行政院國家科學委員會專題研究計畫成果報告
麩胺酸受體對胚胎幹原細胞神經性分化之調控功能研究(2/2)

Glutamate receptor-mediated neuronal differentiation in embryonic stem cells

計畫編號：NSC 90-2320－B－038－031
執行期限：90年 8月1日至 91年7月31日
主持人：李怡萱　臺北醫學大學生理學科

一、 中文摘要

本計畫主要為使用P19細胞株類似幹原細胞的特性，將其分化為神經元，以研究麩胺酸受體在神經分化過程的功能。首先，我們成功地將P19 cells分化成含85%以上神經元的培養。再者，結果發現離子通道型麩胺酸受體次單元GluR5,6,7及GluR2,3的表現分別在分化前及分化後3天出現，顯示不同亞型的麩胺酸受體在不同階段的神經發育可能扮演重要的角色。以三種離子通道型麩胺酸受體催動劑NMDA, AMPA, and 紅藻胺酸KA均可引發P19神經元的內鈣升高，且可升高磷脂腖 C 所引發之多磷酸纖維糖代謝，而代謝型麩胺酸受體催動劑trans-ACPD則否，顯示離子通道型麩胺酸受體在早期神經發育的活性較代謝型受體為主要。進而結果顯示，紅藻胺酸可降低P19神經元在低壓低氧環境下的死亡率，且 AMPA/KA 受體拮抗劑CNQX及磷脂腖C 抑制劑會促進此環境下的死亡率，顯示AMPA/KA受體對神經發育早期存活的重要性。流式細胞儀的研究結果顯示，神經生長因子對P19細胞自然凋亡的保護必需要在AMPA和 KA 的存在下才有顯著的作用，而AMPA和KA會顯著升高神經生長因子受體中促進存活的TrkA而非促進凋亡的$p75^{NTR}$的表現。最後，AMPA受體也會降低神經纖維生長所需的蛋白GAP-43的磷酸化，顯示離子通道型麩胺酸受體對早期發育神經之存活及神經纖維生長均扮演重要的角色。

關鍵詞：麩胺酸，神經生長因子，紅藻酸，P19細胞, 低壓低氧, 鈣離子, 生長相關蛋白

Abstract

We cultured a P19 mouse teratocarcinoma cell line and induced its neuronal differentiation to study the function of ionotropic glutamate receptors in early neuronal development. Immunocytochemical studies showed 85% neuronal population at 5 DIV with microtubule-associated protein 2-positive staining. Cells expressing the α-amino-3-hydroxy-5-methyl-4-isopropinonate (AMPA) receptor subunit, GluR2/3, and the kainate receptor subunit, GluR5/6/7, were 30% and 50%, 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 kainate, but not to the metabotropic glutamate receptor agonist, trans-1-aminocyclopentane-1,3-decarboxylic acid (trans-ACPD), by 5 DIV in terms of increases in intracellular calcium and phospholipase C-mediated poly-phospho-inositide turnover. Furthermore, kainic acid reduced cell death of P19-derived neurons in both atmospheric and hypobaric conditions in a phospholipase C-dependent manner. The AMPA/kainate receptor common antagonist, CNQX, but not the AMPA receptor antagonist, NBQX, profoundly increased hypobaric insult-induced neurotoxicity. In a flow cytometry study, the nerve growth factor-mediated anti-apoptotic effect was facilitated by AMPA, with an induction of TrkA, but not $p75^{NTR}$ expression. Lastly, phosphorylation of GAP-43, which is involved in neuritogenesis during neurogenesis, was reduced by AMPA

receptor antagonist GYKI 52466. Therefore AMPA and kainate receptors might mediate neurotrophic functions to facilitate neurotrophic factor signaling to protect neurons against hypoxic insult in early neuronal development. (Part of this work is accepted by Journal of Biomedical Science on Oct. 7, 2002., and another part will be submitted to *Developmental Biology*)

Keywords: glutamate, neurotrophic, kainic acid, P19 cells, hypobaric hypoxia, calcium, growth-associated protein - 43

