Neurochemistry International 57 (2010) 730–737

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Requirement of both NR3A and NR3B subunits for dominant negative properties on Ca^{2+} mobilization mediated by acquired N-methyl-D-aspartate receptor channels into mitochondria

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ARTICLE INFO

Article history:
Received 30 April 2010
Received in revised form 21 July 2010
Accepted 20 August 2010
Available online 8 September 2010

Keywords:
NMDA receptor
Ca^{2+} influx
NR3 subunit
Acquired channels
Cytotoxicity

ABSTRACT

Conventional N-methyl-D-aspartate (NMDA) receptor (NMDAR) is a heteromeric complex between the essential NR1 subunit and one of NR2A-D subunits toward functional channels permeable to Ca^{2+} rather than Na^{+} ions. Although recent studies identified dominant negative NR3A and NR3B subunits, whether these subunits inhibit Ca^{2+} mobilization through NMDAR channels into mitochondria is not clarified so far. In this study, we investigated Ca^{2+} influx across acquired NMDAR channels composed of different NR subunits artificially expressed in HEK293 cells. The addition of NMDA markedly increased intracellular free Ca^{2+} levels determined by Fluo-3 in cells transfected with either NR2A or NR2B subunit together with NR1 subunit. Further addition of dizocilpine completely inhibited the increase by NMDA in both types of acquired channels, while the NR2B subunit selective antagonist ifenprodil drastically inhibited the increase by NMDA in cells expressing NR1/NR2B, but not NR1/NR2A, subunits. Similar pharmacological profiles were invariably seen with cell death by NMDA. Introduction of both NR3A and NR3B subunits significantly inhibited the increase by NMDA in intracellular free Ca^{2+} levels in both acquired channels, while introduction of either NR3A or NR3B alone was ineffective. Co-expression of both NR3A and NR3B subunits was also required for the prevention of increased mitochondrial free Ca^{2+} levels determined by Rhod-2, as well as decreased cellular viability, in cells expressing NR1/NR2A or NR1/NR2B subunits upon exposure to NMDA. These results suggest that co-expression of both NR3A and NR3B subunits is essential for the dominant negative properties on Ca^{2+} mobilization through acquired functional NMDAR channels into mitochondria.

1. Introduction

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, such as ionotropic (iGluR) and metabotropic receptors, on the basis of intracellular signal transduction systems as well as gene homology. Amongst different iGluR subtypes, N-methyl-D-aspartate (NMDA) receptor (NMDAR) is a subtype consisting of a heteromeric protein complex between the essential NR1 subunit and one of NR2A-D subunits toward the orchestration of a ligand-gated ion channel with higher permeability to Ca^{2+} than Na^{+} ions. The prevailing view is that glycine binding site is located on the essential NR1 subunit with Glu binding site being on NR2 subunit. 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).

Several independent lines of evidence indicate that NR2B subunit is a determinant of the neurotoxicity after activation of NMDAR.

Abbreviations: AM, acetoxymethyl; AP-5, 2-amino-5-phosphonovaleric acid; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; EV, empty vector; FBS, fetal bovine serum; GFP, green fluorescent protein; Glu, glutamate; HEK, human embryonic kidney; iGluR, ionotropic glutamate receptors; MK-801, dizocilpine; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; NMDA, N-methyl-D-aspartate; NMDAR, N-methyl-D-aspartate receptor; PBS, phosphate-buffered saline.

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doi:10.1016/j.neuint.2010.08.009

Calcium entry through extrasynaptic NMDAR containing NR2B subunit is shown to be toxic to neurons, whereas Ca^{2+} influx through synaptic NMDAR containing NR2A subunit is anti-apoptotic (Hardingham and Bading, 2003). Distinct roles are proposed between synaptic and extrasynaptic NMDAR in Ca^{2+}-dependent neurodegeneration toward excitotoxicity (Sattler and Tymianski, 2001). In a previous *in vivo* study (Chen et al., 2008), prior administration of a selective blocker of NR2A-containing NMDAR exacerbates ischemic neuronal damage in rats with transient global ischemia, with concomitant prevention by ifenprodil used for the selective blockade of NR2B-containing NMDAR. Moreover, recent studies have identified and cloned novel NMDAR subunits with dominant negative properties (Ciabarra et al., 1995; Sucher et al., 1995). The introduction of NR3A subunit leads to a significant reduction of unitary conductance in single channel recordings from Xenopus oocytes with acquired NMDAR channels composed of NR1 and NR2 subunits, for example, while marked increases are seen in NMDA responses and the number of dendritic spines in early postnatal cortical neurons of mice defective of NR3A subunit (Das et al., 1998). On *in situ* hybridization and immunohistochemistry analyses, NR3A subunit is widely distributed in rat brain with predominant expression of the novel NR3B subunit by motor neurons (Chatterton et al., 2002). Complementary review articles on NR3 subunits are available in the literature (Cavara and Hollmann, 2008; Low and Wee, 2010).

On the other hand, neuronal cell death is shown to primarily involve the translocation and subsequent excessive accumulation of Ca^{2+} in mitochondria after the influx across NMDAR channels on cell surface (Stout et al., 1998), while 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). In our previous studies (Kambe et al., 2008), brief exposure to either Glu or NMDA leads to subsequent loss of cellular viability in cultured rat hippocampal neurons, without markedly affecting that in cortical neurons. Although NMDA is more efficient in increasing intracellular free Ca^{2+} levels in cortical neurons than in hippocampal neurons, NMDA is more effective in disrupting mitochondrial membrane potential in hippocampal neurons than in cortical neurons (Kambe et al., 2008). On the basis of these findings, we have proposed that hippocampal neurons would be highly vulnerable to the neurotoxicity mediated by NMDAR through a mechanism related to mitochondrial membrane potential disruption, rather than increased intracellular free Ca^{2+} levels. Nevertheless, relatively little attention has been paid to the modulation by NR3 subunits on mitochondrial free Ca^{2+} levels in association with other NR subunits.

