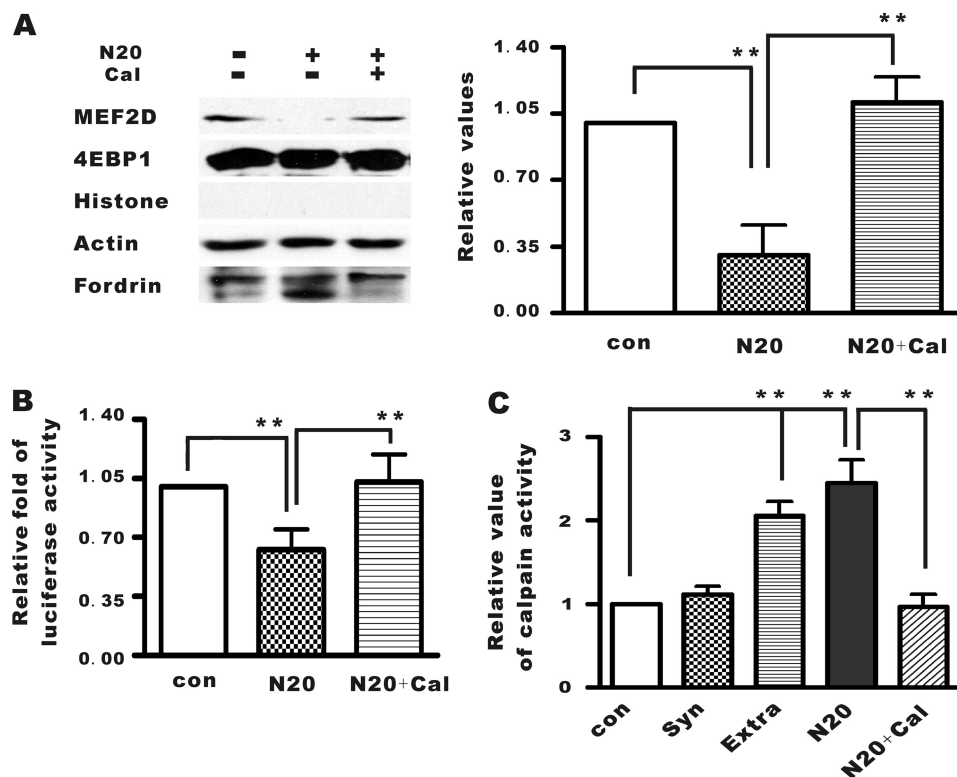
FIGURE 4. **Activation of calpain mediates NMDA toxicity to MEF2D.** *A*, inhibition of calpain activity restored MEF2D level following NMDA treatment. *B*, inhibition of calpain activity restored MEF2D transcription activity reduced by NMDA. *C*, the increase of calpain activity induced by NMDA was inhibited by calpain inhibitor. **, $p < 0.01$ compared with the indicated groups ($n = 4$). *N20*, 20 μM NMDA; *Cal*, calpeptin; *4EBP1*, cytoplasmic marker; and *Histone*, nuclear marker; *con*, control.

29–32). We tested the possibility that calpain may mediate the toxic effect of NMDA on MEF2D. Under our experimental conditions, NMDA induced the activation of calpain, resulting in the cleavage of a well known calpain substrate fodrin, which could be completely blocked by inhibition of calpain (Fig. 4*A*). Consistently, inhibition of calpain activity also restored the levels of MEF2D protein and MEF2 transcription potential (Fig. 4*A*-B). Using Suc-LLVY-AMC as a sensitive calpain substrate, we measured directly caplain activity (Fig. 4*C*). We found that activation of synaptic NMDARs did not alter calpain activity. But activation of extrasynaptic NMDARs alone led to significant increase in calpain activity, as it was bath application of NMDA that resulted in the elevation of calpain activity (Fig. 4*C*).

