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Abbreviations used: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; Ara-C, cytosine arabinoside; DIV, days *in vitro*; DMEM, Dulbecco's Modified Eagle Medium; FCS, fetal calf serum; GAP-43, growth-associated protein 43; GFAP, glial fibrillary acidic protein; Glu, glutamate; MAP-2, microtubule-associated protein-2; NeuN; neuronal nuclei; NMDA, N-methyl-D-aspartate; NSE, neuron specific enolase; PBS, phosphate-buffered saline; SDS, sodium dodecylsulfate.

Abstract

In contrast to other ionotropic glutamate receptors, N-methyl-D-aspartate (NMDA) receptor channels are rather stable after the simulation. Brief exposure to NMDA at 50 μM rapidly increased the fluorescence intensity for increased intracellular free Ca^{2+} levels in a reversible- and concentration-dependent manner in rat cortical neurons cultured for 3 to 15 days in vitro (DIV), while EC_{50} values were significantly decreased in proportion to cellular maturation from 3 to 15 DIV. Although a constant increase was persistently seen in the fluorescence throughout the sustained exposure to NMDA for 60 min irrespective of the cell maturation from 3 to 15 DIV, the second brief exposure for 5 min resulted in a less efficient increase in the fluorescence than that found after the first brief exposure for 5 min in a manner dependent on intervals between the 2 repetitive brief exposures. *In vitro* maturation significantly shortened the interval required for the reduced responsiveness to the second brief exposure, while in immature neurons prolonged intervals were required for the reduced responsiveness to the second brief exposure to NMDA. Brief exposure to NMDA led to a marked decrease in immunoreactivity to extracellular loop of NR1 subunit after brief exposure to NMDA when determined in cultured neurons not permeabilized in proportion to the time after washing. These results suggest that cellular maturation would reduce the responsiveness to repeated stimulation by NMDA, without markedly affecting that to sustained exposure to NMDA, through the decrease in the number of membranous NMDA receptors in cultured rat cortical neurons.

Keywords: NMDA receptors; intracellular Ca^{2+}; *in vitro* maturation; desensitization

1. Introduction

N-Methyl-D-aspartate (NMDA) receptor is a subtype of ionotropic glutamate (Glu) receptors supposed to be preferentially expressed by neurons in the central nervous system. Activation of NMDA receptor channels leads to the influx of Ca^{2+} ions across cell membranes and subsequent elevation of intracellular free Ca^{2+} levels in neurons (Segal and Manor, 1992). Increased intracellular free Ca^{2+} ions are responsible for a variety of subsequent physiological and pathological events in the brain. These include neuronal development (Scheetz and Constantine-Paton, 1994), plasticity (Lisman and McIntyre, 2001), learning and memory (Nakazawa et al., 2002), and cell death (Choi, 1994; Nakamichi et al., 2004). A confocal microscopic imaging study reveals a localized increase in intracellular free Ca^{2+} concentrations following exposure to NMDA in a single cultured hippocampal neuron with relatively large variations (Segal and Manor, 1992; Korkotian and Segal, 1996). Accumulating evidence for an increase in intracellular Ca^{2+} concentrations in response to activation of NMDA receptors in mature cultured neurons (Parks et al., 1991; Dayanithi et al., 1995; Korkotian and Segal, 1997) is also available in the literature to date.

Activity-dependent alterations of synaptic responses are integral to the information processing in the brain. Thus, it is not surprising that synaptic efficacy can be reduced by many different mechanisms including downregulation of postsynaptic receptors (Krupp et al., 2002). On a time scale of minutes such downregulation can involve endocytosis of α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors that are also a subtype of ionotropic Glu receptors (Man et al., 2000; Lin et al., 2000; Ehlers, 2001), and possibly also for NMDA receptors (Roche et al., 2001; Vissel et al., 2001; Nong et al., 2003, 2004). The internalization of Glu receptors from cell surface may underlie long-term changes in synaptic strength (Luscher et al., 1999; Zhu et al., 2000). On the timescale of seconds and

milliseconds receptor desensitization can inhibit synaptic transmission (Trussell and Fischbach, 1989; Trussell et al., 1993; Tong et al., 1995). Desensitization is particularly important in shaping single synaptic events and the integration of high-frequency inputs during sustained firings (Trussell and Fischbach, 1989; Jones and Westbrook, 1996; Krupp et al., 2002).

Although AMPA receptors have been shown to rapidly internalize, NMDA receptors are often considered 'stable' in the plasma membrane (Man et al., 2000; Roche et al., 2001; Nong et al., 2003). However, recent accumulating evidence gives rise to the possibility that NMDA receptors may also undergo internalization (Man et al., 2000; Roche et al., 2001; Vissel et al., 2001; Carroll and Zukin, 2002; Nong et al., 2003, 2004), but mechanisms for internalization of NMDA receptor channels with neuronal development and maturation are not fully clarified so far. In this article, therefore, we have evaluated neuronal responses to NMDA with respect to intracellular free Ca^{2+} levels to investigate mechanisms underlying the possible desensitization of NMDA receptor channels during *in vitro* maturation in primary cultured rat cortical neurons.

2. Materials and methods

2.1. Materials

Antibodies against microtubule-associated protein-2 (MAP-2), glial fibrillary acidic protein (GFAP), synapsin-I, neuronal specific growth-associated protein-43 (GAP-43) and DNase I were purchased from Sigma Chemicals (St. Louis, MO, USA). Mouse monoclonal antibodies against neuronal nuclei (NeuN) and extracellular loop of NR1 subunit, as well as an anti-goat IgG antibody conjugated with peroxidase, were all supplied by Chemicon

International (Temecula, CA, USA). An antibody against neuron specific enolase (NSE) was obtained from Quartett (Berlin, Germany). Versene, Dulbecco's Modified Eagle Medium (DMEM) and DMEM: Nutrient Mixture F-12 (DMEM/F-12) 1:1 Mixture were supplied by GIBCO BRL (Gaithersburg, MD, USA). Fetal calf serum (FCS) was obtained from JRH Biosciences, Inc. (Lenexa, KS, USA). Fluo-3 acetoxymethyl ester was provided by Molecular Probes (Eugene, OR, USA). An anti-mouse IgG antibody conjugated with rhodamine and an anti-rabbit IgG antibody conjugated with fluorescein were obtained from ICN Pharmaceuticals (Aurora, OH, USA). An anti-mouse IgG or anti-rabbit IgG antibody conjugated with peroxidase and ECL^{TM} detection reagents were provided by Amersham Biosciences (Piscataway, NJ, USA). Other chemicals used were all of the highest purity commercially available.

