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Blockade of ionotropic glutamate receptors produces neuronal apoptosis through the Bax-cytochrome C-caspase pathway: the causative role of Ca^{2+} deficiency

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Abstract

Blockade of ionotropic glutamate receptors induces neuronal cell apoptosis. We investigated if mitochondria-mediated death signals would contribute to neuronal apoptosis following administration of glutamate antagonists. The administration of MK-801 and CNQX (MK-801/CNQX), the selective antagonists of *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors, produced widespread neuronal death in neonatal rat brain and cortical cell cultures. MK-801/CNQX-induced neuronal apoptosis was prevented by zVAD-fmk, a broad inhibitor of caspases, but insensitive to inhibitors of calpain or cathepsin D. Activation of caspase-3 was observed within 6–12 h and sustained over 36 h after exposure to MK-801/CNQX, which cleaved PHF-1 tau, the substrate for caspase-3. Activation of caspase-3 was blocked by high K^+ and mimicked by BAPTA-AM, a selective Ca^{2+} chelator. Reducing extracellular Ca^{2+}, but not Na^+, activated caspase-3, suggesting an essential role of Ca^{2+} deficiency in MK-801/CNQX-induced activation of caspases. Cortical neurons treated with MK-801/CNQX triggered activation of caspase-9, release of cytochrome *c* from mitochondria, and translocation of Bax into mitochondria. The present study suggests that blockade of ionotropic glutamate receptors causes caspase-3-mediated neuronal apoptosis due to Ca^{2+} deficiency that is coupled to the sequential mitochondrial death pathway.

Keywords: apoptosis, Bax, calcium, cytochrome *c*, glutamate, mitochondria.

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Excitatory glutamatergic transmission is mediated through ionotropic glutamate receptors sensitive to *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate and abnormally increased under pathological conditions such as hypoxic-ischemia, traumatic brain injury, or epilepsy, which result in influx of Ca^{2+} through the receptors (Choi 1988; Choi and Rothman 1990) and can trigger neuronal death (Nadler *et al*. 1978; Choi *et al*. 1987; Faden *et al*. 1989). NMDA receptor-mediated synaptic transmission is also essential for the process of development and survival of central neurons. Activation of NMDA receptors enhances differentiation and survival of cerebellar granule neurons (Moran and Patel 1989). Administration of NMDA receptor antagonists produces neuronal death in various brain areas of developing rat, olfactory bulb of adult rat and primary cortical cell cultures that reveals hallmark of apoptosis (Hwang *et al*. 1999; Ikonomidou *et al*. 1999; Takadera *et al*.

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Abbreviations used: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Ara C, cytosine arabinofuranoside; Apaf-1, apoptotic protease-activating factor-1; BAPTA-AM, 1,2-bis (2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid, tetrakis (acetoxymethyl) ester; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DEVD-amc, Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarine; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a.d] cyclohepten-5,10-imine-maleate; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; zVAD-fmk, z-Val-Ala-DL-Asp-fluoromethylketone.

1999; Fiske and Brunjes 2001). NMDA receptor antagonists appear to increase slowly progressive neurodegeneration in mature brain (Ikonomidou *et al*. 2000b). Transient exposure to ethanol, an antagonist of NMDA receptors and an agonist of $GABA_A$ receptors, results in widespread apoptotic neurodegeneration in the developing rat brain that may underlie brain damage and dysfunction in patients with human fetal alcohol syndrome (Ikonomidou *et al*. 2000a).

Evidence has accumulated demonstrating that reduced excitatory glutamatergic transmission also contributes to neuronal death in the process of aging and neurodegenerative diseases such as Alzheimer's disease (AD). The binding sites of NMDA receptors are consistently decreased in the cerebral cortex and hippocampal formation with aging (Gonzales *et al*. 1991; Tamaru *et al*. 1991; Wenk *et al*. 1991; Magnusson and Cotman 1993), possibly due to decreased expression of NR1 and NR2B subtypes (Magnusson 2000). The age-related change of NMDA receptor composition can result in reduced NMDA responses (Baskys *et al*. 1990). Levels of mRNA and binding sites for NMDA, AMPA, and kainate receptors are decreased in AD brain (Pellegrini-Giampietro *et al*. 1994; Ulas and Cotman 1997; Wakabayashi *et al*. 1999; Ginsberg *et al*. 2000). AMPA receptors become less responsive in cells expressing a mutant PS1 and can undergo caspase-mediated proteolysis in AD (Yasuda *et al*. 1995; Chan *et al*. 1999; Moerman and Barger 1999). Glutamatergic neurons in layer II of the entorhinal cortex are selectively destroyed at the early stage of AD (Gomez-Isla *et al*. 1996). Accordingly, the level of glutamate is markedly reduced in the target areas of the entorhinal cortex in subjects with AD (Hyman *et al*. 1987).

We reasoned that dysfunction of excitatory transmission through both NMDA and AMPA/kainate receptors would contribute to neuronal death associated with development, aging, and AD. The present study was performed to examine how blockade of ionotropic glutamate receptors, turning to (+)-5-methyl-10,11-dihydro-5H-dibenzo[a.d] cyclohepten-5,10-imine-maleate (MK-801) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) induces neuronal death *in vivo* and *in vitro.* Moreover, we investigated the possibility that mitochondria-dependent apoptosis pathway and Ca^{2+} would mediate MK-801 and CNQX-induced neuronal death.

