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Effects of inhibitory amino acids on expression of GABA_A R α and glycine R α 1 in hypoxic rat cortical neurons during development

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Abstract

Recent studies suggest that GABA and glycine are protective to mature but toxic to immature cortical neurons during prolonged hypoxia. Since the action of these inhibitory amino acids is mediated by GABA and glycine receptors, the expression of these receptors is a critical factor in determining neuronal response to GABA_A and glycine in hypoxia. Therefore, we asked whether in rat cortical neurons, 1) hypoxia alters the expression of the GABA and glycine receptors; 2) inhibitory amino acids change the course of GABA and glycine receptor expression; and 3) there are any differences between the immature and mature neurons. In cultured rat cortical neurons from day 4 (4 Days in Vitro or DIV 4) to day 20 (DIV 20), we observed that 1) GABA_AR α and GlyR α 1 underwent differential changes in expression during the development in-vitro; 2) hypoxia for 3 days decreased GABA_AR α and GlyR α 1 density in the neurons in-between DIV 4 and DIV 20, but did not induce a major change in immature (DIV 4) and mature (DIV 20) neurons; 3) during normoxia GABA, glycine and taurine decreased GABA_AR α and GlyR α 1 density in the immature neurons, but had a tendency to increase the density in the mature neurons, except for taurine; 4) under hypoxia, all these amino acids decreased GABA_AR α and GlyR α 1 density in most groups of the immature neurons with a slight effect on the mature neurons; and 5) δ -opioid receptor activation with DADLE increased GABA_AR α and GlyR α 1 density in both the immature and mature neurons under normoxia and in the mature neurons under hypoxic condition. These data suggest that inhibitory amino acids differentially regulate the expression of GABA_A and glycine receptors in rat cortical neurons in normoxic and hypoxic conditions with major differences between the immature and mature neurons.

Keywords

GABA; glycine; taurine; opioid; receptors; cortex; hypoxia

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1. Introduction

Gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter in the adult brain, exerts its action via an interaction with specific receptors such as GABA_A receptor (GABA_AR) (Magnaghi *et al.*, 2006). The GABA_A receptors (ionotropic GABA receptors) are formed upon co-assembly of α -, β - and γ -subunits (Macdonald & Olsen, 1994). Substantial evidence has shown that GABA_A receptors mediate the majority of fast inhibitory neurotransmission in the central nervous system (Steiger and Russek, 2004).

Glycine is another major inhibitory amino acid in the brain (Kirsch, 2006) and binds to glycine receptor (GlyR) in the post-synaptic membranes. The glycine-mediated inhibitory neurotransmission is essential for voluntary motor control and the reflex responses (Lynch, 2004; Webb & Lynch, 2007). Besides its inhibition of neurotransmission, glycine also regulates interneuron differentiation during development of the central neural network (McDermid *et al.*, 2006) and depresses neurite outgrowth by shunting the neuronal excitability (Tapia *et al.*, 2001). Till date, four alpha-subunits ($\alpha 1$ - to $\alpha 4$) and one beta-subunit have been identified. The differential expression of subunits underlies the diversity in GlyR pharmacology. At present, most of the information is obtained from homomeric GlyR $\alpha 1$ (Lynch, 2004).

GABA_A and glycine receptors play an important role in the neuronal responses to environmental stress (Chen *et al.*, 1999; Zhao *et al.*, 2005). Hypoxic/ischemic stress causes excessive accumulation of excitatory amino acids, which initiate the process of neuronal injury via neurotoxicity (Banasiak *et al.*, 2000). It has been proposed that inhibitory amino acids may antagonize this neurotoxicity in the brain. There are many studies that support this view and have shown that inhibitory amino acids are neuroprotective during stress. For example, GABA has been demonstrated to be neuroprotective against ischemic injury in various species (Schwartz-Bloom & Sah, 2001). Recent studies, however, suggest that the effect of inhibitory amino acids on neuronal activity differs depending on the neuronal age. For example, electrophysiological experiments have shown that GABA_A is excitatory ("depolarizing") to the immature but inhibitory ("hyperpolarizing") to the mature neurons in normoxia (Ben-Ari, 2002). Glycine shows a similar switch from excitatory to inhibitory during neuronal development (Singer *et al.*, 1998; Betz *et al.*, 1999; Ehrlich *et al.*, 1999). Indeed, our recent work suggests that GABA and glycine are protective to mature but toxic to immature cortical neurons during prolonged hypoxia (Zhao *et al.*, 2005). However, the mechanism of the "age-related" differential neuronal response to inhibitory amino acids is not well understood, though there is evidence suggesting a functional difference in GABA and glycine receptors associated with age, especially in the ionic fluxes mediated by these receptors (Bekar *et al.*, 1999).