2.2. Preparation of neuronal cultures

This study was carried out in compliance with the Guideline for Animal Experimentation at Kanazawa University with an effort to minimize the number of animals used and their suffering. Primary neuronal cultures were prepared from cerebral neocortex of 18-day-old embryonic rats as originally described by di Porzio et al. (1980) with minor modifications (Nakamichi et al., 2002a,b). In brief, cerebral neocortex was dissected from embryonic Wistar rats and incubated with Versene at room temperature for 12 min. Cells were then mechanically dissociated with a fire-narrowed Pasteur pipette in the culture medium, and plated at a density of 2.5 x 10^5 cells/cm^2 in a 6-well dish (NUNC, Roskilde, Denmark) after counting cell numbers with a Trypan Blue exclusion test. Prior to use, dishes were sequentially coated with 7.5 μg/mL poly-L-lysine and 10% FCS. The culture medium contained basal DMEM/F-12 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. Cells were treated with 10 μM cytosine arabinoside (Ara-C) for 24 h during 2 to 3 days *in vitro* (DIV) as needed. The prior treatment with Ara-C indeed led to a drastic decrease in the endogenous level of immunoreactive GFAP in cortical neuronal cells cultured for 3 to 15 DIV, with a slight but statistically significant decrease in the endogenous level of immunoreactive MAP-2 on 15 DIV (data not shown). In the present study, therefore, cortical neuronal cultures were not treated with Ara-C unless otherwise indicated. The culture medium was 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% air-humidified incubator.

2.3. Immunocytochemistry

Cortical neurons cultured for 3, 9 or 15 DIV were washed twice with phosphate-buffered saline (PBS), followed by fixation with 4% paraformaldehyde in PBS for 20 min at room temperature and subsequent blocking with 10% bovine serum albumin in PBS containing 1% Triton X-100. Cells were then reacted with antibodies adequately diluted against the neuronal marker protein MAP-2, the neuron specific nuclear protein NeuN and/or the glial marker protein GFAP for 1.5 h at room temperature. Finally, cells were reacted with the corresponding secondary antibody, an anti-mouse IgG antibody conjugated with rhodamine or an anti-rabbit IgG antibody conjugated with fluorescein, and then observed under a confocal laser-scanning microscope (LSM 510; Carl Zeiss, Jena, Germany).

2.4. Western blotting

Cerebral cortical neurons harvested at 3, 9 or 15 DIV 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 the addition of 10 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% sodium dodecylsulfate (SDS), 0.01% bromophenol blue and 5% mercaptoethanol (SDS sample buffer) at a volume ratio of 4:1 and subsequent boiling at 100°C for 10 min (Manabe et al., 2001). Each aliquot of 10-40 μg proteins was loaded on a 7.5% polyacrylamide gel for electrophoresis at a constant current of 15 mA/plate for 2 h at room temperature and subsequent blotting to a polyvinylidene fluoride membrane previously treated with 100% methanol. After blocking by 5% skimmed milk, and 3% normal horse serum as needed, 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, GFAP, the synapse marker protein synapsin-I, GAP-43 or the neuronal marker proteins NSE and NeuN, adequately diluted with the buffer containing 1% skim milk, followed by a reaction with an anti-mouse IgG, anti-rabbit IgG or anti-goat IgG antibody conjugated with peroxidase. Proteins reactive with those antibodies were detected with the aid of ECL^{TM} detection reagents through exposure to X-ray films.

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

Cortical neurons or astrocytes 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 and incubated at 37°C for 50 min in recording medium containing 30 nM Pluronic

F-127 and 3 μM fluo-3 acetoxymethyl ester that is a membrane-permeable form of the Ca^{2+}-sensitive dye (Nakamichi et al., 2002a). Cultures were then washed with recording medium twice, followed by settlement for at least 1 h in the recording medium and subsequent placement of the 6-well dish under a confocal laser-scanning microscope. Medium was changed once more, followed by the addition of NMDA at 10 times higher concentrations than the final concentrations 5 min later. Cells were invariably used within 1 to 5 h after these procedures for observation with a confocal laser-scanning microscope. Drugs were prepared in recording medium immediately before each use. Dye-loaded cells were monitored for fluorescence visualized with a confocal laser-scanning microscope equipped with an argon laser. Images were obtained by using 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. The parameters of illumination and detection were digitally controlled to keep the same settings throughout the experiments (Nakamichi et al., 2002b). For quantitative analysis, fluorescence images were quantified using Scion Image β 4.02 software (Scion Co., Frederick, MD, USA) as a mean of the whole screen. Fluorescence intensity was calculated in arbitrary digital units according to conversion of the photomultiplier output into numbers from 0 to 255. An image acquired 1 min before medium change was considered to be zero as described previously (Nakamichi et al., 2002a,b).

2.6. Determination of cell viability

Neuronal survivability analysis was performed by means of Trypan blue exclusion. Cortical neurons cultured for 3, 9 or 15 DIV were exposed to 50 μM NMDA for 5 min in the absence of $MgCl_2$, followed by washing and subsequent second exposure to 50 μM NMDA

with intervals of 25 min after washing. Cells were immediately stained with 1.5% Trypan blue for 10 min at room temperature 5 min before or after the first exposure to NMDA, or 5 min after the second exposure to NMDA, followed by fixation with isotonic formalin and subsequent rinsing with PBS (Taguchi et al., 2003). Cells stained with Trypan blue were regarded as non-viable.

2.7. Preparation of astrocyte cultures

Astrocytes were prepared as described previously (Murakami et al., 2003). In brief, brain cortices from 19-day-old embryos of Wistar rats were cleared of meninges, cut into about 1-mm^3 blocks, and treated with 0.25% trypsin in Ca^{2+}, Mg^{2+}-free PBS containing 5.5 mM glucose for 20 min at 37°C with gentle shaking. An equal volume of horse serum supplemented with 0.1 mg/mL of DNase I was added to the medium to inactivate trypsin. Then, tissues were centrifuged at 1,500×*g* for 5 min. The tissue sediments were triturated through a Pasteur pipette with DMEM containing 10% FCS, 100 mg/L streptomycin, and 5×10^4 unit/L penicillin. After filtration of cell suspensions through a lens-cleaning paper (Fuji Photo, Tokyo, Japan), cells were plated on polyethylenimine-coated 100 mm-diameter plastic dishes (NUNC, Roskilde, Denmark) at a density of 0.8-1.3×10^5 cells/cm^2. Cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C with changing medium every 3 days. After one week, astrocytes were replated to remove neurons. On days 12–14, they were replated onto a 6-well dish using an ordinary trypsin-treatment technique at a density of 1.0×10^5 cells/cm^2 and cultured for additional 2 DIV.

2.8. Detection of surface expressed receptors

Cortical neurons cultured for 9 DIV were exposed to 50 μM NMDA for 5 min in the absence of $MgCl_2$, followed by washing and subsequent additional incubation at 37°C for different periods up to 45 min after washing. In immunocytochemical experiments, cells were immediately placed on ice before, immediately, 25 or 45 min after brief exposure to NMDA for 5 min, followed by washing with ice-cold PBS. Cells were then fixed with 4% paraformaldehyde in PBS for 20 min at 4°C, followed by blocking with 10% bovine serum albumin in PBS not containing Triton X-100. Finally, cells were reacted with the primary antibody directed against extracellular loop of NR1 subunit, followed by incubation with the secondary antibody conjugated with rhodamine for detection by a confocal laser-scanning microscope.

