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Roles of Ionotropic Glutamate Receptors in Early Developing Neurons Derived from the P19 Mouse Cell Line

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Key Words
P19 cells · Glutamate receptor · Kainic acid · Hypoxia · Nerve growth factor

Abstract
We cultured a P19 mouse teratocarcinoma cell line and induced its neuronal differentiation to study the function of ionotropic glutamate receptors (GluRs) in early neuronal development. Immunocytochemical studies showed 85% neuronal population at 5 days in vitro (DIV) with microtubule-associated protein 2-positive staining. Thirty percent and 50% of the cells expressed the α-amino-3-hydroxy-5-methyl-4-isopropinonate (AMPA) receptor subunit, GluR2/3, and the kainate (kainic acid; KA) receptor subunit, GluR5/6/7, respectively. In Western blot analysis, the temporal expression of GluR2/3 began to appear at 3 DIV, whereas GluR5/6/7 was already expressed in the undifferentiated cells. P19-derived neurons began to respond to glutamate, AMPA and KA, but not to the metabotropic GluR agonist trans-1-aminocyclopentane-1,3-decarboxylic acid, by 5 DIV in terms of increases in intracellular calcium and phospholipase C-mediated poly-phosphoinositide turnover. Furthermore, KA reduced cell death of P19-derived neurons in both atmospheric and hypobaric conditions in a phospholipase C-dependent manner. The common AMPA/KA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione, but not the AMPA receptor antagonist, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium, profoundly increased hypobaric insult-induced neurotoxicity. In a flow cytometry study, the nerve growth factor-mediated antiapoptotic effect was facilitated by AMPA, with an induction of TrkA, but not $p75^{NTR}$ expression. Therefore, AMPA and KA receptors might mediate neurotrophic functions to facilitate neurotrophic factor signaling to protect neurons against hypoxic insult in early neuronal development.

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Introduction

Excitatory neurotransmission in the mammalian central nervous system is mainly mediated by glutamate receptors (GluRs). GluRs are divided into two distinct categories, called metabotropic GluRs (mGluRs) and ion-

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otropic GluRs [for a review, see ref. 21]. mGluRs are coupled to G protein and mediate phospholipase C activity or regulate adenylate cyclase activity. Ionotropic GluRs are further divided into three subtypes based on their agonist specificities, namely α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate (kainic acid; KA) and N-methyl-*D*-aspartate (NMDA) receptors. In neonatal brain, AMPA/KA receptors and the NMDA receptor display sequential participation in neuronal excitation [4]. Glutamate receptors are believed to play important roles in neural development, i.e. underactivation can retard or disrupt normal development, whereas overexcitation can lead to neuronal injury and destruction [20]. AMPA/KA receptors have been found on the membranes of proliferating ventricular zone cells as well as in cortical neuroepithelial cells undergoing neurogenesis [16, 18]. After mitosis and during migration out of the ventricular zone, immature migrating neurons begin to express the NMDA receptor [15]. After synaptogenesis, mGluRs are expressed at a transient level [1]. The early appearance of AMPA/KA receptors in the developing brain implies that they may play important roles in neuronal differentiation. There has been ample evidence suggesting that GluRs may mediate activity-dependent neurotrophic activities, such as neuronal survival and neurite outgrowth for neuronal plasticity and injury repair in both the developing brain as well as in injured peripheral neurons [14, 28, 29]. A GluR-mediated intracellular calcium increase through extracellular calcium entry or release of intracellular calcium storage, as well as GluR-induced expression and activation of neurotrophin receptors, were reported to be the possible mechanisms involved in these neurotrophic activities [14, 28].

Neurons differentiated from pluripotent teratocarcinoma cell lines, such as the mouse teratocarcinoma cell line P19 and the human teratocarcinoma cell line NT2, have been shown to functionally express all subtypes of GluRs and to show typical responses upon glutamate stimulation, such as glutamate-evoked synaptic potential and elevation of intracellular calcium [7, 11, 17, 30]. Most of these studies used late-developing neurons to match their behavior with mature central neurons. However, how neurons respond to glutamate in early neurogenesis is unknown.

In this article, we examined the temporal expression of AMPA and KA receptors in P19 cells during neurogenesis, and their possible roles in neuronal development, with special focus on neuronal survival. The signaling mechanism responsible for their neuroprotective activity was also examined.

Materials and Methods

P19 Cell Culture and Neuronal Differentiation

The P19 cell line was obtained from the American Type Culture Collection (Rockville, Md., USA). Undifferentiated P19 cells were maintained in alpha-minimal essential medium (α-MEM) supplemented with 2.5% fetal bovine serum (Hyclone, Logan, Utah, USA) and 7.5% calf serum (Biochrom KG, Berlin, Germany). To induce neural differentiation, P19 cells were cultured for 4 days in serum-containing α-MEM medium with 1 μ*M* retinoic acid, and seeded onto a 10-cm bacteriological culture dish with approximately 1×10^6 cells per plate. Cells did not adhere to dishes but instead formed large aggregates in suspension. After 4 days of retinoic acid treatment, aggregates were trypsinized, washed, resuspended in serum-containing α-MEM and plated onto poly-*L*-lysine-coated 24-well plates at a density of approximately 2.5×10^5 cells per well or onto 35-mm dishes at 1×10^6 cells per dish. After a 1-hour incubation at 37°C in a 5% CO_2 incubator, the culture medium was changed to Neurobasal medium containing 0.5 m*M* *L*-glutamine and N2 supplement for the first 4 days, and was then changed to Neurobasal medium containing 0.5 m*M* *L*-glutamine and B27 supplement for up to 14 days. The duration of retinoic acid treatment is represented by days of retinoic acid treatment, and the culture stage of plated cells dissociated from retinoic acid-induced aggregates is represented by days in vitro (DIV). Neurobasal medium, α-MEM medium and N2 and B27 supplements were obtained from Life Technology (Gaithersburg, Md., USA).