二、緣由與目的

Excitatory neurotransmission in the mammalian central nervous system is mainly mediated by glutamate receptors (GluRs). GluRs are divided into two distinct categories, called metabotropic and ionotropic GluRs (for a review see Monaghan et al., 1989). Metabotropic GluRs 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, and N-methyl-D-aspartate (NMDA) receptors. In neonatal brain, AMPA/KA receptors and the NMDA receptor display sequential participation in neuronal excitation (Ben-Ari, 1997). EAA 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 (McDonald, 1990). AMPA/KA receptors were found on the membranes of proliferating ventricular zone cells as well as in cortical neuroepithelial cells undergo neurogenesis (LoTurco et al., 1995; Maric et al., 2000). After mitosis and during migration out of the ventricular zone, immature migrating neurons begin to express the NMDA receptor (LoTurco et al., 1991). Later on after synaptogenesis, mGluRs are expressed at a transient level (Aronica et al., 1993). 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 glutamate receptors may mediate activity-dependent neurotrophic activities, such as neuronal survival and neurite outgrowth for neuronal plasticity and injury repair in both developing brain as well as in injured peripheral neurons (Lee et al., 2000; Wilson et al., 2000; Tabernero et al., 2002). Glutamate receptor-mediated intracellular calcium increase through extracellular calcium entry or release of intracellular calcium storage, as well as glutamate receptor-induced expression and activation of neurotrophin receptors were reported to be the possible mechanisms involved in these neurotrophic activities.

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 glutamate receptors and to exert typical responses upon glutamate stimulation, such as glutamate-evoked synaptic potential and elevation of intracellular calcium (Younkin et al., 1993; Magnuson et al., 1995; Canzoniero et al., 1996). Most of these studies used late-developing P19 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 and neurite growth-

associated protein phosphorylation. The signaling mechanism responsible for their neuroprotective activity was also examined.

三、結果與討論（第一年及第二年）

Modified culture conditions produce more than 80% neurons in P19-differentiated cells

Application of RA 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 high 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 glutamate receptors are expressed with differential temporal patterns

To examine whether P19-derived neurons express glutamate receptors similar to central neurons, we immunostained the neurons to identify the population of the AMPA receptor- and the KA receptor-positive neurons in P19 neurons at 3 and 5 DIV. Figure 2A shows that at both 3 and 5 DIV of P19-derived neurons, the immunoreactivity of the KA receptor subunit, GluR5/6/7, was significantly higher than that of the AMPA receptor subunit, GluR2/3. Percentage 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 RA induction, then was re-elevated after 3 DIV. Expression levels of glutamate receptors coincided with their functional characterization, in which the response of P19 neurons to EAA agonists at 50 μM in terms of intracellular calcium increase, a common feature of glutamate receptor activation, appeared after 3 DIV and gradually increase toward maturation (Fig. 2C). Lastly, KA increased phospholipase C-mediated poly-PI turnover in 5 DIV P19-derived neurons (Fig. 2D), similar to the response of primary cultured cortical neurons during early days in vitro (Lee et al., 1994). However, the mGluR agonist, trans-ACPD, failed to increase poly-PI turnover, coinciding with its lack of a calcium response, suggesting that excitatory mGluR is not expressed at this early stage of developing neurons.

Neuroprotective effect of the KA receptor against hypobaric insult in P19 neurons

Roles of glutamate receptors 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 kainate of up to 1 mM, a neurotoxic concentration to adult central neurons, had negligible cell damage as determined by LDH release assay (Fig 3A). We further challenged P19 neurons with a hypobaric atmosphere (HBA) down to 200 mmHg for a brief 2 min. Under this condition, P19 neurons showed a significant increase in LDH release, which was reduced by kainate at the concentration range of 1-100 μM. Furthermore, the AMPA/KA receptor antagonist, CNQX, but not the AMPA receptor antagonist, NBQX, significantly increased susceptibility of P19 neurons to hypobaric insult (Fig. 3B). A similar

phenomenon was observed in primary cultured cortical neurons during early days in vitro (3 DIV). The phospholipase C inhibitor, U73122, reversed the kainate-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 PLC signaling could be involved in KA receptor-mediated neuroprotection.

AMPA/KA receptors facilitate NGF-mediated anti-apoptotic activity via induction of TrkA expression and alter induce Bcl-2 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 5 DIV P19 cells at the sub G1 phase, which represents apoptotic DNA. However, when NGF at 100 ng/ml was applied to P19 neurons, the percentage of cells at the sub G1 phase was significantly reduced from approximately 30% to 12% when neurons were pretreated with EAAs (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 anti-apoptotic effect by increasing TrkA expression of surviving neurons for development.

In our hypothesis, we proposed that glutamate receptors may exert their neurotrophic function via regulating apoptotic gene expressions. Here we examined if the apoptotic gene bax and antiapoptotic gene Bcl-2 are altered upon EAA receptor activation. Figure 6 shows that KA and AMPA significantly increase Bax expression, whereas only KA significantly increase Bcl2 expression. This preliminary result suggest that AMPA and KA receptors may be responsible for natural occurring cell death during neuronal development, and the KA receptor may also trigger a protective effect to maintain neuronal survival.

