Critical Review

Neurogenesis Mediated by γ-Aminobutyric Acid and Glutamate Signaling

Noritaka Nakamichi¹, Takeshi Takarada¹, and Yukio Yoneda¹,*
¹Laboratory of Molecular Pharmacology, Division of Pharmaceutical Sciences, Kanazawa University Graduate School of Natural Science and Technology, Kanazawa, Ishikawa 920-1192, Japan

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Abstract. In this review, we will summarize our ongoing studies on the functionality of both γ-aminobutyric acid (GABA) and glutamate receptors expressed by undifferentiated neural progenitor cells isolated from embryonic rodent brains. Cells were cultured with growth factors for the formation of round spheres by clustered cells under floating conditions, whereas a reverse transcription polymerase chain reaction analysis revealed expression of mRNA for particular subtypes of different ionotropic and metabotropic GABA and glutamate receptors in undifferentiated progenitors and neurospheres. Moreover, sustained exposure to either GABAergic or glutamatergic agonists not only modulated the size of neurospheres formed, but also affected spontaneous and induced differentiation of neural progenitor cells into particular progeny cell lineages such as neurons and astroglia. Both GABA and glutamate could play a pivotal role in the mechanisms underlying proliferation for self-replication along with the determination of subsequent differentiation fate toward particular progeny lineages through activation of their receptor subtypes functionally expressed by undifferentiated neural progenitor cells. Accordingly, neurogenesis seems to be also under control by GABAergic and glutamatergic signaling in developing brains as seen with neurotransmission in adult brains.

Keywords: neural progenitor, neurosphere, γ-aminobutyric acid (GABA), glutamate

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Abbreviations used (in alphabetical order): AMPA, D,L-α-amino-3-hydroxy-5-methylisoxazole-4-propionate; AMPAR, AMPA receptor; AP1, activator protein-1; ATRA, all-trans-retinoic acid; bFGF, basic fibroblast growth factor; bHLH, basic helix-loop-helix; BrdU, 5-bromo-2'-deoxyuridine; CNTF, ciliary neurotrophic factor; CPPG, RS-α-cyclopropyl-4-phosphonophenyl glycine; CRE, cAMP responsive element; CREB, CRE-binding protein; DCG-IV, (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine; DG, dentate gyrus; DHPG, 3,5-dihydroxyphenylglycine; EGF, epidermal growth factor; FBS, fetal bovine serum; GABA, γ-aminobutyric acid; GABAAR, GABA receptor; GABAAR, GABA receptor; GABAAR, GABA receptor; GABAAR, GABA receptor; GFAP, glial fibrillary acidic protein; GluR, ionotropic glutamate receptor; KA, kainate; KAR, KA receptor; L-AP4, 1,2-amino-4-phosphonobutyrate; LDH, lactate dehydrogenase; MAP2, microtubule-associated protein-2; mGluR, metabotropic glutamate receptor; MK-801, dizocilpine; MTX, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NCAM, neural cell adhesion molecule; NMDA, N-methyl-D-aspartate; NMDAR, NMDA receptor; PACAP, pituitary adenyl cyclase activating peptide; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; PKA, protein kinase A; PTSD, posttraumatic stress disorder; RAR, retinoic acid receptor; RT-PCR, reverse transcription polymerase chain reaction; RXR, retinoid X receptor; SVZ, subventricular zone; WIRS, water-immersion restraint stress.
1. Introduction

The prevailing view is that neural stem cells are primitive progenitor cells with abilities to proliferate for self-renewal and to differentiate into different progeny cell lineages including neurons, astroglia, and oligodendroglia. These progenitor cells are enriched in the SVZ during development of the brain (1, 2), while in the adult brain, progenitor cells are also localized in the DG of the hippocampus as well as the SVZ (3–8). In contrast to other structures in the adult brain, the subgranular cells spontaneously undergo proliferation and migration to the granule cell layer of the DG (9). The fact that these progenitors undergo cellular proliferation, commitment, and differentiation into neurons and glia in vitro (10) is suggestive of the proposal that cells are derived from multi-potential neural stem cells (11). On transplantation of neural stem cells into brains, cells develop into mature neurons or glia with morphological and biochemical features similar to those of neighboring cells, which gives rise to the idea that cellular commitment and/or differentiation is at least in part under control by microenvironments around the stem cells in vivo (12).

Emerging evidence that endogenous factors regulate self-renewal capacity and multi-potentiality of neural progenitors expressed in the developing and matured brains is available in the literature. These endogenous factors are believed to be derived from a variety of adjacent cells around the stem cell niche (13–16). Several extracellular molecules, such as growth factors and neurotransmitters, have been implicated in the extrinsic regulation of cell proliferation in the developing telencephalon. For example, bFGF prolongs proliferation of progenitor cells with a concomitant increase in the number of neurons in rat neocortex when either added to cultured cells in vitro (17) or microinjected into embryonic rat brains in vivo (18). The neurotransmitters GABA and glutamate are shown to reduce the number of proliferating cells in dissociated or organotypic cultures of rat neocortex (19). GABA partially blocks the bFGF-induced increase in cell proliferation (20), but promotes cell proliferation in cultures of rat cerebellar progenitors (21). Signals mediated by ionotropic GABA\_R are shown to promote neuronal differentiation after depolarization in neural progenitors of adult mouse hippocampal slices (22). In our previous study, by contrast, sustained exposure to a GABA\_R agonist leads to increased proliferation along with facilitation of commitment and/or differentiation toward an astroglial lineage in the presence of CNTF in neural progenitor cells isolated from the neocortex of embryonic rats (23) and mice (24).

In this review, therefore, we will mainly summarize our recent findings on the importance of signals mediated by different GABA\_ergic and glutamatergic receptor subtypes in the mechanisms related to self-replication and multi-potentiality in undifferentiated neural progenitor cells isolated from fetal rodent brains. In 1991, Meier et al. published a pioneering review on brain development by neurotransmitters (25).

2. Isolation of neural progenitor cells

Mitotic cells can be clearly separated from postmitotic cells in embryonic brains at the late stage by using density gradients generated with colloidal silica (26, 27). Fetal postmitotic cells have a buoyant density lower than 1.043 g/ml, whereas mitotic and progenitor cells have densities higher than 1.056 g/ml (28). In these previous studies, cells are derived from stem cells and contain a mixture of lineages at various stages of differentiation. The most immature cells have remarkably high densities ranging from 1.065 to 1.075 g/ml, with ability to differentiate to mature cells. The present preparation procedures for neural progenitor cells are in principle based on the aforementioned differential densities of cells at different developmental stages.

In the upper cell layer prepared by Percoll density gradient centrifugation, particular cells were immunoreactive for either the neuronal marker MAP2 or the astroglial marker GFAP, but not for the neural progenitor marker nestin (29). In the lower cell layer, several cells were immunoreactive for nestin, but expression of either MAP2 or GFAP was not detected. The lower cell layer was used as a source of progenitors expressing nestin and cultured in the presence of a growth factor for subsequent double immunocytochemical detection of MAP2 and GFAP. In the presence of EGF, round spheres were formed within 4 days with increasing cluster sizes proportional to culture durations up to 10 days. In these neurospheres cultured for 10 days, cells were immunoreactive for nestin but not for either MAP2 or GFAP.

Floating neurospheres were then dispersed and the cells were seeded on wells previously coated with poly-L-lysine and subsequently cultured in the absence of a growth factor for an additional several days toward spontaneous differentiation. Removal of EGF led to a complete abolition of immunoreactive nestin together with a drastic increase in the number of cells immunoreactive for either MAP2 or GFAP. Dispersed cells were also cultured for an additional 4 days in the presence of either the neuronal inducer ATRA or the astroglial inducer CNTF to promote the commitment toward a neuronal or astroglial lineage, respectively, after the removal of EGF. Sustained exposure to ATRA markedly
increased the number of cells immunoreactive for MAP2 compared to that found in the presence of CNTF. Repetition of the dispersion and culture led to similar expression profiles of different immunoreactive marker proteins (23, 30). Accordingly, the lower cell layer indeed contains neural progenitor cells endowed to proliferate for self-replication in a manner sensitive to a growth factor and to differentiate into neuronal and astroglial lineages in response to the respective differentiation inducers.