Primary Culture of Cortical Neurons

Primary cortical cultures were prepared as described previously [14]. In brief, cerebral cortex was dissected from fetal Sprague-Dawley rats on embryonic day 17. Cortical tissue was mechanically dissociated, washed and plated onto poly-*L*-lysine-coated 24-well culture plates at a density of approximately 5×10^5 cells/well, with basal medium Eagle's culture media (BME) supplemented with sodium bicarbonate (13.1 m*M*), *D*-glucose (13.9 m*M*), *L*-glutamine (2.0 m*M*) and heat-inactivated fetal calf serum (20%). Cells were initially incubated for 45 min in a humidified incubator with 5% CO_2 at 37°C for monolayer attachment, after which the medium was replaced with serum-free BME, and the cells were maintained in 5% CO_2 at 37°C thereafter. Cultures obtained under these conditions appeared to contain mostly neuronal cells (>85%) [14].

Immunocytochemistry

P19 cells at different developmental stages were rinsed twice with a control solution (120 m*M* NaCl, 5.4 m*M* KCl, 8 m*M* $MgCl_2$, 1.8 m*M* $CaCl_2$, 15 m*M* glucose, pH 7.4) and fixed for 30 min at 37°C with 4% paraformaldehyde and 1% H_2O_2 in 20 m*M* phosphate-buffered saline (PBS; 16.4 m*M* Na_2HPO_4, 3.6 m*M* NaH_2PO_4, 0.9% NaCl, pH 7.5). Fixed cells were rinsed with 20 m*M* PBS containing 0.2% Tween 20 and incubated sequentially with blocking solution, primary antibody, biotinylated secondary antibody and avidin-horseradish peroxidase (HRP) reagent as described in the instructions of the Vectastain ABC kit (Vector Laboratories, Burlingame, Calif., USA). The anti-microtubule-associated protein 2 (MAP-2) monoclonal antibody, the anti-glial fibrillary acidic protein (GFAP) monoclonal antibody, the anti-GluR2/3 monoclonal antibody and the anti-GluR5/6/7 monoclonal antibody were obtained from Chemicon (Temecula, Calif., USA), and were properly diluted for primary antibody incubation. Color development of the immuno-

reactive cells was performed using diaminobenzidine tetrahydrochloride (DAB) in an HRP substrate DAB kit (Vector Laboratories). Immunomicrographs were taken using an Olympus (Tokyo, Japan) DP50 digital camera.

Western Blot Analysis

P19 cells at different developmental stages were harvested with ice-cold homogenizing buffer (50 m*M* Tris-HCl, containing 1 m*M* EDTA, 1 m*M* sodium orthovanadate, 10 μg/ml trypsin inhibitor and 10 μg/ml leupeptin, pH 7.4). Cells were sonicated and centrifuged at 100,000 *g* for 30 min to obtain the crude membrane fraction in the pellet. Sixty micrograms of protein per lane of the crude membrane fraction was separated onto 7.5 or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a Hybond enhanced chemiluminscence nitrocellulose membrane (Amersham, Buckinghamshire, UK) and probed with anti-GluR2/3, anti-GluR5/6/7 or $P75^{NTR}$ monoclonal antibodies (Chemicon), or the anti-TrkA polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA). The immune complex was further probed with HRP-conjugated goat anti-rabbit IgG, visualized by HRP-reactive chemiluminescence reagents (Pierce, Rockford, Ill., USA) and developed on autoradiographic film (Kodak BioMax film, Eastman Kodak, Rochester, N.Y., USA). The relative density of the protein band in the Western blot was further analyzed with an electrophoresis image analysis system (Eastman Kodak).

Quantitative Assay of Neuronal Cell Death

Cultured neurons were equilibrated with newly changed Neurobasal medium for 1 h, followed by enzyme inhibitor or antagonist pretreatment for 20 min and KA stimulation for 5–15 or 30–45 min, after which the solution was moved into a 200-mm Hg chamber to undergo hypoxia for 2 min. After stimulation, 0.5 ml of medium was incubated with 0.2 mg of β-dihydronicotinamide adenine dinucleotide in 2.4 ml of 0.1 *M* phosphate buffer for 5–15 min at room temperature. The absorbance at a wavelength of 340 nm was measured for 3 min immediately after 0.1 *M* sodium pyruvate was added to the mixture. The unit activity of lactate dehydrogenase (LDH) released into the culture medium was defined as the decrease in A_{340} in 1 min in 1 ml of sample, multiplied by 1,000.

