MFN2 Couples Glutamate Excitotoxicity and Mitochondrial Dysfunction in Motor Neurons**

Wenzhang Wang,† Fan Zhang,‡§, Li Li,‡ Fangqiang Tang,§ Sandra L. Siedlak,§ Hisashi Fujioka,§ Yingchao Liu,§ Bo Su,† Yan Pi*,** and Xinglong Wang†‡

From the †Department of Pathology and §Electron Microscopy Core Facility, Case Western Reserve University, Cleveland, Ohio 44106, the Departments of ‡Neurosurgery and §Neurobiology, Shandong University, Jinan 250012, China, and the **State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University, Shanghai 200433, China

Background: Mitochondrial function is dependent on mitochondrial fission and fusion dynamics. Calpain-mediated degradation of MFN2 is responsible for glutamate-induced mitochondrial dysfunction and neuronal death in spinal cord motor neurons.

Results: Calpain-mediated MFN2 degradation is a novel mechanism regulating mitochondrial fusion during glutamate excitotoxicity.

Conclusion: Calpain-mediated MFN2 degradation is responsible for glutamate-induced mitochondrial dysfunction and neuronal death.

Significance: MFN2 might be a novel therapeutic target against glutamate excitotoxicity in motor neurons.

Mitochondrial dysfunction plays a central role in glutamate-evoked neuronal excitotoxicity, and mitochondrial fission/fusion dynamics are essential for mitochondrial morphology and function. Here, we establish a novel mechanistic linker among glutamate excitotoxicity, mitochondrial dynamics, and mitochondrial dysfunction in spinal cord motor neurons. Ca²⁺-dependent activation of the cysteine protease calpain in response to glutamate results in the degradation of a key mitochondrial outer membrane fusion regulator, mitofusin 2 (MFN2), and leads to MFN2-mediated mitochondrial fragmentation preceding glutamate-induced neuronal death. MFN2 deficiency impairs mitochondrial function, induces motor neuronal death, and renders motor neurons vulnerable to glutamate excitotoxicity. Conversely, MFN2 overexpression blocks glutamate-induced mitochondrial fragmentation, mitochondrial dysfunction, and/or neuronal death in spinal cord motor neurons both in vitro and in mice. The inhibition of calpain activation also alleviates glutamate-induced excitotoxicity of mitochondria and neurons. Overall, these results suggest that glutamate excitotoxicity causes mitochondrial dysfunction by impairing mitochondrial dynamics via calpain-mediated MFN2 degradation in motor neurons and thus present a molecular mechanism coupling glutamate excitotoxicity and mitochondrial dysfunction.

L-Glutamate is the major excitatory neurotransmitter in the central nervous system (1). Excessive stimulation or overactivation of neurons by glutamate causes neuronal death, a pathological process referred to as glutamate excitotoxicity (2, 3). It has been proposed that glutamate excitotoxicity is an important pathological mechanism in a wide range of neurological disorders, including cerebral ischemia, stroke, brain trauma, and even neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Parkinson disease, and Huntington disease (4–6). The detailed mechanisms underlying glutamate excitotoxicity-induced neuronal death are not fully elucidated. However, mitochondrial dysfunction, including mitochondrial membrane potential (MMP) reduction and ROS overproduction, was reported as a primary event in glutamate-induced neurotoxicity (7, 8).

Mitochondria are highly dynamic organelles that undergo continuous fission/fusion events that play a critical role in maintaining mitochondrial homeostasis (9). Unopposed fission leads to fragmentation whereas unopposed fusion causes elongation, both of which could impair mitochondrial function (10, 11). Mitochondrial fission and fusion events are tightly regulated by mitochondrial fission and fusion proteins as follows: dynamin-like protein 1 (DLP1, also referred to as DRP1) and its recruiting factors on mitochondria such as FIS1, MFF, MID49, and MID51 for fission (12); and mitofusin 1 (MFN1), mitofusin 2 (MFN2), and optic atrophy protein 1 (OPA1) for fusion (13). In addition to regulating mitochondrial morphology, mitochondrial fission and fusion processes are essential for the maintenance of various aspects of mitochondrial function (14). Thus, not surprisingly, emerging studies suggest the altered balance in mitochondrial fission and fusion might be a potential mechanism leading to mitochondrial dysfunction in neurological disorders (15–17).

Around 40–75% of ALS cases demonstrated defects in glutamate transport and elevated extracellular glutamate levels in spinal cord, and abundant evidence suggests an important role of glutamate excitotoxicity in motor neuron degeneration (18–

* This work was supported, in whole or in part, by National Institutes of Health Grants R03AG044680, R21NS085747, and R01NS089604. This work was also supported by Alzheimer’s Association Grant NIGR-11-204281 and China Scholarship Council (to F. Z.).

† This article was selected as a Paper of the Week.

‡ To whom correspondence should be addressed. E-mail: xinglong.wang@case.edu.

The abbreviations used are: ALS, amyotrophic lateral sclerosis; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; LDH, lactate dehydrogenase; OCR, oxygen consumption rate; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetrakis(acetoxymethyl ester); aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; NLS, nuclear localization signal; NGS, normal goat serum; DIV, days in vitro; Tg, transgene-positive; NTg, transgene-negative.
Glutamate Excitotoxicity and Mitochondrial Dysfunction

20). Because mitochondrial dysfunction was reported as a primary event in glutamate-induced neurotoxicity, and mitochondrial function is highly dependent on mitochondrial dynamics, here we sought to investigate whether and how mitochondrial dynamics play a role in glutamate-induced mitochondrial dysfunction and neuronal death in spinal cord motor neurons both in vitro and in mice.

MATERIALS AND METHODS

Embryonic Primary Motor/Cortical Neuron Isolation, Culture, and Transfection—MFN2 floxed male and female mice were the kind gifts of Dr. David Chan (California Institute of Technology). MFN2 transgenic male and female mice were generated by pronuclear injection of the Thy1-MFN2 transgene into C57BL/6N fertilized eggs using the murine Thy-1.2 genomic expression cassette (gift of Dr. Philip C. Wong, Johns Hopkins University). Timed pregnant Sprague-Dawley female rats (Harlan or Charles River) or C57BL/6N female mice were sacrificed following the protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University. Primary motor neurons were isolated from E13 to E15 female rat/E12 to E14 female mouse embryos as we described before (21). Primary cortical neurons were isolated from E18 female rats as described previously (22) but with some modifications.