Materials and methods

Materials

NMDA, MK-801, CNQX, and AMPA were obtained from RBI (Natick, MA, USA); cycloheximide was purchased from Sigma (St Louis, MO, USA); Trolox was obtained from Aldrich (Milwaukee, WI, USA); z-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk) was obtained from Enzyme System Products (Livermore, CA, USA); Trypan blue stain 0.4% was purchased from Gibco-BRL (Grand Island, NY, USA); Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (DEVD-amc) was obtained from Bachem (Bubendorf, Switzerland); terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) kit was purchased from Oncogene (Boston, MA, USA).

Primary mouse cortical cell cultures

Mouse cortical cell cultures were prepared as previously described (Noh and Gwag 1997). Animals were handled in accordance with a protocol approved by our institutional animal care committee. Mouse cerebral cortices were removed from brains of the 14-day-old fetal mice, gently triturated with a large-bore Pasteur pipette three to four times, dissociated into individual cells using a small-bore Pasteur pipette, and plated on six- or 24-well culture plates (five hemispheres/plate, approximately 2.5×10^5 cells/plate) pre-coated with poly D-lysine (100 μg/mL) and laminin (4 μg/mL). Plating media consisted of Eagle's minimal essential media (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% horse serum, 5% fetal bovine serum, 2 mM glutamine, and 21 mM glucose. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Cytosine arabinofuranoside (final concentration, 10 μM) was added to cultures at 7–9 days *in vitro* (DIV 7–9) where glia became confluent underneath neurons. Two days later, cultures were shifted into a growth medium identical to the plating medium but lacking fetal serum. All experiments were performed at DIV 10–12.

Induction and analysis of neuronal injury

Experiments were performed in culture medium (CM) containing MEM (bicarbonate-free, 11700-010) supplemented with 26.6 mM bicarbonate and 21 mM glucose. Cortical cell cultures (DIV 10–12) were washed with CM and then exposed to a sham wash control or 10 μM MK-801 plus 50 μM CNQX (MK-801/CNQX), alone or with additions of various drugs. All experiments were performed in the presence of 100 μM trolox, a vitamin E analogue, that prevents oxidative stress that is often produced 1–2 days following a sham wash. To analyze neuronal death, cultures were stained with 0.4% trypan blue and neuronal death was analyzed by counting viable neurons that exclude trypan blue, mean ± SEM (n = 16–24 fields randomly chosen from four wells per condition), and comparing to sham wash controls (= 0%).

Subcelluar fractionation

Cells were resuspended in an isotonic buffer containing 10 mM HEPES pH 8.0, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DL-dithiothreitol (DTT), 2 mM phenylmethylsulfonyl fluoride (PMSF), 100 μg/mL leupeptin, 10 μg/mL pepstatin A, and homogenized using 26-G syringe needle. Nuclei and unbroken cells were removed by centrifugation at 600 g for 10 min. The supernatant was centrifuged at 100 000 g for 30 min to collect the cytosolic fractions. The cytosolic fraction was subjected to western blot in order to analyze cytoplasmic levels of cytochrome c or Bax.

Western blot analysis

Cultures were lysed in a lysis buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 1 mM PMSF, 10 μg/mL pepstatin A, and 100 μg/mL leupeptin. Tissues were lysed in a

lysis buffer containing 10 mM Tris–HCl pH 7.5, 50 mM NaCl, 1% Triton X-100, 30 mM sodium pyrophosphate, 50 mM NaF, 5 μM $ZnCl_2$, 1 mM PMSF, 10 μg/mL pepstatin A, and 100 μg/mL leupeptin. Cell lysates were centrifuged at 13 000 *g* for 10 min, the supernatants were collected, subjected to electrophoresis on a 10–15% SDS–polyacrylamide gel, and transferred to a nitrocellulose membrane. The blot was incubated in 5% non-fat dry milk for 1 h, reacted with primary antibodies for overnight at 4°C, and then incubated with a biotinylated anti-mouse or rabbit secondary antibodies for 2 h. Signals were detected using the Vectastatin ABC kit (Vector Laboratories, Burlingame, CA, USA) and luminol for enhanced chemiluminescence (Amersham, Buckinghamshire, UK), and then analyzed using Kodak X-Omat film or an image analyzer LAS1000 (Fuji Photo Film, Tokyo, Japan). The PHF-1 tau monoclonal antibody recognizes tau peptide phosphorylated at Ser396 or Ser404 (a gift from Dr Peter Davies, Department of Pathology, Albert Einstein College of Medicine, Bronx, NY, USA). The rabbit polyclonal antibodies for caspase-3 and -9 recognize active fragment of each enzyme (New England Biolabs, Beverly, MA, USA). The cytochrome *c* monoclonal and Bax polyclonal antibodies were obtained from Pharmingen (San Diego, CA, USA). The rabbit polyclonal antibody for actin was purchased from Sigma (St Louis, MO, USA).