Direct Cleavage of MEF2D by Calpain—Previous studies have shown that MEF2D stability is regulated in response to neuronal stress (21). Our above findings raised the possibility that calpain might directly cleave MEF2D in response to NMDA. In cortical neurons at DIV 15, 20 μM NMDA induced degradation of MEF2D in a time-dependent manner as early as 15 min (data not shown). NMDA treatment for 30 min led to a decrease in the full length MEF2D in cortical neurons as revealed by anti-MEF2D C-terminal antibody and generation of two fragments of MEF2D with the molecular weight of ~52 and 30 kDa, respectively (Fig. 5*A*). Inhibition of calpain by several distinct inhibitors prevented NMDA-induced degradation of MEF2D and the appearance of the cleaved molecular bands. To show whether calpain directly degraded MEF2D, we performed direct cleavage assay *in vitro*. Purified GST-MEF2D

N-terminal (1–86 amino acids) or C-terminal (87–507 amino acids) fragments were incubated with purified calpain, and the cleavage was measured using either anti-GST or anti-MEF2D antibodies. These studies showed that calpain cleaved both GST-MEF2D(1–86) and GST-MEF2D(87–507) but not GST, generating several recognizable fragments (Fig. 5, *B* and *C*). We isolated one degraded fragment generated from N-terminal MEF2D from Coomassie Brilliant Blue stained gel and subjected it to mass spectrometry analysis (Fig. 5*C*, *left*). This analysis revealed that one of calpain cleavage sites occurred between MEF2D Ser-78 and Arg-79. When Glu-77 was mutated to Ala-77, the mutated GST-MEF2D(1–86) became resistant to cleavage by calpain (Fig. 5*C*, *right*). Based on data from Fig. 5, *B* and *C*, it appears that the bigger molecular weight fragment in Fig. 5*A* corresponds to the C-terminal portion of MEF2D lacking the first N-terminal 78 amino acid residues. The smaller molecular weight fragment in Fig. 5*A* may correspond to one of the C-terminal fragments generated by calpain *in vitro* (Fig. 5*B*, *middle panel*). The N terminus of MEF2 is involved in DNA binding, which is required for its transactivation activity (33, 34). Consistent with the cleavage of MEF2D at the N terminus by calpain, NMDA treatment inhibited the activity of MEF2D-VP16, whose DNA binding potential relies on the MEF2D DNA binding domain fused to the transactivation domain of viral protein VP16 (Fig. 5*D*).

NMDA and OGD Induce Calpain- and MEF2D-dependent Neuronal Death—We examined whether NMDAR-calpain pathway played a role in neuronal toxicity. Cortical neurons were exposed to NMDA and neuronal viability was determined
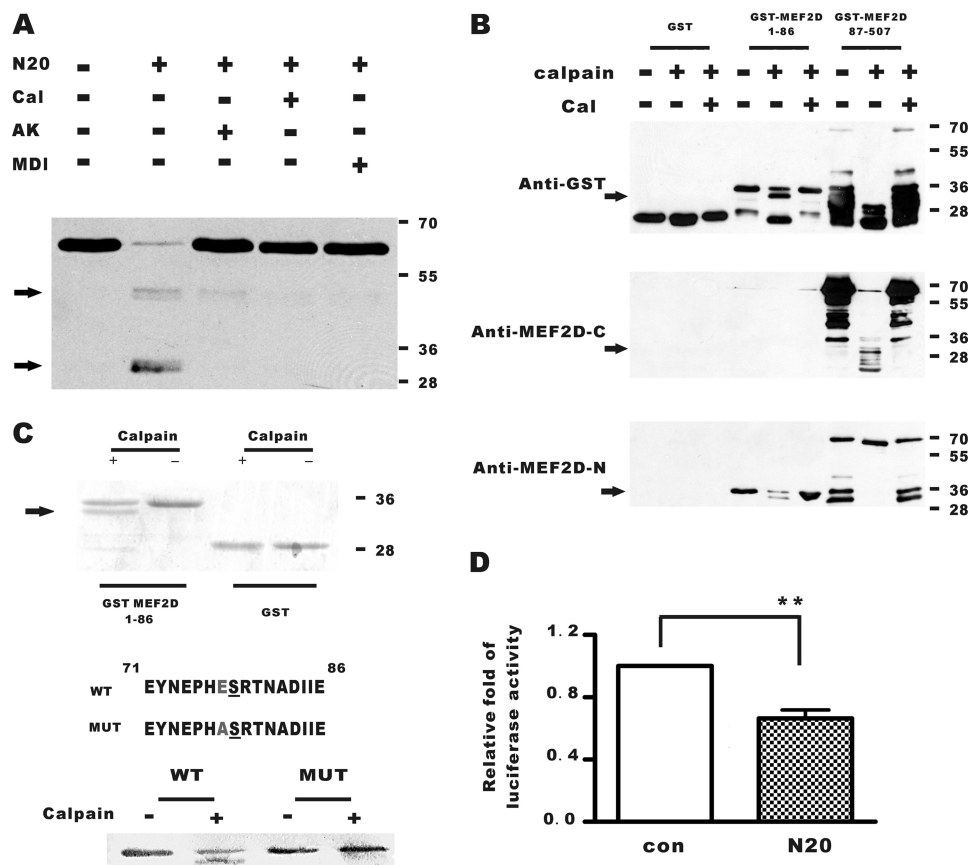
FIGURE 5. **Calpain directly cleaves MEF2D.** *A*, NMDA induced calpain-dependent cleavage of MEF2D in cortical neurons (*arrow* indicated cleaved segments). NMDA at 20 μM was added to neurons for 30 min, and lysates were prepared for Western blotting with MEF2D C-terminal antibody. *B*, calpain directly cleaved recombinant MEF2D *in vitro*. GST, GST-MEF2D N terminus (1–86 residues), and GST-MEF2D C terminus (87–507 residues) recombinant proteins were incubated with purified calpain and probed with indicated antibodies. *Arrows* indicate cleaved segments of MEF2D by calpain. *C*, mass spectrometry analysis identified a cleavage site at the N-terminal MEF2D. Coomassie Brilliant Blue staining of MEF2D N-terminal (1–86 residues) showed the cleaved fragment (indicated by *arrow*) by calpain. Mass spectrometry analysis of the band indicated by *arrow* identified a cleavage site between Ser-78 and Arg-79. GST-MEF2D(1–86) E77A (*MUT*) was resistant to calpain cleavage (*right*). *D*, NMDA inhibited MEF2D-VP16 transcription activity. **, $p < 0.01$ compared with control groups ($n = 5$). *N20*, 20 μM NMDA; *MDL*, MDL28170; *con*, control; *AK*, AK295; and *Cal*, calpeptin.