3. GABAergic signaling

In signal transduction mechanisms mediated by the inhibitory neurotransmitter GABA, extracellular signals are transformed into intracellular signals through ionotropic (GABA\(_A\)R and GABA\(_B\)R) and metabotropic (GABA\(_B\)R) GABAR subtypes in the brain. The GABA\(_A\)R subtype is orchestrated by the heteromeric assembly of 5 different proteins, including \(\alpha\), \(\beta\), and \(\gamma\) subunits, toward anion channels permeable to chloride ions, whereas the GABA\(_B\)R subtype is formed from the homomeric assembly of 5 \(\rho\) subunits for chloride channels. In contrast to other metabotropic receptors, the metabotropic GABA\(_B\)R subtype is a heteromeric dimer from the assembly between GABA\(_B\)R1 and GABA\(_B\)R2 subunits with the functional linkage to the inhibitory G\(_i/o\) protein to negatively regulate the activity of adenylyl cyclase, which in turn leads to a decrease in intracellular concentrations of cAMP, opening of potassium channels, and closing of calcium channels. GABAergic input is shown to partially block the bFGF-induced cell proliferation (20), but promotes cell proliferation in cultures of rat cerebellar progenitors (21). Signals mediated by ionotropic GABA\(_A\)R subtype promote neuronal differentiation after depolarization in neural progenitors of adult mouse hippocampal slices (22). In our previous study, by contrast, sustained activation of the GABA\(_A\)R subtype leads to increased proliferation along with subsequent facilitation of commitment and/or differentiation toward an astroglial lineage in the presence of CNTF in neural progenitor cells isolated from the fetal rat neocortex (23). Moreover, we have attempted to evaluate the possible modulation of the functionality by the metabotropic GABA\(_B\)R subtype expressed in neural progenitor cells prepared from fetal mouse neocortex under the similar conditions (31).

3.1. Expression of GABAergic machineries

In the neocortical lower cell layer before culture, mRNA expression was seen for the \(\alpha1, \alpha2, \alpha3, \alpha4, \alpha5, \beta1, \beta2, \beta3, \gamma2, \gamma3, \) and \(\delta\) subunits of GABA\(_A\)R, GABA\(_B\)R1a, 1b, and GABA\(_B\)R2 subunits of GABA\(_B\)R and for the \(\rho1, \rho2, \) and \(\rho3\) subunits of GABA\(_B\)R, but not for the \(\alpha6\) or \(\gamma1\) subunit of GABA\(_A\)R, on RT-PCR analysis. In neocortical neurospheres grown for 10 days, mRNA expression was seen for the \(\alpha2, \alpha3, \alpha4, \alpha5, \beta1, \beta2, \beta3, \gamma1, \gamma2, \) and \(\gamma3\) subunits of GABA\(_A\)R and GABA\(_B\)R1a, 1b and GABA\(_B\)R2 subunits of GABA\(_B\)R and for the \(\rho1\) and \(\rho2\) subunits of GABA\(_B\)R, but not for the \(\alpha1, \alpha6, \) and \(\delta\) subunits of GABA\(_A\)R or the \(\rho3\) subunit of GABA\(_B\)R. Moreover, in the lower cell layer prepared from fetal mouse neocortex, mRNA expression was seen for mGAT1, mGAT3, and mGAT4, but not mGAT2, isoforms of GABA transporters; vesicular GABA transporter required for the condensation of GABA; and both GAD65 and GAD67 isoforms of glutamate decarboxylase responsible for the synthesis of GABA from glutamate. Similar expression profiles were found with mRNA for these GABAergic signaling molecules, except for the mGAT2 isoform, in neurospheres cultured for 10 days.

3.2. Ionotropic GABA\(_A\)R signals

3.2.1. Proliferation mediated by GABA\(_A\)R: In order to evaluate the role of GAB\(_A\)R expressed by undifferentiated progenitors, neurosphere formation was examined in cells cultured with the endogenous agonist GABA at different concentrations for a period of up to 10 days. The size of clustered neurospheres was drastically increased in proportion to the culture period from 4 to 10 days, while sustained exposure to GABA significantly increased the size of neurospheres in a concentration-dependent manner at concentrations of 10 to 300 \(\mu\)M from 6 to 10 days. The increase by GABA was significantly prevented by either the GABA\(_A\)R antagonist bicuculline or the GABA\(_B\)R antagonist CGP54626. No significant difference was seen in the release of LDH from neocortical neurospheres cultured for 10 days into culture medium irrespective of sustained exposure to any GABAR ligand. A negligibly small number of cells were reactive with the membrane-impermeable dye PI for DNA staining, but most cells were stained with the membrane-permeable dye Hoeschst 33342.

Although neurospheres were proportionally increased with days in culture, the GABA\(_A\)R agonist muscimol significantly increased the neurosphere size in a manner sensitive to the GABA\(_B\)R antagonist bicuculline. Sustained exposure to bicuculline alone significantly and concentration-dependently inhibited the cluster formation in the concentration range of 0.1 – 50 \(\mu\)M in neurospheres, with concomitant significant inhibition of MTT reduction at 10 – 50 \(\mu\)M. The incorporation of the thymidine analog BrdU was found in neurospheres cultured in the presence of EGF for 6 days, and sustained
exposure to muscimol increased the number of cells immunoreactive for BrdU in a manner sensitive to bicuculline. Bicuculline alone decreased the number of cells immunoreactive for BrdU. Repetition of these experiments for quantitative analysis confirmed the significant increase in the number of BrdU-positive cells in neurospheres exposed to muscimol and the prevention by bicuculline. Therefore, tonic activation of GABA_A_R would lead to a significant increase in proliferation activity in neural progenitor cells before commitment without affecting cellular viability.

3.2.2. Differentiation mediated by GABA_A_R: Removal of the growth factor EGF led to an appearance of cells with marked expression of either MAP2 or GFAP 4 days later, with a concomitant complete abolition of immunoreactive nestin. For the induction of cellular differentiation, dispersed cells were cultured for an additional 4 days in the presence of either ATRA or CNTF to facillitate commitment toward a neuronal lineage or an astrogial lineage, respectively. Sustained exposure to ATRA for 4 days increased the number of cells immunoreactive for MAP2 compared to that found in its absence. CNTF was effective in increasing the number of cells immunoreactive for GFAP and decreasing that for MAP2. In cells previously exposed to muscimol for 10 days, a marked decrease was seen in MAP2 expression with a concomitant increase in GFAP expression when cell differentiation was induced by CNTF. Previous exposure to muscimol did not markedly affect the expression of either MAP2 or GFAP in differentiation induced by ATRA as well as spontaneous differentiation.

Quantification of these data revealed that around 50% of the cells were immunoreactive for GFAP and around 30% for MAP2 after spontaneous differentiation. Although cells not immunoreactive for either MAP2 or GFAP were almost 20% of the total cells in spontaneous differentiation, ATRA increased the percentage of MAP2-positive cells with concomitant decreases in those of GFAP-positive cells and cells not immunoreactive for either marker protein. By contrast, CNTF decreased the percentage of MAP2-positive cells along with an increase in that of GFAP-positive cells. Prior sustained exposure to muscimol induced a significant increase in the number of GFAP-positive cells with a concurrent significant decrease in the number of MAP2-positive cells upon differentiation induced by CNTF only. No significant changes were seen in the numbers of cells immunoreactive for either MAP2 or GFAP in cells with prior exposure to muscimol after spontaneous and ATRA-induced differentiation. Prior exposure to muscimol decreased MAP2 expression and increased GFAP expression in cells differentiated by CNTF, but bicuculline alone induced an increase in MAP2 expression with a decrease in GFAP expression. Quantitative calculation showed that muscimol significantly increased GFAP expression with decreased MAP2 expression in the presence of CNTF in a manner sensitive to bicuculline. Bicuculline alone was effective in significantly increasing MAP2 expression and decreasing GFAP expression.