In order to further evaluate dominant negative properties of NR3 subunits, therefore, we have determined mitochondrial free Ca^{2+} levels shown to be responsible for apoptotic cell death, in addition to intracellular free Ca^{2+} levels, in human embryonic kidney (HEK) 293 cells artificially transfected with different NMDAR subunit expression vectors.

2. Materials and methods

2.1. Materials

HEK293 cells were purchased from RIKEN Cell Bank (Saitama, Japan). The plasmid constructs pcDNA1-NR2A and pcDNA3.1-NR1-1a were generous gifts from Dr. Jon W. Johnson (Department of Neuroscience, University of Pittsburgh, Pittsburgh, PA, USA) and pRc-CMV-NR2B plasmid was kindly given by Dr. David M. Lovinger (Laboratory for Integrative Neuroscience, NIAAA/NIH, Rockville, MD, USA). pcDNA3.1-NR3A was kindly provided by Dr. Stuart A. Lipton (University of California, San Diego, USA). pcDNA3.1-NR3B was kindly donated by Dr. Dongxian Zhang (Burnham Institute for Medical Research, La Jolla, CA, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco BRL (Gaithersburgm, MD, USA). Fluo-3 acetoxymethyl (AM) ester and rhodamine-2 (Rhod-2) AM ester were provided by Molecular Probes (Eugene, OR, USA). Both NMDA and Hoechst33342 were purchased from Sigma Chemicals (St. Louis, MO, USA). Other chemicals used were all of the highest purity commercially available.

2.2. Expression of acquired NMDAR channels

In this study, we used rat NMDAR subunits cloned into expression vectors as described previously (Fukumori et al., 2010). HEK293 cells were grown in DMEM supplemented with 5% fetal bovine serum (FBS) before transfection (Taniura et al., 2007). Cells were then transfected at a 1:3 ratio with NR1-1a and either NR2A or NR2B subunit expression vector by the calcium phosphate co-precipitation method in minimum essential medium with 5% FBS, followed by further culture for an additional 24 h unless otherwise indicated. We also introduced the vector pEGFP-C2 (Clontech, Mountain View, CA, USA) with cloned enhanced green fluorescent protein (GFP) to monitor the transfection efficiency (Georgiev et al., 2008). Cells were rinsed with recording medium, followed by loading of Fluo-3 AM and subsequent exposure to NMDA at different concentrations in either the presence or absence of 10 μM dizocilpine (MK-801), 10 μM ifenprodil and 100 μM 2-amino-5-phosphonovaleric acid (AP-5) for determination of intracellular free Ca^{2+} levels.

The transfection efficiency was over 50% as measured by pEGFP-C2 vector with cloned EGFP, while introduction of NR1/NR2A and NR1/NR2B vectors led to a marked decrease in percentages of GFP-positive cells over Hoechst33342-positive cells from 52.9 ± 1.1% to 9.0 ± 1.3% for NR1/NR2A subunits and to 12.2 ± 1.5% for NR1/NR2B subunits, respectively, during culture for 48 h. However, culture with the NMDAR blocker MK-801 significantly prevented the decreases by the introduction of NMDAR subunits in survival ratios of cells transfected with NR1/NR2A (30.0 ± 0.6%) and NR1/NR2B (29.5 ± 1.6%) vectors. As Glu in culture medium could thus induce cell death during culture for 48 h through activation of acquired NMDAR in HEK293 cells artificially expressing NR1/NR2A and NR1/NR2B subunits as described previously (Fukumori et al., 2010), we employed cells cultured for 24 h after transfection in order to reduce the number of dead cells before exposure to NMDA as much as possible in this study.

Under the experimental conditions employed in this study, marked expression was seen with corresponding subunit proteins in homogenates of cells transfected with each subunit expression vector on Western blotting analysis as previously demonstrated (Nakamichi et al., 2010). Both Glu and NMDA invariably increased the number of fluorescent cells due to Fluo-3 for intracellular free Ca^{2+} levels in HEK293 cells transfected with both NR1 and NR2 subunits, while neither Glu nor NMDA was effective in markedly increasing the fluorescence in cells transfected with either NR1 or NR2 subunit alone (data not shown).

2.3. Measurement of intracellular free Ca^{2+} levels

Cultured cells were washed 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) once, followed by incubation at 37 °C for 50 min in recording medium containing 30 nM Pluronic F-127 and 3 μM Fluo-3 AM (Nakamichi et al., 2002). Cultures were then washed with recording medium twice, followed by settlement for at least 1 h in the recording medium and subsequent placement in a confocal laser-scanning microscope for observation. Medium was changed once more, followed by exposure to NMDA at different concentrations for determination of fluorescence image. The calcium ionophore A23187 was then added at 10 μM to obtain the maximal fluorescence for quantitative normalization. Drugs were prepared in recording medium immediately before each use. Fluorescence images obtained with Fluo-3 were collected using an excitation wavelength of 488 nm. The parameters of illumination and detection were digitally controlled to keep the same settings throughout the experiments (Nakamichi et al., 2002). The data obtained were subjected to quantification by normalization on the basis of fluorescence intensity in cells exposed to A23187 at 10 μM.