by LDH assay. Activation of extrasynaptic NMDARs, rather than synaptic NMDARs, resulted in a significant increase in neuronal death (Fig. 6, *A* and *B*). Blocking calpain rescued neurons from NMDA excitotoxicity. To test that NMDA-induced neuronal death was related directly to MEF2D stability and activity, we knocked down MEF2D with MEF2D siRNA lentiviruses in cortical neurons (Fig. 6*C*, *upper panel*). MEF2D knockdown significantly sensitized neurons to NMDA-induced toxicity. Consistent with this, knockdown of MEF2D attenuated calpain inhibitor calpeptin-mediated protection against excitotoxicity (Fig. 6*C*, *lower panel*). Similarly to NMDA treatment, OGD, a condition mimicking stroke, also led to calpain-dependent cleavage of MEF2D (Fig. 7*A*). Inhibition of either NMDARs or calpain not only restored MEF2D levels but also protected cortical neurons from death (Fig. 7, *A* and *B*).

DISCUSSION

Ample evidence supports the role of NMDARs in mediating damaging signals during excitotoxicity (32, 35, 36). The precise molecular targets of the NMDAR damaging signals are not fully defined. In addition, it is not clear whether different NMDAR-based mechanisms may be involved in regulating distinct downstream targets. Our findings demonstrate that survival

factor MEF2D is a molecular target of NMDAR signaling in excitotoxicity. Activation of NMDARs stimulates protease calpain, leading to direct cleavage of MEF2D and down-regulating its transactivation activity.

