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Induced Tolerance to Glutamate Neurotoxicity Through Down-Regulation of NR2 Subunits of N-Methyl-D-Aspartate Receptors in Cultured Rat Striatal Neurons

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We have previously shown differential vulnerabilities to glutamate (Glu) excitotoxicity mediated by the N-methyl-D-aspartate (NMDA) receptor (NMDAR) between rat cortical and rat hippocampal neurons in culture. In this study, we evaluated the possible induced tolerance to NMDA neurotoxicity in cultured rat striatal neurons with prior sustained activation of NMDAR. Brief exposure to Glu or NMDA for 1 hr led to a significant decrease in cellular vitality determined 24 hr later in cultured rat striatal neurons, whereas no marked loss was seen in cellular survival after exposure to Glu or NMDA in striatal neurons previously cultured with Glu or NMDA. Sustained culture with Glu or NMDA invariably led to a significant decrease in protein levels of NR2, but not NR1, subunits without affecting their mRNA levels. Similar induced tolerance was seen to the excitotoxicity of NMDA in hippocampal neurons in a manner sensitive to an NMDAR antagonist. Prior culture with NMDA induced less effective alterations in both intracellular free Ca^{2+} levels and mitochondrial membrane potentials after the addition of NMDA in striatal neurons. However, calpain inhibitor-I significantly prevented the decreased NR2B and NR2C protein levels in striatal neurons cultured with NMDA. These results suggest that prior tonic activation of NMDAR would induce tolerance to the excitotoxicity mediated by NMDAR through a mechanism related to calpain-induced down-regulation of particular NR2 subunits in rat striatal neurons.

Key words: neurotoxicity; tolerance; down-regulation; striatum; NMDA receptor

Glutamate (Glu) is believed to play an excitatory amino acid neurotransmitter role in the mammalian central nervous system (CNS), whereas Glu receptors are categorized into two major subclasses, ionotropic (iGluR) and metabotropic (mGluR) receptors, on the basis of intracellular signal transduction systems as well as gene homology. Among the different iGluR subtypes, N-methyl-D-aspartate (NMDA) receptor (NMDAR) is a subtype consisting of a heteromeric protein complex between different subunits toward the orchestration of a ligand-gated ion channel with higher permeability to Ca^{2+} than Na^{+} ions. Activation of NMDAR leads to an elevation of intracellular free Ca^{2+} concentrations in CNS neurons (MacDermott et al., 1986; Mayer and Westbrook, 1987), which is at least in part responsible for a variety of physiological and pathological events in the brain. These include neuronal development (Scheetz and Constantine-Paton, 1994), neuronal plasticity (Collingridge and Bliss 1995), and delayed neuronal cell death in ischemia (Choi et al., 1988; Lipton, 1999; Sattler and Tymianski, 2000; Bernabeu and Sharp, 2000).

On the other hand, brain ischemia is attributed to blood flow arrest in a variety of pathological situations. Even a brief ischemic insult for 2–3 min could lead to delayed neuronal cell death in certain vulnerable populations of neurons, such as the hippocampal CA1 pyrami-

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dal layer and the amygdala, within several days after the recirculation of blood flow (Smith et al., 1984). The prevailing view is that this delayed neuronal cell death is mediated mainly by excessive accumulation of extracellular Glu through the retrograde operation of different Glu transporters during ischemia (Szatkowski et al., 1990; Mitani et al., 1994). In this ischemic situation, several typical symptoms are seen in a manner dependent on the ischemic severity in vulnerable brain structures (Iritani et al., 1991; Catania et al., 2002). By contrast, a prior mild ischemic insult has been shown to induce ischemic tolerance to subsequent severe ischemia in terms of delayed neuronal cell death in vulnerable hippocampal regions (Kitagawa et al., 1990; Kirino et al., 1991). Although such mild ischemia leads to the neuroprotection through mechanisms relevant to the induction of heat shock protein (Chen et al., 1996), erythropoietin (Ruscher et al., 2002), and hypoxia inducible factor-1 (Bernaudin et al., 2002) in both in vivo and in vitro studies, little attention has been paid to the possible correlation between induced tolerance and desensitization of NMDAR toward internalization after ischemia. In fact, prior activation of NMDAR is shown to result in down-regulation of NMDAR subunit proteins expressed at cellular surfaces in cultured rat cortical neurons (Nakamichi and Yoneda, 2006).

In neuronal circuitry within basal ganglia vulnerable to ischemic insults, moreover, the striatum receives excitatory inputs by Glu from the motor cortex and the thalamus in addition to projecting the major inhibitory output to subcortical structures (Smith and Bolam, 1990). This excitatory Glu input is particularly important for the regulation of dopamine secretion responsible for locomotor activity in the striatum (Burns et al., 1994; Avshalumov et al., 2003), and dopamine could accelerate Glu release for subsequent overactivation of GluR toward neuronal damage in the striatal subfield after brain ischemia (Seki et al., 1999; Misu et al., 2002). Indeed, an intrastriatal injection of Glu results in different biochemical features similar to those associated with Huntington's chorea in the brain (Olney and Gubareff, 1978). These previous findings led us to evaluate the possible occurrence of induced tolerance to the excitotoxicity mediated by NMDAR in rat striatal neurons highly vulnerable to a variety of ischemic insults.

MATERIALS AND METHODS

Materials

Fluo-3 acetoxymethyl ester was provided by Molecular Probes (Eugene, OR). Versene and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco BRL (Grand Island, NY). Poly-L-lysine, cytosine arabinoside (Ara-C), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). The FluorSave reagent was supplied by Calbiochem-Novabiochem (San Diego, CA). Isogen was purchased from Nippon Gene (Tokyo, Japan). Other chemicals used were all of the highest purity available.