Measurement of Intracellular Calcium Concentration

Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured in P19-differentiated neurons on coverslips with a Ca^{2+}-sensitive dye, fura-2 acetoxymethyl ester (Fura-2/AM; Molecular Probes, Eugene, Oreg., USA) using a fluorescence spectrophotometer (F-4500, Hitachi, Japan). Cells were loaded for 45 min at 37°C in Neurobasal medium with 5 μ*M* Fura-2/AM. At the end of the loading period, the coverslip was washed with phocal buffer (125 m*M* NaCl, 5 m*M* KCl, 2 m*M* $MgCl_2$, 1.8 m*M* $CaCl_2$, 15 m*M* glucose, pH 7.4). The fluorescence of Ca^{2+}-bound and Ca^{2+}-free Fura-2 was measured with dual excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The ratios (R) of the fluorescence at the two wavelengths were computed and calculated for changes in $[Ca^{2+}]_i$ using built-in software. Ratios of maximum (Rmax) and minimum (Rmin) fluorescence of Fura-2 were determined by the addition of ionomycin (10 m*M*) in PBS containing 5 m*M* Ca^{2+} and by adding 5 m*M* EGTA and 0.2 m*M* $MnCl_2$ at a pH of 8 in Ca^{2+}-free PBS, respectively. The intracellular calcium concentration was obtained from the following equation: $[Ca^{2+}]_i = Kd \times \beta \times (R - Rmin)/(Rmax - R)$, in which Kd = 224 n*M* and β = 6.9352 were assumed.

Poly-Phosphatidylinositol Turnover Analysis

Poly-phosphatidylinositol (PI) turnover in P19-differentiated neurons was determined by prelabeling cells with 1 μCi of *myo*-[^{3}H] inositol (Amersham) for 6 h in 5% CO_2 at 37°C, followed by 3 washes for 10 min each with Earle's balanced salt solution containing 10 m*M* LiCl and agonist stimulation for 45 min with a final volume of 0.6 ml. All incubations described above were in 5% CO_2 at 37°C. The stimulation was terminated by adding four volumes of chloroform-methanol (2:1 vol/vol) to extract PI and inositol phosphates (IPn). The aqueous phase of the mixture, which contained labeled IPn, was eluted from an AG1-X8 (Bio-Rad, Hercules, Calif., USA) anion exchange column by 400 m*M* sodium formate in 100 m*M* formic acid. The organic phase contained labeled PI, which was dried in air and directly counted for radioactivity with a scintillation counter. Levels of poly-PI turnover were expressed as the ratio of [^{3}H]-IPn to [^{3}H]-PI, which normalizes variations in incorporation of [^{3}H]-inositol in cultures.

Flow Cytometry for Determination of Apoptosis

P19 neurons at 5 DIV were treated with 50 μ*M* GluR agonists for 30 min, followed by washes with Neurobasal medium and incubation with Neurobasal medium containing B27 supplement with or without 100 ng/ml nerve growth factor (NGF) for 24 h. P19 neurons were then harvested with PBS containing 0.1% dextrose and fixed in 70% ethanol at 4°C. The DNA content of the nuclei was determined by staining nuclear DNA with 40 μg/ml propidium iodide (Sigma, St. Louis, Mo., USA) in PBS, 100 μg/ml RNaseA and 0.1% bovine serum albumin, and by measuring with a fluorescence-activated cell sorter. The proportion of nuclei in each phase of the cell cycle was determined using established CellFIT DNA analysis software (Becton Dickinson, San Jose, Calif., USA).

Results

Modified Culture Conditions Produce More than 80% Neurons in P19-Differentiated Cells

Application of retinoic acid resulted in differentiation of P19 cells into neuron-like cells (fig. 1A). However, to enrich the neuronal population in the culture, we used serum-free Neurobasal medium combined with N2 and B27 supplements to consistently obtain large populations of neurons without including cytosine arabinoside in the culture. Immunocytochemistry using the neuronal marker anti-MAP-2 antibody and the astroglial marker anti-GFAP antibody revealed that both neural cell types could be derived, with more than 80% neurons and approximately 15–20% astroglia (fig. 1B).

Functional GluRs Are Expressed with Differential Temporal Patterns

To examine whether P19-derived neurons express GluRs similar to central neurons, we immunostained the neurons to identify the populations of AMPA receptor- and KA receptor-positive neurons in P19 neurons at 3