Because the action of these inhibitory amino acids is mediated by GABA and glycine receptors, the expression of these receptors is a critical factor in determining neuronal response to GABA_A and glycine in hypoxia. Little is known, however, about the expression of GABA_A and glycine receptors in hypoxic cortical neurons and the effect of the inhibitory amino acids on their expression during development. In this work performed on cultured cortical neurons, we measured the expression of GABA_AR α and GlyR $\alpha 1$ and asked 1) how does hypoxia alter their expression with neuronal development? 2) Is such an alteration affected by neuronal treatment with inhibitory amino acids? and, 3) Is there any difference between immature and mature neurons under hypoxia in terms of GABA_AR α and GlyR $\alpha 1$ expression in response to inhibitory amino acids? Since the δ -opioid receptor (DOR) also plays an inhibitory role in neuronal regulation and its activation is neuroprotective against hypoxic/excitatory insults (Zhang *et al.*, 2000, 2002; Ma *et al.*, 2005; Chao and Xia, 2010),

we also determined the effect of DOR activation on GABA_AR α and GlyR α 1 expression for the purpose of comparison.

2. Results

In this work, we recognized the expression of GABA_AR α and GlyR α 1 protein as an index of GABA and glycine receptor expression. Realizing that the receptors are comprised of multiple subunits (Lynch, 2004; McCarson et al., 2006), in the following description we sometimes refer to the data as GABA_A and glycine receptors for the purpose of brevity.

To determine the effects of inhibitory amino acids (GABA, glycine and taurine) on GABA_AR α and GlyR α 1 expression in a dose-response manner, we applied them to immature (DIV 4) and mature (DIV 20) cortical neurons in serial concentrations (10–2000 μ M).

2.1. Major differences in GABA_A R α and Gly R α 1 density during neuronal development

The density of GABA_AR α , (Fig. 1A) was very low in the immature neurons. As shown in Figure 1, the density in DIV-4 neurons was less than one-third of the mature level. The density increased in the culture with days, reaching peak level at DIV 16 and then slightly decreasing to the mature level at DIV 20.

In sharp contrast, the expression of GlyR α 1, a 49-KDa protein (Fig. 1A), had a different pattern with a high density in DIV-4 neurons. Although the density slightly increased with neuronal age between DIV 8 and DIV 16, the changes were not significantly different when compared to the immature level. In DIV-20 neurons, GlyR α 1 density was similar to that of DIV-4 neurons.

2.2. Hypoxic regulation of GABA_AR α and GlyR α 1 expression during neuronal development

Hypoxia slightly increased GABA_AR α density in DIV-4 neurons ($+6.9 \pm 1.3\%$, $p < 0.01$), while it had no appreciable effect on DIV-8 neurons. The same hypoxic stress, however, significantly decreased GABA_AR α density in later ages (e.g., DIV 12 and DIV 16). Interestingly, the hypoxic reduction of GABA_AR α expression was not seen in DIV-20 neurons.

Hypoxia did not significantly alter GlyR α 1 density in DIV-4 neurons, but greatly reduced the density in DIV-8 neurons ($-26.0 \pm 8.9\%$, $p < 0.05$). From DIV 12 to DIV 20, hypoxia tended to decrease GlyR α 1 expression, but the reduction was not statistically significant.

2.3 Effects of GABA, glycine and taurine on GABA_AR α and GlyR α 1 expression in immature and mature neurons in normoxia

GABA_AR α —In the immature neurons, GABA, glycine and taurine induced a modest reduction in GABA_AR α density at certain concentrations (Fig. 3A). For example, 10 μ M of GABA led to $>10\%$ reduction ($p < 0.05$). Glycine at lower concentrations (10–100 μ M) also significantly reduced GABA_AR α density by 13–26% ($p < 0.05$). Similarly, taurine at 100 μ M reduced GABA_AR α density by $10.9 \pm 1.6\%$ ($p < 0.05$).