2.9. Data analysis

Densitometric data were subjected to calculation of the area under the curve by the PC computer. For samples on the same gel, the area was directly used as a densitometric unit for quantitative analysis. Results are all expressed as the mean ± S.E. and the statistical significance was determined by the one-way analysis of variance ANOVA with Bonferroni/Dunnett post hoc test.

3. Results

3.1. In vitro neuronal maturation

Rat cortical neurons were cultured for 3 to 15 DIV, followed by determination of expression of the neuronal marker protein MAP-2 and the neuron specific nuclear protein

NeuN on immunocytochemistry. Immunoreactivities were similarly seen to both MAP-2 and NeuN in cell bodies, with a gradual increase in neurites during *in vitro* culture from 3 to 15 DIV (data not shown). By contrast, the number of neurons rather decreased in proportion to the duration of cultivation. On Western blotting analysis, expression of MAP-2 was not markedly changed between cortical neurons cultured for 3 and 9 DIV, with a significant decrease on 15 DIV (Table 1). The glial marker protein GFAP was highly expressed in cells cultured for 9 DIV in a manner dependent on the duration of culturing (Table 1). Double immunocytochemical analysis using antibodies against MAP-2 and GFAP revealed that the population of MAP-2-positive cells was over 98%, 95% and 90% in cells cultured for 3, 9 and 15 DIV, respectively (data not shown). Expression of the synapse marker protein synapsin I, the neuronal marker protein NSE and NeuN significantly increased in a manner dependent on the culture period up to 15 DIV, while GAP-43 significantly increased in cortical cells cultured for 3 to 9 DIV with a significant decrease in cells cultured for 9 to 15 DIV (Table 1).

3.2. Changes in intracellular Ca^{2+} by NMDA

Cortical neuronal cells were cultured for 3, 9 or 15 DIV, followed by exposure to 50 μM NMDA in the absence of $MgCl_2$, because Mg^{2+} blocked the elevation of intracellular free Ca^{2+} levels by NMDA in previous our study (Nakamichi et al., 2002a,b), for determination of intracellular free Ca^{2+} levels on fluo-3 fluorescence image analysis. The exposure to NMDA markedly increased the number of cells with increased fluorescence intensity 5 min later in cortical neuronal cells cultured for 3, 9 and 15 DIV (Fig. 1a). The two different calcium ionophores ionomycin and A23187 at 10 μM were added 5 min after the addition of NMDA for normalization of the fluorescence by NMDA over the maximal fluorescence by the additions of the ionophores for 5 min. In both cases using ionomycin and A23187 for

normalization, NMDA was more efficient in increasing intracellular free Ca^{2+} ions in cortical neurons cultured for 9 and 15 DIV than in neurons cultured for 3 DIV (Fig. 1b). Values normalized by A23187 were smaller than these normalized by ionomycin, it may be dependent on the difference of efficacy between A23187 and ionomycin at the concentration used. Cortical neurons were exposed to NMDA at different concentrations from 1 to 100 μM, followed by determination of the fluorescence intensity 5 min later. The exposure to NMDA increased the fluorescence intensity in a concentration-dependent manner irrespective of the maturity of neurons used (Fig. 1c). EC_{50} values were calculated according to the Hill plot analysis using the computer program "Origin". The sensitivity to NMDA was higher in cortical neurons cultured for 9 DIV (EC_{50}=7.16±1.37 μM, $P<0.05$) and 15 DIV (EC_{50}=4.89±0.47 μM, $P<0.01$) than in neurons cultured for 3 DIV (EC_{50}=11.64±1.48 μM).

3.3. Reduced responsiveness to repeated stimulation

When cortical neurons cultured for 3, 9 or 15 DIV were continuously exposed to 50 μM NMDA, the fluorescence intensity was immediately increased and the increase was sustained for at least 60 min at constant levels as long as NMDA was present (Fig. 2).

Cultured neurons were briefly exposed to 50 μM NMDA for 5 min, followed by washing and subsequent second brief exposure to NMDA at the same concentration for 10 min with an interval of 25 min. The second exposure to NMDA led to a less efficient increase in the fluorescence intensity than that found after the first exposure in neurons cultured for 9 and 15 DIV, but not in those cultured for 3 DIV (Fig. 3a). When the interval was prolonged from 25 min to over 35 min, however, the second exposure was less effective in increasing the fluorescence intensity than the first exposure even in neurons cultured for 3 DIV as seen in neurons cultured for 9 and 15 DIV (Fig. 3b). When the interval was shortened from 25 to 5

min, by contrast, a similarly efficient increase was seen in the fluorescence intensity even after the second exposure irrespective of the duration of culturing. However, NMDA did not significantly affect cellular viability after the first and the second exposure compared with that before the first exposure (Fig. 3c).

3.4. Possible involvement of astrocytes

As the glial marker protein GFAP was markedly expressed in cells cultured for a period longer than 9 DIV, an attempt was next made to determine whether astrocytes are involved in the decrease by repeated exposure to NMDA. For this purpose, astrocytes prepared from embryonic rat neocortex were cultured and loaded with the fluorescent dye fluo-3 2 days after replating. These cultured cells were exposed to 50 mM KCl in the presence of $MgCl_2$ or to 50 μM NMDA in the absence of $MgCl_2$, followed by determination of fluorescence intensity for 5 min. As shown in Figure 4a (left panel), KCl induced a marked increase in the number of cells with increased fluorescence intensity within 5 min after the addition, but NMDA did not markedly affect the number of fluorescent cells. Quantitative analysis revealed that KCl significantly increased the fluorescence intensity immediately after the addition, followed by a sustained increase during exposure to KCl (Fig. 4a, right panel). However, NMDA failed to increase the fluorescence intensity during exposure for 5 min in cultured cortical astrocytes. In cortical neuronal cells previously treated with Ara-C on 2 to 3 DIV and cultured for up to 15 DIV, moreover, the second exposure to NMDA also led to a less efficient increase in the number of neurons with high fluorescence than that found after the first exposure in neurons cultured for 9 and 15 DIV, but not in those cultured for 3 DIV (Fig. 4b), as seen in cortical cells not treated with Ara-C.

3.5. Changes in membranous NMDA receptors

An attempt was made to determine whether the number of NMDA receptors expressed on cellular surfaces is changed after the stimulation by an agonist. Cortical neurons cultured for 9 DIV were briefly exposed to 50 μM NMDA for 5 min, followed by washing and subsequent additional incubation at 37°C for different periods up to 45 min after washing. Immunocytochemical analysis using an antibody against the extracellular loop of NR1 subunit revealed that surface immunoreactivity to NR1 subunit was gradually attenuated in proportion to the time after the washing (Fig. 5).