Neuronal Cultures

Primary neuronal cultures were prepared from 18-day-old embryonic rat striatum and hippocampus as originally described by di Porzio et al. (1980), with minor modifications. In brief, embryonic rat striatum and hippocampus were dissected and incubated with Versene for 12 min. Cells were then mechanically dissociated with a Pasteur pipette in culture medium and plated at a density of 3.0×10^6 cells on a 9.6-cm^2 well in MS-coated glass-bottom dish (Matsunami Glass, Osaka, Japan) or in a six-well dish (Nunc, Roskilde, Denmark) after counting cell numbers with a Trypan blue exclusion test. Prior to use, six-well dishes were sequentially coated with 75 μg/ml poly L-lysine. Culture medium was DMEM:mutrient mixture F-12 (1:1) with supplementation by 33 mM glucose, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM HEPES, and 10% fetal bovine serum (FBS) for the initial 3 days. To suppress the growth of proliferative cells, Ara-C was added into culture medium from 2–3 days unless otherwise indicated. From day 3, cells were cultured in DMEM with supplementation by 33 mM glucose, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM HEPES, 0.11% sodium bicarbonate, 50 μg/ml transferrin, 500 ng/ml insulin, 1 pM β-estradiol, 3 nM triiodothyronine, 20 nM progesterone, 8 ng/ml sodium seleniate, and 100 μM putrescine in either the presence or the absence of Glu and NMDA at 50 μM for 5 days. Cells were then washed in DMEM with the aforementioned supplementation, followed by exposure to Glu or NMDA at different concentrations for 1 hr. Medium was again changed to DMEM with the routine compositions toward further culture for an additional 24 hr as summarized in Figure 1. The culture medium was usually replaced with freshly prepared culture medium of the same composition every 3 days. Cultures were always maintained at 37°C in a 5% CO_2/95% O_2 air-humidified incubator.

MTT Reduction Assay

Cellular survival was measured by MTT reduction colorimetric assays (Mosmann, 1983). Culture medium was replaced with 10 mM HEPES buffer (pH 7.4) containing 129 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, and 4.2 mM glucose. Cells were then incubated in either the presence or the absence of Glu and NMDA at different concentrations for 1 hr at 37°C unless otherwise indicated, followed by further culture in the routine DMEM medium for an additional 24 hr and subsequent incubation with MTT at 0.05 mg/ml in phosphate-buffered saline (PBS) to form MTT formazan. Then MTT formazan was solubilized by the addition of a lysis solution containing 99.5% isopropanol and 0.04 M HCl. The amount of MTT formazan product was determined by measuring the absorbance at 550 nm on a microplate reader. Relative values were calculated as percentages over the value obtained in the control group.

Immunocytochemistry

For immunocytochemical analysis, cells were plated on a chamber slide (Nunc, Roskilde, Denmark) coated with poly-L-lysine. Cultures were fixed for 20 min with 4% paraformaldehyde in PBS, followed by washing with PBS and

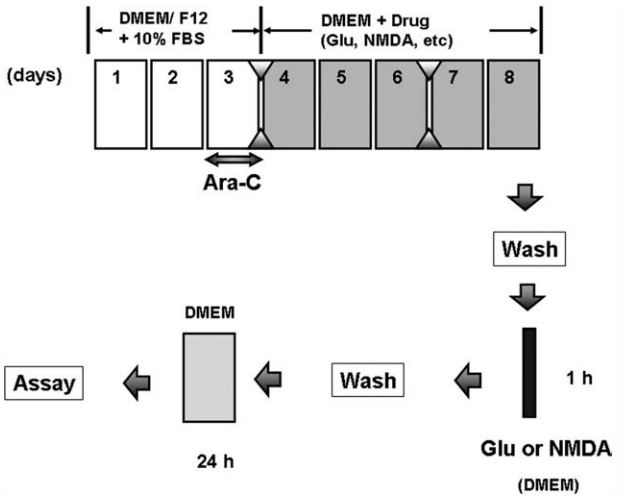

Fig. 1. Experimental protocols. Striatal and hippocampal neurons were cultured in DMEM/F12 containing 10% FBS for the initial 3 days, followed by washing in DMEM and subsequent culture in DMEM in either the presence or tyhe absence of different substances, such as Glu and NMDA, for 5 days. Cells were then washed with DMEM, followed by brief exposure to Glu or NMDA at different concentrations for 1 hr and subsequent washing with DMEM toward further culture for an additional 24 hr.

subsequent treatment with 10% bovine serum albumin (BSA) in PBS containing 0.1% Triton X-100 for 60 min. Cells were then stained with a primary antibody against the neuronal marker protein microtubule-associated protein-2 (MAP-2) diluted with 1% BSA in PBS. Staining was visualized with the anti-mouse IgG conjugated with rhodamine before chamber slides were mounted with the FluorSave reagent. Phase-contrast and fluorescence images were obtained with a Zeiss Axiovert microscope (Carl Zeiss, Thornwood, NY).

Reverse Transcription-Polymerase Chain Reaction Analysis

Total RNA was extracted from rat striatal neurons cultured in either the presence or the absence of Glu and NMDA at 50 μM according to the standard procedure of Isogen, followed by synthesis of cDNA with 500 ng oligo-(dT) primers. The individual cDNA species were amplified in a reaction mixture containing a cDNA aliquot, PCR buffer, dNTPs, the relevant sense and antisense primers, and rTaq DNA polymerase. Primers used were as follows: NR1, 1,033 bp, ACGGAATGATGGGCGAGC, GGCATTCCTTGTGT CGCTTGTAG; NR2A, 422 bp, ATACCGGCAGAACTC CACAC, CTCTTGCTGTCCTCCAGACC; NR2B, 619 bp, CAAAGGAGAACTCGCCTCAC, GTTGGCAAAGG AGCTCTCAC; NR2C, 413 bp, AGACCAATACCC ACCCTTCC, GCCATGTTGTCAATGTCCAG; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 452 bp, ACCACAGTCCATGCCATCAC, TCCACCACCCTGTT GCTGTA. Reactions were initiated by incubating at 94°C for 5 min, and PCR (denaturation at 94°C for 30 sec, annealing at 57°C or 63°C for 1 min, and extension at 72°C for 1 min) was performed for cycles individually determined, with a final extension at 72°C for 7 min. Quantitative analysis was done by the normalization over mRNA expression of the housekeeping gene GAPDH. PCR products were separated on 1.5% agarose gels with ethidium bromide for visualization. The relative abundance of each PCR product was determined by quantitative analysis of digital photographs of gels in Image J software (NIH).

Measurement of Intracellular Free Ca^{2+} Levels

Cultured neurons were washed once with recording medium containing 129 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 4.2 mM glucose, and 10 mM HEPES (pH 7.4), and incubated at 37°C for 1 hr in the recording medium containing 30 nM Pluronic F-127 and 3 μM fluo-3 acetoxymethyl ester, which is a membrane-permeable form of the Ca^{2+}-sensitive dye. Culture dishes were then washed twice with the recording medium and allowed to settle for at least 1 hr in recording medium. The medium was changed once more, followed by addition of NMDA at 100 μM in the absence of $MgCl_2$ and subsequent determination of the fluorescence intensity every 2 min. The fluorescence intensity was normalized after the addition of the Ca^{2+} ionophore A23187 at 10 μM (Nakamichi et al., 2002). Cells were invariably used within 1–5 hr after these procedures for observation of the fluorescence visualized with a confocal laser scanning microscope equipped with an argon laser. Images were obtained with an objective lens with numeral apertures of 0.5 (Plan-Neofluar) for 20-fold magnification. Fluorescence images labeled with fluo-3 were collected using an excitation wavelength of 488 nm. Parameters of illumination and detection were digitally controlled for consistent settings throughout the experiments. Two successive digital images were collected, usually at 512 × 512 pixels in the same visual field (Nakamichi et al., 2002).