2.4. Measurement of mitochondrial free Ca^{2+} levels

In order to produce colorless and non-fluorescent dihydro Rhod-2 AM ester, which is a membrane-permeable form of the Ca^{2+}-sensitive dye selectively accumulated into mitochondria due to its high cationic charge, Rhod-2 AM ester (Molecular Probes) was at first incubated for 10 min with a particular amount of $NaBH_4$ until the red color of Rhod-2 AM ester vanished according to the manufacturer's instruction. Dihydro Rhod-2 AM ester is permeable across cell membranes for the cleavage of AM ester and subsequent oxidization to the fluorescent dye Rhod-2 for Ca^{2+}-dependent fluorescence in the mitochondrial environment. Cultured cells were washed once with recording medium, followed by incubation at 37 °C for 1 h in recording medium containing 0.02% Pluronic F-127 and 3 μM dihydro Rhod-2 AM. Cells were then washed twice with DMEM and cultured for an additional 24 h. Culture medium was changed to recording medium, followed by exposure to NMDA at 100 μM and subsequent determination of the fluorescence intensity every 1 min. The fluorescence intensity was normalized after the addition of the Ca^{2+} ionophore A23187 at 10 μM. Cells were invariably used within 1–5 h after these procedures for observation of the fluorescence visualized by a confocal laser-scanning microscope equipped with an helium–neon laser. Images were obtained using an objective lens with numeral apertures of 0.5 (Plan-Neofluar) for 20-fold magnification. Fluorescence images

labeled with Rhod-2 were collected using an excitation wavelength of 543 nm. Parameters of illumination and detection were controlled digitally for consistent settings throughout the experiments. Two successive digital images were collected usually at 512 × 512 pixels in the same visual field. In order to confirm the accumulation in mitochondria, cells were exposed to Rhod-2 AM for 1 h, followed by further culture for an additional 24 h to allow the dye to be incorporated into mitochondria and subsequent staining with MitoTracker Green FM at 100 nM for 15 min at 37 °C, followed by washing 3 times and subsequent observation of cellular illumination by a fluorescent microscope to visualize mitochondria.

2.5. Determination of cell viability

Cell viability was measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) reduction colorimetric assays (Mosmann, 1983) with minor modifications (Fukumori et al., 2010). Cells were cultured in routine DMEM medium for 24 h after the transfection, followed by washing with phosphate-buffered saline (PBS) once and subsequent incubation with 0.5 mg/ml MTT in PBS for 2 h. Cells were then 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. In addition, the background value obtained under cell-free conditions was invariably subtracted from the total value (Hinoi et al., 2006).

Cell viability was also examined by staining with the membrane-permeable fluorescent dye Hoechst33342 for DNA, in association with measurement of the fluorescence of GFP. Cultured cells were washed by recording medium and incubated with 10 μg/ml Hoechst33342 in recording medium for 10 min. Cells were then observed using an epifluorescent microscope (BZ-8100; Keyence, Osaka, Japan). The numbers of GFP- and Hoechst33342-positive cells were individually counted in five different visual fields chosen at random per each well, for subsequent calculation of percentages of GFP-positive cells over Hoechst33342-positive cells (Kambe et al., 2008).

2.6. Data analysis

Results are all expressed as the mean ± S.E.M. and the statistical significance was determined by the two-tailed and unpaired Students' *t*-test.

3. Results

3.1. Profiles of acquired NMDAR channels

HEK293 cells were transfected with NR1/NR2A or NR1/NR2B expression vectors for 24 h, followed by loading of Fluo-3 AM and subsequent exposure to NMDA at different concentrations up to 1 mM for 5 min. Irrespective of the NR2 subunit expression vector used, exposure to NMDA drastically increased the florescence intensity of Fluo-3 in a concentration-dependent manner (Fig. 1A). The fluorescence intensity was quantified according to the normalization over the maximal fluorescence by A23187. Exposure to NMDA at 100 μM immediately increased the fluorescence intensity by around 60% of that by A23187 irrespective of the expression vectors for NMDAR subunits. Prior addition of either MK-801 or AP-5 resulted in similarly effective decreases in fluorescence intensity elevations by NMDA in both acquired NMDAR channels composed of NR1/NR2A and NR1/NR2B subunits, while the NR2B subunit selective antagonist ifenprodil almost completely inhibited the fluorescence intensity in cells expressing NR2B subunit (Fig. 1A, right panel), without markedly affecting that in cells expressing NR2A subunit (Fig. 1A, left panel).

In order to confirm the orchestration of functional NMDAR channels, cells were transfected with different expression vectors, followed by culture for 24 h in defined DMEM and subsequent determination of MTT reduction. Introduction of NR1, NR2A or NR2B subunit alone did not significantly affect MTT reduction, while the combination of expression vectors such as NR1/NR2A and NR1/NR2B significantly decreased MTT reduction (Fig. 1B). As seen with intracellular free Ca^{2+} levels, MK-801 prevented the decrease in MTT reduction in cells with NR1/NR2A and NR1/NR2B subunits. By contrast, the NR2B subunit selective antagonist ifenprodil prevented the decrease in cells expressing NR1/NR2B subunits without affecting that in cells with NR1/NR2A subunits. Accordingly, the fluorescence by Fluo-3 was employed as a measure of functional acquired NMDAR channels artificially expressed in HEK293 cells in subsequent experiments.

3.2. Intracellular free Ca^{2+} levels

We next introduced NR3A and NR3B expression vectors into HEK293 cells expressing functional NMDAR channels composed of NR1/NR2A or NR1/NR2B subunits. As shown in Fig. 2, the addition of NMDA induced a marked increase in the number of fluorescent cells in HEK293 cells with NR1/NR2A subunits at concentrations over 10 μM. Further introduction of either NR3A or NR3B subunit alone did not prominently affect the number of fluorescent cells in HEK293 cells containing NR1/NR2A subunits after exposure to NMDA, while artificial co-expression of both NR3A and NR3B subunits markedly reduced the number of fluorescent cells after exposure to NMDA.