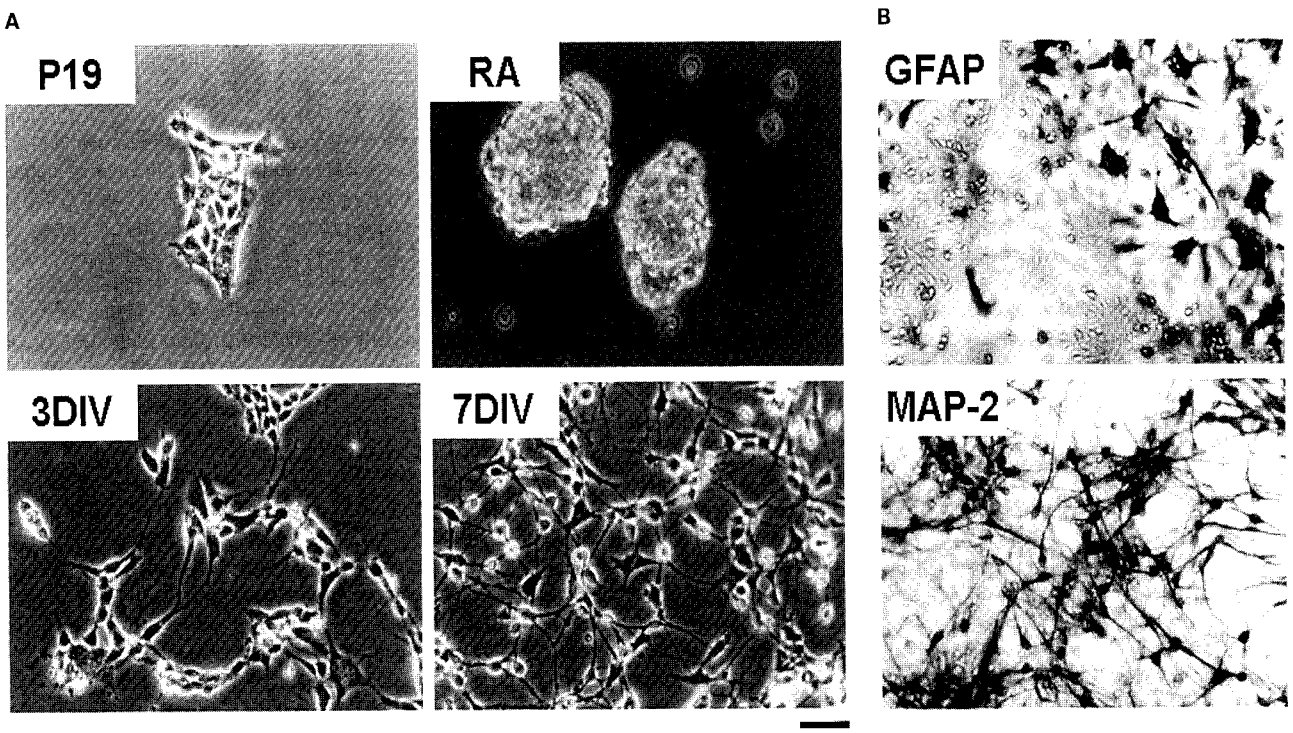


Fig. 1. Differentiation of P19 cells into neurons. **A** Phase contrast photomicrographs show that the undifferentiated P19 cells started to form aggregates during retinoic acid (RA) treatment. Four days after retinoic acid treatment, aggregates were dissociated and plated onto poly-*L*-lysine-coated plates, and started to form neuron-like cells as shown in 3 DIV and 7 DIV. **B** P19 cells at 7 DIV were immunostained with neuron marker MAP-2 and astroglial marker GFAP. Bar = 80 μm.

and 5 DIV. Figure 2A shows that in P19-derived neurons at both 3 and 5 DIV, the immunoreactivity of the KA receptor subunit, GluR5/6/7, was significantly higher than that of the AMPA receptor subunit, GluR2/3. Percentages of GluR5/6/7-positive and GluR2/3-positive neurons were approximately 50 and 30%, respectively. Furthermore, crude membrane fractions of P19 neurons at 3, 5, 7 and 11 DIV were subjected to Western blot analysis for temporal expression profiles of the AMPA and KA receptors. Figure 2B shows that the AMPA receptor subunit GluR2/3 began to appear at 3 DIV and increased toward maturation (11 DIV). On the other hand, the KA receptor subunit GluR5/6/7 had already begun to appear in undifferentiated P19 cells. The expression level of GluR5/6/7 dropped during retinoic acid induction, then was re-elevated after 3 DIV. Expression levels of GluRs coincided with their functional characterization, in which the response of P19 neurons to EAA agonists at 50 μ*M* in terms of intracellular calcium increase, a common feature of GluR activation, appeared after 3 DIV and gradually increased toward maturation (fig. 2C). Lastly, KA increased phospholipase C-mediated poly-PI turnover in P19-derived neurons after 5 DIV (fig. 2D), similar to the response of primary cultured cortical neurons during early DIV [14]. However, the mGluR agonist trans-1-aminocyclopentane-1,3-decarboxylic acid (trans-ACPD) failed to increase poly-PI turnover, coinciding with its lack of a calcium response, suggesting that excitatory mGluRs are not expressed at this early stage of developing neurons.

Neuroprotective Effect of the KA Receptor against Hypobaric Insult in P19 Neurons

The roles of GluRs in P19-derived neurons at stages later than 10 DIV have been demonstrated by their excitotoxic effects in other studies. However, in our study, P19 neurons at 7 DIV subjected to high concentrations of KA of up to 1 m*M*, a concentration neurotoxic to adult central neurons, had negligible cell damage as determined by LDH release assay (fig. 3A). We further challenged P19 neurons in a hypobaric atmosphere down to 200 mm Hg for a brief 2 min. Under this condition, P19 neurons showed a significant increase in LDH release, which was reduced by KA in the concentration range of 1–100 μ*M*. Furthermore, the AMPA/KA receptor antagonist, 6-cya-