In the mature neurons, an increase in the receptor density occurred in response to low concentrations of GABA (10 or 100 μ M) and the highest concentration of glycine (2000 μ M, $p < 0.05$, Fig. 3B). In contrast, taurine at higher concentrations (1000–2000 μ M) reduced GABA_AR α density by $>15\%$ ($p < 0.01$, Fig. 3B).

GlyR α 1—In the immature neurons, the changes in GlyR α 1 density also varied with concentrations of inhibitory amino acids. The highest concentration of GABA (2000 μ M) decreased GlyR α 1 density by 20% ($p < 0.05$). However, a trend towards a decrease in GlyR α 1 density was seen in response to glycine only at its lowest concentration (10 μ M) (Fig. 4A). Neither the increasing concentrations of glycine nor any concentration of taurine induced any significant changes in GlyR α 1 density. In the mature group, in contrast to the immature neurons, increasing concentrations of GABA resulted in an initial increase in GlyR α 1 density by $>40\%$ at a concentration of 100 μ M ($P < 0.05$), followed by a decrease in density at its highest concentration (2000 μ M). Glycine at all concentrations tended to increase GlyR α 1 density, especially at its highest concentration (2000 μ M) where a more than 50% increase was observed ($P < 0.05$). Taurine, unlike GABA and glycine, tended to decrease GlyR α 1 density with a significant change seen only at its highest concentration (Fig. 4B, $P < 0.01$).

2.4 Effects of GABA, glycine and taurine on GABA $_A$ R α and GlyR α 1 expression in immature and mature neurons during hypoxia

GABA $_A$ R α —In DIV-4 neurons, GABA tended to decrease GABA $_A$ R α density (Fig. 5A), with a significant reduction of more than 20% observed at 1000 μ M GABA ($p < 0.05$). On the other hand, glycine at all concentrations significantly decreased GABA $_A$ R α density by $>15\%$ ($p < 0.05$ or 0.01). Taurine at lower concentrations (10–100 μ M) slightly decreased GABA $_A$ R α density, while the higher concentrations (1000–2000 μ M) did not cause an appreciable change in the expression (Fig. 5A).

At DIV-20, GABA induced a slight decrease in GABA $_A$ R α density only at the highest concentration (2000 μ M) ($P < 0.001$), with no effect at lower concentrations (Fig. 5B). The neuronal response to glycine was different from that seen in the immature neurons. As shown in Figure 5B, glycine at all concentrations did not change GABA $_A$ R α density. Similar to glycine, taurine also had no effect on the mature neurons.

GlyR α 1—In the immature neurons, GABA, glycine and taurine reduced GlyR α 1 density at almost all the concentrations (Fig. 6A) except for high concentrations of taurine (1000–2000 μ M). There was no significant difference among the concentrations in terms of amplitude of the reduction.

An opposite effect was seen in the mature neurons (Fig. 6B). GABA at all concentrations (10–2000 μ M) did not significantly decrease GlyR α 1 density whereas glycine induced a reduction of GlyR α 1 expression only with the highest concentration of glycine (2000 μ M). Moreover, 10 μ M of taurine led to an increase in GlyR α 1 density by 35% ($P < 0.05$), while the higher concentrations of taurine had no evident effect on the density (Fig. 6B).

2.5 Increased GABA $_A$ R α and GlyR α 1 expression by DOR activation

To determine if the above results were specific to the inhibitory amino acids, we further investigated if activation of DOR, which is also neuroprotective against hypoxic/excitatory insults (Zhang *et al.*, 2000, 2002; Ma *et al.*, 2005; Chao and Xia, 2010), alters GABA $_A$ R α and GlyR α 1 expression. We treated the cultured neurons with 10 μ M of DADLE, a DOR agonist, and then examined GABA $_A$ R α and GlyR α 1 expression.

In normoxia, DOR activation slightly increased GABA $_A$ R α expression in both immature (DIV 4) and mature (DIV 20) neurons (Fig. 7A). However, during hypoxia DOR increased GABA $_A$ R α density only in the mature neurons.

