Glycine Receptors Mediate Excitation of Subplate Neurons in Neonatal Rat Cerebral Cortex


Institute of Physiology and Pathophysiology, Johannes Gutenberg University, Mainz, Germany; Department of Physiology, Hamamatsu University School of Medicine, Shizuoka; Department of Physiology, Division of Physiome, Hyogo College of Medicine, Hyogo; and Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences at Kagawa Campus, Tokushima Bunri University, Kagawa, Japan

Submitted 14 June 2007; accepted in final form 12 June 2008

The formation of cortical columns (Kanold et al. 2003). SPn receive transient synaptic inputs from thalamus, cortical neurons, and other SPn (Friauf et al. 1990; Hanganu et al. 2002; Kostovic and Rakic 1980), indicating that they are capable of integrating and processing information from cortical and subcortical regions. In addition, SPn play an important role in the generation of transient oscillatory network activity in the newborn rodent cortex (Dupont et al. 2006). These observations indicate that SPn are essential for activity-dependent formation of cortical circuits during early stages of development (Allendoerfer and Shatz 1994; Kanold 2004; Kostovic and Judas 2002).

A variety of functional neurotransmitter receptors have been described on SPn. Ionotropic N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate and γ-aminobutyric acid type A (GABAA) receptors mediate fast synaptic transmission between cortex, thalamus, and within the subplate (Hanganu et al. 2001, 2002). Furthermore, the cholinergic innervation, which transiently accumulates in the subplate, plays a modulatory role via activation of nicotinic (Hanganu and Luhmann 2004) and muscarinic receptors (Dupont et al. 2006). Another neurotransmitter system that plays an important role in early cortical development is the glycineergic system (Flint et al. 1998; Kilb et al. 2002; Okabe et al. 2004). Activation of glycine receptors causes a membrane depolarization in immature neurons (Ehrlich et al. 1999; Flint et al. 1998; Ito and Cherubini 1991; Kilb et al. 2002) and contributes to the generation of correlated neuronal activity (Momose-Sato et al. 2005). Since taurine is much more abundant in the immature cortex than glycine (Cutler and Dudzinski 1974; Davies and Johnston 1974), taurine is presumably the endogenous agonist of glycine receptors (Flint et al. 1998). It has been previously demonstrated that taurine is required for correct neuronal proliferation, migration, and differentiation (Altshuler et al. 1993; Maar et al. 1995; Palackal et al. 1986; Sturman et al. 1994), illustrating the important role of the glycineergic system in cortical maturation.

However, the questions whether the glycineergic system influences SPn and which effects activation of glycine receptors have on these neurons remain unanswered. To address these questions, we studied the effect of glycine and glycine-agonists on SPn using whole cell and gramicidin-perforated patch-clamp recordings. We demonstrate the existence of glycine receptors on SPn and that activation of these receptors by glycine and taurine mediates excitatory effects.

INTRODUCTION

The formation of the cerebral cortex requires an elaborate sequence of developmental events including neurogenesis, neuronal migration, cellular differentiation, and the growth and activity-dependent refinement of synaptic connections. Certain populations of early generated and transiently expressed neurons play a central role in controlling these developmental events (for review see Sarnat and Flores-Sarnat 2002). One important cell type during cortical development is subplate neurons (SPns), which are involved in the pathfinding of corticopetal and corticofugal axonal projections (Ghosh and Shatz 1993; Ghosh et al. 1990; McConnell et al. 1989) and the formation of cortical columns (Kanold et al. 2003). SPn receive transient synaptic inputs from thalamus, cortical neurons, and other SPn (Friauf et al. 1990; Hanganu et al. 2002; Kostovic and Rakic 1980), indicating that they are capable of integrating and processing information from cortical and subcortical regions. In addition, SPn play an important role in the generation of transient oscillatory network activity in the newborn rodent cortex (Dupont et al. 2006). These observations indicate that SPn are essential for activity-dependent formation of cortical circuits during early stages of development (Allendoerfer and Shatz 1994; Kanold 2004; Kostovic and Judas 2002).