Estimation of Mitochondrial Membrane Potential

Measurement of membrane potential ($\Delta\Psi$) was performed according to the procedures previously reported (Duchen and Biscoe, 1992) with minor modifications (Kambe et al., 2008). In brief, striatal cultures were loaded with 10 μg/ml rhodamine-123 for 15 min in recording medium at 37°C prior to extensive washing in the recording medium and mounting on the stage of confocal laser microscope. Medium was replaced with the recording medium without Mg^{2+} for subsequent exposure of cells to 100 μM NMDA for 5 min. The fluorescence intensity was then determined during the exposure to NMDA at an excitation wavelength of 488 nm and an emission wavelength of 530 nm, respectively. Maximum fluorescence intensity was obtained from images by exposing to the mitochondrial oxidative phosphorylation uncoupler 2,4-dinitrophenol at 200 μg/ml for 5 min. Depolarization of mitochondrial membrane was expressed as a function of rhodamine-123 fluorescence $[(F - F_0)/(F_{max} - F_0)]$, where F_{max} is the fluorescence intensity obtained in the presence of 2,4-dinitrophenol at 200 μg/ml.

Western Blotting

Cultured neurons were homogenized in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 1 mM EGTA,

10 mM sodium fluoride, 10 mM sodium β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1 μg/ml of various protease inhibitors [(p-amidinophenyl)-methanesulfonyl fluoride, leupeptin, antipain, and benzamidine], followed by centrifugation at 4°C for 5 min at 15,000*g* as described elsewhere (Manabe et al., 2001). Pellets thus obtained were suspended in 20 mM Tris-HCl buffer (pH7.5). Suspensions were added at a volume ratio of 4:1 with 10 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue and 5% 2-mercaptoethanol, followed by mixing and boiling at 100°C for 10 min. Each aliquot of 10-μg proteins was loaded on a 5% or 7.5% polyacrylamide gel for electrophoresis at a constant current of 15 mA/plate for 2 hr at room temperature and subsequent blotting to a polyvinylidene fluoride membrane previously treated with 100% methanol. After blocking by 5% skimmed milk dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween 20, the membrane was reacted with an antibody against MAP-2, NR1, NR2A, NR2B, NR2C, or β-tubulin diluted with the buffer containing 1% skimmed milk, followed by reaction with an anti-goat IgG antibody conjugated with peroxidase. Proteins reactive with those antibodies were individually detected with the aid of ECL detection reagents through exposure to X-ray films.

Data Analysis

Quantitative densitometric data are expressed as mean ± SEM, and statistical significance was determined by one-way factorial or two-factor repeated-measures of ANOVA with Turkey-Kramer and Student's *t*-test.

RESULTS

Induced Tolerance to Glu Neurotoxicity

Striatal neurons were cultured in DMEM/F12 for the initial 3 days, followed by culture under different conditions for the next 5 days and subsequent brief exposure to Glu at 10–100 μM for 1 hr. Culture medium was then changed to DMEM for determination of MTT reduction 24 hr later. Most cells died during culture in DMEM for the initial 3 days, although no significant cell death was seen after the medium change from DMEM/F12 to DMEM on day 3 for culture up to 8 days (data not shown). In striatal neurons cultured in DMEM for the last 5 days, brief exposure to Glu induced a significant decrease in MTT reduction in a concentration-dependent manner (Fig. 2A, left columns). In cells cultured in DMEM/F12 for the last 5 days, however, Glu was less effective at 100 μM in inhibiting MTT reduction than in cells cultured in DMEM, which occurred irrespective of prior treatment with Ara-C. Brief exposure to Glu at 10 μM did not significantly decrease MTT reduction in striatal neurons with (Fig. 2A, middle columns) or without (Fig. 2A, right columns) Ara-C treatment when cultured in DMEM/F12. Therefore, prior sustained culture in DMEM/F12 would render striatal neurons resistant to the excitotoxicity by subsequent brief exposure to Glu.

Because DMEM/F12 contains Glu at 50 μM, striatal neurons were cultured in DMEM in either the presence or the absence of Glu at 50 μM for the last 5 days. Cells were then exposed to Glu at 10 μM to 1 mM for 1 hr, followed by further culture in DMEM for an additional 24 hr. Brief exposure to Glu led to a significant decrease in MTT reduction in a concentration-dependent manner in striatal neurons cultured in the absence of added Glu but failed to affect MTT reduction significantly in striatal neurons cultured in the presence of 50 μM Glu even at the highest concentration used (Fig. 2B). In striatal neurons cultured in the presence of 50 μM Glu for the last 5 days, a significant decrease was seen in protein levels of NR2 subunits, including NR2A, NR2B, and NR2C, but not in NR1 protein expression, on Western blotting analysis (Fig. 2C). In these cells cultured with Glu, however, no significant changes were found in mRNA expression levels of NR1, NR2A, NR2B, or NR2C subunits on RT-PCR analysis (Fig. 2D).

Possible Involvement of NMDAR

An attempt was next made to determine whether NMDAR is involved in the resistance to Glu excitotoxicity. Striatal neurons were cultured in either the presence or the absence of NMDA at 50 μM for the last 5 days, followed by brief exposure to Glu at 100 μM and subsequent immunocytochemistry for neurons using the anti-MAP-2 antibody. In striatal neurons cultured in the absence of added NMDA, brief exposure to 100 μM Glu markedly reduced the number of MAP-2-positive cells (Fig. 3A, upper panels). In neurons cultured with 50 μM NMDA, however, brief exposure to Glu did not markedly affect the number of MAP-2-positive cells (Fig. 3A, lower panels). Striatal neurons were cultured with NMDA in either the presence or the absence of the NMDAR antagonist MK-801 at 10 nM for the last 5 days, followed by brief exposure to 100 μM Glu for 1 hr and subsequent determination of MTT reduction 24 hr later. As shown in Figure 3B, prior culture with NMDA induced a significant prevention of the loss of MTT reduction in neurons briefly exposed to 100 μM Glu as seen in cells cultured with Glu. This prevention by NMDA was lost in neurons cocultured with MK-801. Brief exposure to Glu led to a significant decrease in MTT reduction in a manner sensitive to antagonism by the NR2B subunit-selective NMDAR antagonist ifenprodil and the general NMDAR antagonist MK-801 in striatal neurons cultured in the absence of added Glu or NMDA (Fig. 3C), whereas brief exposure to NMDA indeed decreased MTT reduction in a concentration-dependent manner, with a maximal decrease of 60% at 100 μM (Fig. 3D). Accordingly, NMDAR seems to be involved in the acquisition of resistance to Glu neurotoxicity in striatal neurons cultured with Glu or NMDA at a relatively low concentration.