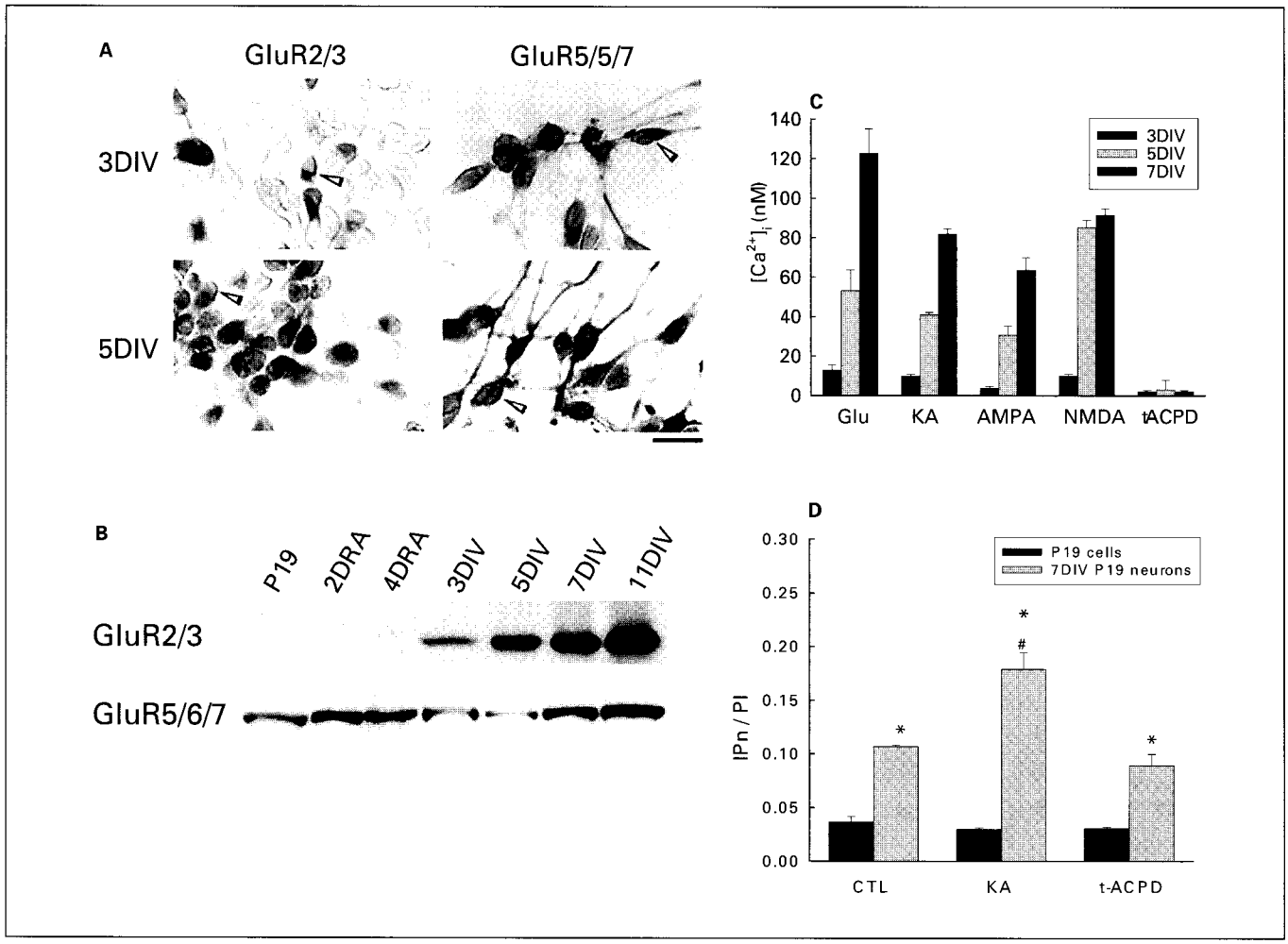


Fig. 2. Expression and basic functional characteristics of EAA receptors in P19-derived neurons. **A** Immunocytochemical staining of P19-derived neurons at 3 and 5 DIV with anti-GluR2/3 antibody for the AMPA receptor and anti-GluR5/6/7 antibody for the KA receptor. Bar = 80 μm. **B** Temporal expression profile of GluR2/3 and GluR5/6/7 during neurogenesis in Western blot analysis using crude membrane fraction of P19 cells. DRA = Days of retinoic acid treatment. **C** EAA agonist-stimulated intracellular calcium increase in P19-derived neurons at 3, 5 and 7 DIV. Glu = Glutamate; t-ACPD = trans-ACPD. **D** KA- and trans(t)-ACPD-stimulated poly-PI turnover in P19 neurons at 7 DIV. Data represent means ± SEM (n = 4). * $p < 0.05$ compared with the respective P19 cells. CTL = Vehicle-treated control.

no-7-nitroquinoxaline-2,3-dione, but not the AMPA receptor antagonist, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium, significantly increased the susceptibility of P19 neurons to hypobaric insult (fig. 3B). A similar phenomenon was observed in primary cultured cortical neurons during early DIV (3 DIV). The phospholipase C inhibitor, 1-[6[[(17beta)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122), reversed the KA-reduced LDH release in P19 neurons, although U73122 by itself reduced LDH release (fig. 3C). Taken together, these results suggest that the KA receptor may play an important role in protecting developing neurons against hypoxic insult, and the mechanism underlying KA receptor-mediated phospholipase C signaling could be involved in KA receptor-mediated neuroprotection.

AMPA/KA Receptors Facilitate NGF-Mediated Antiapoptotic Activity via Induction of TrkA Expression

We further examined the neuroprotective activity of AMPA/KA receptors by flow cytometry analysis. Figure 4A shows that glutamate, AMPA or KA by themselves did not alter the percentage of P19 cells at 5 DIV in the