A variety of functional neurotransmitter receptors have been described on SPn. Ionotropic N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate and γ-aminobutyric acid type A (GABAA) receptors mediate fast synaptic transmission between cortex, thalamus, and within the subplate (Hanganu et al. 2001, 2002). Furthermore, the cholinergic innervation, which transiently accumulates in the subplate, plays a modulatory role via activation of nicotinic (Hanganu and Luhmann 2004) and muscarinic receptors (Dupont et al. 2006). Another neurotransmitter system that plays an important role in early cortical development is the glycineergic system (Flint et al. 1998; Kilb et al. 2002; Okabe et al. 2004). Activation of glycine receptors causes a membrane depolarization in immature neurons (Ehrlich et al. 1999; Flint et al. 1998; Ito and Cherubini 1991; Kilb et al. 2002) and contributes to the generation of correlated neuronal activity (Momose-Sato et al. 2005). Since taurine is much more abundant in the immature cortex than glycine (Cutler and Dudzinski 1974; Davies and Johnston 1974), taurine is presumably the endogenous agonist of glycine receptors (Flint et al. 1998). It has been previously demonstrated that taurine is required for correct neuronal proliferation, migration, and differentiation (Altshuler et al. 1993; Maar et al. 1995; Palackal et al. 1986; Sturman et al. 1994), illustrating the important role of the glycineergic system in cortical maturation.

However, the questions whether the glycineergic system influences SPn and which effects activation of glycine receptors have on these neurons remain unanswered. To address these questions, we studied the effect of glycine and glycine-agonists on SPn using whole cell and gramicidin-perforated patch-clamp recordings. We demonstrate the existence of glycine receptors on SPn and that activation of these receptors by glycine and taurine mediates excitatory effects.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
METHODS

Slice preparation

Coronal slices with a thickness of 400 μm were prepared as described previously (Luhmann et al. 2000). Animal handling was performed in accordance with EU directive 86/609/EEC for the use of animals in research and approved by the local ethical committee (approval number 23 177-07/G 07-1-001 from the Landesuntersuchungsanstalt RLP, Koblenz, Germany). The brains from neonatal (postnatal days P0–P5, with P0 being the day of birth) Wistar rats that had been deeply anesthetized by hypothermia or enflurane (Abbot, Wiesbaden, Germany) were quickly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 26 NaHCO3, 1.25 NaH2PO4, 1.8 MgCl2, 1.6 CaCl2, 3 KCl, 20 glucose (equilibrated with 95% O2-5% CO2), and sectioned on a Vibratome (HR2, Sigmann Elektronik, Huf#enhardt, Germany).

Electrophysiological procedure

All experiments were performed at 32 ± 1°C in a submerged-type recording chamber. Whole cell patch-clamp recordings were performed as described previously (Hanganu et al. 2001; Kilb and Luhmann 2000). Patch pipettes (5–12 MΩ) were filled with pipette solution containing (in mM) 86 K-gluconate, 44 KCl, 1 CaCl2, 2 MgCl2, 11 EGTA, 10 HEPES, 2 Na2-ATP, 0.5 Na-GTP, pH adjusted to 7.4 with KOH and osmolarity to 306 mOsm with sucrose. Experiments under gabazine-perforated patch conditions were performed as described previously (Kilb et al. 2002), using 6–12 μg/ml gramicidin D (Sigma, Taufkirchen, Germany) in pipette solutions containing (in mM) 126 K-gluconate, 4 KCl, 1 CaCl2, 2 MgCl2, 11 EGTA, and 10 HEPES. Recording started at access resistances <60 MΩ, which in any case was <5% of input resistance (Rm).

Subplate neurons (SPn) were visualized by infrared differential interference contrast videomicroscopy and were identified according to their location, morphology, and electrophysiological properties. Neurons were excluded from analysis if their morphological and electrophysiological properties did not correspond to those reported previously (Friauf et al. 1996; Hanganu et al. 2001, 2002). All potentials were corrected for liquid-junction potentials of 9.1 and 9.6 mV for whole cell and perforated-patch recordings, respectively (Kilb et al. 2002). For determination of current densities peak current amplitudes were divided by surface area of the cells, which was estimated from input capacitance, assuming a specific membrane capacitance of 2 μF/cm2 (Barrett and Crill 1974). The decay of inward currents was fitted by a monoexponential function using WINTIDA 4.11. For the estimation of binding constants datapoints were fitted by the equation \( I/I_{\text{max}} = c^h/(c^h + EC_{50}^h) \) (where \( c \) is agonist concentration, \( EC_{50} \) is half-maximal response concentration, and \( h \) is the Hill coefficient) using a least-squares algorithm. Microglia glutamatergic postsynaptic currents (mPSCs) were isolated in 0.2–1 μM tetrodotoxin (TTX) and 10 μM gabazine. These mPSCs were detected in continuous recordings lasting ≥5 min using MiniAnalysis software (Synaptosoft, Decatur, GA) according to their amplitude (>0.5 pA), rise time (>0.5 ms), and shape (as inspected by eye).