4. Discussion

The data provided above clearly indicate that cellular maturation increased the possibility for NMDA receptor channels to undergo desensitization in cultured rat neocortical neurons. In contrast to non-NMDA receptors that rapidly desensitize, NMDA channels are shown to have rather long opening times (Ascher and Nowak, 1987; Gasic and Hollmann, 1992), as revealed by intracellular free Ca^{2+} levels in the present study where a sustained increase was seen at the constant level during continuous exposure to NMDA. Compared to studies using electrophysiological techniques, the present investigation has an advantage that desensitization of NMDA receptor channels could be monitored in terms of the level of intracellular free Ca^{2+} ions directly permeable to the channels. The possibility that sustained exposure to NMDA could lead to a persistent increase in intracellular free Ca^{2+} levels through facilitation of both the influx across voltage-sensitive Ca^{2+} channels and the release from intracellular Ca^{2+} stores as well as the activation of NMDA receptor channels to this divalent cation in cultured neurons as shown previously (Nakamichi et al., 2002a), by contrast, is a

disadvantage not ruled out in the present analysis. Our previous findings that the addition of an NMDA receptor antagonist completely abolishes the increased fluorescence in the presence of NMDA (Nakamichi et al., 2002b), however, argue in favor of an idea that activation of NMDA receptor channels are essentially required for the sustained increase in intracellular free Ca^{2+} levels in cultured neurons. The possible developmental desensitization shown here could involve mechanisms underlying altered subunit compositions (Monyer et al., 1994; Dingledine et al., 1999; Perez-Otano and Ehlers, 2004) and/or amino acid substitutions (Sakurada et al., 1993) of NMDA receptor channels during maturation of cultured cortical cells from 3 to 15 DIV. Nevertheless, the significant decrease in responsiveness to the second exposure to NMDA with a prolonged interval gives rise to an idea that reduced responsiveness of NMDA-gated Ca^{2+} channels would occur even in immature cultured neurons.

It should be emphasized that NMDA responsiveness underwent possible desensitization with regard to intracellular free Ca^{2+} ions in cultured cortical neurons following repeated brief exposure to NMDA in a manner dependent on intervals between the 2 brief exposures, but not after sustained continuous exposure to NMDA. Reduced responsiveness could lead to protection against the excitotoxicity mediated by NMDA receptor channels even when overactivation occurs with the channels following a massive increase in extracellular glutamate in particular pathological situations. Moreover, the brief exposure to NMDA led to decreased immunoreactivity to extracellular loop of NR1 subunit in proportion to the time after washing. Although NMDA receptors are often considered ‘stable’ in the plasma membrane (Man et al., 2000; Roche et al., 2001; Nong et al., 2003), there is accumulating evidence that NMDA receptors may also undergo internalization (Man et al., 2000; Roche et al., 2001; Vissel et al., 2001; Carroll and Zukin, 2002; Nong et al., 2003, 2004). In different metabotropic receptor systems, an agonist binds to surface receptor

proteins leading to initiate the internalization and subsequent desensitization processes (Claing et al., 2002; Dale et al., 2002). If NMDA receptors should internalize according to this model, NMDA receptors would have been internalized and desensitized following sustained exposure to an agonist. The possibility that the dissociation, but not association, of an agonist may prime the internalization of subunit proteins required for functional heteromeric assemblies of NMDA receptor channels is thus conceivable. Reduced responsiveness would rescue neurons from cell death due to the overload of free Ca^{2+} ions after overactivation of NMDA receptor channels under particular pathological conditions.

It thus appears that reduced responsiveness could lead to a decrease in intracellular free Ca^{2+} levels after repeated stimulation by NMDA through the internalization of NMDA receptor channels which could be primed by the dissociation, but not association, of an agonist in cultured neocortical neurons. Desensitization of NMDA receptors during repetitive firing is shown to contribute to shaping synaptic responses and neuronal activity (Tong et al., 1995; Jones and Westbrook, 1996). Mechanisms for regulated desensitization of NMDA receptors may be responsible for several forms of long-term depression in the brain. As neuronal cell death would undoubtedly involve mechanisms relevant to overshooting of intracellular free Ca^{2+} levels, elucidation of the reduced responsiveness processes could give us a new point of view toward the therapy and treatment of a variety of neurodegenerative disorders in human beings.

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Figure legends

Fig. 1. Effects of NMDA on intracellular Ca^{2+} ions in cultured neurons. Cortical neurons cultured for 3 to 15 DIV were loaded with fluo-3, followed by the exposure to 50 μM NMDA in the absence of $MgCl_2$. a). A representative observation is shown with similar results in 3 independent experiments. b). Two different calcium ionophores ionomycin and A23187 at 10 μM were added 5 min after the addition of NMDA. Values are the mean±S.E. of percentages over the maximal value (maximum) obtained 5 min later in neurons exposed to calcium ionophores in 8 to 9 independent determinations. *P<0.05, **P<0.01, significantly different from the value obtained in cells cultured for 3 DIV. c). Cortical neurons were exposed to NMDA at different concentrations from 1 to 100 μM in the absence of $MgCl_2$. Values are the mean±S.E. of percentages over the maximal value (maximum) obtained 5 min later in cells exposed to 100, 50 and 20 μM NMDA in neurons cultured for 3, 9 and 15 DIV, respectively, in 9 to 13 independent determinations. *P<0.05, **P<0.01, significantly different from the value obtained in neurons cultured for 3 DIV.

Fig. 2. Sustained exposure to NMDA. Cortical neurons cultured for 3 to 15 DIV were loaded with fluo-3, followed by the exposure to 50 μM NMDA in the absence of $MgCl_2$. Values are the mean±S.E. of percentages over the value (control) obtained 5 min later in neurons exposed to NMDA in 6 independent determinations. **P<0.01, significantly different from the value obtained in neurons cultured for 3 DIV.

Fig. 3. Repeated stimulation by NMDA. Cortical neurons cultured for 3 to 15 DIV were loaded with fluo-3. a). Cells were exposed to 50 μM NMDA for 5 min in the absence of $MgCl_2$, followed by washing and subsequent second exposure to 50 μM NMDA with

intervals of 25 min after washing. Fluorescence intensity was determined 5 min after the second exposure to NMDA for quantitative analysis. Values are the mean±S.E. of percentages over the value (control) obtained 5 min later in neurons exposed to first NMDA in 6 to 8 independent determinations. **P<0.01, significantly different from the value obtained in neurons cultured for 3 DIV. b). Intervals between first and second exposure to NMDA were changed from 5 to 45 min. *P<0.05, **P<0.01, significantly different from the value obtained in neurons exposed to second NMDA with interval of 5 min after washing. c). Cortical neurons cultured for 3 to 15 DIV were exposed to 50 μM NMDA for 5 min in the absence of $MgCl_2$, followed by washing and subsequent second exposure to 50 μM NMDA with intervals of 25 min after washing. Cell viabilities were determined by Trypan blue exclusion 5 min before or after first exposure to NMDA, or 5 min after second exposure to NMDA. Values are the mean±S.E. of percentages over the value (control) obtained 5 min before first exposure to NMDA in 20 different observations from 4 independent preparations.