Repetition and quantification of these experiments clearly revealed that NMDA significantly increased fluorescence intensity due to intracellular free Ca^{2+} levels in a concentration-dependent manner at concentrations of 1 μM to 1 mM in HEK293 cells with acquired NMDAR channels composed of NR1/NR2A (EC_{50} = 7.9 ± 0.6 μM), NR1/NR2A/NR3A (EC_{50} = 8.9 ± 0.7 μM) and NR1/NR2A/NR3B (EC_{50} = 8.4 ± 1.2 μM) subunits (Fig. 3, left panel). However, further introduction of both NR3A and NR3B subunits (EC_{50} = 12.2 ± 1.1 μM, $^{*}P < 0.05$) significantly attenuated the increased fluorescence intensity by NMDA in acquired NMDAR channels composed of NR1/NR2A subunits. Further introduction of either NR3A (EC_{50} = 8.6 ± 0.9 μM) or NR3B (EC_{50} = 11.7 ± 0.8 μM) subunit did not significantly affect the fluorescence intensity increased by NMDA in acquired NMDAR channels composed of NR1/NR2B subunits (EC_{50} = 7.1 ± 0.9 μM), similarly, whereas co-introduction of both NR3A and NR3B (EC_{50} = 12.4 ± 1.1 μM, $^{*}P < 0.05$) subunits significantly diminished the NMDA-induced increase in fluorescence intensity in acquired NMDAR channels composed of NR1/NR2B subunits (Fig. 3, right panel). Therefore, co-expression of both NR3A and NR3B subunits would be required for the inhibition of Ca^{2+} influx across acquired NMDAR channels irrespective of the type of NR2 subunits tested here.

3.3. Mitochondrial free Ca^{2+} levels

We have previously shown a predominant role of free Ca^{2+} ions in mitochondria rather than cytoplasm in molecular mechanisms underlying cell death in cultured hippocampal neurons exposed to NMDA (Kambe et al., 2008). Accordingly, cells with overexpression of NR subunits were loaded with the dye dihydro Rhod-2 AM ester for mitochondrial free Ca^{2+} determination. Localization of Rhod-2 fluorescence was entirely merged with the fluorescence of the mitochondrial marker MitoTracker in HEK293 cells transfected with NR1/NR2A subunit expression vectors (data not shown). In cells transfected with NR1/NR2A vectors, both NMDA and A23187 similarly increased the fluorescence intensity of Rhod-2 (Fig. 4). Further overexpression of either NR3A or NR3B subunit did not markedly affect the fluorescence in cells with acquired NMDAR channels composed of NR1/NR2A subunits, while further introduction of both NR3A and NR3B subunits led to a marked diminution of the increased number by NMDA of cells with high fluorescence of Rhod-2 accumulated in mitochondria. However, A23187 was invariably effective in increasing the number of fluorescent cells irrespective of the introduction of different NR3 subunits in HEK293 cells.

The fluorescence by NMDA was quantified according to the normalization over the maximal fluorescence by A23187. Exposure to 100 μM NMDA immediately increased the fluorescence