MEF2 proteins are known to support neuronal survival. Many studies have reported that a decrease in MEF2 comprises the viability of cultured primary neurons in several experimental paradigms. Enhancing MEF2 activity has been shown to protect dopaminergic neurons from 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine-induced toxicity in animal models of Parkinson disease (18). Several important signaling pathways are known to regulate MEF2 proteins in neurons. For example, survival-promoting signals such as membrane depolarization potentiate MEF2 activity, whereas death-inducing signals such as neurotoxins or oxidative stress inhibit MEF2 function (13, 21, 37). Importantly, recent studies have revealed MEF2 protein stability as a key point of regulation. Recently, chaperone-mediated autophagy has also been shown to directly regulate MEF2D turnover, which is interrupted under pathological conditions (16). Moreover, phosphorylation of MEF2 proteins by Cdk5 leads to their enhanced cleavage by caspases and inhibition of their protective effects in cerebellar granule neurons in response to excessive glutamate (21). How-
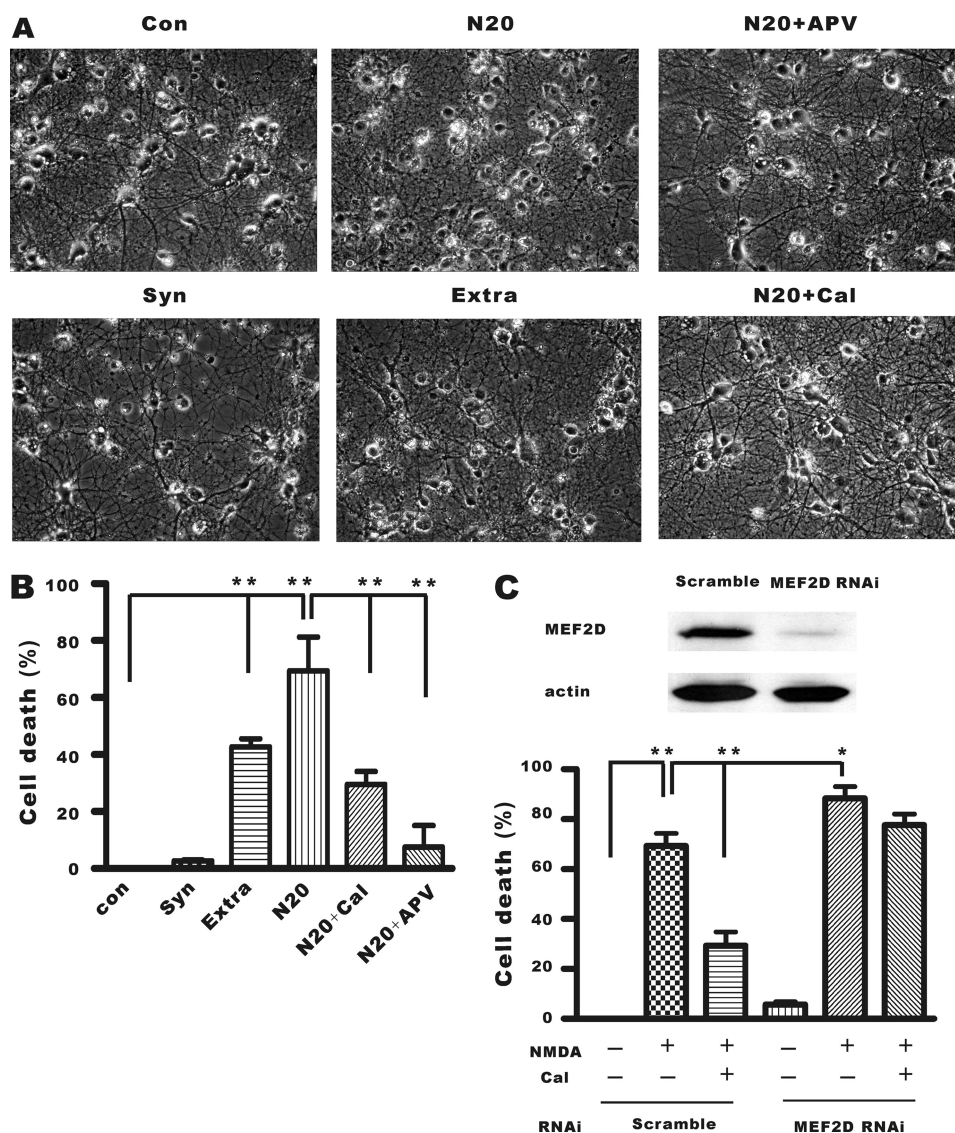
FIGURE 6. **Analysis of NMDA-dependent excitotoxic death of cortical neurons.** *A*, NMDA induced morphological change in neurons. Microscopic photos were taken 8 h following different treatments in cortical neurons. Nuclei shrinking and body swelling occurred in cortical neurons exposed to extrasynaptic or total NMDA activation. The excitotoxicity was protected by both NMDA receptor antagonist and calpain inhibitor. *B*, release of LDH was measured at 8 h after NMDA treatments. *C*, knocking down MEF2D increased NMDA-induced neuronal death. Cortical neurons were infected with lentivirus-MEF2D RNAi or control lentivirus for 72 h and exposed to 20 μM NMDA with or without 10 μM calpeptin for 8 h for LDH assay. The *top panel* shows the efficiency of MEF2D knockdown. *, $p < 0.05$ and **, $p < 0.01$ compared with indicated groups ($n = 4$). *N20*, 20 μM NMDA; *Syn*, synaptic NMDA stimulation; *Extra*, extrasynaptic NMDA stimulation; *Cal*, calpeptin; *con*, control; *APV*, DL-2-amino-5-phosphonopentanoic acid.