Immunocytochemistry

Cortical cell cultures (DIV 10–12) grown on a glass-bottom dish were washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde, washed three times with Tris-buffered saline (TBS; 100 mM Tris–HCl, pH 7.6, and 0.9% NaCl), and incubated with 5% goat serum and 0.3% Triton X-100 in TBS for 30 min at room temperature (RT). Cultures were then reacted with anticleaved capase-3 antibody (1 : 100 dilution) for overnight at 4°C. Cultures were incublated in a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1 : 150 dilution) for 2–4 h and then examined using confocal microscope (Bio-Rad, North Yorkshire, UK).

Caspase activity assay

Cortical cell cultures were incubated in an assay buffer (50 mM Tris–HCl, 4 mM DTT, 2 mM EDTA, 10% glycerol, and 0.1% Triton X-100) containing 20 μM DEVD-amc, a fluorogenic substrate for caspase-3-like proteases. Activity of caspase-3-like proteases was determined by measuring a fluorescence signal (Ex = 360 nm and Em = 460 nm) of the cleaved substrate using a FL-600 microplate fluorescence reader (Bio-TEK, Winooski, VT, USA).

Calcium imaging

Levels of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) were analyzed using a calcium-sensitive fluorescence dye fura-2 (Seo *et al*. 1999). Cortical cell cultures (DIV 11) grown on a glass-bottom dish were loaded with 5 μM fura-2 AM plus 2% Pluronic F-127 for 30 min at RT. Cultures were washed with a slat solution containing 120 mM NaCl, 5 mM KCl, 2.3 mM $CaCl_2$, 15 mM glucose, 20 mM HEPES, and 10 mM NaOH, pH 7.4. Cultures were then treated with 10 μM MK-801 and 50 μM CNQX for the indicated points of time. The fluorescent signal was observed on the stage of a Zeiss Axiovert S100 inverted microscope equipped with a 40× objective lens and a filter (Ex = 340/380 nm, Em = 520 nm) at interval of 3 min. Fura-2 ratio ($F340/F_{380}$) images were acquired from a digital camera (DVC-1310, DVC Co., Austin, TX, USA) and were processed using an imaging system (Empix, Mississauga, ON, USA).

Neurotoxicity of MK-801 and CNQX *in vivo*

Post-natal 7-day-old Sprague–Dawley rats (15–20 g, Korea Institute of Science and Technology, Seoul, Korea) were intraperitoneally injected with saline or a combination of MK-801 (1 or 10 mg/kg) and CNQX (5 or 50 mg/kg). Blood gases, pH, and glucose were determined at 0 min, 10 min, 30 min, and 24 h after the injection of MK-801 and CNQX. Animals were decapitated 24 h later. Brains were removed, quickly frozen on dry ice, stored at −70°C, and sectioned at a thickness of 16 μm on a cryostat (Leica, Nussloch, Germany). Sections were thaw-mounted onto gelatin-coated slides and stored at −70°C.

To identify degenerating cells after the systemic administration of MK-801 and CNQX, the TUNEL method was applied (Won *et al*. 2000). Brain sections were fixed in 3% paraformaldehyde, and reacted with digoxigenin–dUTP and terminal deoxynucleotidyl transferase in a humid incubator, and then incubated with antidigoxigenin–peroxidase antibody. The degenerating cells were visualized using 0.05% diaminobenzidine and methyl green.

Results

Administration of MK-801 and CNQX induces neuronal death *in vivo* and *in vitro*

We first examined if systemic administration of NMDA and AMPA/kainate antagonists would produce neuronal death in neonatal rat (post-natal day 7). The intraperitoneal injections of 10 mg/kg MK-801 plus 50 mg/kg CNQX showed TUNEL-positive neurons throughout forebrain 24 h later. In particular, massive neuronal death was prominent in the cingulate cortex, hippocampal formation, and thalamus (Fig. 1). There were no changes in O_2, CO_2, pH, and glucose concentration in blood following administration of MK-801 and CNQX (Table 1). We performed additional

Table 1 Physiologic variables following the systemic injections of MK-801 and CNQX

Physiologic parameter	0 min	10 min	30 min	24 h
PaO_2 (mmHg)	112.35 ± 3.67	120.93 ± 5.08	120.57 ± 11.74	118.93 ± 5.71
$PaCO_2$ (mmHg)	44.15 ± 1.954	4.15 ± 0.834	5.88 ± 0.70	41.10 ± 1.60
pH	7.42 ± 0.03	7.33 ± 0.05	7.31 ± 0.04	7.34 ± 0.05
Blood glucose (mg/dL)	141.25 ± 15.35	120.80 ± 7.48	135.00 ± 10.42	112.50 ± 5.55

Values are mean ± SD (n = 3–6). MABP, mean arterial blood pressure.