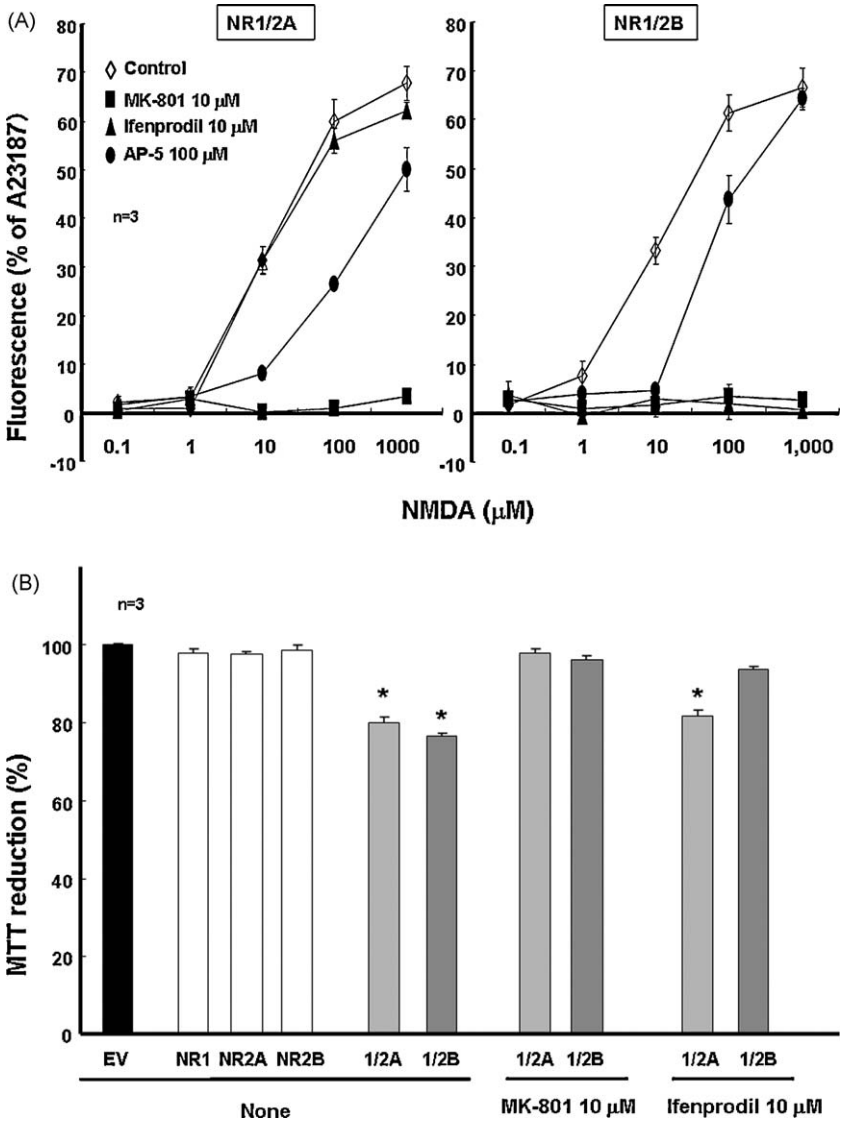

Fig. 1. Effects of NMDAR ligands on Ca^{2+} levels and cell viability. (A) HEK293 cells were transfected with expression vectors of different NR2 subunits along with the essential NR1 subunit. Cells were rinsed with recording medium, followed by loading of Fluo-3 and subsequent exposure to NMDA at different concentrations from 0.1 μM to 1 mM in either the presence or absence of 10 μM MK-801, 10 μM ifenprodil and 100 μM AP-5. The calcium ionophore A23187 was then added at 10 μM for quantitative normalization. Values are the mean ± S.E.M. of percentages over the maximal value obtained 5 min after the addition of 10 μM A23187 in 3 different experiments. (B) HEK293 cells were transfected with NMDAR subunit vectors at different combinations as described in the figure, followed by further culture for an additional 24 h in either the presence or absence of 10 μM MK-801 and 10 μM ifenprodil for subsequent determination of MTT reduction. Values are the mean ± S.E.M. in 3 different experiments. $^*P < 0.05$, significantly different from each control value in cells transfected with empty vector (EV) alone. The statistical significance was determined by the two-tailed and unpaired Students' *t*-test.

intensity for 5 min by around 70% of that by A23187 in acquired NMDAR channels composed of NR1/NR2A subunits, while further co-introduction of both NR3A and NR3B subunits resulted in a drastic decrease in the fluorescence to around 30% of maximal fluorescence by A23187 in cells exposed to NMDA (Fig. 5A). The increase by NMDA occurred in a concentration-dependent manner at concentrations over 10 μM in acquired NMDAR channels composed of NR1/NR2A subunits (EC_{50} = 11.4 ± 1.6 μM), whereas further overexpression of NR3A (EC_{50} = 11.7 ± 2.1 μM), NR3B (EC_{50} = 11.4 ± 1.2 μM) or NR3A/NR3B (EC_{50} = 14.1 ± 0.4 μM) did not significantly affect the half-maximal concentration for NMDA to increase Rhod-2 fluorescence with a significant inhibition of the fluorescence in cells with acquired NR1/NR2A channels (Fig. 5B). Thus, it seems likely that both NR3A and NR3B subunits should be simultaneously expressed for efficient inhibition of the increase in mitochondrial free Ca^{2+} levels by NMDA in cells with acquired NR1/NR2A channels.

3.4. Protection of cell death

For evaluation of pharmacological properties of NR3 subunit on cell death, cells were transfected with different expression vectors for NR subunits and GFP, followed by further culture for an additional 24 h and subsequent determination of cellular vitality with counting the number of GFP-positive cells. The transfection efficiency was over 50% as measured by pEGFP-C2 vector with cloned EGFP, while introduction of NR1/NR2A or NR1/NR2B subunits led to a marked decrease in percentages of GFP-positive cells over Hoechst33342-positive cells during culture for 24 h (Fig. 6A). Further co-introduction of both NR3A and NR3B subunits significantly prevented the decrease in survival ratios of cells transfected with NR1/NR2A vectors. In cells transfected with both NR3A and NR3B vectors along with NR1/NR2B vectors, no significant decrease was seen in percentages of GFP-positive cells over Hoechst33342-positive cells. However, further introduction

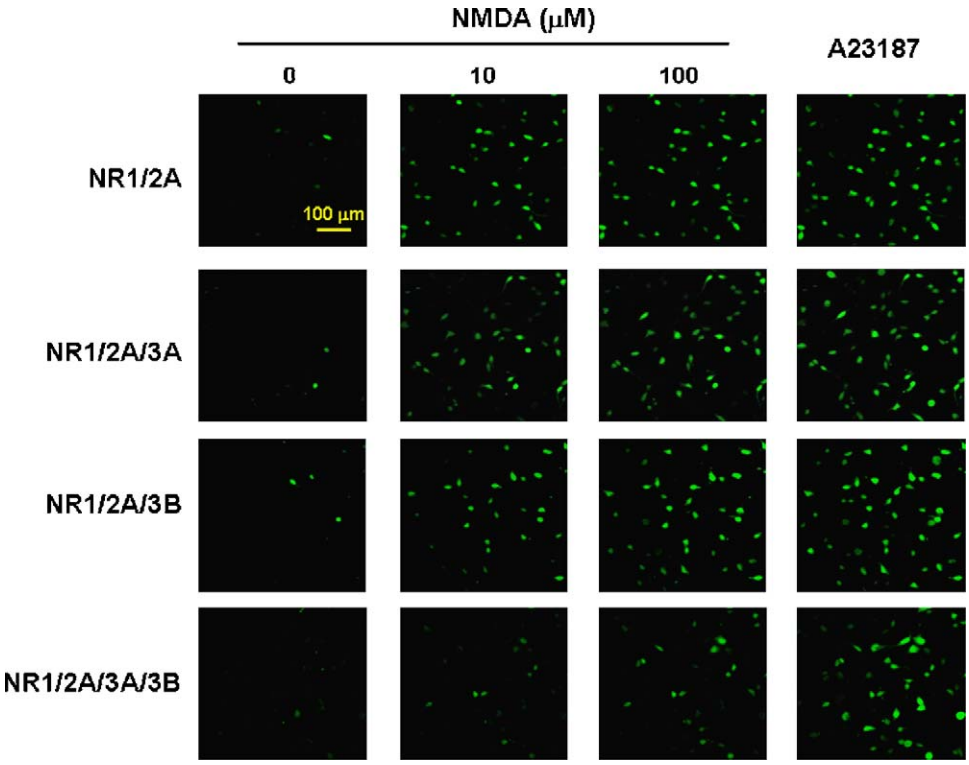

Fig. 2. Effects of NR3 subunit transfection on intracellular free Ca^{2+} levels. HEK293 cells were transfected with expression vectors of NR1 and NR2A subunits along with different NR3 subunits, followed by further culture for an additional 24 h and loading of Fluo-3 for subsequent exposure to NMDA at 10–100 and 10 μM A23187. Typical micrographs are shown with similar results in 3 separate experiments.