ever, the underlying mechanisms by which excitotoxicity regulates MEF2 proteins remain unclear. Our current data define a molecular pathway involving activation of extrasynaptic NMDARs, activation of calpain, and direct cleavage of MEF2D by calpain. Moreover, knockdown of MEF2D sensitizes neurons to NMDA-mediated excitotoxicity. These findings provide a direct link between NMDAR overactivation and the suppression of neuronal survival machinery, suggesting that inhibition of survival factor MEF2D is part of the toxic repertoire of NMDAR excitotoxicity.

One of the major advances in recent years is the realization that NMDAR localization affects the nature of signals originating from the receptor. It has been shown that extrasynaptic NMDAR activation is coupled with an inhibitory pathway leading to dephosphorylation at the active site on CREB and repression of the activity of survival signal from ERK. On the contrary,

activation of synaptic NMDARs clearly promotes CREB and ERK activities (9, 27). In accordance with the classical examples of opposing effects of synaptic and extrasynaptic NMDARs, inhibition of survival factor MEF2D requires activities originated from extrasynaptic NMDARs.

Our findings indicate that toxic NMDA dysregulates calpain through extrasynaptic NMDARs. The overactivation of calpain appears to be required for degradation of MEF2D under the excitotoxic condition. Previous reports have shown that the caspases may mediate the degradation of MEF2 in rat cerebellar granule neurons treated with low potassium and in cerebrocortical neurons exposed to NMDA or glutamate (20, 21). Increasing evidence shows that toxic NMDA and glutamate can activate both calpain and caspases (38), and there is cross-talk between calpain and caspases during excitotoxicity. Calpain is directly activated by over-flux of calcium through NMDA
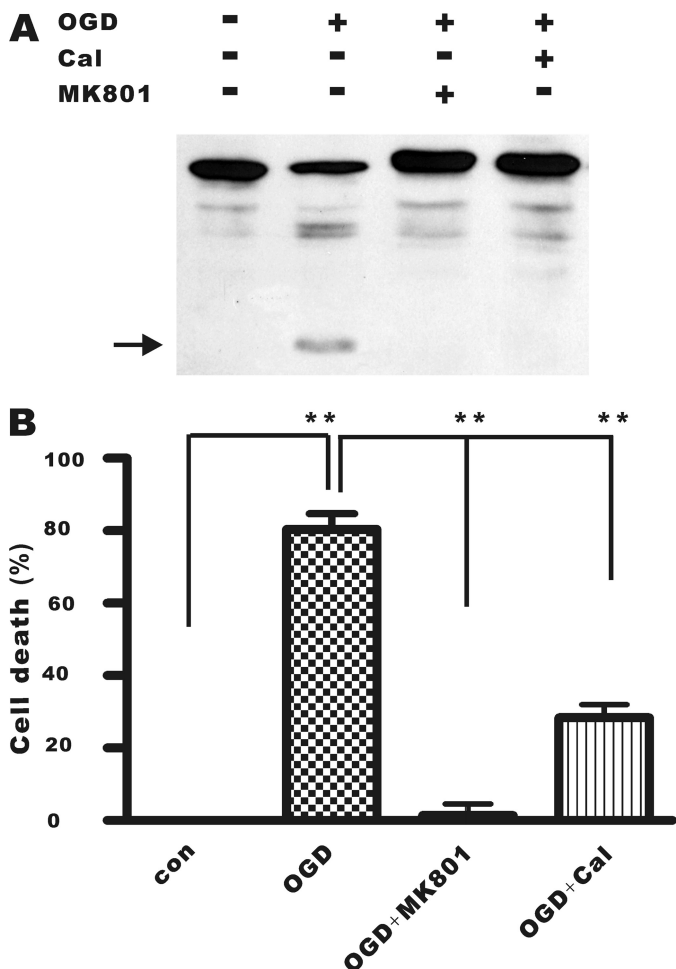
FIGURE 7. OGD induces NMDAR- and calpain-dependent degradation of MEF2D and LDH release. *A*, MEF2D degradation was inhibited by NMDAR and calpain inhibitor with treatment of 1 h of OGD. *B*, LDH levels were measured at 4 h following various treatments. **, $p < 0.01$ compared with indicated groups ($n = 4$).