For dose–response and kinetic analysis experiments, the agonists were applied semifocally for 2 or 20 s by a fine capillary (ID: 250 μm; Microfil, WPI, Sarasota, FL) placed at a distance of 200–600 μm upstream of the investigated cell. This semifocical application system allows the delivery of drugs within 0.4–1 s. In all other experiments, glycine was applied focally to the soma of SPn via a patch pipette for 2–100 ms with a pressure of 0.4 bar using a pressure application system (PDES 02T, npi electronic, Tamm, Germany; LHDA0533115H, Lee, Westbrook, CT). For the analysis of glycine reversal potentials short (2- to 10-ms) puffs of 1 mM glycine were applied focally to the soma of SPn. In these experiments holding potential was set to –60 mV, which was close to the resting membrane potential (RMP) measured under this condition, thus providing conditions under which minimal disturbances of the Cl– homeostasis occur. For extracellular stimulation a bipolar tungsten electrode (tip separation of 150–200 μm; 5 MΩ; FHC, Bowdoinham, ME) was placed in the deeper cortical layers including the SP. Stimuli were delivered at a frequency of 0.033 Hz using a purpose-built isolation unit. Antagonists were applied for ≥3 min prior to agonist application. For experiments involving a low-Ca2+ solution, [Mg2+]o was increased to 3.4 mM, whereas [Ca2+]o was omitted. TTX was purchased from RBI (Natick, MA), glycine and taurine from Tocris (Ballwin, MO), guanidinoethyl sulfonate (GES) from TRC (North York, Canada), and all other substances from Sigma. Cyanotriphenylborate (CTB) was a kind gift from Dr. Till Opitz (Institute of Organic Chemistry, Univ. of Mainz, Germany). Taurine and β-alanine were dissolved directly in ACSF, whereas glycine, CTB, (±) R(−)-3-(2-carboxyoxipryazin-4-yl)-propyl-1-phosphonic acid (CPP), GES, and TTX were used from an aqueous stock solution; picrotoxin and n-2-amino-5-phosphopentanonic acid (APV) from a stock solution in 0.1 M NaOH, and gabazine, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and strychnine from a stock solution in DMSO. The final DMSO concentration never exceeded 0.5%. For hypoosmolar solutions ACSF was diluted with distilled water and the osmolality was checked using a freezing point osmometer (Knauer, Berlin, Germany).

All values are expressed as means ± SE. For statistical analysis of independent data sets two-sample Kolmogorov–Smirnov and for paired data sets Wilcoxon tests were used (Systat 11, Systat, Point Richmond, CA) and results were designated significant at a level of \( P < 0.05 \).

RESULTS

Morphology and membrane properties

Whole cell patch-clamp recordings were performed on 112 visually identified SPn (Fig. 1A). Characterization of the somatic and dendritic organization (Fig. 1B) of these 112 SPn showed that 33 cells were horizontal bitufted, 20 horizontal monufted, 7 inverted pyramidal, 6 tripolar, and 46 multipolar, in agreement with our previous data (Hanganu et al. 2002). The mean RMP measured under whole cell conditions was –62.5 ± 1.1 mV and the mean input resistance (Rm) was 1.245 ± 52.7 MΩ (n = 112). On depolarization above a threshold of –45.6 ± 0.5 mV (n = 112) repetitive action potentials (APs) with a mean amplitude of 54 ± 1.0 mV could be elicited (inset in Fig. 1A). Under gabazine-perforated patch-clamp conditions RMP was –61.7 ± 2.1 mV and Rm was 1.496 ± 138 MΩ (n = 23). In these 23 cells, repetitive APs with amplitudes of 40.7 ± 2.5 mV could be elicited on a membrane depolarization above –44.5 ± 1.3 mV.