To analyze the regional selectivity, both striatal and hippocampal neurons were cultured with NMDA in ei-

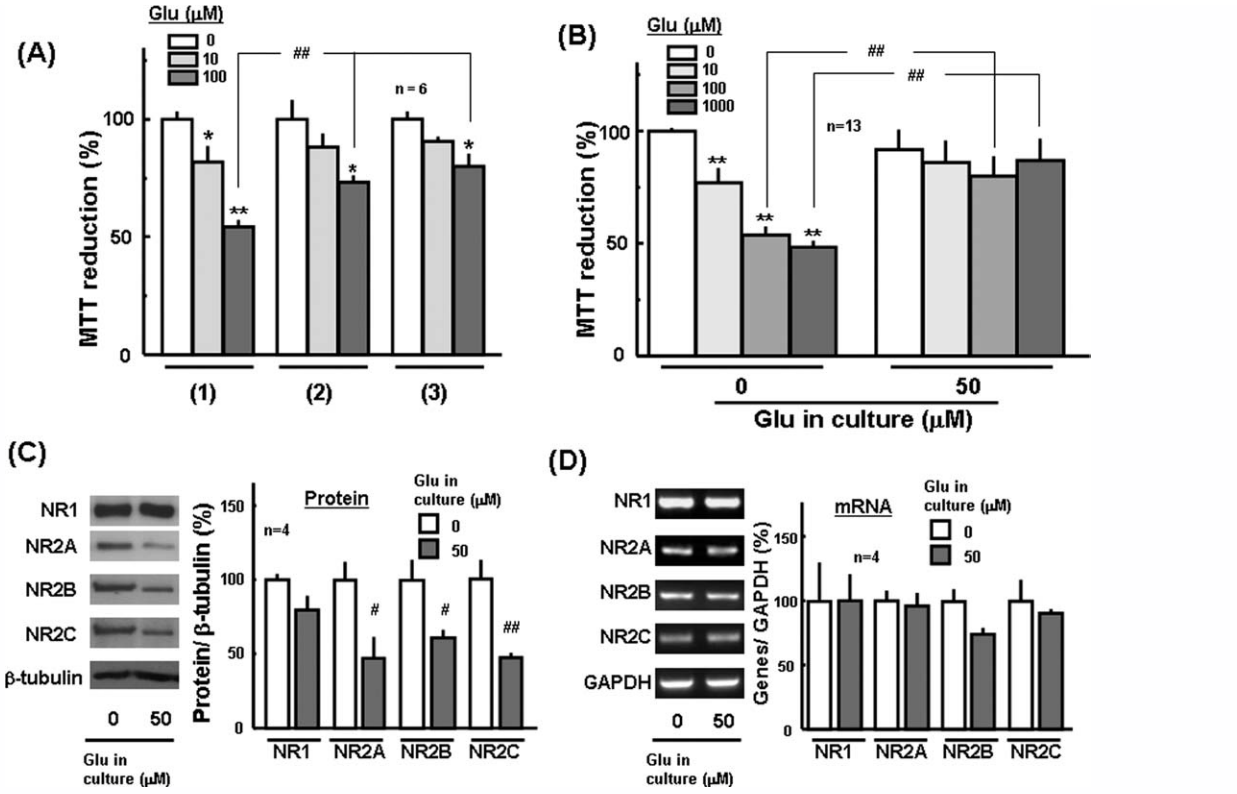

Fig. 2. Effects of culture with Glu on Glu toxicity in striatal neurons. **A:** Striatal neurons were cultured in DMEM/F12 for the initial 3 days, followed by culture in DMEM (1) or DMEM/F12 with (2) or without (3) Ara-C treatment, for the next 5 days. Cells were then exposed to Glu at 10 to 100 μM for 1 hr, followed by determination of MTT reduction 24 hr later. **B:** Striatal neurons were cultured in DMEM in either the presence or the absence of 50 μM Glu for the last 5 days, followed by exposure to Glu at 10–1,000 μM for 1 hr. Striatal neurons were also cultured in either the presence or the absence of 50 μM Glu, followed by Western blotting (**C**) and RT-PCR (**D**) analyses. Values are the mean ± SEM from separate experiments, with the repetition number shown in each panel. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, significantly different from each control value obtained in cells not exposed to Glu after culture. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different from the value obtained in cells cultured in the absence of added Glu.

ther the presence or the absence of MK-801 for the last 5 days, followed by brief exposure to 100 μM NMDA for 1 hr and subsequent determination of MTT reduction 24 hr later. In both striatal and hippocampal neurons cultured with 10 μM NMDA, brief exposure to NMDA inhibited MTT reduction similarly to the inhibition seen in neurons cultured without NMDA (Fig. 4A). When both striatal and hippocampal neurons were cultured with 50 μM NMDA, however, subsequent brief exposure to NMDA led to less effective inhibition of MTT reduction than in neurons cultured without NMDA (Fig. 4A). The resistance by NMDA was significantly prevented in both striatal and hippocampal neurons cocultured with MK-801 at 0.01–0.1 μM (Fig. 4B). Accordingly, the NMDAR-mediated resistance could be seen in both striatal and hippocampal neurons.

Downstream Signals After Activation of NMDAR

Intracellular free Ca^{2+} ions are the major second messenger of NMDA signaling (Manev et al., 1989; Tymianski et al., 1993; Pivovarova et al., 2004), and mitochondria are one of the key mediators leading to neuronal death by Glu in vitro and by brain ischemia in vivo (Stout et al., 1998; Schinzel et al., 2005). From this point of view, we evaluated Ca^{2+} influx and $\Delta\Psi$ in striatal neurons cultured with and without NMDA. The addition of NMDA rapidly increased the fluorescence intensity of fluo-3 as a rexsult of increased intracellular free Ca^{2+} levels, whereas prior culture with NMDA led to a significant decrease in the maximum fluorescence normalized by A23187 from 80% to 60% (Fig. 5A). The addition of NMDA rapidly increased the fluorescence intensity of rhodamine-123 resulting from the disruption of $\Delta\Psi$, similarly, whereas a significantly less effective increase was seen in rhodamine-123 fluorescence in striatal neurons cultured with NMDA for the last 5 days than that seen in cells cultured without NMDA (Fig. 5B).