of either NR3A or NR3B subunit alone failed to significantly prevent the decreased survival ratios in cells expressing NR1/NR2A or NR1/NR2B subunits. Similarly, co-introduction of both NR3A and NR3B subunits was effective in significantly preventing the decreased MTT reduction in cells expressing NR1/NR2A or NR1/NR2B subunits (Fig. 6B). Introduction of either NR3A or NR3B subunit alone was again ineffective in significantly affecting MTT reduction in cells transfected with NR1/NR2A and NR1/NR2B vectors. Therefore, simultaneous expression of both NR3A and NR3B subunits would be required for the prevention of cell death during culture in HEK293 cells with acquired NMDAR channels.

4. Discussion

The essential importance of the present findings is that introduction of either NR3A or NR3B subunit alone was ineffective in preventing the increases in mitochondrial Ca^{2+} levels determined by Rhod-2 and intracellular free Ca^{2+} levels determined by Fluo-3, in

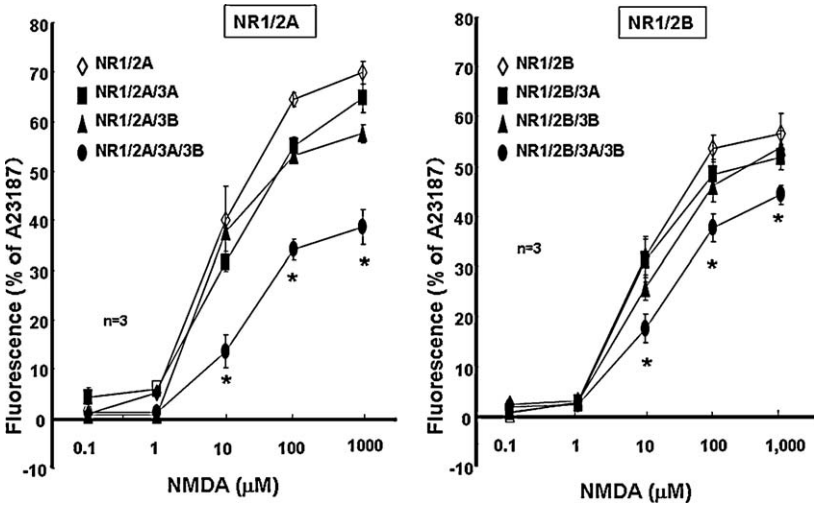

Fig. 3. Effects of NMDA on intracellular free Ca^{2+} levels in cells with acquired NMDAR channels composed of different NR subunits. HEK293 cells were transfected with expression vectors of NR2A or NR2B subunit, along with NR1 subunit, in either the presence or absence of different NR3 subunit vectors. Cells were further cultured for an additional 24 h, followed by loading of Fluo-3 and subsequent exposure to NMDA at 0.1 μM to 1 mM. The calcium ionophore A23187 was then added at 10 μM for quantitative normalization. Values are the mean ± S.E.M. of percentages over the maximal value obtained 5 min after the addition of 10 μM A23187 in 3 different experiments. $^{*}P < 0.05$, significantly different from each control value obtained in cells not transfected with NR3 subunit vectors. The statistical significance was determined by the two-tailed and unpaired Students' *t*-test.