receptors. In our model system, calpain was activated as early as 30 min after NMDA or glutamate (data not shown), whereas caspases were usually activated at 2 h (20, 21). Consistent with our findings, calpain has been reported to be activated in <30 min after glutamate challenge (26, 30). These findings suggest that calpain is activated earlier than caspases during excitotoxicity. Therefore, inhibition of calpain may represent a more effective strategy in maintaining the levels of MEF2 in neurons and protecting them from excitotoxicity.

Our findings that degradation of MEF2D requires the activation of extrasynaptic components of NMDAR signaling support the significant role of extrasynaptic NMDAR signal NMDARs in inhibiting survival factors/pathways such as CREB and ERK. They highlight the importance in targeting extrasynaptic NMDARs, calpain, and MEF2D for neuroprotection. Although normal levels of calpain activity are involved in many neuronal physiological processes, including synaptic plasticity and gene transcription (39, 40), the clear dysregulation of calpain in various pathological conditions may offer an opportunity for selective inhibition of the pathological effects of calpain without significant interference with its physiological functions. Therefore, we conjecture that inhibition of calpain-me-

diated degradation of MEF2D using highly specific techniques may be beneficial for excitotoxicity-associated diseases.

REFERENCES

1. Hardingham, G. E. (2009) Coupling of the NMDA receptor to neuroprotective and neurodestructive events. *Biochem. Soc. Trans* **37,** 1147–1160
2. Arundine, M., and Tymianski, M. (2004) Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury. *Cell Mol. Life Sci.* **61,** 657–668
3. Pohl, D., Bittigau, P., Ishimaru, M. J., Stadthaus, D., Hübner, C., Olney, J. W., Turski, L., and Ikonomidou, C. (1999) *N*-Methyl-D-aspartate antagonists and apoptotic cell death triggered by head trauma in developing rat brain. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 2508–2513
4. Gould, E., Cameron, H. A., and McEwen, B. S. (1994) Blockade of NMDA receptors increases cell death and birth in the developing rat dentate gyrus. *J. Comp. Neurol.* **340,** 551–565
5. Ikonomidou, C., Bosch, F., Miksa, M., Bittigau, P., Vöckler, J., Dikranian, K., Tenkova, T. I., Stefovska, V., Turski, L., and Olney, J. W. (1999) Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science* **283,** 70–74
6. Monti, B., and Contestabile, A. (2000) Blockade of the NMDA receptor increases developmental apoptotic elimination of granule neurons and activates caspases in the rat cerebellum. *Eur. J. Neurosci.* **12,** 3117–3123
7. Adams, S. M., de Rivero Vaccari, J. C., and Corriveau, R. A. (2004) Pronounced cell death in the absence of NMDA receptors in the developing somatosensory thalamus. *J. Neurosci.* **24,** 9441–9450
8. Ikonomidou, C., Stefovska, V., and Turski, L. (2000) Neuronal death enhanced by *N*-methyl-D-aspartate antagonists. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 12885–12890
9. Hardingham, G. E., Fukunaga, Y., and Bading, H. (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat. Neurosci.* **5,** 405–414
10. Yang, Q., and Mao, Z. (2009) Regulation of MEF2 by chaperone-mediated autophagy. *Cell Cycle* **8,** 1304
11. Yang, Q., and Mao, Z. (2010) Dysregulation of autophagy and Parkinson disease: The MEF2D link. *Apoptosis* **15,** 1410–1414