Fig. 4. Involvement of astrocytes. a). Cultured cortical astrocytes were loaded with fluo-3 at 2 days after replating, followed by exposure to 50 mM KCl in the presence of $MgCl_2$ or to 50 μM NMDA in the absence of $MgCl_2$. A representative observation is shown for pictures obtained 5 min after the addition of KCl or NMDA with similar results in 3 independent experiments. Quantitative data are shown as the mean ± S.E. in 3 separate measurements. b). Cortical neurons were treated with 10 μM Ara-C on 2 to 3 DIV and cultured for 3 to 15 DIV. Cells loaded with fluo-3 were exposed to 50 μM NMDA for 5 min in the absence of $MgCl_2$, followed by washing and subsequent second exposure to 50 μM NMDA with intervals of 25 min after washing. Fluorescence intensity was determined 5 min after the second exposure to NMDA for quantitative analysis. Values are the mean±S.E. of percentages over the value (control) obtained 5 min later in neurons exposed to first NMDA in 8 to 11 independent

determinations. *P<0.05, significantly different from the value obtained in neurons cultured for 3 DIV.

Fig. 5. Changes in membranous NMDA receptors after NMDA stimulation. Cortical neurons cultured for 9 DIV were exposed to 50 μM NMDA for 5 min in the absence of $MgCl_2$, followed by washing and subsequent additional incubation at 37°C for different periods after washing. A representative observation is shown with similar results in 3 independent experiments.

Table 1. *In vitro* neuronal maturation.

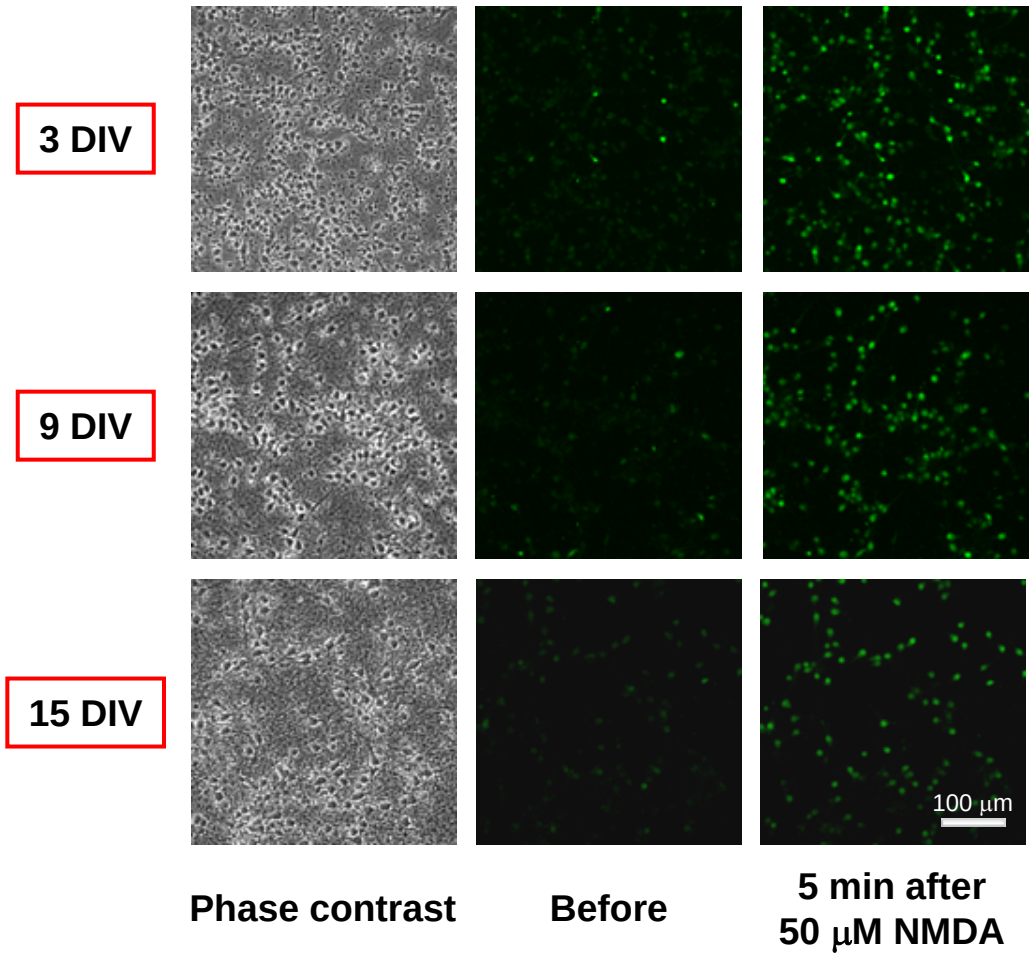
Cortical cultures were harvested on 3, 9 or 15 DIV, followed by homogenization and subsequent SDS-PAGE for immunoblotting using an antibody against MAP-2, GFAP, synapsin I, GAP-43, NSE or NeuN. Values are the mean±S.E. of percentages over the value obtained in neurons cultured for 3 DIV in 6 independent determinations. **P<0.01, significantly different from the value obtained in cells cultured for 3 DIV.

Table 1

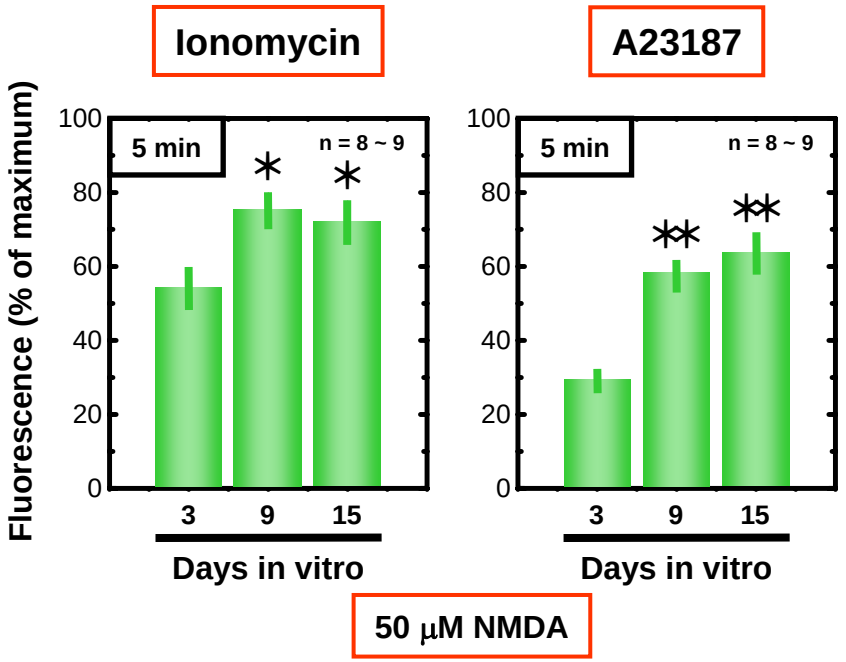
	3 DIV	9 DIV	15 DIV
MAP-2	**100.00 ± 10.89**	**84.04 ± 6.59**	**52.60 ± 3.93****
GFAP	**100.00 ± 17.40**	**318.88 ± 25.08****	**696.59 ± 52.30****
Synapsin I	**100.00 ± 9.31**	**334.32 ± 41.42****	**487.85 ± 42.63****
GAP-43	**100.00 ± 12.36**	**285.41 ± 32.61****	**132.85 ± 16.90**
NSE	**100.00 ± 12.70**	**379.47 ± 52.19****	**715.40 ± 71.10****
NeuN	**100.00 ± 10.62**	**339.87 ± 34.40****	**658.32 ± 52.87****