Expression Vectors, Antibodies, Chemicals, and Measurements—MitoDsRed2 (Clontech), Case12 construct (Evrogen, Moscow, Russia), GFP-Cre (Addgene, Cambridge, MA), and GFP/Myc-tagged MFN2 (gift of Dr. Margaret T. Fuller, Stanford University) were obtained. MitoDsRed2/Cre dual promoter regular construct was generated by replacing the neoycin/canamycin resistance gene in MitoDsRed2 construct with sequence of recombinase Cre with nuclear localization signal (NLS-Cre from GFP-Cre construct). MitoDsRed2/Cre dual promoter lentiviral construct was generated by cloning mitoDsRed2 into pLVX-Puro (Clontech) and replacing puromycin resistance gene with NLS-Cre, whereas the Cre lentiviral construct was generated by cloning NLS-Cre into pLVX-Puro. Lentivirus were generated using the third generation packaging system (packaging plasmid, pMDLg/pRRE and pRSV-Rev; envelope plasmid, pMD2.G from Addgene, Cambridge MA) as described previously (23). The primary antibodies used included the following: mouse anti-VDAC1/rabbit anti-Mff/Spectrin (Abcam, Cambridge, MA); rabbit anti-calpain 1, calpain 2, and cleaved caspase3 (Cell Signaling, Danvers, MA); rabbit anti-Mfn1/mouse anti-Mfn2 (Santa Cruz Biotechnology, Dallas, TX); mouse anti-HB9 (DSHB, Iowa City, IA); mouse anti-glial fibrillary acidic protein (Invitrogen); rabbit anti-lba1 (Wako, Richmond, VA); mouse anti-DLP1/OPA1/Tom20 (BD Biosciences); and mouse anti-actin/rabbit anti-MAP2 (Millipore, Billerica, MA). Glutamate (Sigma) and rotenone (1 μM) were injected at the indicated times. After measurements, cells were lysed, and OCR data were normalized by total protein. Cell death and viability was measured by the cytotoxicity detection kit (LDH; Roche Applied Science) or the cell proliferation kit (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Roche Applied Science). Neuronal viability propidium iodide assay in positively transfected neurons was performed as we described (25). Neurons with propidium iodide-positive nuclei and/or obvious fragmented nuclear/neurites were counted as nonviable neurons, whereas neurons without propidium iodide-positive nuclei and clear nuclear contour/neurites were counted as viable neurons.

Mitochondrial Isolation and Western Blot Analysis—Crude and purified mitochondria were isolated as described before (21). Purified mitochondria from cells were lysed with 1× cell lysis buffer (Cell Signaling, Danvers, MA) plus 1 mM PMSF (Sigma) and protease inhibitor mixture (Sigma). Equal amounts of total protein extract were resolved by SDS-PAGE and transferred to Immobilon-P (Millipore, Billerica, MA). Following blocking with 10% nonfat dry milk, primary and secondary antibodies were applied as described previously (24), and the blots were developed with Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA).

Animal Surgery and Glutamate Infusion—Mouse surgery/procedures were performed according to the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University. One day before implantation, mini-osmotic pumps (model 2001, Alzet, Cupertino, CA; flow rate of 1 μl/h) and brain infusion cannulae attached with 2-cm catheter tubes (brain infusion kit 3, Alzet, Cupertino, CA) were filled with artificial cerebrospinal fluid (aCSF) (124 mM NaCl, 25 mM NaHCO3, 10 mM d-glucose, 2.5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, and 1 mM NaH2PO4 adjusted to pH 7.2–7.4 using NaOH) or aCSF containing 10 mM glutamate followed by pump incubation in phosphate-buffered saline (PBS) at 37 °C overnight according to the manufacturer’s instructions. For stereotaxic surgery, male mice were anesthetized with avertin and placed on a stereotaxic frame. A small incision was first made to expose skull and bregma, and both the catheter and the connected mini-osmotic pump were implanted subcutaneously. A hole was drilled in the skull (relative to bregma, anterior–posterior −0.2 mm; medial–lateral, 1 mm), and the cannula was positioned 2 mm above the lateral ventricle. Another two holes were drilled by the edge of cannula, and self-tapping bone screws (MD-1310, BASi, West Lafayette, IN) were screwed into the holes. The cannulae and screws were finally secured by cement. 7 days after surgery, male mice were deeply anesthetized with avertin and transcardially perfused with cold PBS, and the spinal cord/brain were collected for further analysis.

Immunocytochemistry and Immunofluorescence of Mouse Spinal Cord—To investigate the mitochondrial morphology in motor neurons in male mice, we also developed an optimized
Glutamate Excitotoxicity and Mitochondrial Dysfunction

immunofluorescence-based imaging technique that could directly visualize individual filamentous mitochondria in paraffin-embedded buffered formalin-fixed spinal cord tissues using specific antibodies against mitochondrial protein Tom20 or VDAC1. Taken briefly, deparaffinized and re-hydrated tissue sections were washed briefly three times with distilled H₂O and placed in 1× antigen decloaker (Biocare, Concord, CA). The sections were then subjected to antigen retrieval under pressure using Biocare’s Decloaking Chamber by heating to 125 °C for 10 s and cooling to 90 °C for 30 s followed by heating to 22 p.s.i. at 128 °C and cooling to 0 p.s.i. at 94 °C. After the temperature decreased to 30 °C, the sections were gradually rinsed with distilled H₂O five times. The sections were then blocked with 10% normal goat serum (NGS) (Sigma) for 30 min at room temperature and incubated with primary antibodies in PBS containing 1% NGS overnight at 4 °C. After three washes with PBS, the sections were incubated in 10% NGS for 10 min and then with Alexa Fluor-conjugated secondary antibody (Invitrogen) (1:300) for 2 h at room temperature in the dark. Finally, the sections were rinsed three times with PBS, stained with DAPI, washed again with PBS three times, and mounted with Fluoro-mount-G mounting medium (Southern Biotech, Birmingham, AL).

