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Glycine-activated chloride currents of neurons freshly isolated from the prefrontal cortex of young rats

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Abstract

Strychnine-sensitive glycine receptors (GlyR) play a major role in the excitability of CNS neurons and are also a major target of many drugs including some general anesthetics and ethanol. The prefrontal cortex (PFC) is an important substrate responsible for cognitive function and for sedation, as well as hypnosis (unconsciousness) which is induced by general anesthetics and ethanol. However, the functions and the physiological and pharmacological properties of GlyRs in mature PFC neurons have not been well studied. In this study, whole-cell currents induced by glycine (I_{Gly}) were recorded from freshly isolated PFC neurons of Sprague-Dawley rats aged 5 to 39 postnatal days (neonatal, P5–12; weanling, P17–21 and peri-adolescent, P30–39). We found that most of the neurons examined were responsive to glycine and the response was concentration dependent. With the increase of age, the sensitivity to glycine was significantly decreased and the sensitivity to picrotoxin was significantly increased. Conversely, the changes in sensitivity to strychnine were not significant. Interestingly, I_{Gly} of all age groups was suppressed (to different scope) by low concentrations of picrotoxin (≤ 30 μM), which selectively blocked α homomeric GlyRs. Conversely, about 20–65% of I_{Gly} remained in the presence of 300 μM picrotoxin, suggesting the picrotoxin-resistant subtype the αβ heteromeric GlyR, was also present. These data provide the first evidence that there are at least two subtypes of functional GlyRs in the PFC neurons of young rats, and their physiological and pharmacological properties change substantially during maturation.

Keywords

whole cell patch-clamp; prefrontal cortex; glycine receptor; strychnine; picrotoxin

1. Introduction

Strychnine-sensitive glycine receptors (GlyR) mediate inhibitory neurotransmission in the spinal cord and other regions of the CNS (Aprison and Werman, 1965; Betz, 1991), and are a major target of many drugs including some general anesthetics (Nguyen et al., 2009) and

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ethanol (Aguayo and Pancetti, 1994; Ye et al., 2001; Ye et al., 2009). GlyR contains four α-subunits (1–4) and one β-subunit. Previous studies have indicated that in naïve neurons, functional GlyRs are comprised of α-homomers and α-β heteromers with a subunit stoichiometry of 2α3β (Grenningloh et al., 1987; Grenningloh et al., 1990; Grudzinska et al., 2005; Lynch, 2009) and that the subunit composition and their assembly change with development (Lynch, 2009).

The prefrontal cortex (PFC) is one of the last territories of the neocortex in evolution as well as ontogeny (Fuster, 2001). It has long been implicated to play an important role in cognitive control via the ability to orchestrate thought and action in accordance with internal goals (Fuster, 2001). The cortical networks are the major neuroanatomical substrate responsible for the sedation and hypnosis (unconsciousness), among other effects induced by ethanol (Ye et al., 2009) and some general anesthetics such as propofol. Although previous studies using *in situ* hybridization and quantitative PCR (qPCR) have indicated that α and β subunits of the GlyR are expressed in the rats' cerebral cortex during early development and adult stage (Jonsson et al., 2009; Malosio et al., 1991), little is known regarding the physiological and pharmacological properties including the developmental changes of these receptors in the PFC. Although functional GlyRs have been demonstrated in embryonic and P0–P4 cortical neurons of rats (Flint et al., 1998), they have not been demonstrated in more mature animals. Furthermore, since recent clinical research has been testing inhibitors of the glycine transporter 1 as therapeutics for the negative symptoms of schizophrenia; knowledge on GlyRs in PFC is important and timely, since these receptors may also be activated with increased ambient glycine levels in the PFC. To this end, in the present study, we recorded whole-cell glycine currents from neurons freshly isolated from PFC of Sprague-Dawley rats of postnatal days 5–39 (P5–39).

2. Results

Neurons were grouped according to the postnatal age of the animal from which they were obtained (day of birth = P0): neonatal, P5–12; weanling, P17–21; and peri-adolescent, P30–39 (McCutcheon and Marinelli, 2009). These groups were selected in light of the developmental changes associated with GlyR structure and the physiological and pharmacological properties in rat neurons.