Fig. 1

(a)


3 DIV
9 DIV
15 DIV
100 μm
Phase contrast
Before
5 min after
50 μM NMDA

(b)


Ionomycin
A23187
Fluorescence (% of maximum)
5 min
n = 8 ~ 9
100
80
60
40
20
0
3
9
15
Days in vitro
50 μM NMDA

(c)

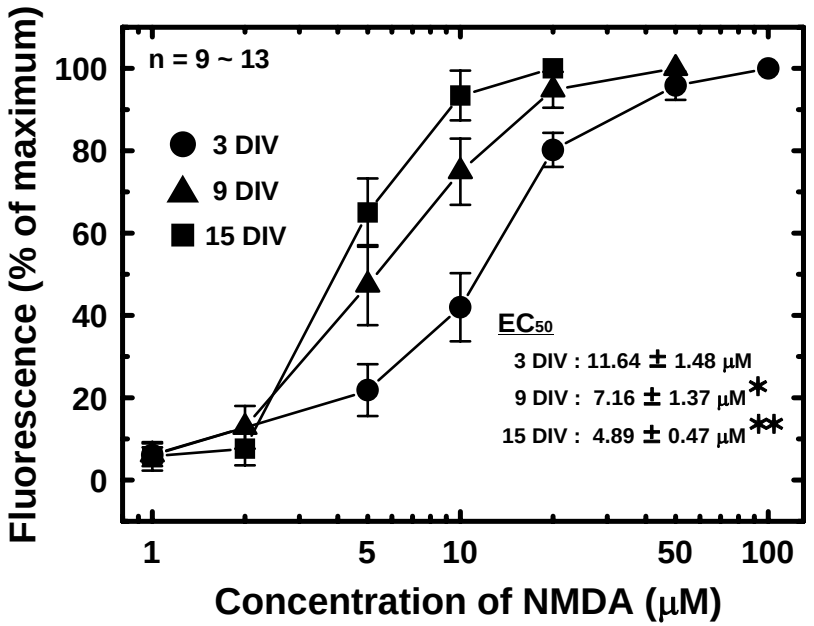

Fluorescence (% of maximum)
n = 9 ~ 13
3 DIV
9 DIV
15 DIV
EC50
3 DIV : 11.64 ± 1.48 μM
9 DIV : 7.16 ± 1.37 μM*
15 DIV : 4.89 ± 0.47 μM**
100
80
60
40
20
0
1
5
10
50
100
Concentration of NMDA (μM)

Fig. 2

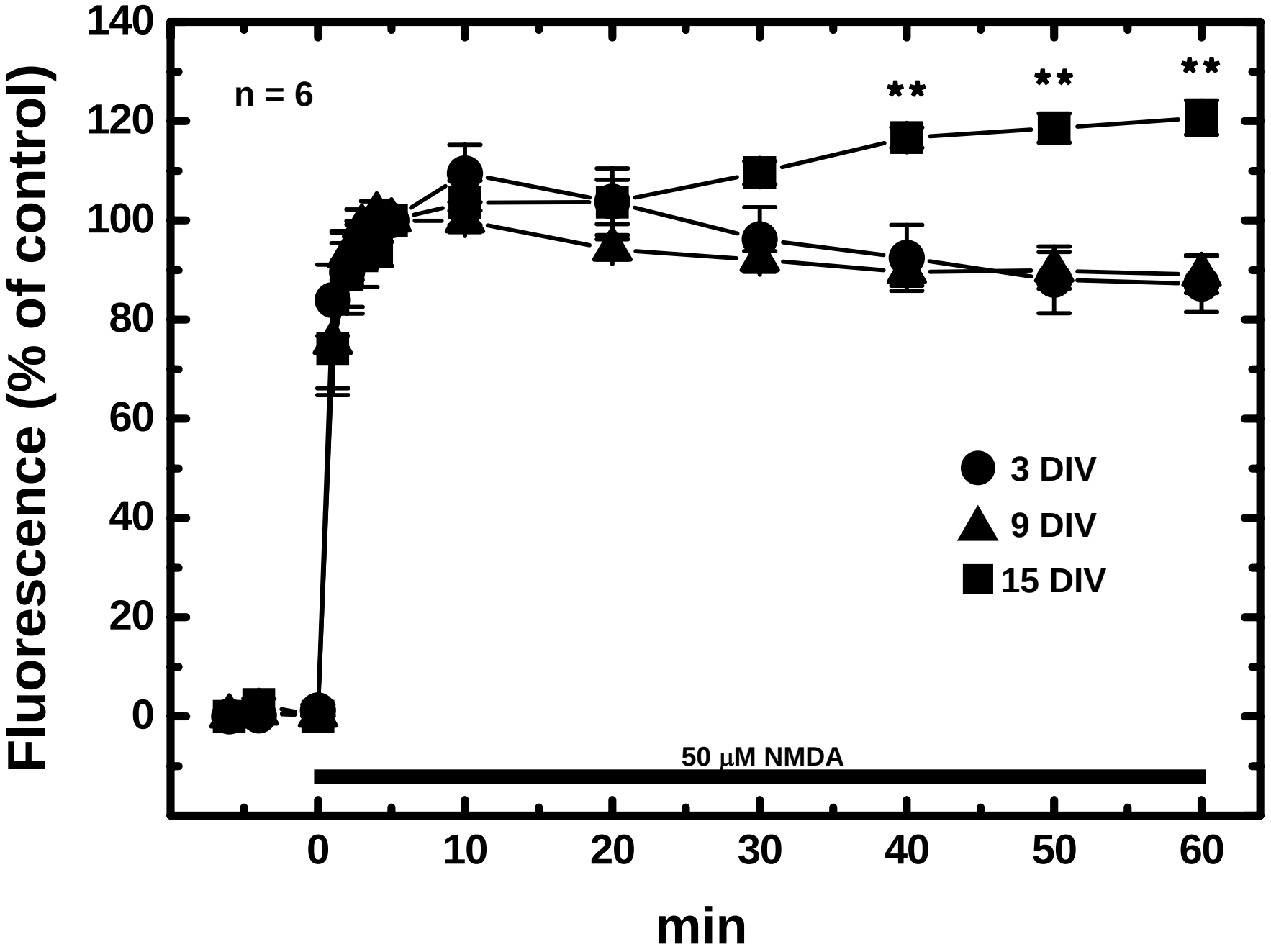
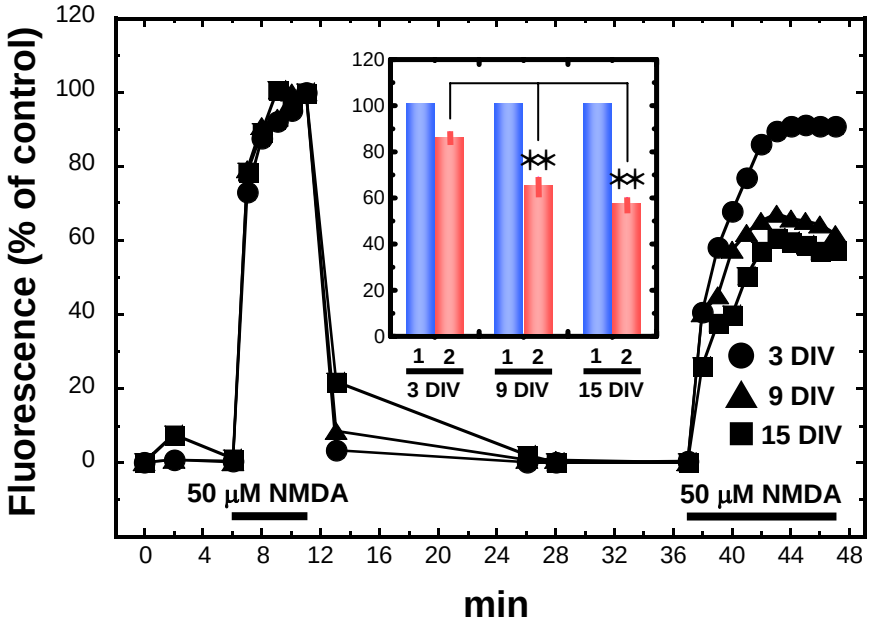
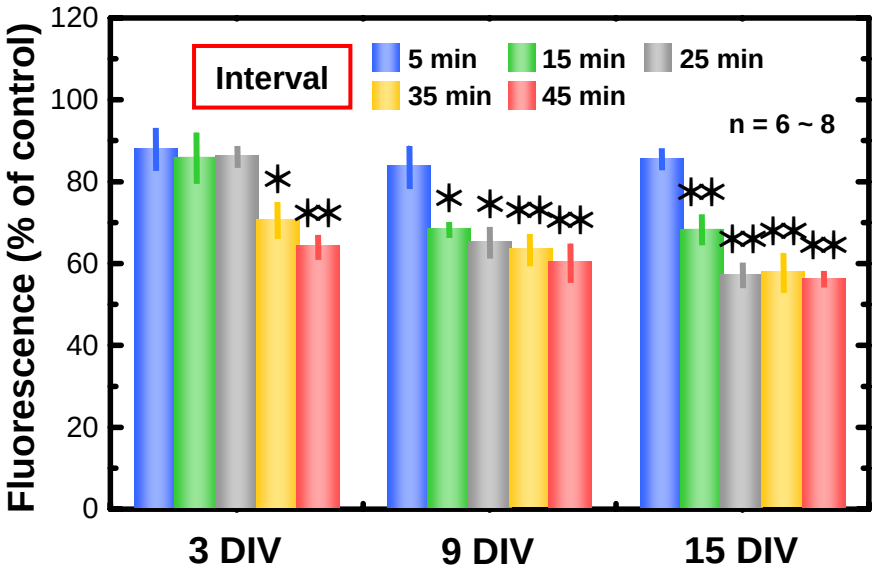