Expression of NMDAR Subunits

To evaluate the mechanism underlying the acquired resistance mediated by NMDAR, striatal neurons were cultured with 50 μM NMDA for the last 5 days, followed by determination of protein levels of dif-

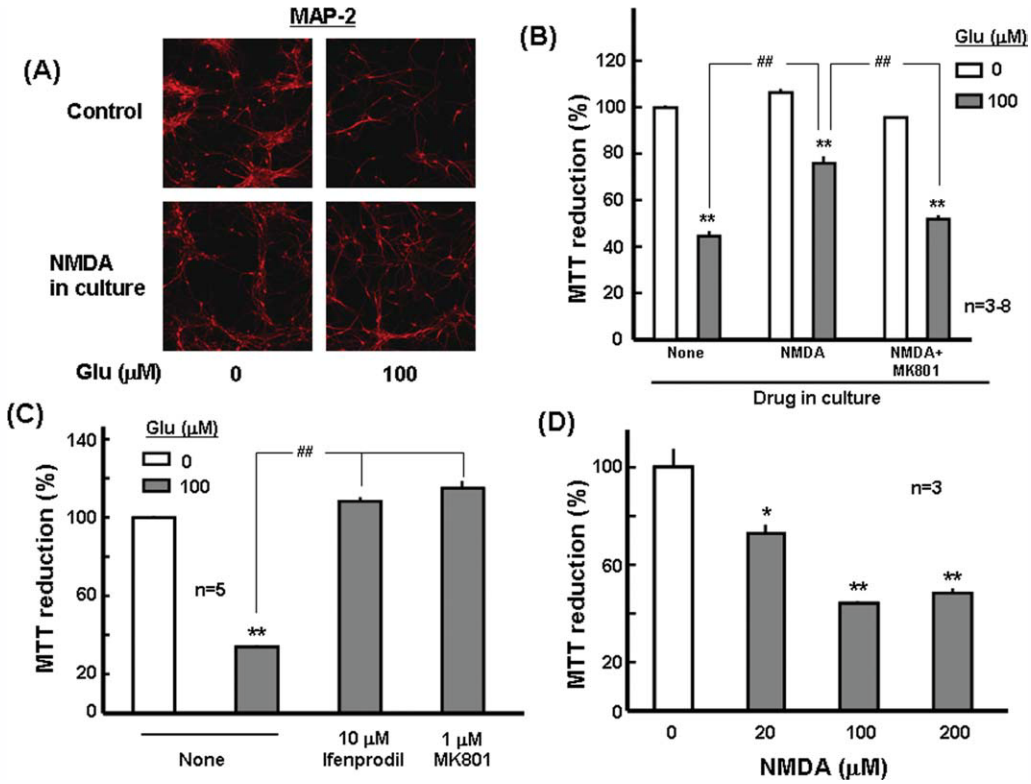

Fig. 3. Effects of culture with NMDA on cell survival in striatal neurons. **A:** Striatal neurons were cultured in either the presence or the absence of 50 μM NMDA, followed by brief exposure to 100 μM Glu for 1 hr and subsequent immunocytochemistry 24 hr later. Typical pictures are shown, with similar results in three separate experiments. **B:** Striatal neurons were cultured with 50 μM NMDA in either the presence or the absence of 10 nM MK-801, followed by exposure to 100 μM Glu for 1 hr. $^{\#\#}P < 0.01$, significantly different from the value in cells cultured in either the presence or the absence of NMDA. Striatal neurons cultured for 8 days were also exposed to 100 μM Glu in either the presence or the absence of 1 μM MK-801 and 10 μM ifenprodil (**C**) or NMDA at 10–200 μM (**D**) for 1 hr. Values are the mean ± SEM from separate experiments, with the repetition number shown in each panel. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from each control value obtained in cells not exposed to Glu or NMDA after culture. $^{\#\#}P < 0.01$, significantly different from the value in cells not exposed to an NMDAR antagonist. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ferent NR subunits in addition to MAP-2. A significant decrease was seen in protein levels of NR2 subunits, including NR2A, NR2B, and NR2C, in striatal neurons cultured with NMDA for 5 days on Western blotting analysis (Fig. 6A). However, prior culture with NMDA induced significant changes in protein expression levels of neither NR1 subunit nor MAP-2. As seen with cells cultured with Glu (Fig. 2D), moreover, no marked changes were found in mRNA expression levels of NR1, NR2A, NR2B, and NR2C subunits in neurons cultured with NMDA for the last 5 days on RT-PCR analysis (Fig. 6B). Therefore, the acquired resistance would at least in part involve the down-regulation of NR2 protein expression in response to prior tonic activation of NMDAR in cultured striatal neutrons.

Effect of A Calpain Inhibitor on NMDAR Subunit Expression

In previous studies using cortical neurons, the Ca^{2+}-dependent protease calpain has been shown to be responsive to Ca^{2+} currents across NMDAR channels to truncate the NMDAR molecule itself (Wu et al., 2005; Amadoro et al., 2006). To evaluate the possible involvement of calpain in the acquired resistance, striatal neurons were cultured with NMDA in either the presence or the absence of calpain inhibitor-I. However, calpain inhibitor-I was quite cytotoxic to striatal neurons when applied from 3 to 8 days at 1 μM (data not shown). To analyze the minimum culture period required for preconditioning by NMDA, NMDA at 50 μM was added to culture medium different days after the initiation of culture up to 8 days. As shown in Figure 7A, NMDA was effective in significantly inducing the acquired resistance to neurotoxicity by subsequent brief exposure to NMDA when incubated with striatal neurons for the last 1–5 days during culture for 8 consecutive days. Neurons were rendered resistant to NMDA excitotoxicity even when previously cultured with NMDA for 1 day. In subsequent experiments, therefore, neurons were cultured with NMDA for 1 day from 7 to 8 days in culture.