Electron Microscopy of Mouse Spinal Cord—Male mice were transcardially perfused with EM fixative (quarter-strength Karnovsky, 1.25% DMSO mixture) for 5 min, and the spinal cords were removed quickly and placed in EM fixative solution for an additional 15 min at room temperature followed by additional fixation in fresh EM fixative for another 2 h at room temperature. After washing, tissue blocks were postfixed in 1% osmium, 1.25% ferrocyanide mixture for two changes of solution of 1 h each (total postfixation time is 2 h) at room temperature. Then the specimens were rinsed and soaked overnight in acidified 0.5% uranyl acetate. After another wash, the blocks were dehydrated in ascending concentrations of ethanol, passed through propylene oxide, and embedded in Poly/Bed 812 embedding resin (Polysciences, Warrington, PA). Thin sections were sequentially stained with 2% acidified uranyl acetate followed by Sato’s triple lead staining as modified by Hanaichi et al. (26) and examined in an FEI Tecnai T12 electron microscope equipped with a Gatan single tilt holder and a Gatan US4000 4kx4k CCD camera (Gatan, Pleasanton, CA).

In Vitro Calpain Cleavage Assay—100 μg of purified mitochondria or 1 μg of recombinant Mfn2 protein (Origene, Rockville, MD) were incubated with purified human erythrocyte μ-calpain (calpain-I) (Biovision, Milpitas, CA) at 30 °C for 30 min according to the manufacturer’s instructions. For inhibition, calpain-I was preincubated with PMSF or calpeptin for 5 min at 30 °C. The reaction was stopped by the addition of SDS sample buffer containing 62.5 mM Tris-HCl, 4% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromphenol blue (pH 6.8). The samples were boiled at 100 °C for 10 min. 1/6 of reaction volume was loaded into SDS-polyacrylamide gel for Western blot.

Time-lapse Imaging and Confocal Microscopy—For time-lapse imaging, neurons were seeded in 35-mm dishes and transfected/co-transfected with mito-DsRed2, GFP-Mfn2, and Case12. 48 h after transfection, neurons were placed in a well equipped environmental chamber with controlled CO₂ content, humidity, and temperature and imaged at 37 °C by an inverted Leica DM16000 fluorescence microscope (Leica, Buffalo Grove, IL) (controlled through Leica LAS AF 3 software) with a 20X/0.7NA Plan Apochromat dry objective. During time-lapse imaging, frames were captured every 1 min for at least 6 h without phototoxicity or photobleaching. For confocal microscopy, neurons cultured on coverslips were fixed and stained as we described previously (24). All other fluorescence images were captured at room temperature with a Zeiss LSM 510 inverted laser-scanning confocal fluorescence microscope (controlled through Zeiss LSM 510 confocal software, Zeiss, Peabody, MA) equipped with a C-Apochromat 40×/1.2-watt water objective or αPlan-Fluar 100×/1.45 oil objective as described previously (24). Confocal images of far-red fluorescence were collected using 633 nm excitation light from a HeNe laser and a 650-nm long pass filter; images of red fluorescence were collected using 543-nm excitation light from an argon laser and a 560-nm long pass filter; and green fluorescence images were collected using 488 nm excitation light from an argon laser and a 500–550-nm bandpass barrier filter.

Image Analysis—Morphology of mitochondria was quantified as we described previously (21). All single plane or series of z-stacks of raw images were background-corrected, linearly contrast-optimized, applied with a 7 × 7-nm “top hat” filter, subjected to a 3 × 3-nm median filter, and then thresholded to generate binary images by ImageJ. Most mitochondria were well separated in binary images, and large clusters of mitochondria were excluded automatically. All binary images were either directly analyzed by ImageJ, or assembled into three-dimensional volumes and quantified by Imaris 7.2 (Bitplane, South Windsor, CT).

RESULTS

Glutamate Induces Mitochondrial Fragmentation in Spinal Cord Motor Neurons—We first investigated the effect of glutamate exposure on mitochondrial morphology in cultured rat primary embryonic spinal cord motor neurons. Consistent with previous studies (27), glutamate treatment caused a dose- and time-dependent increase of cell death in motor neurons, which could be blocked by glutamate receptor blocker MK-801 (Fig. 1, A–C). To examine the potential effect of glutamate on mitochondrial morphology, primary motor neurons (DIV5) were first transfected with mitoDsRed2 and then treated with a sublethal dose of glutamate (25 μM) for 24 h at DIV7. Treatment with glutamate resulted in significant mitochondrial fragmentation (Fig. 1, D and E). In accordance with previous studies (28, 29), the glutamate-induced mitochondrial fragmentation as well as neuronal death were completely prevented by MK-801 (Fig. 1, D and E).

We further sought to determine whether glutamate-induced mitochondrial fragmentation was due to changes in the expression of mitochondrial fission and fusion proteins in motor neurons. Interestingly, glutamate treatment caused a significant and specific decrease of the expression of DLP1 and Mfn2 in motor neurons, although it had no effect on the expression of OPA1, Mfn1, and Mff (Fig. 1, F and G). As expected, the glutamate-induced DLP1/Mfn2 reduction could be completely blocked by MK-801. DLP1 is a cytosolic protein, and its trans-
location from cytosol to mitochondria mediates mitochondrial fission (30). We therefore measured the level of DLP1 in mitochondrial fraction and also found a similar and significant decrease of mitochondrial DLP1 in glutamate-treated neurons (Fig. 1H).

**MFN2 Deficiency Elicits Mitochondrial and Neuronal Toxicity in Spinal Cord Motor Neurons**—Because both DLP1 and MFN2 were reduced by glutamate treatment, we next examined the impact of DLP1 and MFN2 deficiency on mitochondrial dynamics and mitochondrial/neuronal function in *in vitro* cultured primary mouse spinal cord motor neurons. To measure the effect of MFN2 deficiency on mitochondrial morphology, we used mouse primary motor neurons (*Mfn2*^−/−^ neurons) isolated from *Mfn2* floxed mice (31). *Mfn2*^−/−^ motor neurons (DIV4) were transfected with dual promoter constructs encoding both mitoDsRed2 and Cre to genetically label mitochondria and *Mfn2* knock-out genes simultaneously. Cre-mediated ablation of *Mfn2* in *Mfn2*^−/−^ motor neurons was confirmed by Western blot (Fig. 2A). As expected, *Mfn2*^−/−^ motor neurons positively transfected with constructs encoding mitoDsRed2...
only demonstrated normal tubular mitochondrial morphology with an average length of around 1.8 μm, whereas Mfn2Δ/Δ neurons positively transfected with constructs encoding both Cre and MitoDsRed2 showed significant reduction of the average mitochondrial length, suggestive of enhanced mitochondrial fragmentation (Fig. 2, B and C). To investigate the effect of DLP1 deficiency on mitochondrial morphology, primary motor neurons (DIV4) were co-transfected with mitoDsRed2 positively transfected primary mouse spinal cord motor neurons as shown. Red, mito-DsRed2. More than 50 neurons were analyzed in per group. Measurement of intracellular levels of ROS (D), MMP (E), OCR (F and G), ATP (H), and neuronal death by LDH assays (I) are shown. J, quantification of neuronal death by propidium iodide assays in positively transfected neurons 3 days after transfection. More than 200 neurons were analyzed per group. All experiments were repeated three times. Data are means ± S.E. Statistics: one-way ANOVA followed by Tukey’s multiple comparison test. *, p < 0.05; **, p < 0.01; ***, p < 0.001. FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; ns, nonsignificant.