Expression of Growth-Associated Protein GAP-43 is reduced by AMPA/KA receptor antagonist in P19 neurons

Expression of GAP-43 is known to play pivotal role in neurogenesis. Application of AMPA/KA receptor antagonist GYKI 52466 to P19 neurons throughout the neural induction process was performed to investigate the role of glutamate receptors in GAP-43 expression. Figure 7 showed that expression of GAP-43 was not apparent until 3 DIV, reached highest expression at 5 DIV, and declined at 7 DIV. When protein kinase C inhibitor Ro 318220 was present in culture from 0 DRA to 5 DIV and 7 DIV, expression of GAP-43 became higher at 5 DIV but not at 7 DIV. Application of AMPA receptor antagonist GYKI52466 in culture also increase GAP-43 expression, but reduced its phosphorylation suggesting that blockade of AMPA/KA receptor activities during neuronal differentiation may indeed reduced functional GAP-43 and in turn hampered or disrupted neurogenesis process.

四、計畫成果自評

1. 研究內容與原計畫相符程度: 90%。
2. 達成預期目標情況:綜合第一年和第二年的結果，預期目標約達成90%。
3. 是否適合在學術期刊發表或申請專利: 本計畫成果之其中一部份已投稿至 Journal of Biomedical Sciences, 並已被接受。另一部份將投稿至 *Developmental Biology*。

4. 主要發現或學術或應用價值: 本計畫成果主要為成功建立由老鼠幹原細胞分化為類似中樞神經元的模式，且其glutamate receptor的活性特性與primary cultured brain neurons 相同，對於將來應用於研究glutamate receptor在早期發育所扮演的角色，以及應用此培養技術於人類幹原細胞之神經分化,以做為篩選對glutamate receptor作用之新藥研發，將有重大助益。

5. 學生畢業論文: 本計畫部份為臺北醫學院醫研所碩士班學生許玉黎文之碩士論文，其論文已於八十九年六月完成。GAP-43 expression and phosphorylation的部份正由醫研所碩士班學生許敏靖進行最後機轉的探討，預計將於明年三月間完成。

五、參考文獻

1. Aronica E, Dell'Albani P, Condorelli DF, Nicoletti F, Hack N, Balazs R. Mechanisms underlying developmental changes in the expression of metabotropic glutamate receptors in cultured cerebellar granule cells: homologous desensitization and interactive effects involving N-methyl-D-aspartate receptors. Mol Pharmacol 44: 981-9; 1993.
2. Ben-Ari Y, Khazipov R, Leinekugel X, Caillard O, Caiarsa JL. $GABA_A$, NMDA and AMPA receptors: a developmentally regulated 'ménage à trois'. Trends Neurosci 20: 523-529; 1997.
3. Canzoniero LM, Sensi SL, Turetsky DM, Finley MF, Choi DW, Huettner JE. Glutamate receptor-mediated calcium entry in neurons derived from P19 embryonal carcinoma cells. J Neurosci Res 45: 226-236; 1996.
4. Lee YH, Fang KM, Yang CM, Hwang HM, Chiu CT, Tsai W. Kainic acid-induced neurotrophic activities in developing cortical neurons. J Neurochem 74: 2401-2411; 2000.
5. LoTurco JJ, Blanton MG, Kriegstein AR. Initial expression and endogenous activation of NMDA channels in early neocortical development. J Neurosci 11: 792-799; 1991.
6. LoTurco JJ, Owen DF, Heath MJ, Davis MB, Kriegstein AR. GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. Neuron 15: 1287-1298; 1995.
7. Maric D, Liu QY, Grant GM, Andreadis JD, Hu Q, Chang YH, Barker JL, Joseph J, Stenger DA, Ma W. Functional ionotropic glutamate receptors emerge during terminal cell division and early neuronal differentiation of rat neuroepithelial cells. J Neurosci Res 61: 652-662; 2000.
8. Magnuson DS, Morassutti DJ, McBurney MW, Marshall KC. Neurons derived from P19 embryonal carcinoma cells develop responses to excitatory and inhibitory neurotransmitters. Brain Res Dev Brain Res 90: 141-150; 1995.
9. Monaghan D, Bridges RJ, Cotman CW. The excitatory amino acid receptors: their classes, pharmacology and distinct properties in the function of the central nervous system. Annu Rev Pharmacol Toxicol 29: 365-402; 1989.
10. McDonald JW, Johnston MV. Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. Brain Res Brain Res Rev 15: 41-70; 1990.
11. Tabernero A, Granda B, Medina A, Sanchez-Abarca LI, Lavado E, Medina JM. Albumin promotes neuronal survival by increasing the synthesis and release of glutamate. J Neurochem 81: 881-91; 2002.
12. Wilson MT, Kisaalita WS, Keith CH. Glutamate-induced changes in the pattern of hippocampal dendrite outgrowth: a role for calcium-dependent pathways and the microtubule cytoskeleton. J Neurobiol

43, 159-72; 2000.
13. Younkin DP, Tang CM, Hardy M, Reddy UR, Shi QY, Pleasure SJ, Lee VM, Pleasure D. Inducible expression of neuronal glutamate receptor channels in the NT2 human cell line. Proc Natl Acad Sci USA 90: 2174-2178; 1993.