3.2.3. Expression of CNTF receptor: Sustained exposure to muscimol alone resulted in a significant and predominant increase in mRNA expression of CNTF-α receptors in undifferentiated neurospheres, without significantly affecting that of other relevant molecules. These included N-cadherin; β-1 integrin; NCAM; RAR-α, -β, and -γ; and RXR-α. Simultaneous exposure to bicuculline significantly prevented the increase in CNTF-α–receptor mRNA expression induced by muscimol, but was ineffective in altering mRNA expression of other molecules tested.

3.3. A role of GABA_A_R in neurogenesis

As mentioned above, tonic activation of GABA_A_R led to a significant increase in the capabilities to form neurospheres and to express the astrogial marker GFAP in response to CNTF through upregulation of CNTF-receptor expression in cultured neural progenitor cells (Table 1). Significant inhibition was also seen in the expression of the neuronal marker protein MAP2 in the presence of CNTF in cells previously exposed to muscimol, but muscimol failed to significantly affect the expression of both GFAP and MAP2 in the presence of ATRA. Our data clearly confirm the proposal that functional heteromeric GABA_A_R channels are constitu-

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Abbreviations: AST, astrocyte; NEU, neuron.
tively expressed to play a role in mechanisms related to both self-replication and multi-potentiality in undifferentiated neural progenitor cells before commitment to any neural lineages. Activation of GABA_A receptors would play a key role in the mechanisms associated with proliferation, commitment, and differentiation of neural progenitor cells at an early developmental stage in embryonic neurogenesis in both mouse and rat brains.

Evidence is accumulating for the functional expression of GABA_A receptors in neural progenitor cells before commitment and differentiation. In the ventricular zone of rat embryonic neocortex, GABA induces depolarization with decreased DNA synthesis through activation of GABA_A receptors (19). Activation of GABA_A receptors not only leads to the inhibition of proliferation of cortical progenitors in the absence of bFGF with concomitant facilitation of differentiation to neurons (20), it also promotes neuronal differentiation after depolarization in neural progenitors of adult mouse hippocampal slices (22). Although these previous findings disagree with the present significant increase in proliferative activity of neurospheres by tonic activation of GABA_A receptors, GABA was also shown to stimulate proliferation of cultured immature granule cells of the rat cerebellum (21). Opposite effects of GABA are also reported in the proliferation of neural progenitors expressed in the ventricular zone and SVZ of mouse neocortex (32). Exposure to GABA increases neural progenitor proliferation in the ventricular zone, but significantly decreases that in the SVZ, in a manner sensitive to a GABA_A receptor antagonist in mouse organotypic slice cultures. The fact that extracellular environments are highly responsible for commitment of stem cells in vivo (12) would account for the paradox. The abundance of cells already differentiated, such as neurons and astroglia, is a determinant of proliferation and differentiation activities of adjacent undifferentiated neural progenitors in vivo and in vitro. From this point of view, the present technique has the advantage that extracellular environments are artificially defined by the use of neural progenitor cells isolated from embryonic brains under the in vitro culture conditions. In the absence of the influences by different endogenous factors in extracellular environments, activation of GABA_A receptors would lead to the stimulation of proliferation activity of undifferentiated neural progenitor cells toward self-replication and subsequent astroglial commitment in the embryonic neocortex.

Our studies confirm the promotion of subsequent commitment and differentiation induced by CNTF toward the astroglial lineage of undifferentiated neural progenitor cells isolated from embryonic rat (23) and mouse (24) neocortex through prior tonic activation of GABA_A receptors. CNTF is required for neural progenitor cells to differentiate into type-II astrocytes, which is specifically mediated via the signal transducer and activator of transcription 3 (33). ATRA is shown to regulate differentiation and/or proliferation of the progeny cells derived from neural stem cells in the CNS. Under conditions that promote differentiation, cultures of dissociated neurospheres show a dose-dependent increase in the number of neurons in response to ATRA (34). These inducers could regulate both proliferation and differentiation of neural stem cells after commitment. The presence of CNTF would be favorable for commitment to astroglial progenitors and that of ATRA for neuronal progenitors, respectively. Moreover, FBS was shown to increase the number of living cells as well as the number of astrocytes during differentiation, but bFGF facilitates proliferation with reduced differentiation to neurons in neural stem cells (35). These previous findings could account for marked expression of MAP2 even in the presence of CNTF in cells previously exposed to muscimol. A large proportion of the neural progenitor cells would be already rendered to differentiate into neurons during culture under the conditions favorable for cell proliferation. Culture conditions would be a determinant crucial for the fate of commitment to neuronal or astroglial progenitors in neural progenitor cells isolated from fetal mouse neocortex. Neural stem cells commit to differentiation into specific functional cells in subregions of the brain at an early developmental stage in vitro (36–38). The fact that muscimol did not affect spontaneous differentiation of neural progenitor cells toward the neuronal or astroglial lineage could be at least in part due to early commitment to specific cells during culture. The data cited above are all suggestive of the proposal that GABA_A receptors may facilitate the CNTF-induced commitment of neural progenitor cells into astroglia.

We have for the first time demonstrated that tonic activation of GABA_A receptors leads to the predominant up-regulation of CNTF-receptor expression during the culture with EGF in undifferentiated neural progenitor cells of fetal mouse neocortex (24). Although differential expression profiles are shown with CNTF and CNTF receptor-α in astrocytes and neurons of the fascia dentate after the entorhinal cortex lesion (39), the exact underlying mechanism is still unclear. An alternative possibility that activation of GABA_A receptors would stimulate the expression of particular adhesion molecules responsible for commitment and/or differentiation toward an astroglial lineage in response to CNTF in undifferentiated neural progenitor cells is not conceivable if you take into consideration the findings cited above. Neural progenitor cells are shown to express different adhesion molecules, including NCAM, cadherin, and integrin.
$\alpha_5\beta_1$, $\alpha_6\alpha_1\beta_1$, $\alpha_\gamma\beta_8$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ (a low level) (40). The cell adhesion molecules NCAM and the integrins play an important role in the mechanisms underlying cellular proliferation (40), migration (41), and differentiation (42) in neural progenitor cells. Notch is a signal of differentiation in cell adhesion (43), but sustained activation of Notch inhibits both commitment and differentiation to either neurons or glia in neural progenitor cells (44). An AP1 site exists on the promoter regions of both NCAM (45) and integrin $\alpha_6$ (46), whereas the administration of an NMDAR antagonist up-regulates NCAM expression in granule cells of the adult rat DG (47). The interaction between CNTF and CNTF receptor-$\alpha$ leads to heightened expression of connexin 43 mRNA through the Janus tyrosine kinase/STAT pathway in cultured murine cortical astrocytes (48). The exact mechanism as well as the physiological significance of the upregulation of CNTF receptors, however, remains to be elucidated in future studies. It thus appears that functional heteromeric GABA$_B$R channels are constitutively expressed in undifferentiated neural progenitor cells for the regulation of subsequent commitment and differentiation to the astroglial lineage in the developing brains.

3.4. Metabotropic GABA$_B$R signals

3.4.1. Proliferation mediated by GABA$_B$R: The increase by GABA was significantly prevented by simultaneous exposure to the GABA$_B$R antagonist CGP54626 with regards to the size of neurospheres and MTT reduction in neurospheres. Both proliferation indices were significantly inhibited in neurospheres exposed to CGP54626 alone, while GABAR ligands failed to significantly alter the number of PI-positive cells in the neurospheres. The GABA$_B$R-subtype agonist baclofen significantly increased MTT reduction and neurosphere size in a manner sensitive to a GABA$_B$R-subtype antagonist. The GABA$_B$R antagonist CGP54626 significantly prevented the increase in neurosphere size by baclofen. In order to confirm the expression of the heteromeric functional GABA$_B$R subtype, neurospheres cultured for 10 days were subjected to double immunostaining for either the GABA$_B$R1 or GABA$_B$R2 subunit along with nestin. Both GABA$_B$R1 and GABA$_B$R2 subunits were clearly co-localized to cells expressing nestin (31). Baclofen markedly increased the number of cells immunoreactive for BrdU in a fashion sensitive to CGP54626 when exposed to neurospheres cultured for 6 days. Moreover, CGP54626 alone profoundly decreased the number of cells immunoreactive for BrdU. Repetition of these experiments for quantitative analysis clearly confirmed a significant increase in the number of BrdU-positive cells in neurospheres exposed to baclofen alone and the prevention by CGP54626.