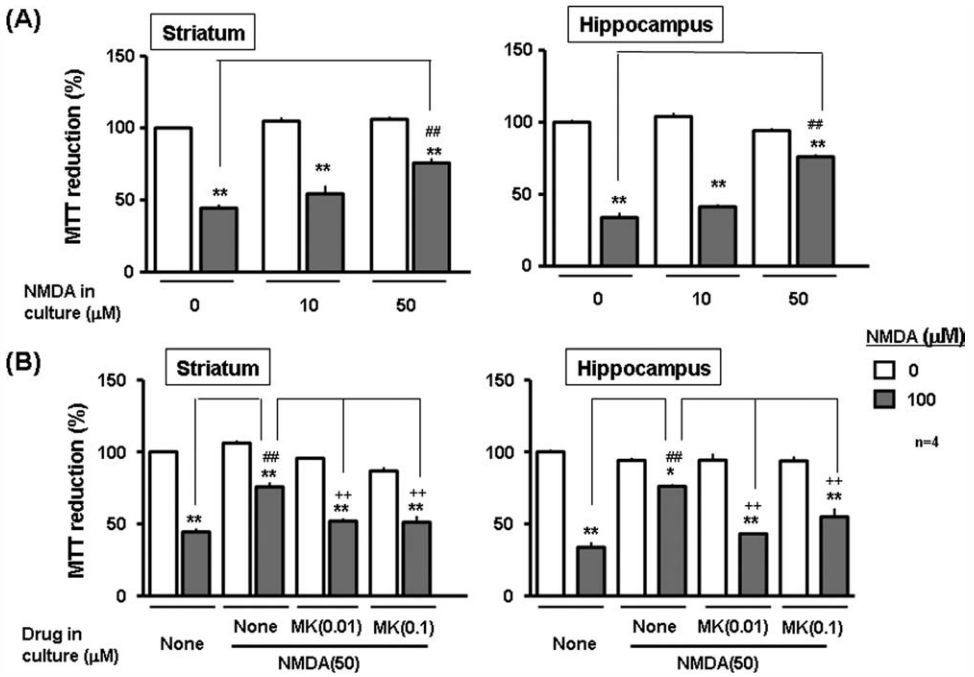

Fig. 4. Effects of culture with MK-801 on cellular survival in striatal and hippocampal neurons. **A:** Striatal and hippocampal neurons were cultured in either the presence or thde absence of NMDA at 10–50 μM, followed by brief exposure to 100 μM NMDA for 1 hr. **B:** Striatal and hippocampal neurons were cultured in either the presence or the absence of NMDA at 50 μM and MK-801 at 10–100 nM, followed by brief exposure to 100 μM NMDA for 1 hr. Values are the mean ± SEM from four separate experiments. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, significantly different from each control value obtained in cells not exposed to NMDA after culture. $^{\#\#}P < 0.01$, significantly different from the value in cells not cultured with NMDA. $^{++}P < 0.01$, significantly different from the value in cells cultured with NMDA alone.

Under these culture conditions, NMDA failed to affect significantly the expression levels of NR1 and NR2A subunits in striatal neurons (Fig. 7B), in contrast to cells cultured with NMDA for the last 5 days (Fig. 6A). By contrast, both NR2B and NR2C subunit protein levels were significantly decreased in neurons cultured with NMDA for the last 1 day during culture for 8 days, whereas calpain inhibitor-I significantly prevented this down-regulation of NR2B and NR2C subunit protein expression in a concentration-dependent manner at concentrations of 1–10 μM. (Fig. 7C). Insofar as a significant loss was seen by 30% in MTT reduction in striatal neurons cultured with 5 μM calpain inhibitor-I alone for 1 day from 7 to 8 days (data not shown), however, we were unable to determine further whether a calpain inhibitor indeed prevents the acquired resistance to excitotoxicity mediated by NMDAR when previously cocultured with NMDA.

DISCUSSION

The essential importance of the present findings is that prior sustained culture with Glu and NMDA invariably led to the acquisition of resistance to excitotoxicity by subsequent brief exposure to Glu and NMDA in association with decreased expression levels of particular NR2 subunit proteins in cultured rat striatal neurons. The prevention by NMDAR antagonists gives support to the involvement of NMDAR in mechanisms underlying the acquired resistance to the neurotoxicity mediated by activation of NMDAR. One possible interpretation is that prior tonic mild activation of NMDAR could lead to acquired resistance to subsequent severe excitotoxicity of NMDAR in striatal and hippocampal neurons. Accumulating evidence for similar preconditioning and/or induced tolerance with ischemic neuronal cell death is available in the literature. Short-term mild ischemic insult has been shown to lead to neuroprotection against subsequent severe ischemia through mechanisms relevant to the induction of heat shock protein (Chen et al., 1996), erythropoietin (Ruscher et al., 2002), and hypoxia-inducible factor-1 (Bernaudin et al., 2002) in both in vivo and in vitro studies.

The mechanism seems to at least in part involve down-regulation of corresponding proteins, but not mRNA, for NR2 subunits required for heteromeric assemblies with the essential NR1 subunit toward functional NMDAR channels (Nakamichi et al., 2009). Several independent lines of evidence indicate that NR2B subunit is a determinant of the neurotoxicity after activation of NMDAR. Calcium entry through extrasynaptic NMDAR containing NR2B subunit has been shown to be toxic to neurons, for instance, whereas Ca^{2+} influx through synaptic NMDAR containing NR2A subunit is antiapoptotic (Hardingham and Bading, 2003). Distinct roles are proposed between synaptic and extrasynaptic