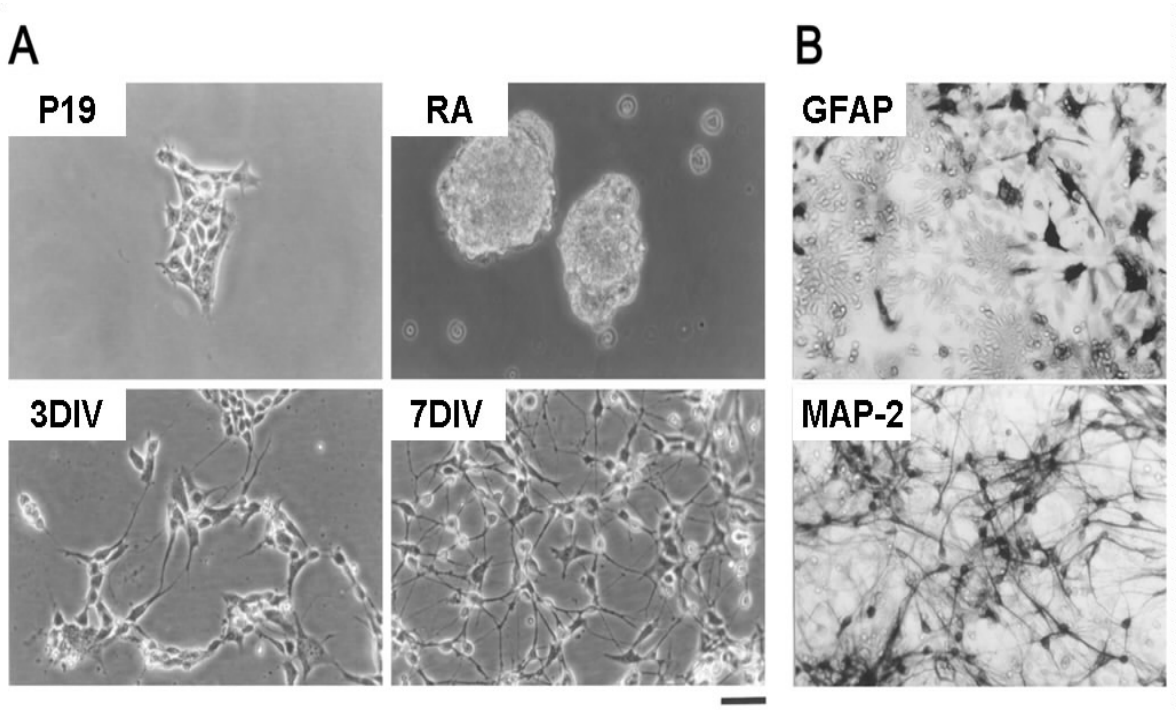


Figure 1. Differentiation of P19 cells into neurons. (A) Phase contrast photomicrographs show the undifferentiated P19 cells started to form aggregates during retinoic acid treatment (DRA). Four days after retinoic acid treatment, aggregates were dissociated and plated onto poly-L-lysine-coated plates,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