Cells were cultured for 10 days in the presence of pertussis toxin, which is responsible for the uncoupling of GABA$_B$R to inhibitory G$_i/o$ proteins toward the negative regulation of adenylyl cyclase. Sustained exposure to the toxin significantly decreased the neurosphere size at a concentration range of 0.02 to 1.0 $\mu$g/ml. MTT reduction was slightly but significantly increased in neurospheres cultured in the presence of baclofen alone, but decreased in those exposed to CGP54626 alone. No significant difference was seen in the release of LDH from neurospheres into culture medium irrespective of the sustained exposure to baclofen or CGP54626. A negligibly small number of cells were reactive with the membrane-impermeable dye PI for DNA staining in neurospheres exposed to a GABA$_B$R ligand, but most cells were stained with the membrane-permeable dye Hoechst 33342. Therefore, tonic activation of GABA$_B$R would lead to a significant increase in proliferation activity in neural progenitor cells before commitment and/or differentiation without affecting cellular viability.

3.4.2. Proliferation of GABA$_B$R1-null progenitors: Neurospheres were prepared from whole brain of fetal mice defective of the gene for the GABA$_B$R1 subunit essential for the orchestration of functional GABA$_B$R (31). Genotyping by RT-PCR clearly revealed the complete absence of GABA$_B$R1-subunit mRNA from particular embryos delivered after the mating of mice with genetically heterozygous genes. Double immunocytochemical analysis using the antibody against GABA$_B$R1 subunit or GABA$_B$R2 subunit together with the anti-nestin antibody confirmed the complete absence of GABA$_B$R1 subunit protein from these neurospheres prepared from GABA$_B$R1-null mice with expression of nestin protein.

The size of clustered cells was invariably smaller with neurospheres prepared from GABA$_B$R1-null mice (gb1$^+/-$) than those prepared from heterozygous (gb1$^+/-$) and wild-type (gb1$^+/+$) mice. In fact, the size of clustered cells was significantly smaller in neurospheres prepared from GABA$_B$R1-null mice (gb1$^+/-$) than in those from heterologous (gb1$^+/-$) and wild-type (gb1$^+/+$) mice during culture for 4 to 10 days. The GABA$_B$R antagonist CGP54626 significantly decreased the size of neurospheres prepared from wild-type mice without significantly affecting that in GABA$_B$R1-null mice. Accordingly, GABA$_B$R could be functionally expressed by undifferentiated neural progenitors before cellular commitment and/or differentiation to stimulate proliferation activity toward self-replication in fetal mouse brain.

3.4.3. Differentiation of GABA$_B$R1-null progenitors: Neural progenitor cells were prepared from GABA$_B$R1-
null mice, followed by culture in the presence of EGF for 8 to 12 consecutive days and subsequent dispersion after the removal of EGF to initiate spontaneous differentiation. Irrespective of the presence of the GABA$_β$R1-subunit gene, cells cultured for an additional 4 days were highly immunoreactive to either the neuronal marker MAP2 or the astroglial marker GFAP. Both the size and shape of the GFAP-positive cells of GABA$_α$R1-null mice were somewhat different from those of wild-type mice under the culture conditions employed. On spontaneous differentiation, almost 40% of the cells were immunoreactive to either MAP2 or GFAP in neurospheres prepared from wild-type animals irrespective of the culture duration. In cells prepared from GABA$_α$R1-null mice, however, a significant decrease was seen in the number of cells immunoreactive to MAP2 with a significant increase in the number of cells immunoreactive to GFAP on spontaneous differentiation.

The addition of ATRA markedly increased the number of cells expressing MAP2 with a concomitant decrease in the number of GFAP-expressing cells in preparations from wild-type mice, while CNTF decreased MAP2 expression with a concurrent increase in GFAP expression. In cultures prepared from GABA$_α$R1-null mice, a significant decrease was seen in the number of cells immunoreactive to MAP2 with a significant increase in the number of cells immunoreactive to GFAP on differentiation by ATRA. In cells after differentiation induced by CNTF, knockout of the GABA$_α$R1 gene failed to significantly affect the number of cells immunoreactive to either MAP2 or GFAP. Accordingly, GABA$_α$R could play a role in the mechanisms related to subsequent differentiation into neurons and astroglia in mouse neural progenitor cells.

We analyzed the expression profiles of mRNA for adhesion molecules and differentiation inducer receptors in the neurospheres. There was marked expression of the mRNAs for RAR-$\alpha$, RAR-$\beta$, RAR-$\gamma$, RXR-$\alpha$, CNTF receptor-$\alpha$, N-cadherin, $\beta$-1 integrin, NCAM, and laminin-$\beta$1 in the undifferentiated neurospheres prepared from wild-type mice. In neurospheres prepared from GABA$_α$R1-null mice, however, a slight but significant increase was seen in the mRNA expressions of both RAR-$\alpha$ and RAR-$\gamma$, with those of the other molecules being unchanged.

### 3.5. A role of GABA$_α$R in neurogenesis

Tonic activation of GABA$_α$R led to a significant increase in the capability to form neurospheres in cultured neural progenitor cells isolated from fetal mouse brain (Table 2). The data from GABA$_α$R1-null mice give support to this proposal. We have already shown that sustained activation of GABA$_α$R leads to increased proliferation activity for self-renewal along with promoted differentiation into an astroglial lineage in response to CNTF (23, 24). Therefore, GABA could play a pivotal role in the positive regulation of cellular proliferation through activation of both GABA$_α$R and GABA$_β$R expressed by undifferentiated neural progenitors endowed with self-replication and multipotentiality activities. Furthermore, our findings from experiments using GABA$_α$R1-null mice argue in favor of the idea that tonic activation of GABA$_α$R would lead to the promotion of both cellular proliferation and subsequent differentiation toward a neuronal lineage irrespective of the presence of differentiation inducers in neural progenitors. At any rate, GABA$_α$R as well as GABA$_β$R would play a key role in the mechanisms associated with both proliferation and differentiation of neural progenitor cells at an early developmental stage in embryonic neurogenesis in the brain. The possible differences in animal species and/or ages would at least in part account for the paradoxical data on the modulation of neural progenitors by different signal mediators in the literature. Although the inhibition by a GABA$_α$ antagonist alone clearly gives support to the presence of endogenous GABA released from neurospheres prepared from embryonic brains, the possibility that GABA$_α$R displays low-level and ligand-independent background activity in a manner sensitive to antagonists as seen in other ionotropic receptors is not excluded. However, the effective inhibition by CGP54626 alone would account for the weak promotion by baclofen of proliferation in neural progenitor cells, in contrast to the full efficiency of GABA that is an endogenous agonist for both GABA$_α$R and GABA$_β$R subtypes.