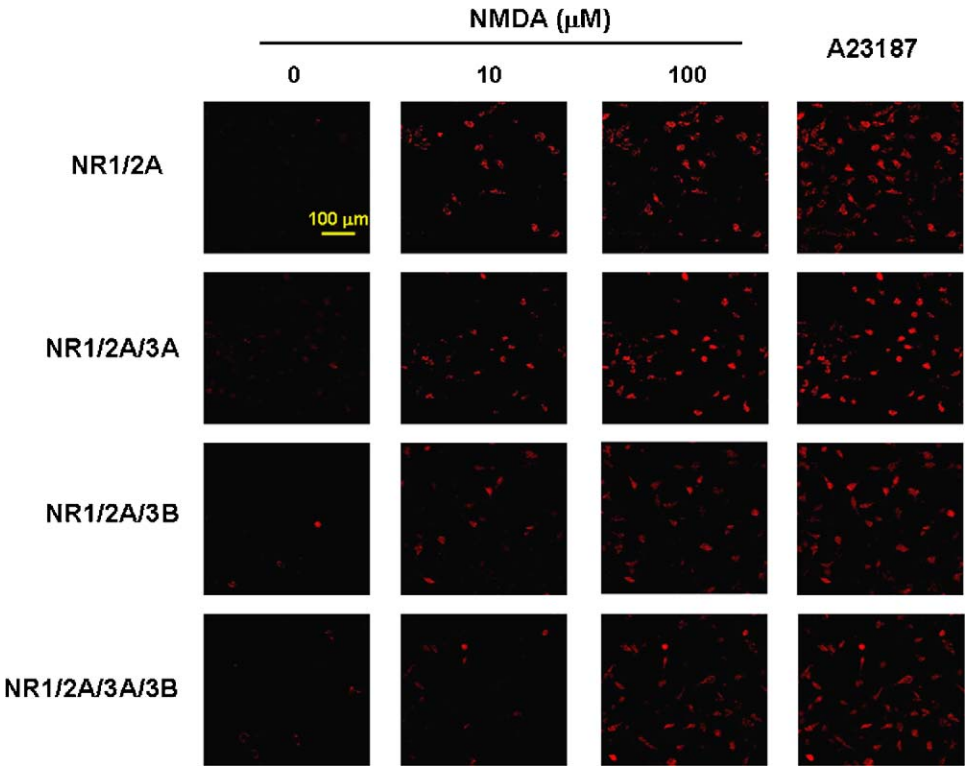

Fig. 4. Effects of NR3 subunit transfection on mitochondrial free Ca^{2+} levels. HEK293 cells were transfected with expression vectors of NR1 and NR2A subunits along with different NR3 subunits, followed by further culture for an additional 24 h and loading of Rhod-2 for subsequent exposure to NMDA at 10–100 and 10 μM A23187. Typical micrographs are shown with similar results in 3 separate experiments.

addition to the increased cell death, in HEK293 cells with acquired NMDAR channels composed of NR1/NR2A and NR1/NR2B subunits. The findings that co-introduction of both NR3A and NR3B subunits invariably prevented the increases in those 3 biological responses mediated by NMDAR channels give strong support to the proposal that simultaneous co-expression of both NR3A and NR3B subunits is absolutely required for the efficient elicitation of dominant negative properties on Ca^{2+} influx across functional NMDAR channels in cell surface. Several independent lines of evidence indicate that incorporation of NR3 subunits into conventional NMDAR leads to smaller unitary conductance states and decreases in current amplitude, Ca^{2+} permeability and Mg^{2+} sensitivity in Xenopus oocytes (Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998). By taking into consideration the fact that cells were exclusively

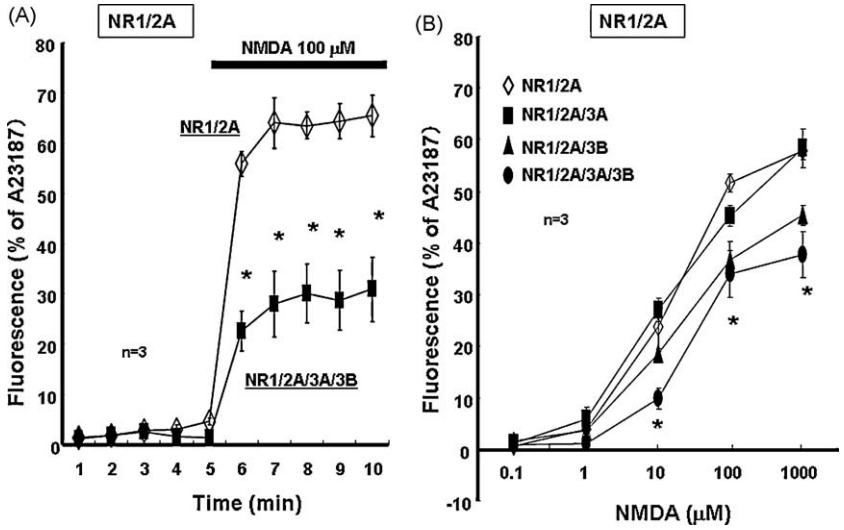

Fig. 5. Effects of NMDA on mitochondrial free Ca^{2+} levels in cells with acquired NMDAR channels composed of different NR subunits. HEK293 cells were transfected with expression vectors of NR1 and NR2A subunits along with different NR3 subunits, followed by further culture for an additional 24 h and loading of Rhod-2 for subsequent exposure to NMDA at 0.1 μM to 1 mM. The calcium ionophore A23187 was then added at 10 μM for quantitative normalization. (A) Fluorescence was measured every 1 min before and after exposure to 100 μM NMDA in cells with NR1/NR2A or NR1/NR2A/NR3A/NR3B subunits. (B) Fluorescence was measured 5 min after the addition of NMDA at different concentrations in cells with different combinations of NR subunits. Values are the mean ± S.E.M. of percentages over the maximal value obtained 5 min after the addition of 10 μM A23187 in 3 different experiments. $^{*}P < 0.05$, significantly different from each control value obtained in cells not transfected with NR3 subunit vectors. The statistical significance was determined by the two-tailed and unpaired Students' *t*-test.

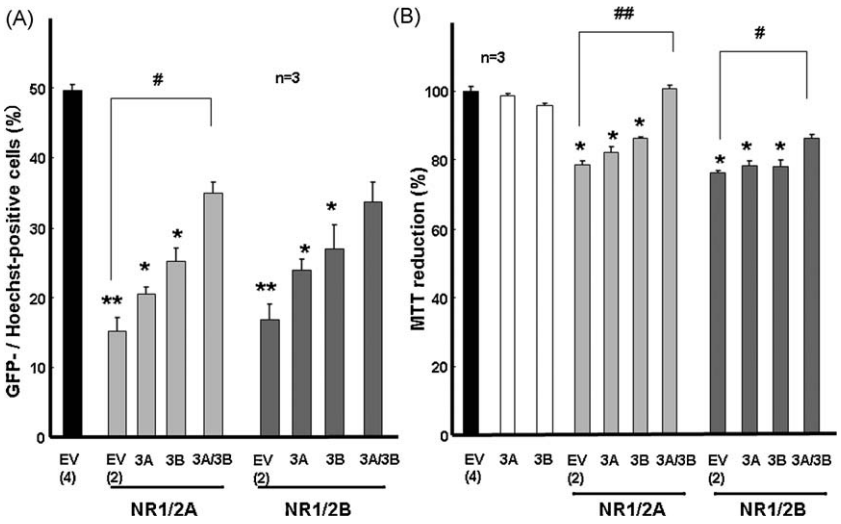

Fig. 6. Viability of cells with acquired NMDAR channels composed of different NR subunits. HEK293 cells were transfected with expression vectors of different NR subunits along with GFP expression vector, followed by further culture for an additional 24 h and subsequent determination of (A) percentages of GFP-positive cells over cells stained with Hoechst33342 or (B) MTT reduction as an index of cellular viability. Values are the mean ± S.E.M. in 3 different experiments. Control cells were transfected with either 2.0 or 4.0 μg of pcDNA3.1 vector. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from each control value obtained in cells not transfected with any NR subunit vectors. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different from each control value obtained in cells transfected with EV alone. The statistical significance was determined by the two-tailed and unpaired Students' *t*-test.