Fluorescence (% of control)
140
120
100
80
60
40
20
0
n = 6
**
**
**
3 DIV
9 DIV
15 DIV
50 μM NMDA
0
10
20
30
40
50
60
min

Fig. 3

(a)


Fluorescence (% of control)
min
50 μM NMDA
50 μM NMDA
3 DIV
9 DIV
15 DIV
1 2
3 DIV
9 DIV
15 DIV

(b)


Fluorescence (% of control)
Interval
5 min
15 min
25 min
35 min
45 min
n = 6 ~ 8
3 DIV
9 DIV
15 DIV

(c)

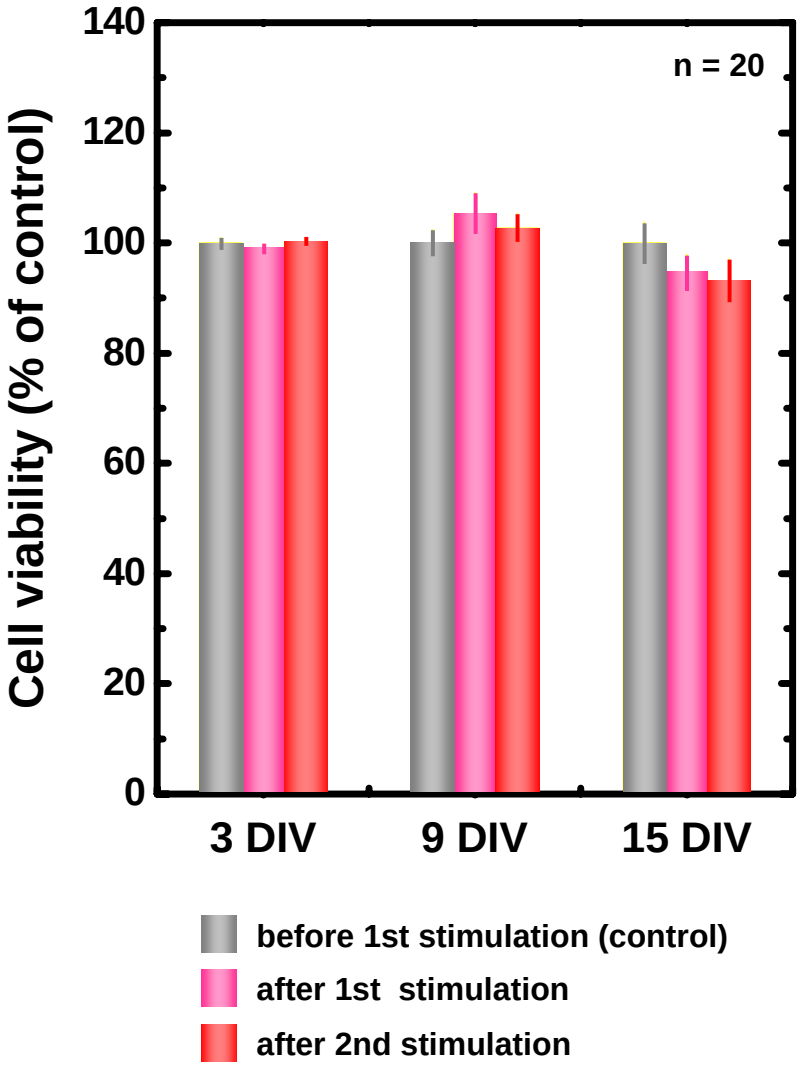
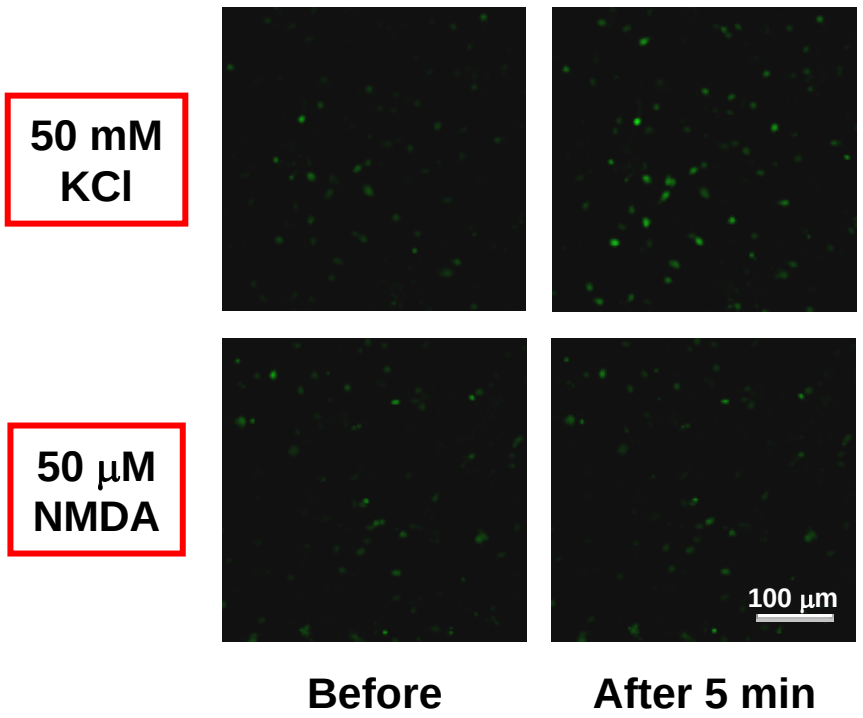
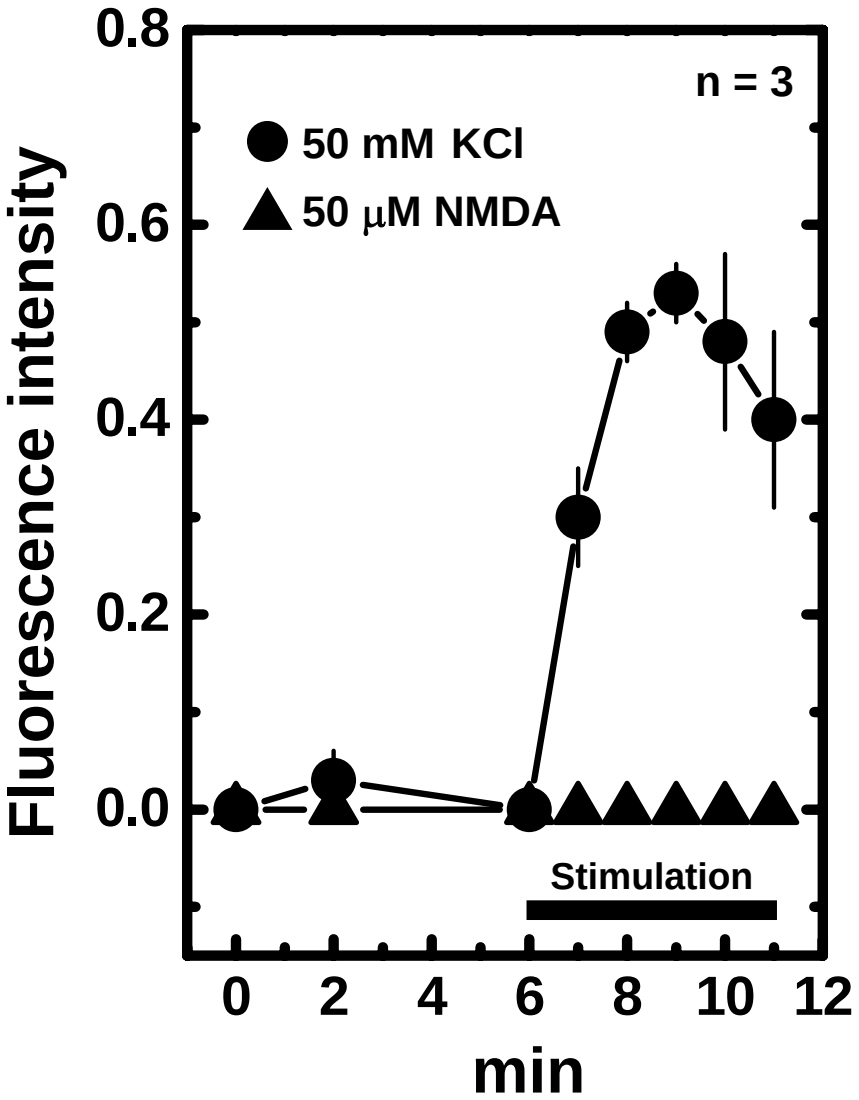

Cell viability (% of control)