Because mitochondrial function is highly dependent on mitochondrial morphology, intracellular levels of ROS and MMP were next measured. Compared with control neurons, motor neurons with Mfn2 deficiency or motor neurons with both Mfn2 and DLP1 deficiency demonstrated a comparably significant increase of ROS and decrease of MMP, whereas motor neurons with DLP1 deficiency showed unchanged ROS and MMP (Fig. 2, D and E). The high infection efficiency (>95%) of motor neurons with lentivirus encoding Cre enabled the performance of bulk measurement of the effect of Mfn2
deficiency on other mitochondrial functions such as OCR and ATP production. As expected, MFN2 deficiency caused significant reduction of basal/maximal OCR and ATP levels (Fig. 2, F–H). In addition to the change of mitochondrial function, it was noted that MFN2-deficient motor neurons demonstrated significantly increased basal neuronal death (Fig. 2, I and J). On the contrary, DLP1 RNAi had no significant effect on neuronal viability.

**MFN2 Overexpression Prevents Glutamate-induced Mitochondrial Dysfunction and Motor Neuron Death in Vitro**—To determine the specific role of DLP1 and MFN2 in glutamate-induced mitochondrial fragmentation, rat primary motor neurons were co-transfected with Myc-tagged MFN2 and mitoDsRed2 or transfected with mitoDsRed2 only and treated with/without 25 μM glutamate at DIV7 for 24 h. Representative confocal images (A) and quantification (B) of mitochondrial length in mitoDsRed2 positively transfected primary rat spinal cord motor neurons overexpressing MFN2 (DIV8) are shown. Primary rat motor neurons were also transfected with mitoDsRed2 and Myc-tagged DLP1 at DIV5 and treated with 25 μM glutamate at DIV7 for 24 h. Neurons were double-stained with specific antibodies against Myc and DLP1. Representative confocal images (C) and quantification (D) of mitochondrial length in mitoDsRed2 positively transfected primary spinal cord motor neurons overexpressing DLP1 (DIV8). Red, mito-DsRed2; green, Myc; white, DLP1. More than 50 neurons were analyzed in per group. All experiments were repeated more than four times. Data are means ± S.E. Statistics: one-way ANOVA followed by Tukey’s multiple comparison test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, nonsignificant.

![Image](image.jpg)

**FIGURE 3. MFN2 but not DLP1 overexpression (OE) prevents glutamate-induced mitochondrial fragmentation in primary spinal cord motor neurons.** To overexpress MFN2, primary rat spinal cord motor neurons (DIV5) were co-transfected with Myc-tagged MFN2 and mitoDsRed2 or transfected with mitoDsRed2 only and treated with/without 25 μM glutamate at DIV7 for 24 h. Representative confocal images (A) and quantification (B) of mitochondrial length in mitoDsRed2 positively transfected primary rat spinal cord motor neurons overexpressing MFN2 (DIV8) are shown. Primary rat motor neurons were also transfected with mitoDsRed2 and Myc-tagged DLP1 at DIV5 and treated with 25 μM glutamate at DIV7 for 24 h. Neurons were double-stained with specific antibodies against Myc and DLP1. Representative confocal images (C) and quantification (D) of mitochondrial length in mitoDsRed2 positively transfected primary spinal cord motor neurons overexpressing DLP1 (DIV8). Red, mito-DsRed2; green, Myc; white, DLP1. More than 50 neurons were analyzed in per group. All experiments were repeated more than four times. Data are means ± S.E. Statistics: one-way ANOVA followed by Tukey’s multiple comparison test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, nonsignificant.

![Diagram](diagram.png)

deficiency on other mitochondrial functions such as OCR and ATP production. As expected, MFN2 deficiency caused significant reduction of basal/maximal OCR and ATP levels (Fig. 2, F–H). In addition to the change of mitochondrial function, it was noted that MFN2-deficient motor neurons demonstrated significantly increased basal neuronal death (Fig. 2, I and J). On the contrary, DLP1 RNAi had no significant effect on neuronal viability.

**MFN2 Overexpression Prevents Glutamate-induced Mitochondrial Dysfunction and Motor Neuron Death in Vitro**—To determine the specific role of DLP1 and MFN2 in glutamate-induced mitochondrial fragmentation, rat primary motor neurons were co-transfected with Myc-tagged MFN2 and mitoDsRed2 or transfected with mitoDsRed2 only and treated with/without 25 μM glutamate at DIV7 for 24 h. Representative confocal images (A) and quantification (B) of mitochondrial length in mitoDsRed2 positively transfected primary rat spinal cord motor neurons overexpressing MFN2 (DIV8) are shown. Primary rat motor neurons were also transfected with mitoDsRed2 and Myc-tagged DLP1 at DIV5 and treated with 25 μM glutamate at DIV7 for 24 h. Neurons were double-stained with specific antibodies against Myc and DLP1. Representative confocal images (C) and quantification (D) of mitochondrial length in mitoDsRed2 positively transfected primary spinal cord motor neurons overexpressing DLP1 (DIV8). Red, mito-DsRed2; green, Myc; white, DLP1. More than 50 neurons were analyzed in per group. All experiments were repeated more than four times. Data are means ± S.E. Statistics: one-way ANOVA followed by Tukey’s multiple comparison test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, nonsignificant.
OCR, and ATP levels to untreated control neurons, strongly suggesting that MFN2-mediated mitochondrial fragmentation plays an essential role in glutamate-induced mitochondrial dysfunction.