Glycine-induced membrane responses

Under whole cell conditions, focal application of 1 mM glycine induced a membrane depolarization of 33.4 ± 2.3 mV (n = 6) and reduced Rm by 85 ± 4.9%. Similar results were obtained by focal application of the glycnergic agonist β-alanine (5 mM), which caused a depolarization by 30.3 ± 2.8 mV (n = 6) and reduced Rm by 80.6 ± 6.8%. Focal application of 5 mM taurine caused a membrane depolarization of 27.2 ± 3.1 mV (n = 6) and a decrease of Rm by 71.1 ± 7.1%. Under voltage-clamp conditions, focal glycine application induced in all investigated SPn inward currents with a mean peak amplitude of 505 ± 51.2 pA (n = 31), corresponding to a current density of 16.1 ± 2.2 μA/cm². Although β-alanine (5 mM) caused inward currents with a mean peak amplitude of 479.9 ±
91.3 pA ($n = 13$), corresponding to a comparable current density (17.3 ± 4.9 μA/cm²), membrane currents evoked by focal application of 5 mM taurine were significantly ($P = 0.025$) smaller (263.9 ± 34.4 pA and 9.1 ± 2.0 μA/cm², $n = 15$). The peak amplitudes of the membrane currents evoked by the glycine agonists glycine, taurine, and β-alanine showed a clear dose dependence (Fig. 2A). The corresponding dose–response curves (Fig. 2B) revealed that glycine receptors on SPn displayed a higher affinity for glycine (0.25 mM) than for taurine and β-alanine (1.67 and 1.14 mM, respectively). In these experiments taurine and β-alanine were applied in the continuous presence of 20 μM bicuculline methiodide. Because bicuculline at this concentration was previously shown to partially block glycine receptors (Shirasaki et al. 1991), the currents induced by β-alanine and taurine may be underestimated.

To investigate the kinetics of current desensitization, glycine receptors were applied semifocally for 20 s (Fig. 2C). Under these conditions, glycine currents desensitized to 24.9 ± 3.4% of the peak amplitude with a time constant of 6 ± 4.9 s.

FIG. 1. Identification of subplate neurons (SPn) in coronal slices of the newborn rat cerebral cortex by morphological and electrophysiological properties. A: videomicroscopic image of a postnatal day 3 (P3) SPn. The cell was recorded with the patch-clamp pipette displayed to the right, whereas the pipette for focal glycine application is visible to the left. Inset shows current-clamp recordings from this cell. Injection of a depolarizing current pulse elicits repetitive action potentials, which represent the typical firing pattern of SPn. B: photomicrograph of the SPn shown in A after staining and histological processing for biocytin. The cell displays the typical appearance of SPn with inverted pyramidal morphology.

FIG. 2. Membrane responses mediated by different glycine receptor agonists in SPn. A: inward currents evoked by various concentrations of glycine (top trace), β-alanine (middle trace), and taurine (bottom trace). B: dose–response curve of the peak currents induced by various concentrations of glycine (black squares), β-alanine (gray circles), and taurine (open squares). Taurine and β-alanine were applied in the presence of 20 μM bicuculline methiodide. Data points represent means ± SE of 6 experiments. C: inward currents elicited by a 20-s application of 1 mM glycine, 5 mM β-alanine, and 5 mM taurine. Note smaller peak amplitudes and smaller degree of desensitization of the taurine response. Application of 100 μM taurine induced non-desensitizing currents with small amplitude.
0.5 s (n = 13, Fig. 2C). Similar values were obtained for currents evoked by 5 mM β-alanine, which desensitized to 27.6 ± 2.8% of the peak amplitude with a time constant of 6.9 ± 0.7 s (n = 12). The currents induced by 5 mM taurine showed significantly (P = 0.002) less desensitization to 41.5 ± 3.5% (n = 14) with a time constant of 6.9 ± 0.8 s (Fig. 2C). Determination of the glycine, β-alanine, and taurine reversal potentials revealed that the reduction in current amplitude was partially related to an attenuation of the Cl− gradient. For all three agonists, the estimated reversal potentials after 15-s applications were negative to the reversal potential calculated for the pipette solution, but there were no significant differences between the agonists. Lowering the taurine concentration to 0.3 mM reduced the degree of desensitization to 66.9 ± 10.3% (n = 5), whereas with 100 μM taurine no significant desensitization (5 ± 4.9%, n = 4) was observed (Fig. 2C).