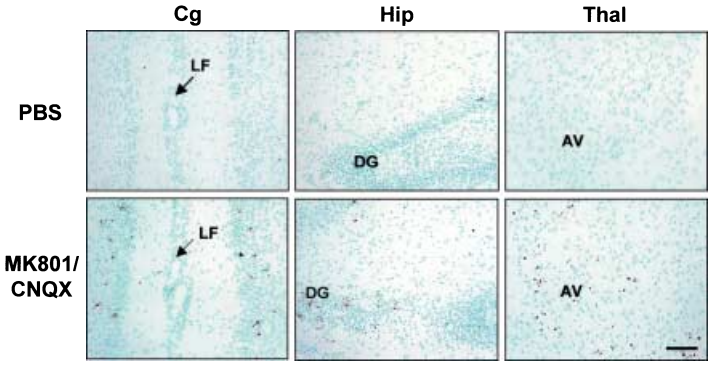

Fig. 1 Cell death following administration of MK-801 plus CNQX in neonatal rats. Bright field photomicrographs of rat brain sections stained by the TUNEL method at 1 day after the intraperitoneal injections of PBS or 10 mg/kg MK-801 plus 50 mg/kg CNQX (MK-801/CNQX). Cg, cingulate cortex; LF, longitudinal fissure; DG, dentate gyrus; Hip, hippocampus; Thal, thalamus; AV, anteroventroal thalamic mucleus. Bar denotes 200 μm.

experiments to delineate mechanisms underlying neurotoxic effects of MK-801 or CNQX in cortical cell cultures. Cortical neurons revealed widespread neuronal death 24 h following exposure to 10 μM MK-801 (Fig. 2a). CNQX alone did not cause neuronal death but significantly increased MK-801-induced neuronal death. Mixed cortical cell cultures (DIV 10–12) exposed to 10 μM MK-801 and 50 μM CNQX (MK-801/CNQX) for 24 h produced neuronal apoptosis that was accompanied by shrinkage of cell body and blocked by cycloheximide, a protein synthesis inhibitor in the absence of glial damage (Fig. 2b). We further tested the possibility that proteases would mediate the glutamate antagonists-induced neuronal apoptosis. Inclusion of 100 μM zVAD-fmk, a broad inhibitor of caspases, significantly reduced glutamate antagonists-induced neuronal apoptosis. However, inclusion of calpain inhibitor III or pepstatin A at various doses that were

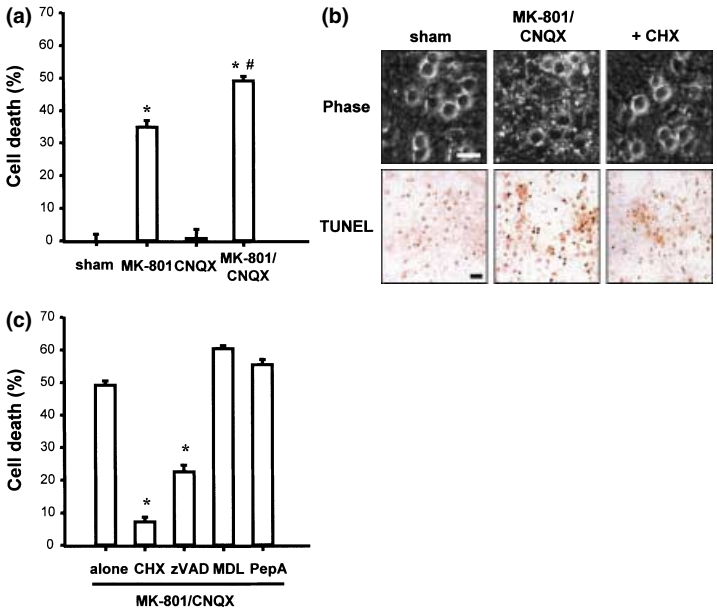

Fig. 2 Neuronal apoptosis following blockade of ionotropic glutamate receptors in cortical cell cultures. (a) Neuronal death was analyzed 24 h after exposure to 10 μM MK-801, 50 μM CNQX, or 10 μM MK-801 plus 50 μM CNQX (MK-801/CNQX) by counting viable neurons excluding trypan blue, mean ± SEM ($n = 32$ fields randomly chosen from eight culture wells per condition). *Significant difference from sham control (sham); #significant difference between groups treated with MK-801 and MK-801 plus CNQX, at $p < 0.05$ using analysis of variance and Student–Neuman–Keuls test. (b) Photomicrographs (upper panel, phase-contrast; lower panel, TUNEL staining) of mixed cortical cell cultures exposed to a sham wash (sham) or 10 μM MK-801 plus 50 μM CNQX for 24 h, alone (MK-801/CNQX) or with 1 μg/mL cycloheximide (+ CHX). Note the shrunken and TUNEL-positive neurons in cortical cell cultures treated with 10 μM MK-801 plus 50 μM CNQX. Bar denotes 30 μm. (c) Neuronal death was analyzed 24 h after exposure to 10 μM MK-801 plus 50 μM CNQX (MK-801/CNQX), alone or with inclusion of 1 μg/mL cycloheximide (CHX), 100 μM zVAD-fmk (zVAD), 1 μM calpain inhibitor III (MDL), or 3 μM pepstatin A (PepA) by counting viable neuron excluding trypan blue, mean ± SEM ($n = 32$ fields randomly chosen from eight culture wells per condition). *Significant difference from relevant control group (MK-801/CNQX, alone), at $p < 0.05$ using analysis of variance and Student–Neuman–Keuls test.

shown to block activity of calpains and cathepsin D, respectively, did not reduce glutamate antagonists-induced neuronal apoptosis (Fig. 2c).