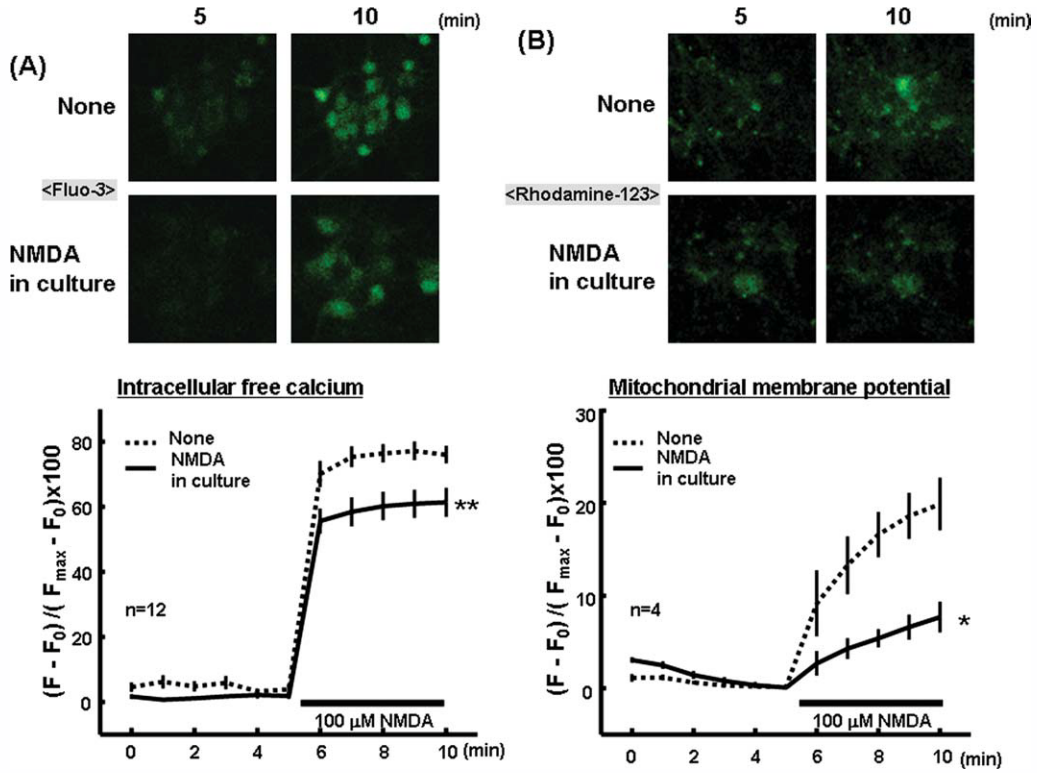

Fig. 5. Effects of culture with NMDA on intracellular signals in striatal neurons. Striatal neurons were cultured in either the presence or the absence of 50 μM NMDA, followed by loading of fluo-3 for intracellular free Ca^{2+} (**A**) or rhodamine-123 for $\Delta\Psi$ (**B**) and subsequent addition of 100 μM NMDA for determination of the fluorescence intensity every 1 min. The individual fluorescence intensity was normalized with the maximal fluorescence obtained 5 min after the addition of 10 μM A23187 for fluo-3 or 200 μg/ml 2,4-dinitrophenol for rhodamine-123. Typical photomicrographs are shown in the upper panels. Values are the mean ± SEM in separate experiments, with the repetition number shown in each panel. $\star P < 0.05$, $\star\star P < 0.01$, significantly different from each control value obtained in cells not cultured with NMDA. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

NMDAR in the Ca^{2+}-dependent neurodegeneration toward excitotoxicity (Sattler and Tymianski, 2001). Given these previous findings along with the present data on the complete prevention by ifenprodil, it is likely that NR2B subunit would be more responsible than NR2A subunit for the neurotoxicity seen after activation of NMDAR in striatal neurons. The fact that short-term preconditioning with NMDA for 1 day led to significant degradation of NR2B and NR2C subunit proteins gives rise to the idea that induced tolerance could at least in part involve the rapid and preferential down-regulation of NR2B subunit in response to prior short-term activation of NMDAR-cultured striatal neurons. The possible role of NR2C subunit in the mechanism underlying the acquisition of induced tolerance after short-term activation of NMDAR, however, was not clarified in the present study.

To our knowledge, this is the first direct demonstration of induced tolerance to the severe excitotoxicity mediated by NMDAR through preferential down-regulation of NR2B subunit expression after preconditioning with prior tonic activation of NMDAR in cultured rat striatal neurons in vitro. The data from experiments on corresponding mRNA expression argue in favor of the idea that NR2 subunit proteins would be predominantly degraded after the entry of Ca^{2+} across NMDAR channels. In a previous in vivo study (Chen et al., 2008), prior administration of a selective blocker of NR2A-containing NMDAR was shown to exacerbate ischemic neuronal damage in rats with transient global ischemia, with prevention by ifenprodil used for the selective blockade of NR2B-containing NMDAR. The present findings that long-term culture with NMDA similarly decreased expression levels of NR2A, NR2B, and NR2C subunit proteins without altering that of NR1 subunit give support to the proposal that rapid responsiveness would be an upstream determinant of induced tolerance against subsequent severe neurotoxicity mediated by NMDAR with regard to down-regulation of particular subunits. The slow down-regulation of antiapoptotic NR2A subunit could lead to a variety of neuronal dysfunctions and/or abnormalities other than induced tolerance after tonic activation of NMDAR for a relatively long period.

From this point of view, it should be emphasized that a calpain inhibitor restored the loss of NR2B

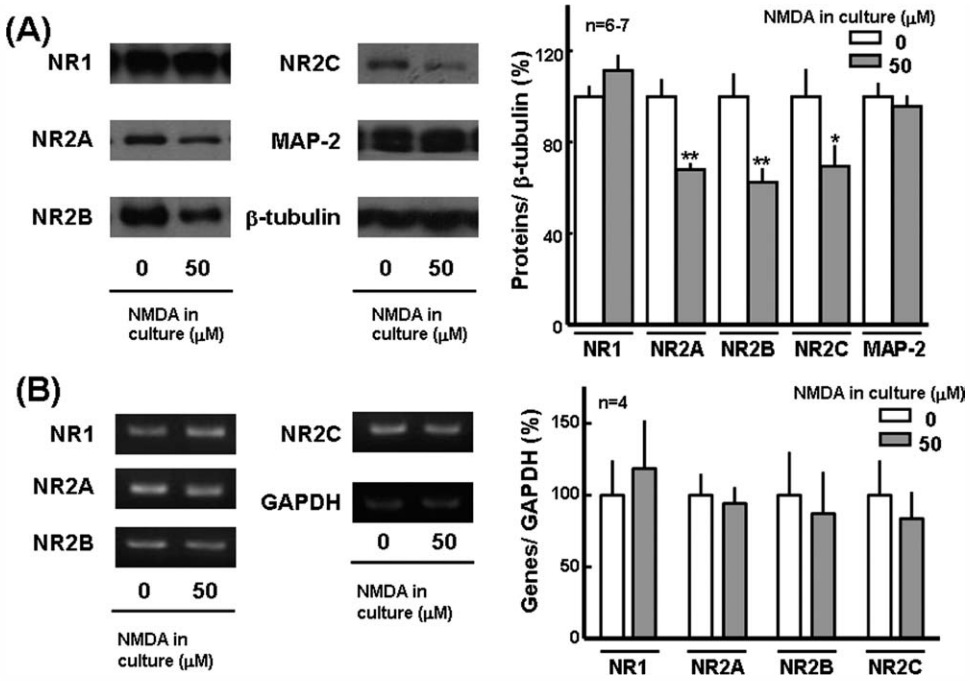

Fig. 6. Effects of culture with NMDA on NMDAR subunit expression in striatal neurons. Striatal neurons were cultured in either the presence or the absence of 50 μM NMDA, followed by Western blotting (**A**) and RT-PCR (**B**) analyses. Typical pictures are shown in the left panels, and quantitative data are shown in the right panels as the mean ± SEM of percentages over expression of β-tubulin or GAPDH in six or seven separate experiments. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, significantly different from each control value in cells not cultured with NMDA.