The effect of DOR activation on glycine receptors was similar to that on GABA receptor, with a significant increase found only in immature neurons in normoxia. (Fig. 7B).

3. Discussion

The main findings in the present work are that in the rat cortical neurons in-vitro: 1) GABA_AR α and GlyR α 1 protein expression underwent differential changes during development; 2) 3-day hypoxia decreased GABA_AR α and GlyR α 1 density in the neurons at a point in-between DIV 4 and DIV 20, but did not induce a major change in immature (DIV 4) and mature (DIV 20) neurons; 3) in normoxia, GABA, glycine and taurine decreased GABA_AR α and GlyR α 1 density in the immature neurons, but tended to increase the density in the mature neurons, except for taurine; 4) under hypoxia, all these amino acids decreased GABA_AR α and GlyR α 1 density in most groups of the immature neurons with a slight effect on the mature neurons (DIV 20). In contrast to the effects of the amino acids, DOR activation increased GABA_AR α density in both the immature and mature neurons in normoxia, but only increased it in the mature under hypoxia. DOR activation induced a similar change in GlyR α 1 density. These observations provide novel insights into GABA_A and glycine receptor expression in the cortical neurons in response to inhibitory amino acids under hypoxia and during development.

The developmental expression of GABA_A and glycine receptors in the cortical neurons was unclear in the past. The multiple subunits of GABA_A and glycine receptors may have different patterns of development with subunit switching (Culiat *et al.*, 1994; Günther *et al.*, 1995). This switch is possibly related to its functional maturation (Liu & Wong-Kiley, 2004) and may vary from region to region in the brain. The developmental pattern of GABA_AR α , seen in this work may reflect the general development of GABA_A in the cortex. This view is supported by our previous work showing that GABA_A receptor expression, detected by receptor binding autoradiography, increases with age in the cortex during postnatal development (Xia & Haddad, 1992). Furthermore, a functional study on the rat cerebral cortex demonstrates that the size of the GABA-induced current increased progressively with age with a maximum in the mature (Carpenter *et al.*, 1988). This functional observation is well in accordance with our results obtained from the in-vivo and in-vitro studies.

Recent information has emerged concerning molecular structure and function of the glycine receptor (Lynch, 2004). Although glycine receptor is best known for its role in mediating inhibitory neurotransmission in the spinal cord and brain stem, accumulating evidence has shown its important role in the majority of regions in the brain, including the cortex at different stages of the development. Till date, most of the information has been obtained from homomeric GlyR α 1 in spite of the fact that 4 α -subunits (α 1 to α 4) and one β -subunit have been identified (Lynch, 2004). The past studies suggest that a developmental switch from GlyR α 2 to GlyR α 1 occurs, which is completed around the 20th postnatal day in the rat (Lynch, 2004). In humans, the composition of the pentamer changes from α 2 subunits in the fetal CNS to α 1- and β -subunits in the adult CNS. Embryonic receptors comprise α 2 homomers whereas adult receptors comprise predominantly α - and β -heteromers in a 2:3 stoichiometry (Webb & Lynch, 2007). However, there is no direct evidence for the development of GlyR α 1 in the cortical neurons. Our work serves as a pioneer source of information on this issue. Interestingly, our data show that the expression of GlyR α 1, shown as a clear signal band at 49 KDa, was not low at all in the immature neurons. In fact, their density already reached the adult level at day 4 in culture (approximately equal to the day at birth in-vivo). Since the size of GlyR α 1 protein is similar to that of other glycine receptor α -subunits (e.g., GlyR α 2), one may argue if we have detected (or mixed) the signals of other glycine receptor α -subunits in this work. This is unlikely based on the epitope sequence of the antibody. It has 100% sequence similarity with the mouse GlyR α 1 protein and does not

pick any of the other forms of GlyR α -subunits, or have any cross-reactivity in the house tests (Julia Tinder, Santa Cruz Biotechnology, personal communication). Therefore, our current data suggest that immature cortical neurons in culture are actually “glycine” cells with high density of GlyR α 1 protein and low density of GABA $_A$ receptors. There is evidence showing that whereas the neonatal glycine receptor isoform in vivo is completely replaced by the adult isoform within 3 weeks after birth, this exchange of subtypes is not seen in primary culture of the mouse spinal cord culture (Hoch et al., 1989). Therefore, there is a possibility that the culture conditions alter the pattern of glycine receptor expression during development, which needs further clarification in the future.