To investigate whether glutamate-induced mitochondrial fragmentation was downstream or upstream of neuronal death, we next measured neuronal viability in neurons with manipulated MFN2 expression after glutamate treatment. MFN2-deficient cells (i.e., Cre:MFN2fl/fl neurons) demonstrated significantly higher vulnerability to glutamate treatment (Fig. 2I). On the contrary, MFN2 overexpressing motor neurons were resistant to glutamate-induced cell death (Figs. 2I and 4H). These data suggested that mitochondrial fragmentation is upstream of glutamate-induced cell death. Indeed, a detailed time lapse study demonstrated that mitochondrial length and MFN2 expression were decreased within 30 min following glutamate treatment (Fig. 4).

**FIGURE 4. Protection from glutamate-induced mitochondrial dysfunction and neuronal death by MFN2 in primary spinal cord motor neurons.** Primary mouse spinal cord motor neurons (DIV7) isolated from Tg neurons and NTg neurons of transgenic mice overexpressing human MFN2 were treated with or without 25 μM glutamate for 24 h. A, representative immunoblot and quantification analysis confirming the overexpression of MFN2 (relative to actin) in Tg motor neurons are shown. Equal protein amounts (10 μg) were loaded and confirmed by actin. Measurement of mitochondrial length by mitoDsRed2 labeling (B) and intracellular levels of ROS (C), MMP (D), OCR (E and F), ATP (G), and neuronal death by LDH assays (H). All experiments were repeated three times. Data are means ± S.E. Statistics: one-way ANOVA followed by Tukey’s multiple comparison test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, nonsignificant.
treatment (Fig. 5, A–C), although significant neuronal death was only observed 8 h later (Fig. 1B).

**MFN2 Overexpression Protects Mitochondria and Motor Neurons from Glutamate Excitotoxicity in Vivo**—Because glutamate causes MFN2-dependent mitochondrial fragmentation and neuronal death in vitro, we further sought to determine whether these events happen in spinal cord motor neurons in vivo. Nontransgenic control mice (NTg mice) or transgenic mice overexpressing MFN2 (Tg mice) received continuous administration of glutamate into the left lateral ventricle via osmotic mini-pumps. According to previous studies (33–35), a low dose of glutamate (10 mM at a flow rate of 1 μl/h) was employed to minimize side effects. 7 days of continuous glutamate infusion did not cause the death of mice or histological abnormalities in brain, spinal cord, and muscle (data not shown). The overexpression of MFN2 in spinal cords of Tg mice was confirmed by Western blot (Fig. 6A) and immunocytochemistry (Fig. 6B). Compared with untreated or aCSF-infused control NTg mice, NTg mice exposed to low dose glutamate demonstrated significant reduction of MFN2 in spinal cords (Fig. 5, A and B). Similar to what we observed in in vitro cultured Tg motor neurons, the MFN2 level is also reduced by glutamate infusion in Tg mice spinal cords but to a level comparable with control NTg mice without glutamate infusion.

Because of the relatively large size of spinal cord motor neurons and the use of the specific antibody against the outer mitochondrial protein TOM20, individual filamentous mitochondria could be discriminated and imaged by immunofluorescence confocal microscopy (Fig. 6C). Unlike neurons cultured in vitro, mitochondria in neurons in vivo are not in single focal plane.

Thus, the reconstructed three-dimensional images were used to measure mitochondrial morphology in spinal cord motor neurons in vivo. As shown in single plane and reconstructed three-dimensional images, mitochondria of spinal cord motor neurons from normal NTg subjects demonstrated typical tubular network-like morphologies similar to what we observed in in vitro cultured mouse primary motor neurons and mouse spinal cord motor neurons in vivo (21), and glutamate caused significant mitochondrial fragmentation (Fig. 6, C and D). Importantly, glutamate-induced mitochondrial fragmentation was completely blocked in MFN2 Tg mice. Consistent with confocal microscopic results, electron microscopic analysis confirmed the significant protective effect of Mfn2 overexpression against glutamate-induced mitochondrial fragmentation in spinal cord motor neurons in Tg mice (Fig. 6E).

We also determined the effects of glutamate-induced neuronal death in spinal cord by double immunostaining using motor neuronal marker HB9 and cleaved caspase-3, the central executive molecule implicated in excitotoxic neuronal death in vivo (36) and the ALS mouse model (37). Glutamate infusion led to a significantly increased number of cleaved caspase-3-positive motor neurons in the spinal cords of NTg mice, which also exhibited clumping or fragmented nuclei (7.5 ± 1.57% of total) (Fig. 6F). In contrast, Tg mice infused with glutamate did not show any cleaved caspase-3-positive motor neurons in the spinal cord, indicating the complete blockage of glutamate-induced neuronal death by MFN2 overexpression. There were no cleaved caspase-3 positively stained neurons observed in the brain of either genotypes exposed to continuous infusion of glutamate (data not shown). Although non-neuronal cells such...
as astrocytes and microglia have been reported to affect the survival of motor neuron in mice (38–41), no increase of astrocytes or microglia was observed in either NTg or Tg spinal cords after glutamate infusion (Fig. 6G).

Calpain Cleaves MFN2 in Spinal Cord Motor Neurons Following Glutamate Exposure—Given the critical role of MFN2 reduction in glutamate-induced mitochondrial fragmentation and dysfunction, we sought to determine the mechanism by which glutamate caused MFN2 reduction in spinal cord motor neurons. Real time PCR revealed no changes in the mRNA levels of MFN2 in neurons treated with glutamate (Fig. 7A), suggesting that glutamate causes MFN2 reduction likely through post-translational protein degradation. Because glutamate exposure resulted in increase of intracellular Ca\(^{2+}\) (42, 43), we first examined the effect of calcium on glutamate-induced MFN2 reduction by treating neurons with a cell-permeable calcium chelator, BAPTA-AM. BAPTA-AM totally prevented the glutamate-induced MFN2 reduction, suggesting the dependence of MFN2 degradation on calcium signaling (Fig. 7, B and C). Following glutamate receptor activation, calpain is the major neuronal cysteine protease activated by calcium and is critical for neuronal function (44). We confirmed that calpain was activated by glutamate in motor neurons as evidenced by the appearance of the 150-kDa cII-spectrin breakdown product (SBDP150) (Fig. 7D), a calpain cleavage product that has been widely used as a direct measurement of calpain activity in neurons (45). To determine whether calpain is involved in MFN2 reduction, motor neurons at DIV7 were co-treated with glutamate and calpeptin, a cell-permeable calpain-specific inhibitor. Calpeptin completely prevented both glutamate-induced calpain activation and MFN2 reduction (Fig. 7, D and E), suggesting the involvement of calpain in glutamate-induced MFN2 degradation. Not surprisingly, along with the prevention of MFN2 degradation, the calpain inhibitor calpeptin resulted in significant inhibition of glutamate-induced mitochondrial fragmentation (Fig. 7, J and K), the increase of ROS, the reduction of MMP/ATP production, and neuronal death (Fig. 7L).