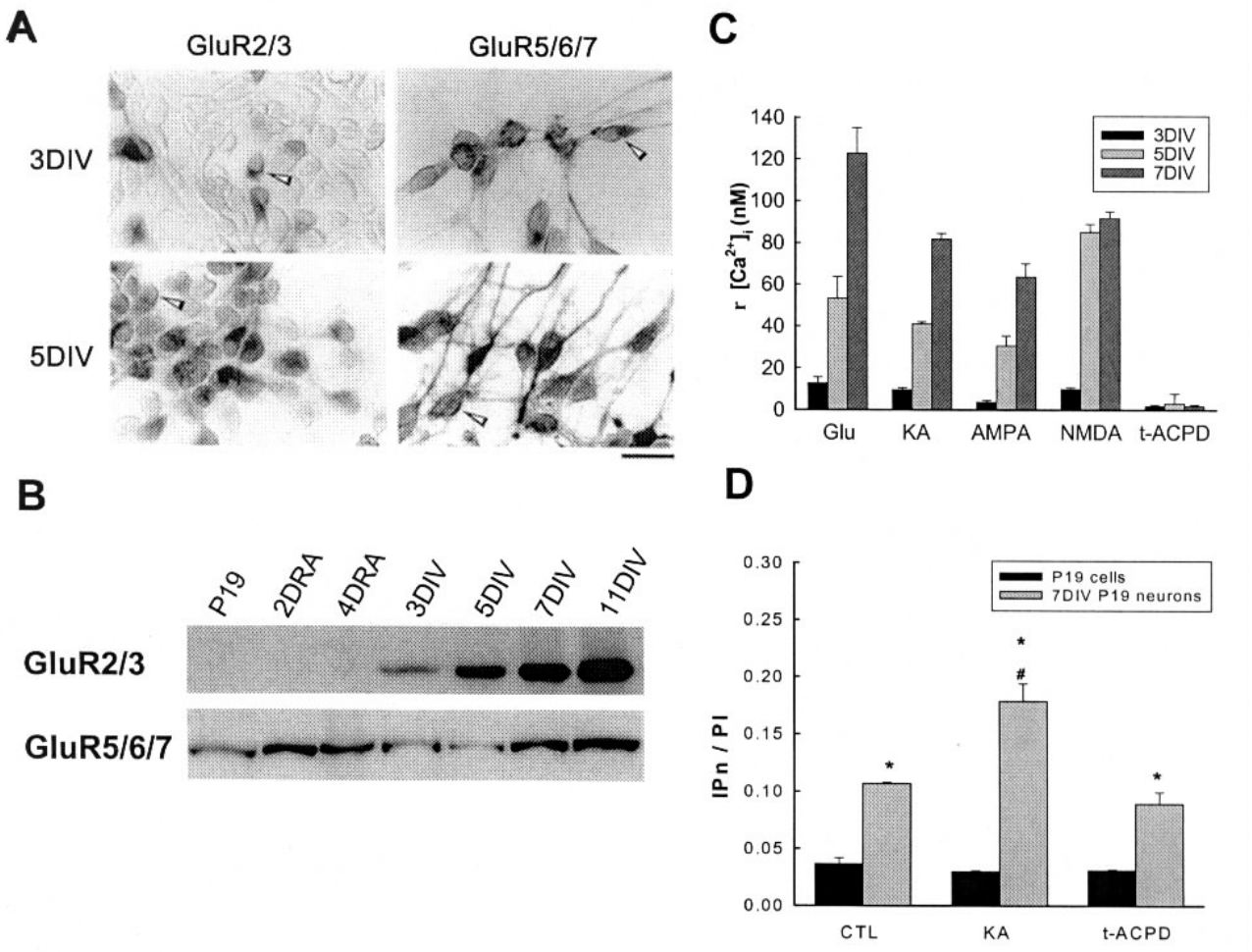


Figure 2. Expression and basic functional characteristics of EAA receptors in P19-derived neurons. (A) Immunocytochemical staining of P19-derived neurons at 3 and 5 DIV with anti-GluR2,3 antibody for the AMPA receptor and anti-GluR5,6,7 antibody for the KA receptor. Bar = 80μm. (B) Temporal expression profile of GluR2,3 and GluR5,6,7 during neurogenesis in western blot analysis using crude membrane fraction of P19 cells. (C) EAA agonist-stimulated intracellular calcium increase in P19-derived neurons at 3, 5, and 7 DIV. (D) KA and trans-ACPD-stimulated poly-PI turnover in 7 DIV P19 neurons. Data represents means ± S.E.M. (n=4) *p<0.05 as compared with the respective P19 cells.