Blockade of ionotropic glutamate receptors induces activation of caspase-3

Additional experiments were undertaken to analyze activity of caspase-3, the downstream caspase responsible for execution of apoptosis, following exposure to MK-801/CNQX. Treatment with the glutamate antagonists increased activation of caspase-3 within 12 h as determined by cleavage of DEVD-amc, a fluorescent substrate of caspase-3-like proteases (Fig. 3a). This activation of caspase-3 was blocked by addition of 100 μM DEVD-CHO or zVAD-fmk (data not shown). Moreover, the active fragment of caspase-3 was observed within 6 h and lasted over the next 36 h following exposure to MK-801/CNQX (Fig. 3b). Immunocytochemistry with anti-activated caspase-3 antibody also revealed activation of caspase-3 in cortical neurons exposed to MK-801/CNQX (Fig. 3c).

We analyzed if treatment with MK-801/CNQX would cleave PHF-1 tau that contains 'DMVD' at amino acid 418–421 sensitive to active caspase-3 (Ko *et al.* 2000). Western blot analysis showed cleavage of PHF-1 tau for 6–36 h following administration of MK-801/CNQX that was blocked by inclusion of z-VAD-fmk, but not by other protease inhibitors (Figs 4a and b). This implies that

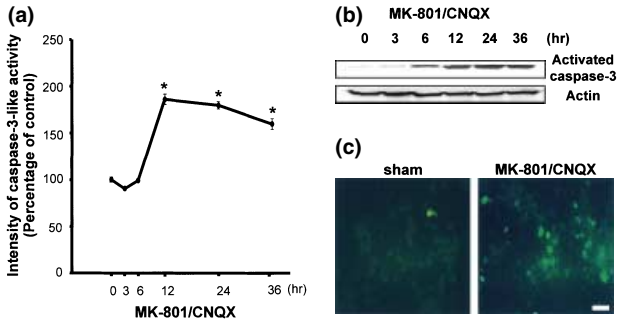

Fig. 3 Activation of caspase-3 following blockade of ionotropic glutamate receptors. (a) Cortical cell cultures (DIV 11) were exposed to 10 μM MK-801 plus 50 μM CNQX (MK-801/CNQX) for indicated points of time. Activity of caspase-3-like proteases was analyzed by measuring fluorescence intensity of amc, the proteolytic fragment of Ac-DEVD-amc, mean ± SEM ($n = 8$ cultures per condition). *Significant difference from control group (0 h), at $p < 0.05$ using analysis of variance and Student–Neuman–Keuls test. (b) Western blot analysis showing activation of caspase-3 with anti-activated caspase-3 (17 kDa) antibodies in cortical cell cultures (DIV 11) treated with 10 μM MK-801 plus 50 μM CNQX (MK-801/CNQX) for indicated points of time. (c) Fluorescence photomicrographs of cortical cell cultures (DIV 11) immunolabeled with anti-activated caspase-3 antibody following exposure to a sham control (sham) or 10 μM MK-801 plus 50 μM CNQX (MK-801/CNQX) for 12 h. Bar denotes 50 μm.

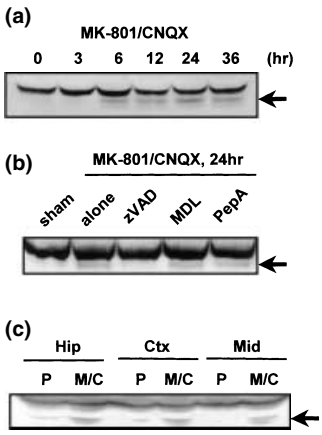

Fig. 4 Cleavage of PHF-1 tau following blockade of ionotropic glutamate receptors. Western blot analysis showing cleavage of PHF-1 tau in cortical cell cultures (a and b) or in various brain areas (c). Arrows indicate cleaved product of PHF-1 tau. (a) Cortical cell cultures (DIV 11) were exposed to 10 μM MK-801 plus 50 μM CNQX (MK-801/CNQX) for indicated points of time. (b) Cortical cell cultures (DIV 11) were exposed to a sham wash (sham) or 10 μM MK-801 plus 50 μM CNQX (MK-801/CNQX) for 24 h, alone or with 100 μM zVAD-fmk (zVAD), 1 μM calpain inhibitor III (MDL), or 3 μM pepstatin A (PepA). (c) Neonatal rats received the intraperitoneal injections of PBS or 10 mg/kg MK-801 plus 50 mg/kg CNQX. After 1 day, the hippocampal formation, neocortex, and midbrain were dissected out. Abbreviations: P, phosphate-buffered saline; M/C, MK-801/CNQX; Ctx, cortex; Hip, hippocampus; Mid, midbrain. Western blot was performed as described in Methods and similar results were obtained from three separate experiments.

MK-801/CNQX-induced activation of caspase-3 induces cleavage of PHF-1 tau protein phosphorylated at Ser396 or Ser404. The proteolytic fragment of PHF-1 tau was also observed in the cingulate cortex, hippocampal formation, and midbrain at 24 h following systemic injections of 10 mg/kg MK-801 plus 50 mg/kg CNQX (Fig. 4c).