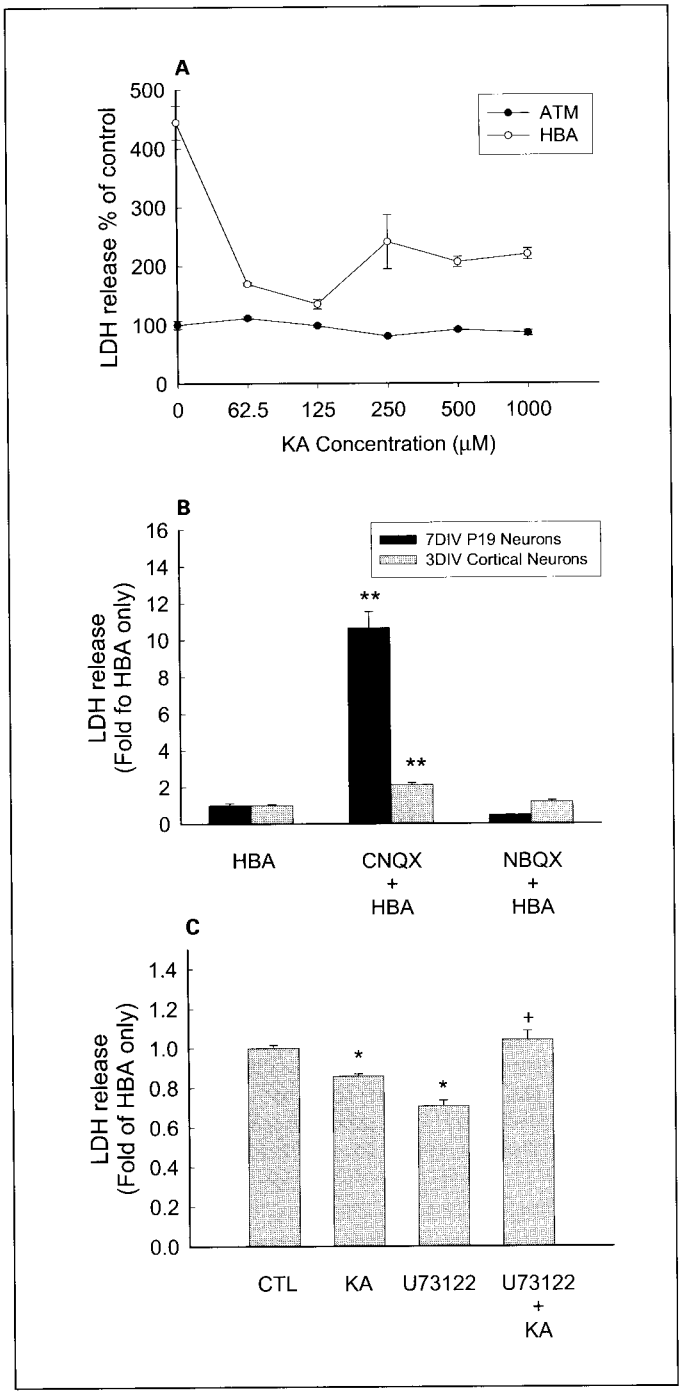


Fig. 3. KA attenuates hypobaric insult-induced cell death in P19-derived neurons. **A** P19 neurons at 7 DIV were subjected to 200-mm Hg hypobaric atmosphere (HBA) for 2 min, and the culture medium was subjected to LDH activity assay. KA at concentrations of 0–1,000 μ*M* was applied to the P19 neurons 30 min prior to the atmospheric (ATM) or HBA conditions. Data represent means ± SEM (n = 4). **B** AMPA/KA receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium (NBQX) at 0.5 m*M* were applied to P19 neurons at 7 DIV and cortical neurons at 3 DIV 10 min prior to hypobaric atmosphere (HBA). Data represent means ± SEM. ** $p < 0.01$ compared with the other two groups by one-way ANOVA with Newman-Keuls multiple comparison posttest. **C** Phospholipase C inhibitor U73122 at 20 μ*M* was applied to P19 neurons at 7 DIV for 30 min, followed by a 5-min treatment with 0.5 m*M* KA. LDH release assay was performed 24 h after the treatment. Data represent means ± SEM (n = 4). * $p < 0.05$ compared with the control; $^{+}$ $p < 0.05$ compared with the KA-treated group by unpaired t test. CTL = Vehicle-treated control.

sub-G1 phase, which represents apoptotic DNA. However, when NGF at 100 ng/ml was applied to P19 neurons, the percentage of cells in the sub-G1 phase was significantly reduced from approximately 30% to 12% when neurons were pretreated with GluR agonists (fig. 4B, C). Western blot analysis showed that glutamate, AMPA and KA, but not NMDA or trans-ACPD, significantly increased the expression of the high-affinity NGF receptor TrkA, whereas that of the low-affinity NGF receptor, $p75^{NTR}$, was not increased by glutamate (fig. 5). These results suggest that AMPA/KA receptors may facilitate the NGF-mediated antiapoptotic effect by increasing TrkA expression of surviving neurons for development.

Discussion

P19-derived neurons have been used in several studies on the characterization of glutamate-mediated neuronal excitation and excitotoxicity [7]. In our study, the most interesting feature of GluRs, especially the KA receptor, is that their expression may precede neurogenesis, and they may play protective roles during neuronal development. We also demonstrated that GluR subtypes were functionally expressed with a distinct temporal sequence during P19 neurogenesis. Interestingly, the ionotropic receptor seems to contribute to GluR-mediated phospholipase C signaling, but the metabotropic receptor in P19 neurons does not seem to. However, these results are somewhat contradictory to a study reported by Morley et al. [22] in 1995, in which they stated that AMPA or KA cannot elicit intracellular calcium elevation in P19-derived neurons, although their subunit transcripts were already expressed. One of their explanations was that P19 neurons might not have voltage-sensitive calcium channels which surge with AMPA/KA receptor activation [13]. Although these discrepancies may result from the culture conditions and the stage of differentiated neurons, in our study, a temporal