It is noteworthy that GABA $_A$ R α and GlyR α 1 expression in the immature (DIV 4) and mature (DIV 20) neurons was relatively resistant to hypoxic stress in spite of the major difference in their developmental patterns in normoxia. Since the immature neurons are tolerant to hypoxic stress, it was not out of our expectation that 3-day hypoxia had no appreciable effect on the neurons at DIV 4. It is also easy for us to understand that the same stress did not reduce GABA $_A$ expression at DIV 20 because it is needed for neuroprotection against hypoxic insult in the mature neurons. However, it is interesting to note that hypoxia tended to decrease GABA $_A$ R α and GlyR α 1 expression in neurons between the immature and mature stages (DIV 8–16). There is no information available to interpret the down-regulation of GABA $_A$ and glycine receptors during this period. Indeed, it was surprising to observe that hypoxia reduced GABA $_A$ and glycine receptor expression at DIV 8–16, but did not affect the expression at DIV 4 or DIV 20 in most of the groups studied. One of the possibilities is that at a rapidly developing stage between DIV 4 and 20, neuronal process of GABA $_A$ and glycine receptor expression is sensitive to oxygen deprivation.

In general, membrane receptor expression is negatively regulated in response to long-term exposure to the receptor agonists (Kouznetsova *et al.*, 2002; Hoffman *et al.*, 2003). In this work, we saw the down-regulation of GABA $_A$ R α expression in the immature neurons after exposure to GABA or glycine, in either hypoxic or normoxic condition. In the mature neurons, however, GABA $_A$ R α expression was not down-regulated in response to GABA or glycine under the hypoxic condition. Moreover, GABA or glycine exposure even induced an up-regulation of GABA $_A$ R α expression in normoxia. In the case of GlyR α 1 expression, a similar phenomenon was also observed except for neurons exposed to glycine at the highest concentration (2000 μ M). This age-difference can be attributed to differences in the regulation of GABA $_A$ and glycine receptor expression and in neuronal sensitivity to hypoxic stress between ages.

Although the precise mechanism of age-dependant difference in the GABA $_A$ and glycine receptor expression in response to inhibitory amino acids under hypoxia is unknown at present, one thing is for sure: the differential expression of GABA $_A$ and glycine receptors has a major impact on neuronal survival in hypoxia. In particular, hypoxic neurons benefit from such a differential regulation. Since GABA and glycine signals are toxic to immature neurons in hypoxia (Zhao *et al.* 2005), a decrease in GABA $_A$ and glycine receptor expression is beneficial to the survival of immature neurons under hypoxia. Initially, GABA or glycine may injure immature neurons, thus reducing the expression of GABA and glycine receptors, which in turn may prevent the neurons from further injury. On the other hand, a sustained or increased level of GABA $_A$ and glycine receptor expression, especially with an increase in inhibitory amino acids, may help neurons better survive hypoxic environment since the inhibitory amino acids are protective to the mature neurons in hypoxia (Zhao *et al.* 2005).

Currently, there is no specific receptor(s) identified for taurine yet. It has been thought that taurine may, at least partially, activate glycine and/or GABA to exert cellular action (Wu &

Xu, 2003). Our data suggest that the action of taurine on GABA_A R α and GlyR α 1 expression is different from that of GABA or glycine. In the mature neurons during normoxia, for example, taurine did not up-regulate GABA_AR α and GlyR α 1 expression although both GABA and glycine tended to increase the expression. It seems that taurine may have a different mechanism in the regulation of GABA_A and glycine receptor expression.