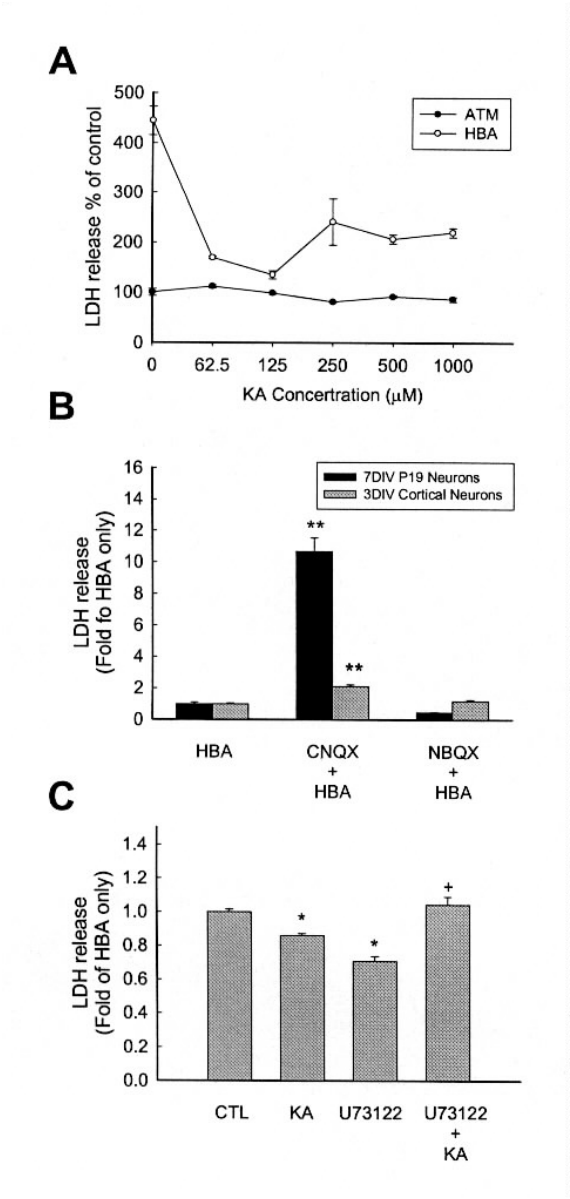


Figure 3. KA attenuates hypobaric insult-induced cell death in P19-derived neurons. (A) P19 neurons at 7 DIV were subjected to 200 mmHg hypobaric atmosphere (HBA) for 2 min, and the culture medium was subjected to LDH activity assay. KA at concentration of 0 to 1000 μM was applied to the P19 neurons 30 min prior to the atmospheric (ATM, closed circle) or HBA (open circle) conditions. Data represents means ± S.E.M. (n=4) $*P<0.05$ as compared with the respective concentration of KA-treated group under ATM by unpaired t-test. (B) AMPA/KA receptor antagonists CNQX and NBQX at 0.5 mM were applied to 7 DIV P19 neurons and 3 DIV cortical neurons 10 min prior to HBA. Data represents means ± S.E.M. $**P<0.01$ as compared with the other two groups by one-way ANOVA with Newman-Keuls multiple comparison posttest. (C) PLC inhibitor U73122 at 20 μM was applied to 7 DIV P19 neurons for 30 min, followed by 5 min treatment of 0.5mM KA. LDH release assay was performed 24 hrs after the treatment. Data represents means ± S.E.M. (n=4) $*P<0.05$ as compared with the control; and $^{+}p<0.05$ as compared with the KA-treated group by unpaired t-test.