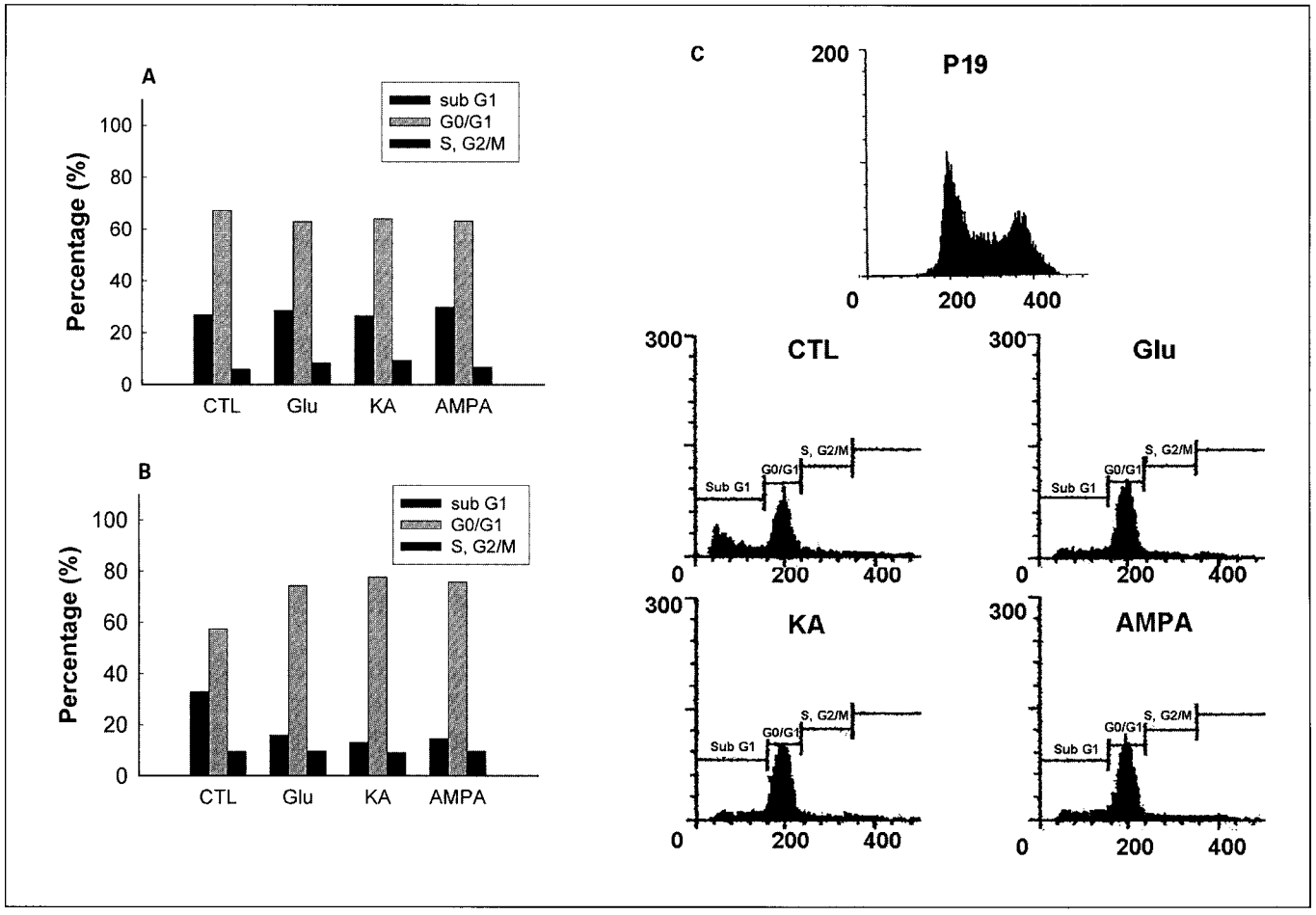


Fig. 4. Flow cytometry analysis of apoptosis of P19-derived neurons. P19-derived neurons at 5 DIV were subjected to 50 μ*M* EAA agonist treatment for 30 min (**A**), or followed by 100 ng/ml NGF treatment for 24 h (**B**, **C**). Percentages of cells at sub-G1, G0/G1 and S, G2/M phases were as indicated. CTL = Vehicle-treated control; Glu = glutamate.

increase in the AMPA/KA-stimulated calcium response toward maturation correlated more closely with the physiological conditions of P19 neuronal culture. In fact, Canzoniero et al. [7] in 1996 also demonstrated a KA-induced calcium current in P19 neurons. Although the authors were discussing the possible contribution of astrocyte feeder layers to the KA response, we found that the major point could be the concentration of retinoic acid used for neuronal differentiation. Studies using 0.5–1 μ*M* retinoic acid to induce P19 differentiation produced a functional AMPA/KA response, whereas the use of 17 μ*M* retinoic acid resulted in no physiological response by AMPA/KA receptors. This means that P19 neurons should be derived under well-defined culture conditions to perform functions similar to central neurons.

Expression of AMPA and KA receptors in P19 neurons as early as 5 DIV, at which stage neurogenesis is only 60% complete, seems to correlate well with their expression in brain development [2, 19]. KA receptor expression as well as the high-affinity [^{3}H]-KA binding sites were detected as early as embryonic day 14 in rat brains, which is the stage of onset of neurogenesis. AMPA receptor subunits, GluR1 and GluR2/3, were also detected in fetal rat brains by midgestation [9]. Although several studies have provided ample evidence for our understanding of distinct temporal and regional expression profiles of GluR subtypes during brain development, how they perform their functions in early neuronal development remains to be investigated. On the other hand, we could not demonstrate the activity of the mGluR in P19 neurons up to 7 DIV with respect to the intracellular calcium surge as well