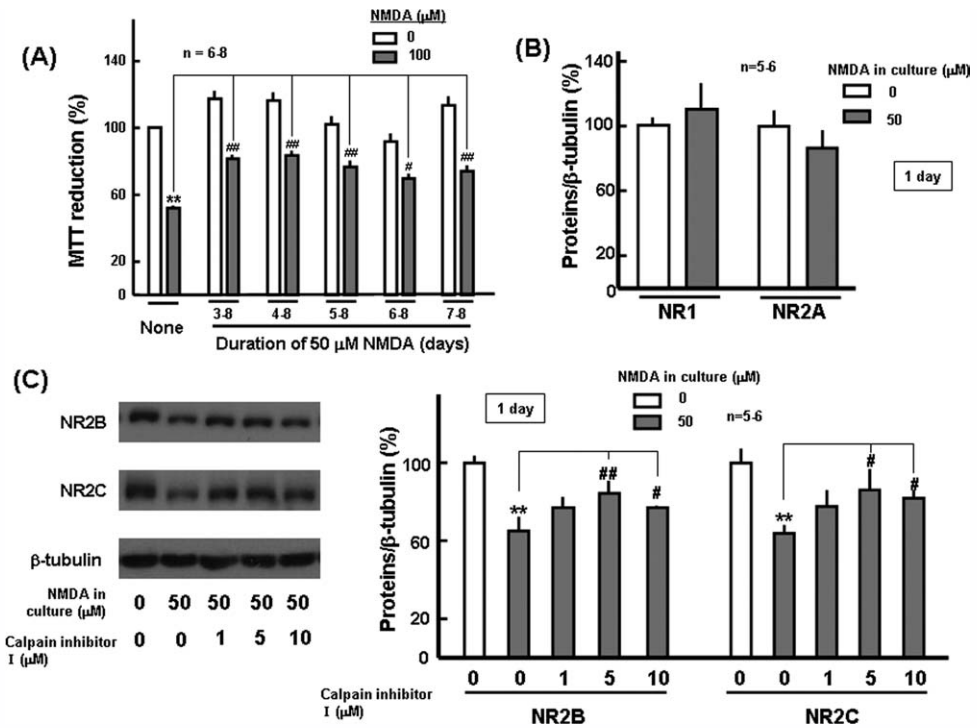

Fig. 7. Effects of calpain inhibitor on NMDAR subunit expression in striatal neurons. **A:** Striatal neurons were cultured with 50 μM NMDA for different periods of from 1 to 5 days up to 8 days, followed by brief exposure to 100 μM NMDA for 1 hr. $^{\#\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different from the value obtained in cells not cultured with NMDA. Striatal neurons were also cultured with 50 μM NMDA in either the presence or the absence of calpain inhibitor-I at 1–10 μM for the last 1 day during the culture period of 8 days, followed by Western blotting analysis of NR1 and NR2A subunits (**B**) and NR2B and NR2C subunits (**C**). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different from the value obtained in cells cultured with NMDA alone. Values are the mean ± SEM in separate experiments, with the repetition number shown in each panel. $^{\star\star}P < 0.01$, significantly different from each control value obtained in neurons not exposed to NMDA after culture.

subunit protein in a concentration-dependent manner in striatal neurons cultured with NMDA for 1 day. Although NMDAR protein expression is down-regulated in response to Ca^{2+} influx across NMDAR channels via activation of the Ca^{2+}-dependent protease calpain (Wu et al., 2005), the NR2B subunit is a selective substrate for calpain rather than NR1 and NR2A subunits (Simpkins et al., 2003; Wu et al., 2007). The present findings that prior culture with NMDA for 1 day selectively decreased protein expression of both NR2B and NR2C subunits without affecting NR2A subunit protein expression clearly give support to this proposal. Calpain is responsible for proteolysis of the C-termini of NR2, but not NR1, subunits (Guttmann et al., 2001), although this site is the domain essential for an interaction with postsynaptic density (PSD)-95, which contributes to stabilize NMDAR on neuronal membranes. Truncated NR2 subunit loses the property of binding to PSD-95 protein (Dong et al., 2004), which promotes the internalization of NMDAR (Roche et al., 2001). In an electrophysiological study, indeed, NMDAR was shown to be more stable on neuronal membranes than other iGluR and mGluR subtypes (Man et al., 2000). Whether the down-regulation of NR2B subunit following cleavage by calpain mediates the internalization of NMDAR, however, remains to be elucidated in future studies.

When neurons are exposed to NMDA in Ca^{2+}-free buffer, NMDA fails to kill neurons (Manev et al., 1989; Tymianski et al., 1993; Pivovarova et al., 2004). Furthermore, mitochondria are one of the key mediators of neuronal death after activation of NMDAR in vitro and in vivo (Stout et al., 1998; Schinzel et al., 2005). The present findings that NMDA induced significantly less effective increases in both intracellular free Ca^{2+} levels and $\Delta\Psi$ depolarization in striatal neurons previously cultured with NMDA could be accounted for by taking into consideration that NMDAR channels are not functionally expressed in the absence of NR2 subunits on cellular surfaces in HEK293 cells (McIlhinney et al., 1996). Moreover, NMDAR containing NR2B subunit exhibits higher conductance and slower desensitization compared with NMDAR containing NR2A or NR2C subunits (Cull-Candy et al., 2001). It is thus conceivable that decreased NR2B subunit expression may at least in part play a role in the mechanisms underlying decreased Ca^{2+} influx after activation of NMDAR in striatal neurons cultured with NMDA. Moreover, neuronal cell death has been shown primarily to involve the translocation and subsequent excess accumulation of Ca^{2+} in mitochondria after the influx across NMDAR channels (Stout et al., 1998), and there is a threshold level of free Ca^{2+} in mitochondria to induce mitochondrial dysfunction (Bambrick et al., 2006). However, ATP synthesis is promoted by free Ca^{2+} ions at levels below threshold in mitochondria, which is trophic rather than toxic for cell survival in neurons (Jouaville et al., 1999). Therefore, the decreased Ca^{2+} entry could lead to suppression of neuronal cell death by NMDA.

It thus appears that prior tonic mild activation of NMDAR could lead to induced tolerance to the neurotoxicity mediated by NMDAR through calpain-mediated down-regulation of NR2B subunit in cultured rat striatal neurons. Elucidation of the underlying mechanism would give us a clue for novel strategies to protect vulnerable populations of neurons from the neurotoxicity relevant to NMDAR in a variety of ischemic neurodegenerative disorders.

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