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Abbreviations: AST, astrocyte; NEU, neuron.
To our knowledge, this is the first direct demonstration of a significant increase in mRNA expression of both RAR-α and RAR-γ in neural progenitor cells prepared from fetal mice devoid of the GABA<sub>B</sub>R1 subunit. In rat neural progenitors that have no GABA<sub>A</sub>R2-subunit mRNA expression, prior tonic activation of GABA<sub>A</sub>R induces the promotion of subsequent differentiation induced by CNTF toward an astroglial lineage (23). In neural progenitor cells of the embryonic rat spinal neural tube ventricular zone (49), activation of RAR-α leads to the predominant formation of both astrocytes and oligodendrocytes with the formation of motorneurons by activation of RAR-β, which is in good agreement with the present findings on expression profiles of RAR isoforms, MAP2, and GFAP in response to ATRA in GABA<sub>B</sub>R1-null mouse progenitor cells. The data cited above are all suggestive of the proposal that signals mediated by GABA<sub>B</sub>R may facilitate differentiation of neural progenitor cells into neurons. It thus appears that functional heteromeric GABA<sub>A</sub>R could be constitutively expressed by undifferentiated neural progenitor cells for the regulation of cellular proliferation and subsequent differentiation in fetal mouse neocortex. Taken together, one fascinating but hitherto unidentified speculation is that self-replication and multi-potentiality would be under control by delicate balancing between ionotropic and metabotropic GABAergic signals through the respective receptors expressed by neural progenitor cells before differentiation as seen in matured neurons. In undifferentiated progenitors, GABA could positively regulate the proliferation through ionotropic GABA<sub>A</sub>R and metabotropic GABA<sub>B</sub>R subtypes, but differentially modulate subsequent differentiation to particular progeny lineages.

4. Glutamatergic signaling

Glutamatergic signals are mediated by iGluR and mGluR glutamate receptors in the developing brain and matured brain. iGluRs are categorized into three different cation channel subtypes according to the nucleotide sequential homology and intracellular signaling systems. These include NMDAR, AMPAR, and KAR subtypes. By contrast, mGluRs are a family of type III G-protein–coupled receptors activated by glutamate and divided into three major subtypes (group I: mGluR1 and 5 isoforms; group II: mGluR2 and 3 isoforms; group III: mGluR4, 6, 7, and 8 isoforms) based on sequence homology, signal transduction pathway, and pharmacology (50 – 52). The group I mGluR subtype is coupled to stimulatory G<sub>q</sub> proteins to activate phospholipase C, which catalyzes the production of diacylglycerol and inositol (1,4,5)-triphosphate for subsequent activation of protein kinase C and release of Ca<sup>2+</sup> from intracellular stores, respectively. Both group II and III mGluR subtypes are coupled to the inhibitory G<sub>i</sub> protein to negatively regulate the activity of adenyl cyclase, which decreases intracellular concentrations of cAMP (53 – 56).

4.1. Glutamatergic machineries

In the rat neocortical lower cell layer before culture, mRNA expression was seen for NR1, NR2A-2C, and NR2D subunits of the NMDAR subtype; GluR1 – 4 subunits of the AMPAR subtype; and GluR5, GluR6, GluR7, KA1, and KA2 subunits of the KAR subtype on RT-PCR analysis (57). In neocortical neurospheres cultured for 12 days, mRNA expression was similarly seen for NR2A-2C subunits of NMDAR; GluR1 – 4 subunits of AMPAR; and GluR5, GluR6, KA1, and KA2 subunits of KAR, but not for NR1 and NR2D subunits of NMDAR and the GluR7 subunit of KAR. In contrast to the temporal expression profile of mRNA for group III mGluR isoforms, transient expression was seen with the mRNA for the NR1 subunit absolutely essential for the heteromeric assemblies toward the functional NMDAR channels in neurospheres cultured for 2 to 6 days with subsequent disappearance during culture with the growth factor bFGF under floating conditions. In dispersed cells cultured for an additional 6 days after the removal of bFGF, however, marked expression was seen with the NR1 subunit mRNA at a level similar to that found in pre-neurospheres before culture. By contrast, NR2A subunit mRNA was not expressed in pre-neurospheres before culture with a gradual increase proportional to the culture duration from 2 to 12 days. Constitutive expression was seen for NR2B-subunit mRNA in neurospheres cultured for up to 12 days with a sudden threefold increase between 2 and 4 days. A gradual decrease was found in the expression of mRNA for both NR2C and NR2D subunits during the culture periods of up to 12 days. Marked mRNA expression was seen for all NMDAR subunits examined in dispersed cells cultured after the removal of bFGF.

Sustained exposure to either the NMDAR agonist NMDA or the group III mGluR agonist L-AP4 led to a significant decrease in MTT reduction, without significantly affecting LDH release during culture. However, other GluR agonists did not significantly affect either MTT reduction or LDH release even when persistently exposed to neurospheres for 12 days. These included the AMPAR agonist AMPA, the KAR agonist KA, the group I mGluR agonist DHPG, and the group II mGluR agonist DCG-IV.
4.2. Ionotropic NMDAR signals

4.2.1. Responses to NMDA: Brief exposure to NMDA led to a marked increase in the fluorescence intensity for Ca\(^{2+}\) imaging analysis in neurospheres cultured for 2 to 6 days in a manner sensitive to the NMDAR antagonist dizocilpine (MK-801), but not in those cultured for a period longer than 8 days. The calcium ionophore A23187 was invariably active in drastically increasing Ca\(^{2+}\) fluorescence in neurospheres cultured for 2 to 12 days, but marked nestin expression was seen in neurospheres throughout the culture period. In cells cultured for 18 days with spontaneous differentiation after the removal of bFGF, NMDA markedly increased the fluorescence in an MK-801–sensitive manner. The addition of A23187 profoundly increased the fluorescence in these differentiated cells, where immunoreactive nestin was not expressed at all.

In order to evaluate the transient expression of functional NMDAR, neurospheres were similarly exposed to 50 mM potassium chloride for determination of intracellular free Ca\(^{2+}\) ions every 2 days. In contrast to the response to NMDA, exposure to potassium chloride at a high concentration invariably increased the fluorescence intensity in neurospheres irrespective of the culture duration up to 12 days. Quantification of these data clearly confirmed the constant responsiveness to depolarization by potassium chloride with respect to the increased intracellular free Ca\(^{2+}\) level in both neurospheres cultured in the presence of bFGF and cells spontaneously differentiated after the removal of bFGF. Accordingly, functional NMDAR seems to be transiently expressed during a culture period of 2 to 6 days, but not for 8 to 12 days, in neurospheres cultured in the presence of bFGF under floating conditions.

4.2.2. Proliferation mediated by NMDAR: Exposure to NMDA alone markedly decreased the size of neurospheres throughout the culture period up to 12 days in a manner sensitive to the prevention by MK-801. In proportion to increasing culture durations, MTT reduction was significantly decreased in neurospheres cultured in the presence of NMDA alone as seen in the size of the clusters on phase contrast micrographs. The incorporation of BrdU was drastically increased in proportion to increasing culture periods from 2 to 12 days in the absence of NMDAR ligands, while sustained exposure to NMDA significantly decreased the activity of BrdU incorporation in neurospheres. The aforementioned decreases by NMDA were not seen in neurospheres cultured in the presence of both NMDA and MK-801. However, no significant difference was seen in the release of LDH from neocortical neurospheres into the culture medium irrespective of the sustained exposure to any NMDAR ligand. Therefore, tonic activation of NMDAR could lead to a significant decrease in proliferation activity in neural progenitor cells before commitment and/or differentiation without affecting cellular viability.

4.2.3. Differentiation mediated by NMDAR: In cells previously exposed to NMDA for 2 to 12 days, a significant increase was seen in MAP2 expression with a concomitant significant decrease in GFAP expression irrespective of the presence of any differentiation inducers. Prior exposure to NMDA led to a significant increase in the number of cells with high fluorescence in response to the second brief exposure to NMDA in an MK-801–sensitive manner on spontaneous differentiation compared to neurospheres not exposed to NMDA. Therefore, activation of NMDAR would invariably lead to suppression of the proliferative activity toward self-renewal and subsequent promotion of the neuronal differentiation with a concomitant diminution of the astroglial differentiation in neural progenitors isolated from fetal rat neocortex.

4.3. A role of NMDAR in neurogenesis

As mentioned above, sustained exposure to NMDA markedly inhibited the formation of neurospheres and subsequent facilitation of differentiation to cells immunoreactive for different neuronal marker proteins in cultured neural progenitor cells (Table 3). The data obtained in both immunocytochemistry and RT-PCR analyses gave rise to the idea that functional heteromeric NMDAR channels are expressed by neural progenitor cells before commitment and/or differentiation to neurons. The fact that brief exposure to NMDA induces marked expression of c-Fos, Fos-B, Fra-2, and c-Jun

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Abbreviations: AST, astrocyte; NEU, neuron.
proteins in a manner sensitive to MK-801 (30) is also favorable for the high functionality of NMDAR subunits expressed by neural progenitor cells before differentiation.