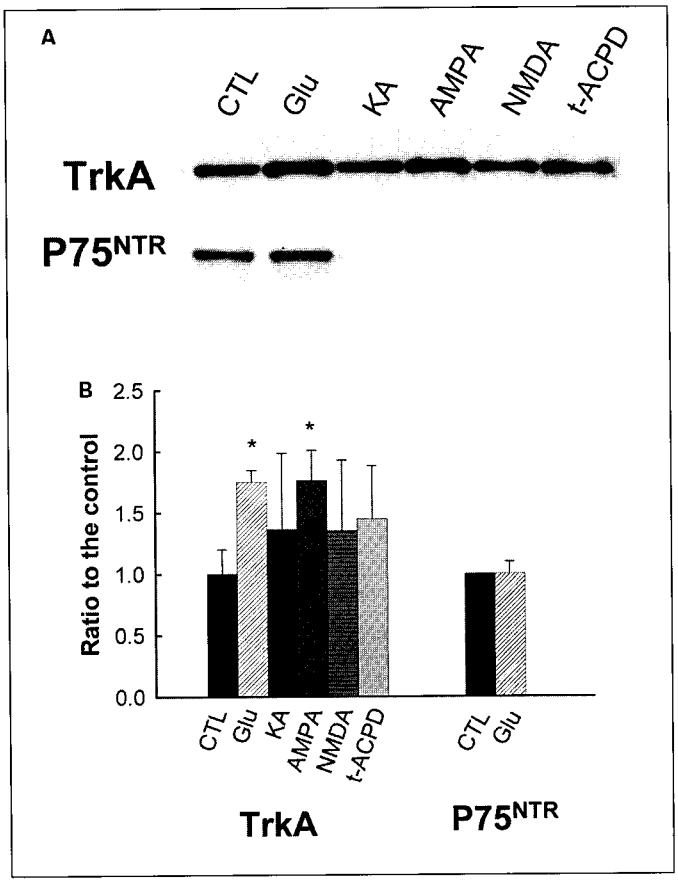


Fig. 5. Effect of EAA agonists on TrkA and p75 expression in P19 neurons. P19-derived neurons at 5 DIV were subjected to 50 μM EAA agonist stimulation for 30 min, and the crude membrane fraction was Western blotted for TrkA and p75NTR. Representative blot results (**A**) and quantitative analysis of relative band density (**B**) are shown. Data represent means ± SEM (n = 3). * $p < 0.05$ compared with the other four groups by one-way ANOVA with Newman-Keuls multiple comparison posttest. CTL = Vehicle-treated control; Glu = glutamate; t-ACPD = trans-ACPD.

as to phospholipase C signaling. Immunocytochemistry data also showed low levels of mGluRs. Compared with other GluR subtypes, expression of mGluRs seemed rather late in our P19 neurons. In the study of Morley et al. [22], P19 neurons at 14 DIV were readily responsive to trans-ACPD stimulation, suggesting that mGluR may indeed require a longer developmental duration in order to be expressed. This phenomenon also reflects the impact of ionotropic GluRs in mediating metabotropic signaling during early neurogenesis.

The neurotrophic functions of GluRs have been demonstrated in primary cultured developing neurons as well as in transgenic animal studies [14, 23]. In P19-derived neurons, we also found that KA can protect against hypobaric insult-induced neuronal death in an AMPA/KA receptor-mediated manner. This result suggests that GluR activation may protect developing neurons from hypoxic insult. It should be noted that P19 neurons prior to 12 DIV do not have functional synapses (data not shown), implying that the neurotrophic effect of GluRs is a nonsynaptic event. Furthermore, our results showed that KA-mediated neuroprotection in P19 neurons seems to be mediated by phospholipase C signaling. Another study also demonstrated that in cultured developing hippocampal neurons, basic fibroblast growth factor potentiates inositol phosphate formation induced by both AMPA/KA receptors and mGluR activation, but only the potentiation of AMPA/KA receptor signaling resulted in an increased neuronal survival rate [6]. Therefore, it is conceivable that certain dynamic changes in intracellular calcium mediated by ionotropic GluRs play an important role in neuronal survival in the developing nervous system.

NGF-mediated neuroprotection was also facilitated by glutamate, AMPA and KA stimulation, with a correlation with increased TrkA expression. The expression of $p75^{NTR}$ was not increased by glutamate stimulation, suggesting that induction of TrkA is specific. NGF is known to be antiapoptotic through TrkA activation. The downstream mechanism includes phosphorylation of the pro-apoptotic protein, Bad, and increased expression of Bcl-2 [5, 10, 26]. In cerebellar Purkinje cells, synergistic interactions between glutamate and NGF to promote neurite growth have been observed [8]. GluR agonists were found to be effective in increasing brain-derived neurotrophic factor and NGF mRNA levels in neurons [31]. In our previous study, KA was also found to initiate a neuroprotective effect by activation of TrkA in developing cortical neurons, and administration of NGF antibody during KA stimulation resulted in the reappearance of KA neurotoxicity [14]. In fact, expression of trk A, B, and C was differentially induced by KA in developing brains [24, 27]. Since neurotrophin signaling is the key determinant in deciding the cell fate during neuronal maturation, in which premature neurons expressing Trk proteins are able to obtain nourishment from the respective neurotrophins and circumvent naturally occurring cell death whereas those without Trks undergo apoptosis [3, 12, 25], it is conceivable that GluR activity may participate in the determination of neuronal fate by mediating specific patterns of neurotrophin expression, release and signaling.

In summary, we herein demonstrate that AMPA/KA receptors are differentially expressed with neuronal dif-

ferentiation of P19 cells, and they may play distinct roles in protecting early developing neurons from hypobaric insult or apoptotic cell death. Furthermore, neuronal differentiation of P19 cells under these modified culture conditions provides an in vitro model comparable to that of primary cultured brain neurons to study the physiological function of GluRs in early neuronal development.

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