It was not extraordinary to see that DOR activation increased GABA_AR α and GlyR α 1 density in both immature and mature neurons in normoxic condition because DOR is involved in neuroprotection under normoxia (Zhang *et al.*, 2000, 2002; Chao and Xia, 2010). DOR activation might improve the living condition of these neurons, thus leading to better expression of GABA_A and glycine receptors. Although hypoxic stress tends to reduce the receptor expression in the cortical neurons, DOR activation prevents the neurons from the reduction of the expression and even increased the expression of both GABA_AR α and GlyR α 1 in the mature neurons. This increase can further improve neuronal survival in hypoxic environment since both GABA_A and glycine activities in the mature neurons are neuroprotective against hypoxic stress. The pattern of the DOR-induced changes in GABA_AR α and GlyR α 1 expression suggests that GABA and glycine-mediated regulation of GABA_A and glycine receptors is relatively specific.

In summary, inhibitory amino acids differentially regulate the expression of GABA_A R α and glycine R α 1 in rat cortical neurons in both normoxia and hypoxia with major differences between the immature and mature.

4. Materials and Methods

4.1. Primary culture of rat cortical neuron

Primary neuron cultures were prepared from the cerebral cortex of pregnant rats (E17–18) (Sprague-Dawley strain; Charles River). The animal protocol was approved by the Animal Care Committee of Yale University School of Medicine, which is accredited by the American Association for Accreditation of Laboratory Animal Care. The embryos were removed from dams and placed into the dish with 100ml Neurobasal medium (GIBCO, Grand Island, NY) containing glucose 0.6 % on ice. Cerebral cortex of the embryos was isolated under the microscope and soaked in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) with 0.6% glucose. The tissue was digested by 2.5 mg/ml trypsin-EDTA buffered with 10 mM HEPES (GIBCO) for 15 min at 37°C and then was incubated with 0.25 mg/ml trypsin inhibitor (Roche, Indianapolis, IN) for 5 min at 37°C. After centrifuging at 1,400 rpm at 4°C for 5 min, the cell pellet was diluted with Neurobasal Medium supplemented with B-27, 0.5 mM glutamine (GIBCO), 25 μ M glutamic acid (GIBCO), and 50U/ml penicillin, 50 μ g/ml streptomycin (GIBCO) and then passed through an 80 μ m nylon mesh. The cells were counted and re-diluted in the same medium to 1×10^6 cells/ml, and plated onto poly-D-lysine coated dishes. Cell dishes were put into a humidified incubator with 5% CO₂ at 37°C for maintenance. The day of cell plating was counted as 0 day *in-vitro* (DIV 0).

4.2. Hypoxia exposure

Neuronal culture dishes were randomly divided into normoxia and hypoxia groups at DIV 4, 6, 12, 16 and 20. For hypoxia exposure, culture dishes were placed into a hypoxia incubator (37°C, 1% O₂, 5% CO₂, 94% N₂) for 72 hours with or without drug treatment.

4.3. Drug application

Gamma-aminobutyric acid (GABA), aminoacetic acid (glycine), and 2-aminoethanesulfonic acid (taurine) (all from Sigma, St. Louis, MO) were separately added to the culture medium

with the final concentrations of 10, 100, 1000, 2000 μM at DIV 4, 8, 12, 16 and 20, respectively. [D-Ala², D-Leu⁵]-enkephalin (DADLE; Sigma) was applied to DIV-4 and DIV-20 dishes at a final concentration of 10 μM . The culture dishes were exposed to normoxia or hypoxia immediately after the drug application. A supplemental dose was added after a 36-hour exposure.

4.4. Western blots

Culture dishes were washed with cold PBS twice after removing the medium. The neurons were collected by cell scrapers and homogenized in a lysis buffer (50 mM Tris-HCl, 150mM NaCl, 0.02% sodium azide, 1% Triton-100, 1 μM pepstatin) containing protease inhibitor cocktail (Roche). After centrifuging at 600g for 10 min at 4 C, the supernatants were placed into a new tube and protein concentrations were determined by the Bradford protein assay (Bio-RAD). Protein samples (30 μg) were subjected to electrophoresis in 10% SDS-containing polyacrylamide gels and then transferred to a polyvinylidene fluoride (PVDF)-membrane (Amersham, Piscataway, NJ). The membrane was pre-incubated with 5% non-fat milk in TBS for 1 hour at room temperature and then incubated overnight at 4°C with rabbit polyclonal anti-GABA_A $\text{R}\alpha$, goat polyclonal anti-Gly $\text{R}\alpha$ 1 or anti- β -Actin (all from Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA), or donkey anti-goat IgG (Santa Cruz) was used as a second antibody for 1 hour. After washing in Tris-Buffered Saline with 0.1% Tween (TTBS), the protein was visualized with chemiluminescence detection ECL (Amersham). All three antibodies detected specific signal bands without non-specific signals in our conditions. According to the manufacturer's information, the size of GABA_A $\text{R}\alpha$, Gly $\text{R}\alpha$ 1 and β -Actin signal band was around 51Kda, 49 KDa and 42 KDa, respectively. All recognized signal bands were quantified by AlphaImager 2200 (Alpha Innotech, San Leandro, CA).