MK-801/CNQX-induced Ca^{2+}-deficiency causes activation of caspase-3

Administration of MK-801/CNQX blocks entry of Ca^{2+} and Na^{+} through NMDA and AMPA/kainate receptors, which likely induces activation of caspase-3. Fura-2 fluorescence microphotometry revealed decrease in neuronal $[Ca^{2+}]_i$ within 5 min following exposure of cortical cell cultures to MK-801 and CNQX (Fig. 5A). Decrease in $[Ca^{2+}]_i$ was maintained up to 50 min when neurons remained viable. We then examined if blockade of Ca^{2+} entry mediated MK-801/CNQX-induced activation of caspase-3. Enhancing Ca^{2+} entry by increasing extracellular concentration of K^{+} to 25 mM abrogated effect of MK-801/CNQX that activated caspase-3 (Fig. 5B). Production of active caspase-3 was observed in cortical cell cultures exposed to 1,2-bis (2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid, tetrakis

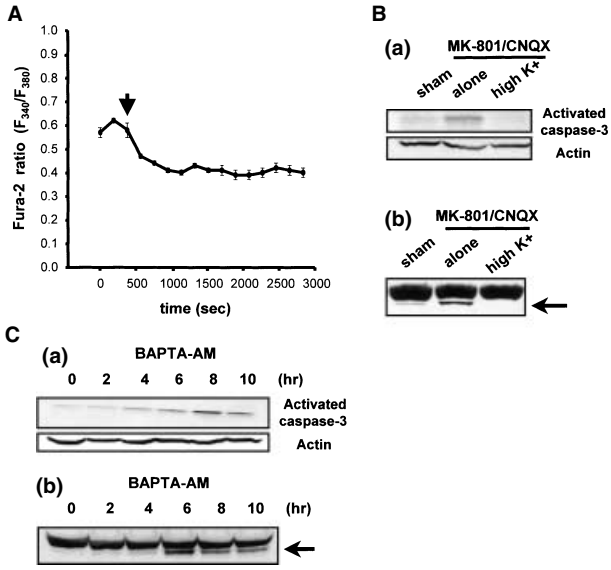

Fig. 5 Decrease in $[Ca^{2+}]_i$ appears to underlie MK-801/CNQX-induced neuronal apoptosis. (A) In cortical cell cultures (DIV 12–14), $[Ca^{2+}]_i$ was analyzed using fura-2 at indicated points of time following exposure to 10 μM MK-801 plus 50 μM CNQX (MK-801/CNQX), mean ± SEM ($n = 50$ randomly chosen neurons from three dishes per condition), scaled to mean neuronal $[Ca^{2+}]_i$ after a sham control. Neuronal $[Ca^{2+}]_i$ after exposure to 10 μM MK-801 and 50 μM CNQX was significantly different from control (t = 0), at $p < 0.05$ using analysis of variance (ANOVA) and Student–Neuman–Keuls test. (B and C) Western blot analysis of cortical cell cultures using anti-activated caspase-3 antibody or anti-actin antibody (a) or anti-PHF-1 antibody (b). Arrows indicate cleaved product of PHF-1 tau. Cultures were exposed to a sham wash (sham) or 10 μM MK-801 plus 50 μM CNQX (MK-801/CNQX), alone or with high K^+ (25 mM) for 12 h. (C) Cultures were exposed to 20 μM BAPTA-AM for indicated points of time. Similar results were obtained from three separate experiments.

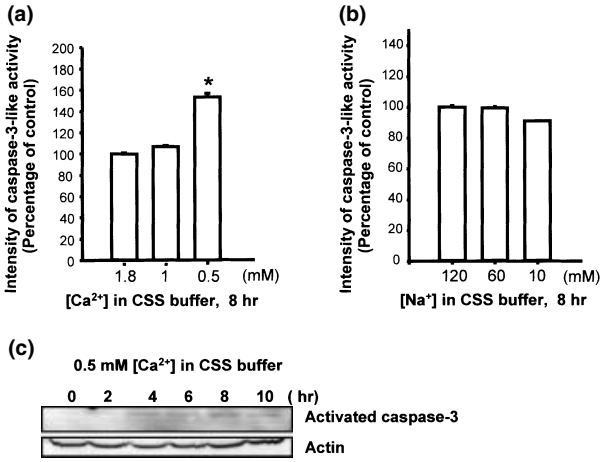

Fig. 6 Low Ca^{2+}-induced activation of caspase-3. (a and b) Cortical cell cultures (DIV 11) were placed in varying concentration of Ca^{2+} (a) or Na^+ (b) for 8 h in CSS buffer and then analyzed for the activity of caspase-3-like proteases, mean ± SEM ($n = 8$ cultures per condition). *Significant difference from relevant control group (1.8 mM Ca^{2+} or 120 mM Na^+), at $p < 0.05$ using analysis of variance and Student–Neuman–Keuls' test. Activity of caspase-3-like proteases was analyzed by measuring fluorescence intensity of amc, the proteolytic fragment of Ac-DEVD-amc. (c) Western blot analysis of activated caspase-3 and actin in cortical cell cultures (DIV 11) after exposure to 0.5 mM $[Ca^{2+}]$ in CSS buffer for indicated points of time.