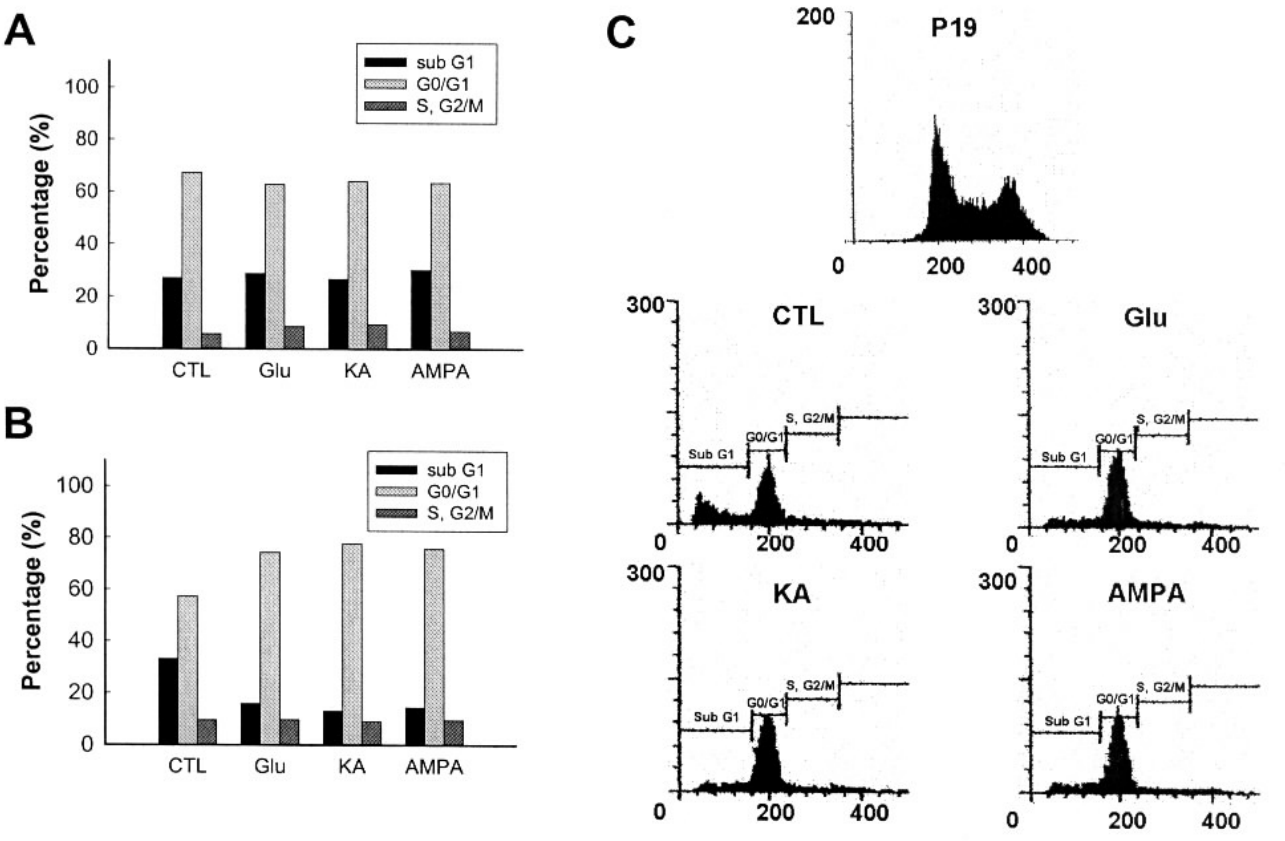


Figure 4. Flow cytometry analysis of apoptosis of P19-derived neurons. P19-derived neurons at 5 DIV were subjected to 50 μM EAA agonists treatment for 30 min (A), or followed by 100 ng/ml NGF treatment for 24 hrs (B and C). Percentage of cells at sub G1, G0/G1, and S,G2/M phases were as indicated.

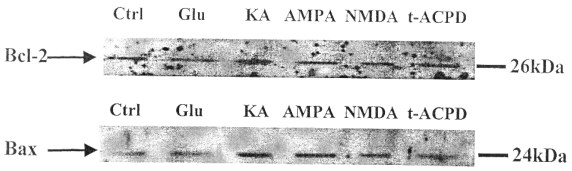


Figure 5. Effect of EAA on Bcl-2 and Bax expressions in P19 neurons.