4.5. Statistical analysis

In all the samples, measured GABA_A $\text{R}\alpha$ 1 and Gly $\text{R}\alpha$ 1 densities were normalized to that of actin. The data (Mean $\pm$ SE, n=sample number of independent cultures) were expressed as signal ratios (GABA_A $\text{R}\alpha$ vs. β -actin or Gly $\text{R}\alpha$ 1 vs. β -actin) or percent change of the control (100%) and subjected to non-paired *t*-test. The difference was considered significant if *P* value was less than 0.05.

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Highlights

- The developing profiles of GABA_AR α and GlyR α 1 vary greatly in cortical neurons.
- GABA and glycine decrease GABA_AR α and GlyR α 1 in the immature, but not mature, neurons.
- 3-day hypoxia significantly reduces GABA_AR α and GlyR α 1 density in developing neurons.
- GABA, glycine and taurine reduce GABA_AR α and GlyR α 1 in the immature neurons in hypoxia.
- DOR activation increases GABA_AR α and GlyR α 1 in both immature and mature neurons.

Fig. 1. Major differences in GABA_A and glycine receptor expression during neuronal development

1A, Representative images of Western blot analysis. GABA_A and glycine receptors were detected at different culture days from DIV 4 to 20 (n=4). **1B**, Summary of changes in GABA_A and glycine receptor levels with increase in days in culture (n=4). The asterisks (*), dagger (◆), ring (●) and square (■) indicate significant differences as compared to DIV 4, DIV 8, DIV 12 and DIV 16, respectively (P<0.05). Note that GABA_A receptor density in immature (e.g., DIV 4) neurons was less than one-third of the mature level. The density increased with culture days, reaching the peak level at DIV 16 and then slightly decreasing to the level at DIV 20. In sharp contrast, glycine receptor had a high density in DIV-4 neurons. There was no significant change in the receptor density from DIV 4 to 20.

Fig. 2. Hypoxic regulation of GABA_A and glycine receptor expression during neuronal development

The control level in each group was expressed as 100% (dashed line). **2A**, Effect of hypoxia on GABA_A receptor expression. Cultured cortical neurons were treated or left untreated (normoxic control) with hypoxia for 72 hours. Top, representative blots at different culture ages. Bottom, statistical summary (n=4) with asterisks indicate a significant difference from the control. Note that hypoxia slightly increased GABA_A receptor density in DIV-4 neurons, while it had no appreciable effect on DIV-8 neurons. The same hypoxic stress, however, significantly decreased GABA_A receptor density in later ages, i.e., DIV 12 and 16 though it did not affect DIV-20 neurons. **2B**, Effect of hypoxia on glycine receptor expression. Top, representative blots at different culture ages. Bottom, statistical data (n=4) with asterisks indicating a significant difference from the control. Note that hypoxia tended to increase glycine receptor expression in DIV-4 neurons, but significantly reduced the expression in DIV-8 neurons.

Fig. 3. Effects of inhibitory neurotransmitters on GABA_A receptor expression of immature and mature cortical neurons in normoxia, c, control

The control level in each group was expressed as 100% (dashed line). **3A**, Effect of inhibitory neurotransmitters on GABA_A receptor expression in immature (DIV-4) neurons. Top, representative blots. Bottom, statistical analysis of the signal density with the asterisks representing significant differences as compared with the control (n=3). Note that in DIV-4 neurons, GABA, glycine and taurine tended to attenuate GABA_A receptor expression. **3B**, Effects of inhibitory neurotransmitters on GABA_A receptor expression in mature (DIV-20) neurons. Top, representative blots. Bottom, statistical analysis of the band density. The asterisks represent significant differences as compared with the control (n=3). Note that GABA_A receptor density was significantly decreased by highest concentration of GABA (2000 μM), and highest concentration of glycine, which was different from changes in DIV-4 neurons. In contrast, taurine showed a tendency similar to that seen in DIV-4 neurons with higher concentrations (1000 μM and 2000 μM) significantly decreased GABA_A receptor density.