2.1. I_{Gly} in the PFC neurons

The application of glycine induced an inward current in the majority of neurons examined at a holding potential of −50 mV: for P5–12 rats, 94% (29/31) of the PFC neurons examined produced I_{Gly} in response to glycine, 83% (40/48) and 53% (30/56) for P17–21 and P30–39 rats, respectively (Table 1). I_{Gly} was characterized by a fast onset followed by a slower decay in the continuous presence of the agonist (Fig.1A, 1B, 1C). I_{Gly} increased in amplitude sigmoidally with the concentration of the agonist (Fig. 1D, 1E, 1F). The EC_{50}s and Hill coefficients were 57.8 ± 3.7 μM (mean ± SEM) and 1.3 for PFC neurons from P5–12 rats (Fig. 1D); 79.9 ± 7.2 μM and 1.4 for P17–21 rats (Fig. 1C); and 116.8 ± 17.9 μM and 1.3 for P30–39 rats (Fig. 1F), respectively. One way ANOVA revealed significant difference between P5–12 and P31–39 groups ($F_{2,12} = 6.9$, $P = 0.008$), and between P17–21 and P31–39 groups ($P = 0.04$), the difference between P5–12 and P17–21 ($P = 0.19$), was not significant. Thus, the EC_{50} of I_{Gly} increased with the age of the rats, indicating that physiological properties of GlyRs in the PFC changed during early life.

2.2. Effects of strychnine on I_{Gly}

The plant alkaloid, strychnine, is a selective antagonist for GlyRs (Young and Snyder, 1973; Young and Snyder, 1974). The α subunits of GlyRs carry the binding site for strychnine. To

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characterize the pharmacological properties of the GlyRs of PFC neurons, we tested the effects of strychnine on I_{Gly} recorded from all three age groups. As illustrated in Fig. 2A, 2B and 2C, glycine (500 μM) was first applied alone to obtain an I_{Gly} baseline. Strychnine (STR, 0.03–1.0 μM) was then applied for 10–20 seconds, followed by a solution containing the mixture of strychnine and glycine. After washout of the mixture, glycine alone was applied again. The peak amplitudes of the I_{Gly} obtained under these different conditions were measured. The percentage of inhibition of I_{Gly} by strychnine was calculated by the formula (B/((A+C)/2) × 100, where (A) is the amplitude of I_{Gly} during baseline conditions, (B) during strychnine application, and (C) after washout of strychnine.

The data showed that strychnine concentration dependently reduced I_{Gly} in neurons of all age groups of rats (Fig. 2A, 2B, 2C). Figures 2D, 2E and 2F showed plots of the relationship between the peak of I_{Gly} (normalized to the control value) and the strychnine concentrations for P5–12, P17–21 and P30–39 neurons, respectively. Inhibition curves fit to these data yielded strychnine IC_{50} of 0.16 ± 0.02 μM (mean ± SEM) for P5–12 neurons, 0.25 ± 0.01 μM for P17–21 neurons and 0.13 ± 0.09 μM for P30–39 neurons. One way ANOVA revealed no significant difference among these three groups ($F_{2,10} = 1.14$, $P = 0.36$).

2.3. Effects of picrotoxin on I_{Gly}

The $GABA_A$ antagonist picrotoxin is a useful tool in differentiating between homomeric and heteromeric GlyRs. Previous studies have shown that low concentrations of picrotoxin suppresses the function of α homomeric receptors, but affects the function of α+β heteromeric receptors less (Chattipakorn and McMahon, 2002; Pribilla et al., 1992). In order to obtain further information for the subunit structure of native GyRs in the PFC, we tested the effect of picrotoxin on I_{Gly} of neurons of all three age groups.

Picrotoxin concentration dependently reduced glycine I_{Gly} in neurons of all age groups (Fig. 3A, 3B and 3C). The relationship between the peak amplitude of I_{Gly} (normalized to the peak amplitude of the control I_{Gly} induced by 500 μM glycine) and the concentrations of picrotoxin for P5–12, P17–21 and P30–39 neurons are illustrated in Fig. 3D, 3E and 3F, respectively. Inhibition curves were fit to these data and the results yielded a picrotoxin IC_{50} of 696.8 ± 97.0 μM (mean ± SEM) for P5–12 neurons; 250.0 ± 41.7 μM for P17–21 neurons; and 67.1 ± 4.8 μM for P30–39 neurons. One way ANOVA revealed significant difference among groups ($F_{2,8} = 39.48$, $P < 0.001$). Post hoc analysis revealed: between P5–12 and P31–39 groups ($P < 0.001$), between P5–12 and P17–21 ($P < 0.001$), and between P17–21 and P31–39 ($P = 0.025$). Thus, the sensitivity of GlyRs to picrotoxin significantly increased with age.