n = 20
3 DIV
9 DIV
15 DIV
before 1st stimulation (control)
after 1st stimulation
after 2nd stimulation

Fig. 4

(a)


50 mM KCl
50 μM NMDA
100 μm
Before
After 5 min


n = 3
50 mM KCl
50 μM NMDA
Fluorescence intensity
0.0
0.2
0.4
0.6
0.8
Stimulation
0
2
4
6
8
10
12
min

(b)

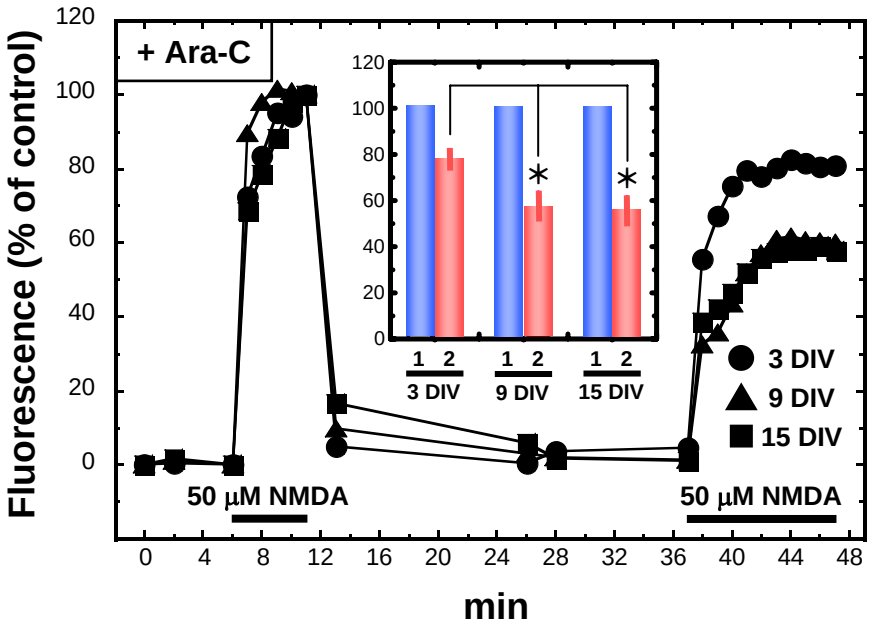

+ Ara-C
Fluorescence (% of control)
120
100
80
60
40
20
0
50 μM NMDA
50 μM NMDA
0
4
8
12
16
20
24
28
32
36
40
44
48
min
3 DIV
9 DIV
15 DIV
1 2
3 DIV
9 DIV
15 DIV

Fig. 5