Although caspases were reportedly activated late after glutamate treatment and caspase inhibitors could alleviate glutamate-induced neuronal death (46), the caspase pan inhibitor Z-VAD-FMK had a slight but not significant effect on glutamate-induced MFN2 degradation (Fig. 7, F and G). Furthermore, proteasome inhibitor lactacystin, autophagy inhibitor bafilomycin A1, and macroautophagy inhibitor 3-methyladenine also failed to significantly block glutamate-induced MFN2 degradation (Fig. 7, H and I).

To prove the direct cleavage of MFN2 by calpain, we performed in vitro calpain cleavage assays using recombinant human MFN2 purified from HEK293 cells. There are two major forms of calpains, i.e. \(\mu\)-calpain (calpain 1) and m-calpain (calpain 2) (47). \(\mu\)-Calpain and/or \(\mu\)-calpain caused both a dose-dependent and time-dependent decrease of recombinant full-length MFN2 and a concurrent increase of many lower molecular mass (<75 kDa) fragments (Fig. 8, A and B), protein degradation of which could be completely blocked by calpeptin. To investigate the cleavage of MFN2 by calpain in physiologically relevant conditions, we took advantage of the purified mitochondria isolated from mouse brain. The purified mitochondria were incubated with calpain in the presence/absence of calpeptin or the broad spectrum serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), followed by immunoblot using antibodies again MFN2 (immunogen spanning last 50 residues). MFN2, but not other mitochondrial fission and fusion proteins such as MFN1 and OPA1, was decreased by calpain in a dose-dependent manner that was effectively prevented by calpeptin but not PMSF (Fig. 8, C and D). Interestingly, significantly fewer fragments were observed using purified mitochondria compared with cleavage using recombinant MFN2. Consistent with in vitro experiments showing calpain-derived MFN2 fragments, examination of neuronal lysates showed the presence of MFN2 fragments ranging from 3 to 75 kDa in control neurons, which accumulated significantly after glutamate treatment and could be greatly suppressed by calpeptin (Fig. 8E).

DISCUSSION

Previous studies demonstrated mitochondrial dysfunction as a primary event in excitatory neurons, yet how glutamate induces mitochondrial dysfunction is not clear. Here, we report that glutamate induced significant mitochondrial fragmentation that mediates glutamate-induced mitochondrial dysfunction in spinal cord motor neurons both in vitro and in vivo. This is likely driven by the glutamate-induced decrease in MFN2 expression because loss of MFN2 caused similar mitochondrial dysfunction and motor neuronal death, and overexpression of MFN2 almost completely alleviates glutamate-induced mitochondrial dysfunction and motor neuronal death. Mechanistically, we found that glutamate caused MFN2 reduction through
FIGURE 7. Degradation of MFN2 by calpain in response to glutamate challenge in primary spinal cord motor neurons. A, motor neurons (DIV7) were treated with 25 μM glutamate for 24 h, and mRNA levels of mitochondrial dynamic proteins were assayed by real time PCR. Representative immunoblot and quantification analysis of the expression of MFN2 (relative to actin) in rat spinal cord motor neurons (DIV7) treated with or without 25 μM glutamate and/or 2.5 μM intracellular calcium chelator BAPTA-AM (B and C), 20 μM calpain inhibitor calpeptin (D and E), and 20 μM pan-caspase inhibitor Z-VAD-FMK (F and G) for 24 h or 5 mM macroautophagy inhibitor 100 nM autophagy inhibitor bafilomycin A1 (BFA), 5 μM proteasome inhibitor lactacystin (LACT), and 3-methyladenine (3MA) (H and I) for 8 h. Equal protein amounts (10 μg) were loaded and confirmed by actin. VDAC1 was used as a mitochondrial specific marker. Representative confocal images (J) and quantification (K) of mitochondrial length in mitoDsRed2 positively transfected primary rat spinal cord motor neurons (DIV8) treated with or without 25 μM glutamate and/or calpain inhibitor calpeptin for 24 h. To label mitochondria, neurons were transfected with mitoDsRed2 2 days before treatment. Red, mito-DsRed2. More than 50 neurons were analyzed in per group. L, measurement of intracellular levels of ROS, MMP, ATP, and neuronal death by LDH assays. All experiments were repeated three times. Data are means ± S.E. Statistics: one-way ANOVA followed by Tukey’s multiple comparison test. *, p < 0.05; ns, nonsignificant.
calpain-dependent cleavage of MFN2. To our best knowledge, these studies for the first time not only provide a mechanism explaining how glutamate induces mitochondrial dysfunction but also present a novel pathway describing how mitochondrial dynamics are regulated in response to stress.