3. Discussion

GlyRs are classically known for mediating inhibitory synaptic transmission in the CNS. In the beginning, on the basis of autoradiography with the GlyR antagonist strychnine, GlyRs were thought to be mainly concentrated in spinal cord, brain stem and other areas of the lower neuraxis and were absent from the cerebral cortex and other rostral regions of the CNS (Frostholm and Rotter, 1985). Using in situ hybridization technique, α2 and β subunits of the GlyR were confirmed in cerebral cortex/PFC during early development (Malosio et al., 1991). While the β subunit persists into adulthood, *a*2 subunit declines sharply following the first postnatal week and remains only at a low level in the adult neocortex (Jonsson et al., 2009; Malosio et al., 1991; Sato et al., 1992). Although a previous electrophysiology study showed that functional GlyRs exist in the PFC neurons of embryonic and early postnatal (P0–P4) (Flint et al., 1998), functional GlyRs in the PFC of older animals have not been demonstrated. In the present study, by using patch clamp technique and pharmacological

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approaches, we have, for the first time, provided strong evidence that functional GlyRs exist in PFC neurons of rats aged 5–39 postnatal days.

Developmental change is one of the important features of GlyRs. Several previous studies have shown that GlyR mRNAs in the fetal rat are predominantly α2 homomers (Becker et al., 1988; Lynch, 2009; Malosio et al., 1991; Watanabe and Akagi, 1995). In the spinal cord and lower brain stem, while α2 expression dramatically subsides between birth and postnatal week three, expression of α1 and β subunits increases over the same period. Thus, it has been proposed that in the maturation of the CNS of the rat, there is a developmental switch from α2 homomers to α1β heteromers (Lynch, 2009). This notion is also supported by the electrophysiological evidence from several brain areas including the medial nucleus of the trapezoid body(Kungel and Friauf, 1997), the VTA (Ye, 2000), and the rat substantia nigra (Mangin et al., 2002).

In the current study, we showed that the glycine sensitivity of PFC neurons decreased with age, which was the opposite to that in the acutely isolated VTA neurons and cultured spinal cord neurons where the agonist sensitivity increased with time (Tapia and Aguayo, 1998; Ye, 2000). However, the sensitivity to glycine may not provide information on the subunit composition of the receptors, since there is no evidence to date for subunit-specific GlyR agonists. All homomeric and heteromeric GlyR subtypes exhibit broadly similar sensitivities to glycine (Pribilla et al., 1992; Yang et al., 2007)

The sensitivity to strychnine in all three age groups did not change with development. This is inconsistent with previous studies in cultured mouse spinal neurons (Tapia and Aguayo, 1998) and in neurons acutely dissociated from the VTA (Ye, 2000). Furthermore, it is worthy noticing that the IC_{50} for strychnine of PFC neurons of peri-adolescent Sprague-Dawley rats is 130 nM, about 11 times of that in VTA neurons of similar age (P24–40), which have an IC_{50} of 12 nM (Ye, 2000). These results indicated that in the peri-adolescent PFC neurons, the α1-containing GlyRs are much lower than that of VTA neurons of the same age. This notion is supported by a recent study that used qPCR comparing the relative GlyR expression in the forebrain of Alko Alchol/Non-Alcohol (AA/ANA) rats, which found that the α1 subunit mRNA expression level in the VTA was the highest, but was not detected in the PFC (Jonsson et al., 2009).

Since α2 is the most abundantly expressed α subunit in adult PFC neurons (Jonsson et al., 2009), it would therefore be reasonable to assume that the I_{Gly} in the PFC neurons is mediated by the α2 subtypes. The existence of α2 homomers in the PFC neurons is also implied by relative high picrotoxin sensitivity of I_{Gly} recorded from these neurons, especially from the peri-adolescent group. In the current study, we found that low concentrations of picrotoxin (30 μM) partially blocked the I_{Gly} recorded from PFC neurons, suggesting that a subpopulation of the GlyRs in the PFC neurons is likely to be α2 homomeric receptors- the immature extrasynaptic form. Conversely, the I_{Gly} which is resistant to the higher picrotoxin concentrations (>300 μM) may be induced by the activation of the αβ heteromeric GlyRs, which have a much lower sensitivity to picrotoxin (Chattipakorn and McMahon, 2002; Pribilla et al., 1994; Ye, 2000; Yoon et al., 1998). Interestingly, the peri-adolescent group has much higher picrotoxin sensitivity than the weaning and the neonatal groups, suggesting that the GlyRs in PFC neurons of the peri-adolescent group contain more α2 homomeric receptors compared to the other two age groups. The other interpretation for this is that the β subunit decreased with increase of age. Furthermore, since previous studies have shown that the β subunit of GlyRs is required for receptor clustering (Kirsch et al., 1993; Meyer et al., 1995), the finding of possible αβ heteromeric GlyRs in PFC raises the possibility that some GlyRs may be synaptically located. According to the present study, this is more in the neonatal and weaning groups

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than in the peri-adolescent group. However, additional experiments will need to be undertaken to test this hypothesis.