Fig. 4. Effects of inhibitory neurotransmitters on glycine receptor expression of immature and mature cortical neurons in normoxia. c, control

The control level in each group was expressed as 100% (dashed line). **4A**, Effect of inhibitory neurotransmitters on glycine receptor expression in immature (DIV-4) neurons. Top, representative blots. Bottom, statistical analysis of the band density with the asterisks representing significant differences as compared with the control (n=3). Note that a high concentration (2000 μM) of GABA and a low concentration (10 μM) of glycine tended to decrease glycine receptor density, while taurine did not cause significant change in glycine receptor density though a low concentration (10 μM) tended to increase it. **4B**, Effect of inhibitory neurotransmitters on glycine receptor expression in mature (DIV-20) neurons. Top, representative blots. Bottom, statistical analysis of the band density. The asterisks represent significant differences as compared with the control (n=3). Note that both GABA and glycine tended to increase glycine receptor density except the highest concentration (2000 μM) GABA, while taurine markedly decreased the glycine receptor density.

Fig. 5. Effects of inhibitory neurotransmitters on GABA_A receptor expression of immature and mature cortical neurons in hypoxia

c, control (hypoxia without inhibitory neurotransmitters). The control level in each group was expressed as 100% (dashed line). **5A**, Effects of inhibitory neurotransmitters on GABA_A receptor expression in DIV-4 neurons. Top, representative blots. Bottom, statistical analysis of the band density. The asterisks represent significant differences as compared with the control (n=3). Note that GABA_A receptor density decreased in response to most concentrations of GABA, glycine and taurine in hypoxic condition. **5B**, Effects of inhibitory neurotransmitters on GABA_A receptor expression in DIV-20 neurons. Top, representative blots. Bottom, statistical analysis of the band signals with the asterisks representing significant differences as compared with the control (n=3). Note that except the high concentration GABA (2000 μM) that reduced GABA_A receptor density, all other concentrations of inhibitory neurotransmitters had no significant effect on the receptor density.

Fig. 6. Effects of inhibitory neurotransmitters on glycine receptor expression of immature and mature cortical neurons in hypoxia

c, hypoxic control (hypoxia without inhibitory neurotransmitters). The control level in each group was expressed as 100% (dashed line). **6A**, Effects of inhibitory neurotransmitters on glycine receptor expression in DIV-4 neurons. Top, representative blots. Bottom, statistical analysis of the band density. The asterisks represent significant differences as compared with the control (n=4). Note that almost all concentrations of inhibitory neurotransmitters induced a decrease in glycine receptor expression. **6B**, Effects of inhibitory neurotransmitters on glycine receptor expression in DIV-20 neurons. Top, representative blots. Bottom, statistical analysis of the band density. The asterisks represent significant differences as compared with the control (n=3). Note that most concentrations of inhibitory neurotransmitters did not cause significant change except the highest concentration (2000 μM) of glycine that significantly decreased the density and the lowest concentration (10 μM) of taurine that increased the density.

Fig. 7. Effect of DOR activation (DOR) on GABA_A and glycine receptor expression in normoxia and during hypoxia

7A, Effect of DOR activation on GABA_A receptor expression. Top, representative Western blots. Bottom, statistical data (n=3) with asterisk indicating a significant difference from the control (no activation of DOR). The control level in each group was expressed as 100% (dashed line). Note that DOR activation with DADLE slightly increased GABA_A receptor density in normoxia and hypoxia except DIV-4 neurons during hypoxia. **7B,** Effect of DOR activation on glycine receptor expression. Top, representative blots. Bottom, statistical data (n=3) with the asterisk indicating significant difference from the control (no activation of DOR). Note that DOR-induced change in glycine receptor density was similar to that of GABA_A receptor density (7A).