Rapid responsiveness of AP1 member proteins (30, 58) supports the speculation that brief exposure to NMDA could induce transient expression of the AP1 complex via functional NMDAR channels assembled by heteromeric subunits expressed by neural progenitor cells, toward the modulation of de novo synthesis of inducible target proteins responsible for the regulation of cellular proliferation, commitment, differentiation, and/or maturation. Activation of NMDAR would play a key role in the mechanisms associated with commitment and differentiation of neural progenitor cells at an early developmental stage in neurogenesis. The exact mechanism as well as physiological significance of early and transient expression of functional heteromeric NMDAR channels in neural progenitor cells before differentiation, however, remains to be elucidated in future studies. The observations in the present study also directly demonstrate facilitation of commitment and differentiation to neurons in neural progenitor cells through prior activation of NMDAR. The data cited above are all suggestive of the proposal that NMDA signal input may suppress proliferation toward self-renewal along with the promotion of subsequent commitment and differentiation to neurons in neural progenitor cells. Thus modulation of NMDAR-channel functionality would be highly beneficial for the regeneration of central neurons without surgical implantations in patients with a variety of neurodegenerative disorders in a particular situation.

4.4. Metabotropic GluR signals

4.4.1. Proliferation mediated by group III mGluR:
In the mouse neocortical lower cell layer before culture, mRNA expression was seen for mGluR1 and mGluR5 isoforms of the group I subtype, mGluR2 and mGluR3 isoforms of the group II subtype, and mGluR4 and mGluR8 isoforms of the group III subtype, but not for mGluR6 and mGluR7 isoforms of the group III subtype, on RT-PCR analysis. In neocortical neurospheres cultured for 10 days, however, mRNA expression was seen for mGluR5, mGluR2, mGluR3, and mGluR8 isoforms, but not for mGluR1, mGluR4, mGluR6, and mGluR7 isoforms. The expression of mGluR4 mRNA was almost constant in neurospheres cultured for up to 12 days, while a drastic increase was seen in mRNA expression of both mGluR6 and mGluR7 isoforms in proportion to increasing culture periods from 2 to 12 days. The expression of mGluR8 mRNA was doubled in neurospheres cultured for 2 days with a constant expression level up to 12 days. Expression levels were not significantly different with mRNA for all group III mGluR subtype isoforms examined in cells further cultured in the absence of bFGF for an additional 6 days from those seen in neurospheres cultured for 12 days.

The group III mGluR subtype agonist L-AP4 was effective in markedly decreasing the size of neurospheres, while sustained exposure to either the group I mGluR subtype agonist DHPG or the group II mGluR subtype agonist DCG-IV did not markedly affect the size of neurospheres. The formation of neurospheres was almost completely abolished after sustained exposure to L-AP4, but not to either DHPG or DCG-IV at the same concentration. At 10 days after plating, MTT reduction was significantly decreased in neurospheres cultured in the presence of L-AP4, but not in those cultured with DHPG or DCG-IV, as assessed by measurement of the total size (59).

L-PA4 at >1 μM was inhibitory when neurospheres were exposed to this compound for 10 consecutive days. Sustained exposure to L-AP4 significantly decreased MTT reduction and the size of neurospheres in a concentration-dependent manner. No neurospheres were formed in the presence of L-AP4 at 50 μM, while no significant difference was seen in the cumulative release of LDH from cells cultured for 10 days into culture medium irrespective of the sustained exposure to L-AP4 at concentrations effective in significantly inhibiting MTT reduction. A negligibly small number of cells were reactive with the membrane-impermeable dye PI for DNA staining in neurospheres exposed to L-AP4 for 10 days, but most cells were stained with the membrane-permeable dye Hoescht33342. A significant decrease was seen in the size of neurospheres exposed to L-AP4 for a period of 4 to 10 days. Therefore, sustained exposure to L-AP4 would lead to a significant decrease in the proliferation activity toward self-replication in neural progenitor cells before commitment and/or differentiation without affecting cellular viability.

Sustained exposure to L-AP4 led to a marked decrease in the size of neurospheres, which was blocked by the group III mGluR antagonist CPPG. Although L-AP4 significantly inhibited MTT reduction in a CPPG-sensitive manner, CPPG alone significantly increased MTT reducing activity and size of the neurospheres. As the group III mGluR subtype is responsible for the negative regulation of adenylyl cyclase through inhibitory G_{i/o} proteins, we attempted to determine whether the adenylyl cyclase activator forskolin affects MTT reduction and size of the neurospheres. Sustained exposure to forskolin significantly increased MTT reduction and neurosphere size, which were both prevented by L-AP4. Accordingly, the inhibition by L-AP4
is indeed mediated by the group III mGluR subtype negatively linked to adenyl cyclase in undifferentiated neural progenitor cells.

4.4.2. cAMP/PKA pathway: In addition to forskolin, both dibutylryl cAMP and PACAP were effective in significantly increasing MTT reduction and the size of the neurospheres, with adrenaline being ineffective. The PKA inhibitor H89 significantly prevented the increases by forskolin in MTT reduction activity and the size in neurospheres. H89 alone was also efficient in significantly inhibiting MITT reduction activity and neurosphere size. L-AP4 markedly decreased the number of cells immunoreactive to BrdU in a manner sensitive to CPPG in the neurospheres. In contrast, forskolin significantly increased the number of cells immunoreactive for BrdU in the neurospheres, which was significantly blocked by L-AP4. The increase by forskolin was significantly inhibited by H89, moreover, while H89 alone significantly decreased the number of cells immunoreactive for BrdU. Thus, the cAMP/PKA pathway would be indeed involved in the mechanisms underlying regulation of proliferation activity toward self-replication in undifferentiated neural progenitor cells.

4.4.3. CyclinD1 gene expression: mRNA expression was altogether assessed with different adhesion molecules, in addition to cyclinD1, a key regulator of the cell cycle. Marked expression was seen for cyclinD1 mRNA in neurospheres cultured for 10 days, while cyclinD1 mRNA expression was significantly inhibited by L-AP4 but promoted by forskolin. The decrease by L-AP4 was significantly prevented by CPPG. Similarly, there were marked expressions of the mRNAs for NCAM, N-cadherin, integrin-β1, and laminin-β1 in neurospheres cultured for 10 days. However, no significant alterations were found in mRNA expression of these adhesion molecules in neurospheres cultured for 10 days irrespective of the presence of the tested drugs.

4.4.4. CyclinD1 promoter activity: In silico analysis clearly showed the presence of particular nucleotide sequences recognized by the nuclear transcription factors AP1 and CREB upstream of the cyclinD1 promoter region. Therefore, we first examined the similarity of the murine embryonic carcinoma stem cell line P19 cells to murine neural progenitor cells (59). P19 cells cultured with ATRA within 4 days showed formation of neurospheres immunoreactive for nestin, but neither MAP2 nor GFAP. RT-PCR analysis indicated that P19 cells before culture with ATRA expressed mRNA for the mGluR4 and mGluR8 isoforms of the group III subtype, but not for mGluR1 and mGluR5 isoforms of the group I subtype, mGluR2 and mGluR3 isoforms of the group II subtype, or mGluR6 and mGluR7 isoforms of the group III subtype. In P19 cells cultured for 4 days with ATRA, however, mRNA was expressed for the mGluR5, mGluR2, mGluR4, and mGluR7, and mGluR8 isoforms, but not for the mGluR1, mGluR3, or mGluR6 isoform. Therefore the mRNA expression pattern for the group III mGluR subtype in undifferentiated P19 cells was similar to that in the neural progenitor cells.