In summary, results from the present study indicated that: (1) functional GlyRs exist in the PFC neurons of young rats, (2) their physiological and pharmacological properties changed substantially during maturation, and (3) there are at least two subtypes of GlyRs in the PFC neurons.

4. Materials and methods

4.1. Isolation of neurons and electrophysiological recording

PFC neurons were prepared as previously described (Ye and Akaike, 1993). Briefly, Sprague-Dawley rats (P5–P39) were anesthetized with ketamine/xylazine (80 mg/5 mg/kg, IP) and then sacrificed by decapitation. Coronal slices (250 μm thick) containing PFC were cut using a Compresstome™ VF-200 slicer (Precisionary Instruments Inc., Greenville, NC) in ice-cold glycerol-based artificial cerebrospinal fluid (GACSF) containing: 250 mM glycerol, 1.6 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11 mM glucose, and saturated with 95% O_2−5% CO_2 (carbogen) (Ye et al., 2006). Slices were then transferred to a standard external solution containing 3 mg/ml protease XIII (Sigma-Aldrich Chemical Company, St Louis, MO, US) and incubated (in 32°C) for 8 min. The standard external solution contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, and saturated with 100% O_2. The pH of the solution was adjusted to 7.4 with Tris Base and the osmolarity to 320 mM/kg with sucrose. When the incubation was completed, these slices were washed six times with the oxygenated standard external solution. Then the PFC was identified according to the stereotaxic coordinates (Paxinos and Watson, 2007), isolated with a blade and transferred to a 10 ml tube filled with oxygenated standard external solution. Mild trituration of these tissues through heat polished pipettes of progressively smaller tip diameter served to dissociate single neurons. A drop of solution containing PFC neurons was transferred to a 35 mm cultured dish. After 20 min, isolated neurons that adhered to the bottom of the dish were used for electrophysiological recordings. The patch electrode had a resistance between 3 and 5 MΩ when filled with solution containing 120 mM CsCl, 21 mM tetraethylammonium chloride, 4 mM $MgCl_2$, 11 mM EGTA, 1 mM $CaCl_2$, 10 mM HEPES, and 2 mM Mg-ATP. The pH was adjusted to 7.2 with Tris-Base and the osmolarity to 280 mM/kg with sucrose.

Whole-cell I_{Gly} was recorded in a conventional whole-cell configuration with an Axopatch 1D amplifier (Molecular Devices Co., Union City, CA), a Digidata 1320A digitizer (Molecular Devices Co.) and pClamp 10.2 software (Molecular Devices Co.). Data was filtered at 1 kHz and sampled at 5 kHz. Junction potential was nulled immediately before forming a Gigaseal. PFC contains both pyramidal and interneurons. All recordings were from pyramidal neurons judging by their shape.

4.2. Chemicals and application

Chemicals, including glycine, strychnine, and picrotoxin were purchased from Sigma Aldrich (St. Louis, MO, USA). The solutions of these chemicals were prepared on the day of the experiment. Picrotoxin was dissolved in methanol; the final concentration of methanol in test solution was <0.1% (v/v), which has no effect on I_{Gly} (Ye, 2000). The drugs were added to the superfusate and were applied to the cell using a fast perfusion system (Y tube). Solutions in the vicinity of a neuron can be completely exchanged within 40 ms without damaging the seal (Zhou et al., 2006). After each application of the agonist and the antagonist(s) of GlyRs, a > 2 min washout time was followed to ensure that the receptors recovered completely before the next drug application.

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4.3 Data analysis

The date was measured by pClamp10.2 (Molecular Devices Co.). Graphics and statistical data analysis were carried out by Sigmaplot 11.0 (SPSS Inc, Chicago, IL, USA). Data is presented as mean ± standard error of the mean (S.E.M) when appropriating. Statistical analysis was performed using one way ANOVA, and significant main effect was further analyzed using Student-Newman-Keuls Method, and values of $P<0.05$ were considered significant.

Research Highlights

We show that functional glycine receptors exist in cortical neurons of young rats.

The properties of cortical glycine receptors change with development.

The cortical neurons contain at least two subtypes of glycine receptors.