(acetoxymethyl) ester (BAPTA-AM), an intracellular Ca^{2+} chelator, for 4–10 h. Treatment with BAPTA-AM also caused cleavage of PHF-1 tau within 4 h (Fig. 5C). This suggests that deficiency in $[Ca^{2+}]_i$ is required for activation of casapse-3 in the MK-801/CNQX-induced neuronal apoptosis. This was further supported by increased activity and cleavage of caspase-3 in cortical cell cultures exposed to low concentrations of extracelluar Ca^{2+} for 8 h (Figs 6a and c). In contrast, activity of caspase-3 was not increased in cortical cell cultures treated with low concentrations of extracelluar Na^+ (Fig. 6b).

Involvement of mitochondria in MK-801/CNQX-induced neurotoxicity

Finally, we tested if mitochondria-associated pro-apoptotic molecules would mediate activation of caspase-3. The pro-apoptotic protein Bax is required for neuronal apoptosis that is induced by trophic factor deprivation (Deckwerth *et al.* 1996), oxygen-glucose deprivation (Gottron *et al.* 1997), or β-amyloid (Selznick *et al.* 1999). Bax is translocated from the cytoplasm into mitochondria during apoptosis, which then activates release of cytochrome *c* and target caspases. To examine a potential role of mitochondrial death pathway for MK-801/CNQX-induced neuronal apoptosis, immunoassay for mitochondrial molecules and caspase-9 was performed. Levels of cytochrome *c* in the cytosolic fraction were barely detectable in sham-operated cortical cell cultures. The cytosolic fractions of cytochrome *c* were increased within 6–12 h following exposure to MK-801/CNQX (Fig. 7a). Immunocytochemical analysis demonstrated release of cytochrome *c* from mitochondria as shown by disappearance of punctated forms of cytochrome *c* signal (data not shown). Additional experiments were performed to determine if blockade of ionotropic glutamate receptors could induce translocation of Bax, a pro-apoptotic member of the Bcl-2 family, into mitochondria in cortical cell cultures that would activate release of cytochrome *c*. In normal cortical cell cultures, Bax was highly expressed in the cytosolic fraction. Cortical neurons exposed to MK-801/CNQX showed translocation of Bax into cytosol after 6 h (Fig. 7a). Administration of MK-801/CNQX to cortical cell cultures caused proteolytic cleavage of caspase-9, a downstream substrate of Apaf-1 that is activated by cytochrome *c*, over the next 6–36 h (Fig. 7b).

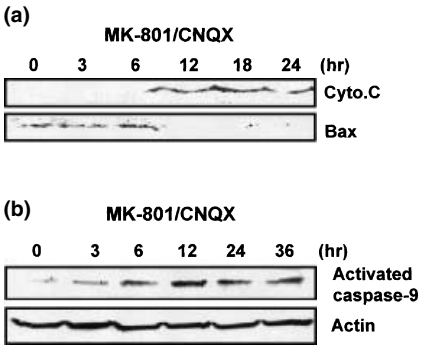

Fig. 7 Translocation of cytochrome *c* and Bax and activation of caspase-9. (a) Western blot analysis of the cytosolic fractions of cortical cell cultures (DIV 11) treated with 10 μM MK-801 plus 50 μM CNQX (MK-801/CNQX) for indicated points of time, using anti-cytochrome *c* (Cyto. *c*) antibody and anti-Bax antibody. (b) Western blot analysis of cleaved caspase-9 (34 kDa) and actin showing proteolytic activation of caspase-9 in cortical cell cultures (DIV 11) treated with 10 μM MK-801 plus 50 μM CNQX (MK-801/CNQX) for indicated points of time. Similar results were obtained from three separate experiments.

Discussion

We report here that administration of ionotropic glutamate receptor antagonists (MK-801/CNQX) induces neuronal apoptosis *in vivo* and *in vitro* through activation of caspase-9 and caspase-3 that is preceded by release of mitochondrial cytochrome *c* and translocation of Bax into mitochondria. Intracellular Ca^{2+} deficiency plays a causative role in MK-801/CNQX-induced activation of caspase-3 and neuronal apoptosis (Fig. 8).

Administration of NMDA receptor antagonists produces neuronal vacuolization and death in adult rats (Fix *et al*. 1993). Apoptosis appears to prevail in NMDA antagonist-induced neuronal death as shown by shrinkage of cell body, aggregated condensation of nuclear chromatin, and sensitivity to inhibitors of protein synthesis (Hwang *et al*. 1999; Ikonomidou *et al*. 1999; Takadera *et al*. 1999). While CNQX alone did not cause neuronal death in cortical cell cultures, it significantly enhanced neuronal apoptosis following exposure to MK-801. In addition, systemic injections of MK-801 and CNQX produced widespread neuronal death in neonatal rats.