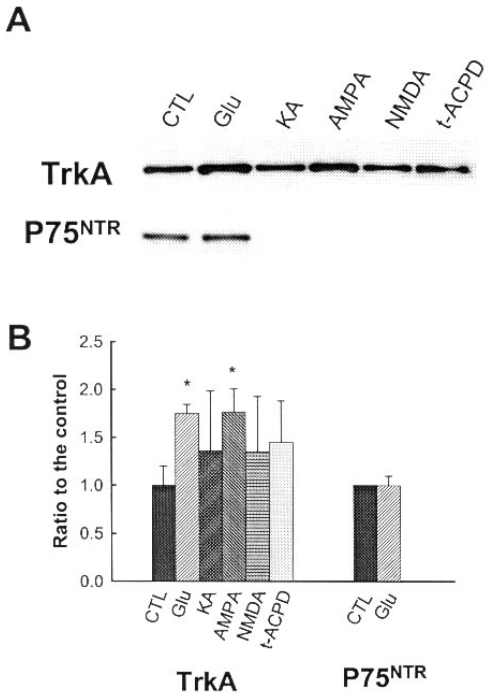
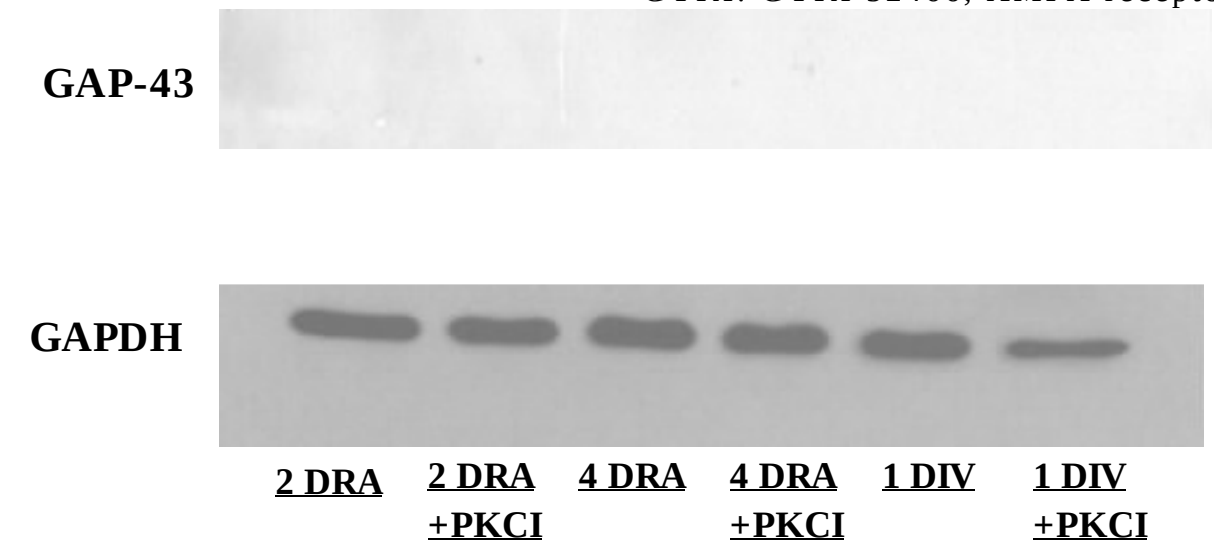
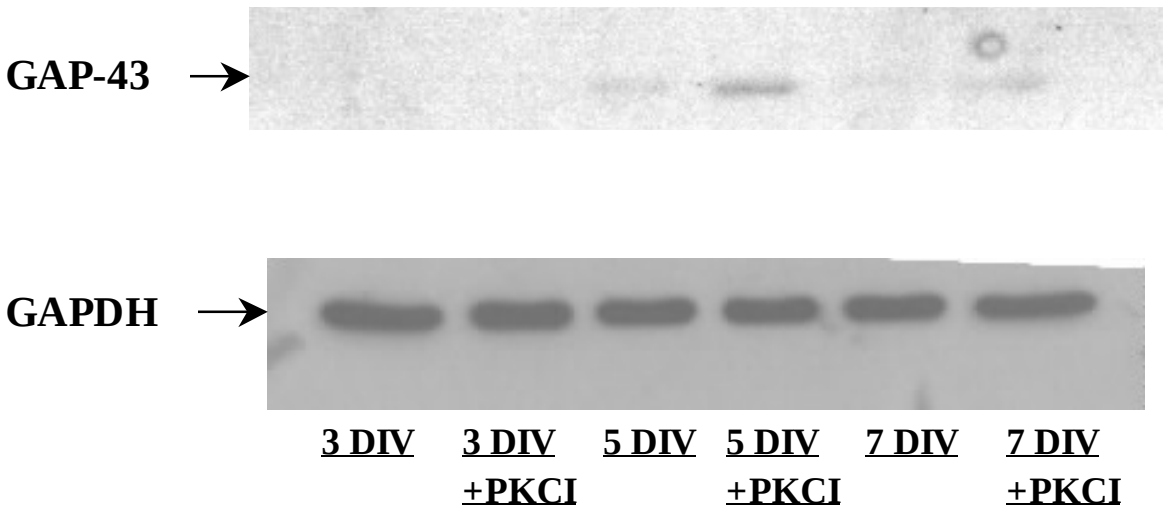
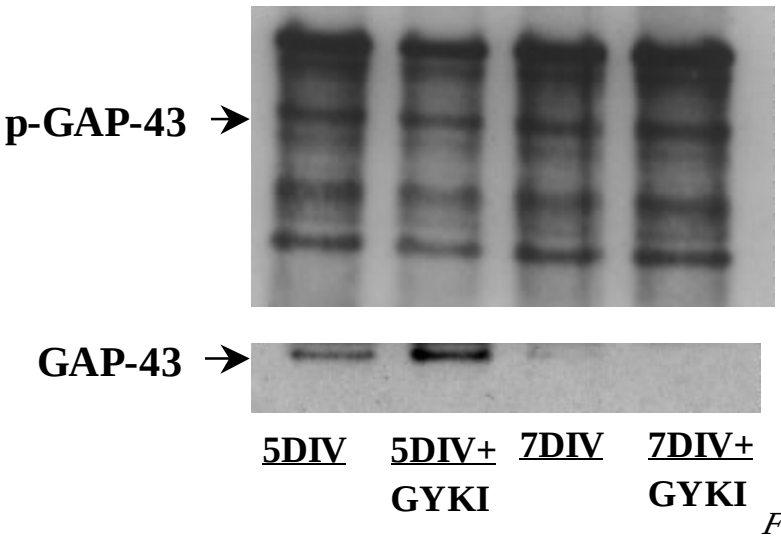


Figure 6. Effect of EAA agonists on TrkA and p75 expressions in P19 neurons. P19-derived neurons at 5 DIV were subjected to 50 M EAA agonist stimulation for 30 min, and crude membrane fraction was western blotted for TrkA and $p75^{NTR}$. Representative blot results (A) and quantitative analysis of relative band density (B) were shown. Data represents means ± S.E.M. (n=3) *P<0.05 as compared with the other four groups by one-way ANOVA with Newman-Keuls multiple comparison posttest.

GYKI: GYKI 52466, AMPA receptor







PKCI: PKC inhibitor Ro 318220

Figure 7. Expression of GAP-43 and immunoprecipitation of GAP-43 and Western

Blotted with anti-phosphoserine for GAP phosphorylation in P19 neurons.