Normal mitochondrial morphology is regulated by the delicate balance of fission and fusion processes (48). Glutamate-induced mitochondrial fragmentation observed in this study suggested a tipped balance of these processes toward excessive fission that can be due to either enhanced mitochondrial fission, reduced mitochondrial fusion, or both. The level of mitochondrial fusion protein MFN2 and mitochondrial fission protein DLP1 were both reduced, suggesting the possible impairment of both fission and fusion. Despite the fact that decreased expression of DLP1 caused mitochondrial elongation and decreased expression of MFN2 led to mitochondrial fragmentation, mitochondria became predominantly fragmented when the expression of DLP1 and MFN2 were both decreased in motor neurons, which is consistent with our prior observation in cortical neurons (49). Further functional measurements showed that DLP1 RNAi had no significant effect on mitochondrial dysfunction caused by MFN2 in motor neurons. Therefore, our data suggest that MFN2 reduction supersedes DLP1 reduction on the regulation of mitochondrial morphology and mitochondrial function. This is somewhat expected because MFN2 is a predominantly mitochondrially localized protein, whereas DLP1 mainly resides in cytosol and is demonstrated to be involved in other cellular functions such as endoplasmic reticulum distribution (50) and vesicle membrane dynamics (51). Nevertheless, because MFN2 but not DLP1 deficiency was sufficient to cause mitochondrial fragmentation/dysfunction and neuronal death, similar to glutamate-induced changes, mitochondrial fragmentation and hence mitochondrial dysfunction during glutamate excitotoxicity appeared to be primarily caused by the impairment of mitochondrial fusion via MFN2 reduction. It is still unclear why DLP1 is reduced and what may be the functional consequence of DLP1 reduction. Of interest, DLP1 interacts with Bax, which may play a critical role in apoptosis (52, 53), and inactivation of DLP1 renders cells more resistant to stresses such as ROS (54). It is possible that DLP1 reduction may be an adaptation or defense mechanism against glutamate-induced cell death.

Calpain overactivation through glutamate receptor-mediated intracellular calcium influx has been regarded as the major cause of glutamate-induced dying-back axonopathy and neuronal death, but the mechanism is still unclear (55–57). Unlike the most recent study demonstrating the late stage reduction of MFN2 during glutamate excitotoxicity in cortical neurons (58), our detailed time lapse study in motor neurons demonstrated that, concurrent with mitochondrial fragmentation, MFN2 was...
Glutamate Excitotoxicity and Mitochondrial Dysfunction
decreased rapidly following glutamate treatment. This discrepancy could be due to either different responses to or sensitivity of motor and cortical neurons to glutamate excitotoxicity. MFN2 reduction and mitochondrial fragmentation well preceded neuronal death during glutamate excitotoxicity, suggesting glutamate-induced mitochondrial fragmentation was unlikely a secondary effect of glutamate-evoked neurotoxicity. In support of this notion, overexpression of MFN2 significantly inhibited neuronal death caused by glutamate treatment. Therefore, MFN2-mediated mitochondrial fragmentation is a prerequisite for glutamate-evoked motor neuronal death. We further demonstrated that calpain was responsible for MFN2 degradation following glutamate exposure. Because the inhibition of calpain could also effectively block glutamate-induced mitochondrial fragmentation, mitochondrial dysfunction, and neuronal death, our data suggest that calpain and MFN2 act in a linear pathway, with calpain-induced MFN2 degradation impairing mitochondrial fusion to cause mitochondrial dysfunction and neuronal death during glutamate challenge. Indeed, MFN2 ablation alone was sufficient to induce motor neuronal death, further supporting the critical role of MFN2-mediated mitochondrial fragmentation during glutamate excitotoxicity. It is still unclear how MFN2-dependent mitochondrial fragmentation leads to mitochondrial dysfunction and neuronal death. Previous studies showed that mitochondrial fission/fusion proteins are involved in the assembly of respiratory complexes (59, 60). Therefore, glutamate-induced mitochondrial fragmentation probably impairs mitochondrial function and causes subsequent neuronal death through the regulation of the mitochondrial respiratory complex assembly. Nevertheless, the exact underlying mechanism(s) still remains to be determined.

We demonstrated that calpain was responsible for MFN2 degradation following glutamate exposure and calpain-cleaved MFN2 in vitro. Compared with recombinant MFN2, MFN2 in mitochondria was more resistant to calpain and generated less calpain-derived fragments. Because recombinant MFN2 was purified from HEK293 cells, it had similar post-translational modifications as MFN2 in mitochondria. So, the relative resistance of MFN2 in mitochondria to calpain was probably due to either the binding of other proteins/factors or the protein conformation that inhibited or masked potential calpain cleavage sites. Our in vitro cleavage assay using isolated mitochondria found that neither MFN1 nor OPA1 in mitochondria could be cleaved by calpain that could efficiently cleave MFN2. This is consistent with our finding that levels of full-length OPA1 and MFN1 remained unchanged in neurons exposed to glutamate. Although caspases were reported to be activated late after glutamate treatment (46) and caspase inhibitors could alleviate glutamate-induced neuronal death, we found that caspases were not involved in glutamate-induced MFN2 degradation. A previous study showed that MFN2 could be degraded through the ubiquitin-proteasome system during apoptosis (61). However, similar to caspases, the inhibition of either proteasome or the autophagy/lysosomal degradation pathway failed to prevent MFN2 degradation in response to glutamate treatment in motor neurons. The most recent study demonstrated that MFN2 was regulated at the transcriptional level at late stage during glutamate excitotoxicity in cortical neurons. However, we did not observe significant changes in the mRNA level of MFN2 and other fission/fusion proteins during glutamate excitotoxicity in motor neurons. Calpain is the major protease reported to be activated following glutamate challenge, and indeed, glutamate-induced MFN2 reduction could be completely blocked by calpain inhibition. Thus, our study suggests that the calpain-mediated MFN2 reduction might be the major pathway responsible for MFN2 degradation during glutamate excitotoxicity at least in motor neurons.

The inhibition of calpain, however, did not completely block glutamate-induced mitochondrial fragmentation and dysfunction, suggesting the possible existence of other pathways contributing to mitochondrial abnormalities during glutamate excitotoxicity. For example, MIRO-1 has been reported to regulate mitochondrial dynamics in response to calcium signaling (62, 63). MIRO-1 is a calcium-binding GTPase, the activity of which could be directly regulated by calcium. It is possible that the inhibition of calpain has no effect on mitochondrial fragmentation and dysfunction mediated by MIRO-1 or other proteins following glutamate-evoked calcium signaling. Nevertheless, overexpression of MFN2 could completely prevent glutamate-induced mitochondrial fragmentation and dysfunction, indicating that MFN2 might compensate for the effect of other pathway(s). In addition to MFN2, calpain has other targets such as CAIN/CABIN1 (64), collapsin response mediator proteins (55), and SRC protein kinase (65) during glutamate-induced excitotoxicity. Considering the involvement of mitochondria in almost all forms of cell death (66) and cellular processes, future studies are expected to address the interplay between MFN2 and other calpain targets in response to glutamate excitotoxicity.