responsive to extracellular Glu and NMDA after the transfection of both NR1 and NR2B subunits in a manner sensitive to different NMDAR antagonists, it is highly likely that both NR1 and NR2 subunits would be expressed at cellular surfaces, but not at membranes of particular intracellular organelles such as mitochondria, for the orchestration of functional NMDAR channels permeable for extracellular Ca^{2+} ions in HEK293 cells transfected with both expression vectors.

Although recombinant NR1/NR3 subunits orchestrate functional glycine-activated receptors in oocytes, by contrast, currents generated by these subunits are not unequivocally observed in neurons (Chatterton et al., 2002; Tong et al., 2008). In mammalian cells with expression of NR1/NR3A or NR1/NR3B subunits no measurable currents are generated after exposure to NMDA (Matsuda et al., 2002), whereas robust responses are produced in cells transfected with expression vectors of both NR3A and NR3B subunits together with NR1 subunit in HEK293 cells (Smothers and Woodward, 2007). The paradox could be at least in part accounted for by taking into consideration possible differences in receptor expression or trafficking properties between oocytes and mammalian cells (Smothers and Woodward, 2008). Co-expression of NR1, NR2 and NR3 subunits altogether leads to separate populations of dimeric NR1/NR2 and NR1/NR3 channels in oocytes, while transfection with NR1, NR3A and NR3B subunits yields tri-heteromeric NMDAR channels with a fixed stoichiometry of two NR1 subunits and one NR3A and NR3B subunit (Ulbrich and Isacoff, 2008). However, differential distribution profiles between NR3A (Wong et al., 2002) and NR3B (Wee et al., 2008) subunit proteins in the rat CNS are still in disagreement with the requirement for co-expression of both NR3 subunits in mammalian CNS. To our knowledge, at any rate, this is the first direct demonstration of dominant negative properties of NR3 subunits against mitochondrial Ca^{2+} increases mediated by activation of acquired NMDAR comprised of NR1/NR2A and NR1/NR2B subunits in HEK293 cells in association with the prevention of cell death.

Calcium entry through extrasynaptic NMDAR containing an NR2B subunit is shown to be toxic to neurons, whereas Ca^{2+} influx through synaptic NMDAR containing an NR2A subunit is anti-apoptotic (Hardingham and Bading, 2003). Both synaptic and extrasynaptic NMDAR are proposed to play different roles in Ca^{2+}-dependent neurodegeneration toward excitotoxicity (Sattler and Tymianski, 2001). The reason why NMDA was similarly effective in increasing intracellular free Ca^{2+} levels in HEK293 cells artificially expressing either NR1/NR2A or NR1/NR2B subunits is not fully clarified so far, while the findings obtained in this study clearly give rise to an idea that simultaneous co-expression of both NR3A and NR3B subunits would rescue neurons from glutamate excitotoxicity mediated by NMDAR through interference with Ca^{2+} influx across membrane ion channels composed of extrasynaptic NR1/NR2B, as well as synaptic NR1/NR2A, subunits. The rescue by MK-801 and ifenprodil argues in favor of an idea that the mechanism involves activation of acquired NMDAR channels permeable to Ca^{2+} ions by endogenous glutamate present in culture medium in HEK293 cells, which occurs irrespective of the subunit composition. Furthermore, recent studies are suggestive of the possible relevance between mitochondrial Ca^{2+} translocation and cell death (Bathori et al., 2006; Hajnoczky et al., 2006). Mitochondrial free Ca^{2+} ions are highly responsible for cell death after activation of NMDAR through disruption of mitochondrial membrane potential in cultured rat hippocampal neurons (Kambe et al., 2008). Although Rhod-2 fluorescence was determined under equilibrium conditions in this study, the fluorescence should be responsive to alterations of a variety intermediate signaling processes after the influx via NMDAR channels expressed at cell surface. One possible but unidentified intermediate molecule is the mitochondrial calcium uniporter, which is a carrier of cytoplasmic free Ca^{2+} ions into the matrix across inner membranes in mitochondria (Kirichok et al., 2004). From the data cited above, it is not clear that mitochondrial free Ca^{2+} ions also play a role in the differential properties between trophic NR1/NR2A and toxic NR1/NR2B subunit channels in neurons. The exact mechanism underlying similar cytotoxicity between NR1/NR2A and NR1/NR2B subunit channels in HEK293 cells, however, remains to be elucidated in future studies.

It thus appears that co-expression of both NR3A and NR3B subunits could prevent Ca^{2+} mobilization into mitochondria after activation of NMDAR channels composed of NR1/NR2A and NR1/NR2B subunits in cell surface, in association with the rescue from cell death. Future elucidation of underlying mechanisms would give us a clue for the discovery and development of a novel drug useful for the therapy and treatment of a variety of neurodegener-

ative and neuropsychiatric disorders associated with the neurotoxicity mediated by NMDAR in human beings.

Acknowledgements

This work was supported in part by Grants-in-Aids for Scientific Research to T.T. (No. 22500330) and Y.Y. (No. 21659018) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and in part by a Research Grant to Y.Y. from the Smoking Research Foundation, Japan.

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