Accordingly, P19 cells were exposed to the group III mGluR subtype agonist L-AP4 in the presence of ATRA for 4 days. Sustained exposure to L-AP4 invariably led to a decreased size of neurospheres in a CPPG-sensitive manner, while a marked increase was seen in the size of neurospheres exposed to forskolin. The increase by forskolin was significantly decreased by L-AP4 and H89. H89 alone significantly decreased the size of neurospheres formed. These data clearly indicate the high similarity between neural progenitors and P19 cells in terms of the responsiveness to intracellular cAMP signals.

Therefore, cells were transfected with a reporter plasmid containing the full-length promoter region of cyclinD1 (60) or four tandem copies of CRE along with the TK-Renilla luciferase construct, with the internal control vector pRL-SV40, by the calcium-phosphate method. Approximately 30% of cells expressed green fluorescent protein in P19 cells transfected with the EGFP-C2 plasmid under the transfection condition employed. The mouse embryonic carcinoma stem cell line P19 cells transfected with a reporter plasmid of the full-length promoter region of cyclinD1, or four tandems of CRE, were thus exposed to a test drug in the presence of ATRA. Exposure to L-AP4 significantly decreased the cyclinD1 promoter activity in a CPPG-sensitive manner. Forskolin alone significantly increased the promoter activity, while the increase by forskolin was significantly decreased by L-AP4 and H89. Moreover, H89 alone was effective in significantly decreasing the promoter activity of cyclinD1. Similarly significant alterations were invariably seen with the promoter activity in P19 cells transfected with a reporter plasmid containing the CRE tandem construct. Therefore, activation of the group III mGluR subtype would lead to suppression of the proliferation activity toward self-replication through downregulation of the cell cycle regulator cyclinD1 at the level of gene transcription in neural progenitor cells.

4.4.5. Differentiation mediated by group III mGluR: Neural progenitor cells were cultured with EGF in either the presence or absence of a test drug, followed by further culture in the absence of those test drugs for an additional 4 days toward spontaneous differentiation and subsequent double immunocytochemical detection of both MAP2 and GFAP. Prior exposure to L-AP4
induced a marked increase in the number of cells immunoreactive for GFAP with a concomitant decrease in that for MAP2, whereas forskolin increased the number of cells immunoreactive for MAP2 together with a decrease in that for GFAP. Around 60% of cells were immunoreactive for GFAP and less than 40% for MAP2 on spontaneous differentiation. Prior exposure to L-AP4 alone significantly increased the number of cells immunoreactive for GFAP with a significant decrease in that for MAP2 in a CPPG-sensitive fashion. In contrast, forskolin significantly increased the number of cells immunoreactive for MAP2 along with a significant decrease in that for GFAP.

As seen with spontaneous differentiation, prior sustained exposure to L-AP4 alone significantly increased the number of cells immunoreactive for GFAP with a concomitant significant decrease in that for MAP2 in a CPPG-sensitive manner irrespective of the differentiation inducers added. Forskolin alone significantly increased the number of cells immunoreactive for MAP2 along with a significant decrease in that for GFAP in cells differentiated by either ATRA or CNTF. Accordingly, the group III mGluR subtype could be functionally expressed to play a pivotal role not only in proliferation toward self-replication through a mechanism relevant to a cAMP/PKA signaling to cyclinD1 gene expression, but also in subsequent differentiation toward particular cell lineages, in undifferentiated neural progenitor cells.

**4.4.6. Expression of regulatory genes:** Next, we attempted to determine whether sustained exposure to L-AP4 modulates the expression profile of a variety of bHLH genes responsible for the critical control of cellular differentiation in neural stem cells. There is accumulating evidence that timing and regulation of cell differentiation are critically controlled by diverse bHLH genes in neural stem cells. These include the activator type (Mash1, Math, NeuroD, and Neurogenin) and the repressor type (Hes1 and Hes5) of bHLH genes (61, 62). In embryonic mouse whole brain, RT-PCR analysis indicated marked mRNA expression was seen for Mash1, Neurogenin2, Math3, NeuroD, and Hes1, but not for Hes5. In neural progenitor cells before culture with EGF, marked expression was similarly seen with mRNA for Mash1, Neurogenin2, and Hes1, but not for Math3, NeuroD, or Hes5. In neural progenitors cultured with EGF for 10 days, Hes5 mRNA was highly expressed in addition to Mash1 and Hes1 mRNA. RT-PCR analysis clearly revealed that neither L-AP4 nor forskolin significantly affected mRNA expression of the activator-type Mash1 or the repressor-type Hes1 in neuropheres cultured for 10 days. The present findings do not support the possible involvement of these regulatory bHLH genes in the mechanisms relevant to the promotion of subsequent astroglial differentiation in neural progenitor cells exposed to the group III mGluR–subtype agonist L-AP4.

**4.5. A role of group III mGluR in neurogenesis**

Tonic activation of the group III mGluR subtype leads to a significant decrease in the capability to form neurospheres in cultured neural progenitor cells (Table 4). The data cited above all support the idea that activation of the group III mGluR subtype leads to suppression of proliferation activity in neural progenitors through inhibition of cAMP/PKA signaling processes as shown in the literature (53 – 56). As the group III mGluR subtype is negatively linked to adenylyl cyclase through the inhibitory G\textsubscript{i/o} protein, however, the formation of cAMP should be under the basic and/or tonic stimulation by some endogenous signals for a group III mGluR agonist to elicit its pharmacological actions in undifferentiated neural progenitor cells.

From this point of view, it should be noted that PACAP significantly increases both MTT reduction and neurosphere size. This polypeptide was shown to regulate the proliferation and differentiation in neural progenitor cells during embryonic development (63). In our hands, mRNA expression was not detected for any isoforms of adrenergic β-receptors known to be positively coupled to adenylyl cyclase in undifferentiated neural progenitor cells. Accordingly, the group III mGluR subtype could play a pivotal role in mechanisms underlying the regulation of cellular proliferation toward self-replication, in association with particular endogenous signals positively coupled to cAMP formation, in

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Abbreviations: AST, astrocyte; NEU, neuron.

**Table 4. Group III mGluR signaling to neurogenesis**
undifferentiated neural progenitor cells.

To our knowledge, this is the first direct demonstration of a significant decrease in mRNA expression of cyclinD1 after sustained activation of the group III mGluR subtype in neural progenitors. CyclinD1 is a major cell-cycle regulator responsible for the promoted transition to a proliferative stage, while overexpression of cyclinD1 leads to shortening of the G1 phase and subsequent rapid entry into the S phase (64). CyclinD1 gene expression is mainly regulated at the transcriptional level with less involvement of the posttranscriptional processes (65). In fact, CRE is shown to be required for the basal and inducible expression of cyclinD1 gene in association with the cAMP/PKA signaling pathway in the pancreatic β-cell line INS-1 cells (66). Our findings from promoter analysis thus argue in favor of the proposal that activation of the group III mGluR subtype suppresses self-replication through decreased cyclinD1 gene expression due to reduction of the promoter activity in response to the attenuation of the cAMP/PKA signaling pathway after decreased intracellular cAMP levels in undifferentiated neural progenitors.

Nevertheless, the reason why group II and group III mGluR subtypes differentially affect self-replication activity in undifferentiated neural progenitor cells is not clear so far. The view that both mGluR subtypes are negatively coupled to adenyl cyclase through the inhibitory G protein in almost all cells is prevailing, whereas several independent lines of evidence indicate differential properties between group II and group III mGluR subtypes (67, 68). Similar differentiation between group II and group III mGluR subtypes is also seen with the inducible expression of the API complex in cultured rat neocortical neurons (69). The present data are suggestive of the proposal that signals mediated by group III mGluR subtype may promote subsequent differentiation of neural progenitor cells into an astroglial lineage. The group III mGluR subtype is not required for the expression of particular adhesion molecules responsible for cellular proliferation, commitment, and/or differentiation toward a particular lineage in undifferentiated neural progenitor cells. Functional group III mGluR subtype could be constitutively expressed in undifferentiated neural progenitor cells before commitment for the regulation of cellular proliferation toward self-replication and subsequent differentiation into particular lineages in fetal mouse brains. Sustained activation of the group III mGluR subtype would result in the suppression of proliferation activity toward self-renewal together with the facilitation of subsequent differentiation into astroglia.