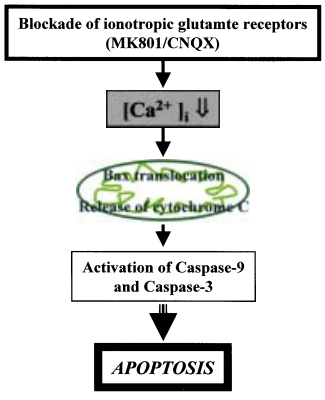

Fig. 8 Scheme of MK-801/CNQX-induced neuronal apoptosis.

Caspase-3, a member of the family of cysteine proteases, mediates neuronal apoptosis that is induced following trophic factor withdrawal, exposures to inhibitors of protein kinases or phosphatases, or hypoxic-ischemic brain injury (Koh *et al*. 1995; Milligan *et al*. 1995; Chen *et al*. 1998; Velier *et al*. 1999). Activated caspase-3 also mediates NMDA receptor antagonist-induced neuronal apoptosis (Hwang *et al*. 1999; Takadera *et al*. 1999; Olney *et al*. 2002). In the present study, activation of caspase-3 was shown to mediate MK-801/CNQX-induced neuronal apoptosis. While influx of Ca^{2+} and Na^{+} through NMDA and AMPA/kainate receptors is interrupted by treatment with MK-801/CNQX, we provide evidence that deficiency in $[Ca^{2+}]_i$ triggers activation of caspase-3 and neuronal apoptosis following blockade of NMDA and AMPA/kainate receptors. First, administration of MK-801/CNQX showed sustained decrease in neuronal $[Ca^{2+}]_i$. Second, raising $[Ca^{2+}]_i$ with treatment of extracellular high $[K^{+}]$ prevented activation of caspase-3, cleavage of PHF-1 tau, and neuronal apoptosis following exposure to MK-801/CNQX. Third, administration of the intracellular Ca^{2+} chelator, BAPTA-AM, resulted in activation of caspase-3. Maneuvers reducing $[Ca^{2+}]_i$, BAPTA-AM or blockers of voltage-gated Ca^{2+} channels, were shown to produce neuronal apoptosis (Koh and Cotman 1992; Han *et al*. 2001). Fourth, activity of caspase-3 was increased by lowering concentration of extracellular Ca^{2+} but not influenced by lowering concentration of extracellular Na^{+}.

Increase in mitochondrial membrane permeabilization leads to release of mitochondrial cytochrome *c* into cytosol where it binds to the CED-4 analog Apaf-1, resulting in proteolytic processing and activation of pro-caspase-9. Active caspase-9 then cleaves pro-caspase-3, which catalyzes degradation of proteins essential for cell survival and function (Li *et al*. 1997; Zou *et al*. 1999). Confocal scanning microscopy and western blotting analysis of cytosolic fraction showed translocation of cytochrome *c* from mitochondria into cytoplasm and subsequent activation of caspase-9 in the early phase of MK-801/CNQX-induced neuronal apoptosis. Furthermore, Bax, a pro-apoptotic member of the Bcl-2 family, was translocated into mitochondria before the translocation of cytochrome *c* in cortical neurons treated with MK-801/CNQX. Mitochondrial Bax was then known to induce release of cytochrome *c* through mitochondrial permeability transition pore (PTP; Jurgensmeier *et al*. 1998). Thus, blockade of ionotropic glutamate receptors induces caspase-3-mediated apoptosis that appears to involve translocation of Bax and cytochrome *c* for activation of the mitochondrial apoptosis pathway.

Although Bax translocation into mitochondria constitutes a key step to neuronal apoptosis, the underlying mechanisms remain to be resolved. In a recent report, increase in intracelluar pH was shown as an essential event for conformational change and mitochondrial translocation of Bax following cytokine withdrawal (Khaled *et al*. 1999). Activation of p38-mitogen-activated protein kinase or cytosolic factors has been proposed to mediate Bax translocation (Nomura *et al*. 1999; Ghatan *et al*. 2000). We found that MK-801/CNQX-induced Bax translocation and neuronal apoptosis were mimicked by BAPTA-AM and prevented by high concentration of extracellular K^+. The latter also blocked Bax translocation into mitochondria and cytochrome *c* release into cytoplasm in sympathetic neurons deprived of nerve growth factor (Putcha *et al*. 1999). This raises a hypothesis that deficiency in $[Ca^{2+}]_i$ may be a key step leading to Bax translocation into mitochondria for execution of apoptosis.

While antagonists of ionotropic glutamate receptors stand out as a promising therapy intervening excitotoxicity or Ca^{2+} overload that leads to neuronal death in various neurological disorders including hypoxic-ischemia and traumatic brain injury (George *et al*. 1988; Tecoma *et al*. 1989), their therapeutic potential should be compromised with pro-apoptotic effects of the antagonists to central neurons. Extensive loss of excitatory glutamate neurotransmission has been well documented at the early stage of AD and may be associated with ongoing neuronal death (Gomez-Isla *et al*. 1996). It is intriguing to postulate that pharmacologically or pathologically interrupted excitatory neurotransmission can result in $[Ca^{2+}]_i$ deficiency-induced neuronal apoptosis that involves activation of mitochondria-mediated pro-apoptotic proteins such as Bax, cytochrome *c*, caspase-9, and caspase-3.

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