12. Mao, Z., Bonni, A., Xia, F., Nadal-Vicens, M., and Greenberg, M. E. (1999) Neuronal activity-dependent cell survival mediated by transcription factor MEF2. *Science* **286,** 785–790
13. Gong, X., Tang, X., Wiedmann, M., Wang, X., Peng, J., Zheng, D., Blair, L. A., Marshall, J., and Mao, Z. (2003) Cdk5-mediated inhibition of the protective effects of transcription factor MEF2 in neurotoxicity-induced apoptosis. *Neuron* **38,** 33–46
14. Liu, L., Cavanaugh, J. E., Wang, Y., Sakagami, H., Mao, Z., and Xia, Z. (2003) ERK5 activation of MEF2-mediated gene expression plays a critical role in BDNF-promoted survival of developing but not mature cortical neurons. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 8532–8537
15. Wang, X., Tang, X., Li, M., Marshall, J., and Mao, Z. (2005) Regulation of neuroprotective activity of myocyte enhancer factor 2 by cAMP protein kinase A signaling pathway in neuronal survival. *J. Biol. Chem.* **280,** 16705–16713
16. Yang, Q., She, H., Gearing, M., Colla, E., Lee, M., Shacka, J. J., and Mao, Z. (2009) Regulation of neuronal survival factor MEF2D by chaperone-mediated autophagy. *Science* **323,** 124–127
17. She, H., Yang, Q., Shepherd, K., Smith, Y., Miller, G., Testa, C., and Mao, Z. (2011) Direct regulation of complex I by mitochondrial MEF2D is disrupted in a mouse model of Parkinson disease and in human patients. *J. Clin. Invest.* **121,** 930–940
18. Smith, P. D., Mount, M. P., Shree, R., Callaghan, S., Slack, R. S., Anisman, H., Vincent, I., Wang, X., Mao, Z., and Park, D. S. (2006) Calpain-regulated p35/cdk5 plays a central role in dopaminergic neuron death through modulation of the transcription factor myocyte enhancer factor 2. *J. Neurosci.* **26,** 440–447
19. Li, M., Linseman, D. A., Allen, M. P., Meintzer, M. K., Wang, X., Laessig, T., Wierman, M. E., and Heidenreich, K. A. (2001) Myocyte enhancer factor 2A and 2D undergo phosphorylation and caspase-mediated degradation during apoptosis of rat cerebellar granule neurons. *J. Neurosci.* **21,**

6544–6552

20. Okamoto, S., Li, Z., Ju, C., Scholzke, M. N., Mathews, E., Cui, J., Salvesen, G. S., Bossy-Wetzel, E., and Lipton, S. A. (2002) Dominant-interfering forms of MEF2 generated by caspase cleavage contribute to NMDA-induced neuronal apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 3974–3979

21. Tang, X., Wang, X., Gong, X., Tong, M., Park, D., Xia, Z., and Mao, Z. (2005) Cyclin-dependent kinase 5 mediates neurotoxin-induced degradation of the transcription factor myocyte enhancer factor 2. *J. Neurosci.* **25,** 4823–4834

22. Mao, Z., and Wiedmann, M. (1999) Calcineurin enhances MEF2 DNA binding activity in calcium-dependent survival of cerebellar granule neurons. *J. Biol. Chem.* **274,** 31102–31107

23. Flavell, S. W., Cowan, C. W., Kim, T. K., Greer, P. L., Lin, Y., Paradis, S., Griffith, E. C., Hu, L. S., Chen, C., and Greenberg, M. E. (2006) Activity-dependent regulation of MEF2 transcription factors suppresses excitatory synapse number. *Science* **311,** 1008–1012

24. Tian, B., Yang, Q., and Mao, Z. (2009) Phosphorylation of ATM by Cdk5 mediates DNA damage signalling and regulates neuronal death. *Nat. Cell Biol.* **11,** 211–218

25. Léveillé, F., El Gaamouch, F., Gouix, E., Lecocq, M., Lobner, D., Nicole, O., and Buisson, A. (2008) Neuronal viability is controlled by a functional relation between synaptic and extrasynaptic NMDA receptors. *FASEB J.* **22,** 4258–4271