4.6. Adult neurogenesis

4.6.1. Involvement of NMDAR in adult neurogenesis: Adult neuronal proliferation is susceptible to the inhibition by excitatory inputs from the entorhinal cortex via the perforant path in a manner prevented by NMDAR antagonists (70). Systemic administration of NMDA not only increases DNA binding of the transcription factor AP1 (58, 71), but, in fact, also decreases cellular proliferation activity determined by the incorporation of BrdU in a manner sensitive to the NMDAR antagonist MK-801 (29) in the DG of the adult murine hippocampus. Systemic administration of NMDA markedly reduces expression of the neural progenitor marker protein nestin as well as PCNA in the DG, without significantly affecting that in the SVZ (29). Moreover, sustained exposure to an NMDAR agonist not only decreases the size of neurospheres formed by clustered cells, but also facilitates neuronal commitment induced by ATRA, in cultured neural progenitor cells isolated from the adult mouse hippocampus (30). In our hands, astroglial differentiation predominates in neural progenitor cells isolated from adult mouse hippocampus irrespective of the presence of differentiation inducers in vitro.

4.6.2. Involvement of NMDAR-mediated neurogenesis in a psychiatric disease: PTSD is a long-lasting psychiatric disease after the traumatic experience of severe fatal stress with the consequence of hippocampal atrophy. Accumulating evidence that glutamatergic signals are involved in the mechanisms relevant to the crisis of a variety of symptoms caused by stress is available in the literature. WIRS leads to significant alterations of endogenous levels of both glutamate and GABA in particular discrete brain structures in rats (72), for example, while stress is believed to promote the excessive release of glutamate at glutamatergic synapses in patients with depression and PTSD (73). A single immobilization stress induces changes in expression levels of mRNA for particular subunits of ionotropic glutamate–receptor subtypes, such as NMDAR and AMPAR, in selected regions of the rat hippocampus and hypothalamus (74). Restraint and tailshock stress impairs long-term potentiation but enhances long-term depression through activation of NMDAR in the hippocampal CA1 pyramidal subfield (75). Moreover, an antagonist for NMDAR, but not for AMPAR, prevents the dendritic atrophy induced by daily restraint stress in hippocampal CA3 pyramidal neurons, which is a highly replicable consequence of chronic stress (76).

In order to test the possible involvement of NMDAR responsible for long-term memory as well as proliferation of neural progenitor cells, mice were intra-peritoneally administered the NMDAR antagonist MK-
of the mechanisms relevant to the input and exposed to WIRS. Thus, NMDAR could play a role in positive clustered cells to the control level in mice reversed the significant decrease in the number of BrdU-positive clustered cells in the DG 5 days after administration, not significantly affect the number of BrdU-positive clustered cells 5 days later. The administration of MK-801 alone did not significantly decrease in the number of BrdU-positive clustered cells to the control level in mice exposed to WIRS. Prior single administration of MK-801 was also effective in significantly preventing the decrease in the number of BrdU-positive clustered cells in mice exposed to WIRS when determined 5 days after the flashback stress by forced swimming. Similarly, mice were administered with MK-801, followed by WIRS for 3 h, and subsequent measurement of BrdU incorporation 5 days later. The administration of MK-801 alone did not significantly affect the number of BrdU-positive clustered cells in the DG 5 days after administration, while prior administration of MK-801 significantly reversed the significant decrease in the number of BrdU-positive clustered cells to the control level in mice exposed to WIRS. Thus, NMDAR could play a role in the mechanisms relevant to the input and/or consolidation of fatal traumatic stress signals in the DG.

To our knowledge, this is the first direct demonstration of the prevention by prior administration of MK-801 of the suppressed incorporation of BrdU in the DG along with the improvement of behavioral abnormalities in mice exposed to WIRS. In our previous studies, an intraperitoneal injection of NMDA led to marked decreases in the incorporation of BrdU and the expression of both nestin and PCNA (24), in addition to the predominant induction of AP1 (23), in the DG of adult mice. These alterations are all antagonized by the prior administration of MK-801 (29, 58). Thus, one possible but hitherto unidentified speculation is that WIRS would facilitate the release of glutamate to activate NMDAR toward subsequent suppression of the proliferation of neural progenitor cells in the DG. Stress is shown to promote glutamate release in patients with depression and PTSD (73), indeed, while systemic administration of NMDA induces the suppression of BrdU incorporation, nestin expression, and PCNA expression in the DG 2 days later (29). Similarly, flashback by forced swimming significantly decreases the number of BrdU-positive clustered cells in the DG (77). The memory acquired in contextual fear-conditioning is shown to be associated with the hippocampal functions (78). Taken together, NMDAR would mediate the prolonged and repeated suppression of progenitor cell proliferation in the DG toward long-lasting and bidirectional abnormal behaviors in patients with PTSD in a particular situation.

5. Amino acid signaling to neurogenesis

Our studies on neurogenesis have begun with the findings that systemic administration of NMDA predominantly increases AP1 DNA binding in a manner sensitive to MK-801 in the DG of the adult murine hippocampus (58, 71). On the basis of these previous in vivo studies, we have analyzed the mechanisms underlying amino acid signaling to neurogenesis in the developing brain in vitro. It is likely that self-renewal activity would be under control by the delicate balancing between GABAergic and glutamatergic signals through the respective receptors expressed by neural progenitor cells before differentiation as seen in matured neurons. In undifferentiated progenitors expressed in the developing and adult brains, GABA could positively regulate proliferation for self-replication through activation of either the GABA_\text{A}R or GABA_\text{B}R subtype, but glutamate would negatively modulate proliferation through activation of either NMDAR or group III mGluR subtype (Fig. 1). Activation of the GABA_\text{A}R subtype could lead to the increased self-replication activity in neural progenitor cells along with the promotion of subsequent differentiation into astroglia (23, 24), while activation of the GABA_\text{B}R subtype would result in the similarly efficient promotion of self-renewal activity together with the facilitation of subsequent differentiation into neurons (31). On the other hand, activation of the NMDAR subtype could lead to the suppressed self-renewal capacity along with the promotion of subsequent differentiation into neurons (57), while activation of the group III mGluR subtype would result in the similarly efficient suppression of self-renewal activity together with the facilitation of subsequent differentiation into astroglia (59). Therefore, modulation of the functionality of particular GABA and/or glutamate receptor subtypes would be of a great benefit for the regeneration and supplementation of neuronal and/or astrogial lineages without surgical implantations of neural progenitor cells in patients suffering from a variety of brain diseases relevant to neuronal and/or astrogial dysfunctions. Alternatively, prior modulation of the activity of GABAergic and glutamatergic receptors could be a novel strategy for subsequent regulation of the differentiation toward a particular progeny cell lineage of implanted neural progenitor cells.
Fig. 1. Hypothetical GABAergic and glutamatergic signaling to neurogenesis. Although GABAergic signal inputs would similarly lead to the stimulation of proliferation for self-replication through activation of either the ionotropic GABA$_{	ext{A}}$R or the metabotropic GABA$_{	ext{B}}$R subtype expressed by undifferentiated neural progenitor cells, GABA$_{	ext{A}}$R signals could promote subsequent differentiation to an astroglial lineage and GABA$_{	ext{B}}$R signals could induce facilitation of subsequent differentiation to neurons, respectively. Glutamatergic signal inputs could invariably result in the suppression of proliferation for self-renewal of undifferentiated neural progenitor cells, by contrast, while prior activation of the ionotropic NMDAR subtype would lead to the promotion of subsequent differentiation into neurons. Activation of the metabotropic group III mGluR subtype would promote the subsequent differentiation to an astroglial lineage. Accordingly, neurogenesis may be under control by the delicate balancing between GABAergic and glutamatergic signal inputs in neural progenitor cells as seen in matured neurons. In the absence of GABAergic and glutamatergic signals, neural progenitor cells are undoubtedly endowed to proliferate for self-renewal and differentiate into progeny lineages.

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References