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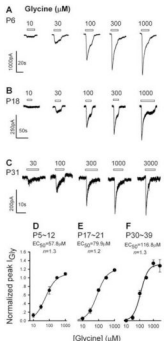

Fig. 1.

I_{Gly} of PFC neurons. Typical I_{Gly} traces of PFC neurons from an 6-day-old rat (A), a 18-day-old rat (B) and a 31-day-old rat (C). Holding potential V_H was −50 mV in all cells. Concentration-response curves of I_{Gly} for the 5–12day-old (D), 17–21-day-old (E) and 30–39-day-old (F) neurons. All points were normalized to the peak response elicited by 300 μM glycine. Each point is the mean of three to seven cells and the vertical bars show ± S.E.M. The glycine concentration producing a half-maximal response (EC_{50}) and the Hill coefficient (n) were estimated using the Michaelis-Menten equation: $I= (I_{max}C^n)/(C^n + (EC_{50})^n)$, where I is the observed I_{Gly}, I_{max} the maximum current, and C the glycine concentration. The EC_{50} value and the Hill coefficient were 57.8 μM and 1.3, 79.9 μM and 1.2 for the 17–21-day-old, and 116.8 μM and 1.3 for the 30–39-day-old neurons.

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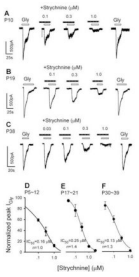

Fig. 2.

Strychnine suppression of I_{Gly}. Typical I_{Gly} traces of PFC neurons from a 10-day-old rat (A), a 19-day-old rat (B) and a 38-day-old rat (C) in response to 500 μM glycine in the absence and presence of strychnine. Strychnine was applied a few seconds before the application of glycine. (D, E, F) concentration-response relationships of strychnine blockage of I_{Gly}. After normalizing the peak I_{Gly} in the presence of strychnine to the control value, the mean ± S.E.M. was calculated and plotted as a function of strychnine concentrations. Each point represents the mean of three to four cells. V_H is −50 mV. For estimation of the dissociation constant (K_d) and the Hill coefficient (n) of the concentration-response curve, the following form of the Logistic equation was fit to the data, $I/I_{Gly}=1/(1+(C+(K_d)^n))$. Where I is the current with strychnine, I_{Gly} is the control current, C is the concentration of strychnine. The IC_{50} and the Hill coefficient were calculated to be 0.16 μM and 1.0 for the 5–12-day-old, 0.25 μM and 1.4 for the 17–21-day-old, and 0.13 μM and 1.3 for the 30–39-day-old neurons, respectively.

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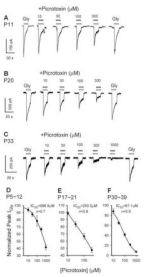

Fig. 3.

Picrotoxin suppression of I_{Gly}. Typical I_{Gly} traces of PFC neurons from a 11-day-old rat (A), a 20-day-old rat (B) and a 33-day-old rat (C) in response to 500 μM glycine in the absence and presence of picrotoxin. V_H was −50 mV. Picrotoxin was co-applied with glycine. (D, E, F) concentration-response relation of picrotoxin blockage of I_{Gly}. After normalizing the peak I_{Gly} in the presence of picrotoxin to the control value, the mean ±S.E.M. was calculated and plotted as a function of picrotoxin concentrations. Each point represents the mean of three to six cells. The dissociation constant (K_d) and the Hill coefficient (n) of the concentration-response curve were estimated using the Logistic equation described above in the legend of Fig. 2, where I is the current with picrotoxin, I_{Gly} is the control current, C is the concentration of picrotoxin. The IC_{50} and the Hill coefficient were 690.1 μM and 0.7 for the 5–12-day-old, 250.0 μM and 0.8 for the 17–21-day-old, and 67.1 μM and 0.9 for the 30–39-day-old neurons, respectively.

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Table 1

EC_{50} or IC_{50} (mean ±SEM, μM) and Hill coefficient (n) of three age groups

	P5–12	**P17–21**	**P30–39**
Glycine response	94% (29/31)	83%(40/48)	53%(30/56)
Glycine EC_{50}	57.8±3.7 (n=1.3)	79.9±7.2(n=1.4)	116.8±17.9(n=1.3)
Strychnine IC_{50}	0.16±0.02(n=1.0)	0.25±0.01(n=1.4)	0.13±0.09(n=1.3)
Picrotoxin IC_{50}	690.1±91.2(n=0.7)	250.0±41.7(n=0.8)	67.1±4.8(n=0.9)