26. Xu, J., Kurup, P., Zhang, Y., Goebel-Goody, S. M., Wu, P. H., Hawasli, A. H., Baum, M. L., Bibb, J. A., and Lombroso, P. J. (2009) Extrasynaptic NMDA receptors couple preferentially to excitotoxicity via calpain-mediated cleavage of STEP. *J. Neurosci.* **29,** 9330–9343

27. Ivanov, A., Pellegrino, C., Rama, S., Dumalska, I., Salyha, Y., Ben-Ari, Y., and Medina, I. (2006) Opposing role of synaptic and extrasynaptic NMDA receptors in regulation of the extracellular signal-regulated kinases (ERK) activity in cultured rat hippocampal neurons. *J. Physiol.* **572,** 789–798

28. Xia, P., Chen, H. S., Zhang, D., and Lipton, S. A. (2010) Memantine preferentially blocks extrasynaptic over synaptic NMDA receptor currents in hippocampal autapses. *J. Neurosci.* **30,** 11246–11250

29. Bano, D., Young, K. W., Guerin, C. J., Lefeuvre, R., Rothwell, N. J., Naldini, L., Rizzuto, R., Carafoli, E., and Nicotera, P. (2005) Cleavage of the plasma membrane Na^+/Ca^{2+} exchanger in excitotoxicity. *Cell* **120,** 275–285

30. Xu, W., Wong, T. P., Chery, N., Gaertner, T., Wang, Y. T., and Baudry, M.

31. Vosler, P. S., Brennan, C. S., and Chen, J. (2008) Calpain-mediated signaling mechanisms in neuronal injury and neurodegeneration. *Mol. Neurobiol.* **38,** 78–100

32. Bevers, M. B., Lawrence, E., Maronski, M., Starr, N., Amesquita, M., and Neumar, R. W. (2009) Knockdown of *m*-calpain increases survival of primary hippocampal neurons following NMDA excitotoxicity. *J. Neurochem.* **108,** 1237–1250

33. Yu, Y. T., Breitbart, R. E., Smoot, L. B., Lee, Y., Mahdavi, V., and Nadal-Ginard, B. (1992) Human myocyte-specific enhancer factor 2 comprises a group of tissue-restricted MADS box transcription factors. *Genes Dev.* **6,** 1783–1798

34. Heidenreich, K. A., and Linseman, D. A. (2004) Myocyte enhancer factor-2 transcription factors in neuronal differentiation and survival. *Mol. Neurobiol.* **29,** 155–166

35. Cui, H., Hayashi, A., Sun, H. S., Belmares, M. P., Cobey, C., Phan, T., Schweizer, J., Salter, M. W., Wang, Y. T., Tasker, R. A., Garman, D., Rabinowitz, J., Lu, P. S., and Tymianski, M. (2007) PDZ protein interactions underlying NMDA receptor-mediated excitotoxicity and neuroprotection by PSD-95 inhibitors. *J. Neurosci.* **27,** 9901–9915

36. Khosravani, H., Zhang, Y., Tsutsui, S., Hameed, S., Altier, C., Hamid, J., Chen, L., Villemaire, M., Ali, Z., Jirik, F. R., and Zamponi, G. W. (2008) Prion protein attenuates excitotoxicity by inhibiting NMDA receptors. *J. Cell Biol.* **181,** 551–565

37. Wiedmann, M., Wang, X., Tang, X., Han, M., Li, M., and Mao, Z. (2005) PI3K/Akt-dependent regulation of the transcription factor myocyte enhancer factor-2 in insulin-like growth factor-1- and membrane depolarization-mediated survival of cerebellar granule neurons. *J. Neurosci. Res.* **81,** 226–234

38. Lau, A., and Tymianski, M. (2010) Glutamate receptors, neurotoxicity, and neurodegeneration. *Pflugers Arch.* **460,** 525–542

39. Battaglia, F., Trinchese, F., Liu, S., Walter, S., Nixon, R. A., and Arancio, O. (2003) Calpain inhibitors, a treatment for Alzheimer disease: Position paper. *J. Mol. Neurosci.* **20,** 357–362

40. Schölzke, M. N., Potrovita, I., Subramaniam, S., Prinz, S., and Schwaninger, M. (2003) Glutamate activates NF-κB through calpain in neurons. *Eur. J. Neurosci.* **18,** 3305–3310