Taken together, this study presents how calpain-mediated MFN2 degradation may act as the novel mechanistic linker among glutamate excitotoxicity, mitochondrial dynamic abnormalities, mitochondrial dysfunction, and neuronal death in spinal cord motor neurons. As our understanding of the important role of mitochondrial dynamics in pathophysiology continues to expand, this study implicates that this newly identified MFN2 degradation pathway through calpain activation might be a potential novel therapeutic target worthy of validation in disease models that show either elevated glutamate and/or calpain activation.

REFERENCES
1. Sommer, B., Keinänen, K., Verdoorn, T. A., Wisden, W., Burnashev, N., Herb, A., Köhler, M., Takagi, T., Sakmann, B., and Seeburg, P. H. (1990) Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. Science 249, 1580–1585.
2. Olney, J. W. (1969) Brain lesions obesity and other disturbances in mice treated with monosodium glutamate. Science 164, 719–721.
3. Olney, J. W., and Sharpe, L. G. (1969) Brain lesions in an infant rhesus monkey treated with monosodium glutamate. Science 166, 386–388.
4. Choi, D. W. (1988) Glutamate neurotoxicity and diseases of the nervous system. Neuron 1, 623–634.
5. Lau, A., and Tymianski, M. (2010) Glutamate receptors, neurotoxicity and neurodegeneration. Pflugers Arch. 460, 525–542.
6. Mehta, A., Prabhakar, M., Kumar, P., Deshmukh, R., and Sharma, P. L. (2013) Excitotoxicity: bridge to various triggers in neurodegenerative disorders. Eur. J. Pharmacol. 698, 6–18.
Glutamate Excitotoxicity and Mitochondrial Dysfunction

cells. Mol. Biol. Cell 10, 4403–4417

51. Li, H., Alavian, K. N., Lazzaro, E., Mehta, N., Jones, A., Zhang, P., Licznar-
ski, P., Graham, M., Uo, T., Guo, J., Rahner, C., Duman, R. S., Morrison,
R. S., and Jonas, E. A. (2013) A Bcl-xL-Drp1 complex regulates synaptic
vesicle membrane dynamics during endocytosis. Nat. Cell Biol. 15,
773–785

52. Frank, S., Gaume, B., Bergmann-Leitner, E. S., Leitner, W. W., Robert,
E. G., Catez, F., Smith, C. L., and Youle, R. J. (2001) The role of dynamin-
related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev.
Cell 1, 515–525

53. Karbowski, M., Lee, Y. I., Gaume, B., Jeong, S. Y., Frank, S., Nechushtan, A.,
Santel, A., Fuller, M., Smith, C. L., and Youle, R. J. (2002) Spatial and
temporal association of Bax with mitochondrial fission sites, Drp1, and
Mfn2 during apoptosis. J. Cell Biol. 159, 931–938

54. Yu, T., Robotham, J. L., and Yoon, Y. (2006) Increased production of
reactive oxygen species in hyperglycemic conditions requires dynamic
change of mitochondrial morphology. Proc. Natl. Acad. Sci. U.S.A. 103,
2653–2658

55. Hou, S. T., Jiang, S. X., Desbois, A., Huang, D., Kelly, J., Tessier, L.,
Karchewski, L., and Kappler, J. (2006) Calpain-cleaved collapsin response
mediator protein-3 induces neuronal death after glutamate toxicity and
cerebral ischemia. J. Neurosci. 26, 2241–2249

56. 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+/Ca2+ exchanger in excitotoxicity. Cell 120, 275–285

57. Hou, S. T., and MacManus, J. P. (2002) Molecular mechanisms of cerebral
ischemia-induced neuronal death. Int. Rev. Cytol. 221, 93–148

58. Martorell-Riera, A., Segarra-Mondejar, M., Muñoz, J. P., Ginet, V., Ollo-
quequé, J., Pérez-Clausell, J., Palacín, M., Reina, M., Puyal, J., Zorzano, A.,
and Soriano, F. X. (2014) Mfn2 downregulation in excitotoxicity causes
mitochondrial dysfunction and delayed neuronal death. EMBO J. 33,
2388–2407

59. Liu, W., Acin-Perez, R., Geggman, K. D., Manfredi, G., Lu, B., and Li, C.
(2011) Pink1 regulates the oxidative phosphorylation machinery via mi-
 tochondrial fission. Proc. Natl. Acad. Sci. U.S.A. 108, 12920–12924

60. Cogliati, S., Frezza, C., Soriano, M. E., Varanita, T., Quintana-Cabrera, R.,
Corrado, M., Cipolat, S., Costa, V., Casarin, A., Gomes, L. C., Perales-
Clemente, E., Salvati, L., Fernandez-Silva, P., Enriquez, J. A., and Scorr-
rano, L. (2013) Mitochondrial cristae shape determines respiratory chain
supercomplexes assembly and respiratory efficiency. Cell 155, 160–171

64. Kim, M. J., Jo, D. G., Hong, G. S., Kim, B. J., Lai, M., Cho, D. H., Kim, K. W.,
Bandyopadhyay, A., Hong, Y. M., Kim, D. H., Cho, C., Liu, J. O., Snyder,
S. H., and Jung, Y. K. (2002) Calpain-dependent cleavage of cain/cabin1
activates calcineurin to mediate calcium-triggered cell death. Proc. Natl.
Acad. Sci. U.S.A. 99, 9870–9875

65. Hossain, M. I., Roulston, C. L., Kamaruddin, M. A., Chu, P. W., Ng, D. C.,
Dusting, G. J., Bjorge, J. D., Williamson, N. A., Fujita, D. J., Cheung, S. N.,
Chan, T. O., Hill, A. F., and Cheng, H. C. (2013) A truncated fragment of
Src protein kinase generated by calpain-mediated cleavage is a mediator of
neuronal death in excitotoxicity. J. Biol. Chem. 288, 9696–9709

66. Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, I., Alnemri, E. S.,
Baehrecke, E. H., Blagosklonny, M. V., El-Deiry, W. S., Golstein, P., Green,
D. R., Hengartner, M., Knight, R. A., Kumar, S., Lipton, S. A., Malorni, W.,
Nuñez, G., Peter, M. E., Tschopp, J., Yuan, J., Piacentini, M., Zhivotovsky,
B., Melino, G., and Nomenclature Committee on Cell Death 2009 (2009)
Classification of cell death: recommendations of the Nomenclature Com-
mittee on Cell Death 2009. Cell Death Differ. 16, 3–11