To elucidate whether taurine indeed activates glycine receptors, cross-desensitization experiments (Wang et al. 2005) were performed in the presence of 3 μM gabazine. These experiments revealed that bath application of 5 mM taurine nearly completely abolished (to 1.2 ± 1%; n = 8) inward currents induced by focal application of 1 mM glycine (Fig. 3A). Similarly, inward currents induced by focal taurine application were also massively suppressed (by 98 ± 2%; n = 6) during bath application of 1 mM glycine (Fig. 3B). Since this reduction may be caused by a decline in Cl− gradient and/or space-clamp errors in distal parts of dendrites, control experiments analyzing the effect of bath-applied glycine on GABAergic currents were performed. In these experiments GABAergic currents were only partially reduced (by 56.6 ± 10.3%; n = 5) in the presence of 1 mM glycine (Fig. 3C), indicating that only a fraction of the cross-desensitization between glycine and taurine can be due to a decline in Cl− gradient and/or space-clamp errors.

**Pharmacological properties of glycine-induced responses**

To discriminate pre- and postsynaptic effects of glycine on SPn, action potentials (APs) were blocked by bath application of 1 μM TTX and a low extracellular Ca2+ concentration was used to suppress activity-dependent synaptic vesicle release (Fig. 4, A and B). Under these conditions, the amplitude of glycine-induced currents was similar to the glycine-induced control responses obtained in normal ACSF (97.1 ± 5.4% of control values, n = 6), indicating that the glycine-induced currents were predominantly mediated by a direct postsynaptic effect. In the presence of the selective glycine receptor antagonist strychnine (1 μM) the glycine-induced currents were partially inhibited by 92.0 ± 2.1% (n = 7, Fig. 4, A and B), suggesting that focal glycine application activates glycine receptors with relatively low strychnine affinity (Okabe et al. 2004; Ye 2000). Increasing the strychnine concentration to 30 μM completely abolished glycine-induced currents (n = 5, Fig. 4, A and B). The specific GABA_A-receptor antagonist gabazine (10 μM) had no significant effect (97.5 ± 0.9%; n = 5) on the amplitude of glycine-induced currents (Fig. 4, A and C). Because it was previously shown that glycine application is sufficient for NMDA-receptor activation in the absence of an externally applied glutamatergic agonist (Paudice et al. 1998), the effects of NMDA-receptor blockade on the glycine-induced response were also investigated. However, bath application of the selective NMDA-receptor antagonist APV (60 μM) had no significant effect on glycine-induced currents in 7 SPn (99.8 ± 3.8%; Fig. 4, A and C).

In summary, these results indicate that the glycine-induced membrane responses in SPn were mediated by postsynaptically located, classical strychnine-sensitive glycine receptors.
of inward currents evoked by focal application of 1 mM glycine before and after application of gabazine (10 μM) revealed values between −25.8 and −46.4 mV and an average reversal potential of −36.7 ± 2 mV (Fig. 5B). No significant difference in $E_{Gly}$ was observed between P0 and P1 (−34.6 ± 3.31 mV; $n = 7$) or between P3 and P4 (−37.3 ± 3.53 mV; $n = 5$).

Since depolarizing membrane responses do not necessarily mediate excitation but can also lead to shunting inhibition (Lamsa et al. 2000; Staley and Mody 1992), we next investigated the effect of glycine application on excitation thresholds. For this purpose, subthreshold glycine responses were adjusted by decreasing the application time and current pulses were injected simultaneously via the patch pipette. Subthreshold glycine-induced depolarizations significantly ($P = 0.006$) decreased the current amplitude required to elicit an AP from 17.1 ± 2 to 11.1 ± 2.4 pA ($n = 14$; Fig. 5C), without affecting AP threshold (−43.1 ± 1.1 vs. −44.3 ± 1.1 mV). These results suggest that glycine has an excitatory effect on SPn. To further study this issue, we investigated the effect of subthreshold glycine applications on AMPA-receptor–mediated excitatory postsynaptic potentials (AMPA–EPSPs) evoked by electrical stimulation of deeper cortical layers including the subplate. AMPA–EPSPs were isolated by bath application of the NMDA-receptor antagonist (±) CPP (20 μM) and the GABA$_A$-receptor antagonist gabazine (10–20 μM). The combination of subthreshold electrical stimulation and subthreshold glycine application significantly ($P = 0.028$) reduced the stimulation strength required to elicit suprathreshold AMPA–EPSPs from 43 ± 10.8 to 24.3 ± 7.4 V ($n = 6$), corresponding to 56.3 ± 4.9% of the stimulation strength under control conditions (Fig. 5D). In additional five SPn, in which under control conditions maximal stimulation failed to induce APs, a combination of electrical stimulation and subthreshold glycine application was capable of triggering APs.

Since glycinergetic synaptic inputs were not found in SPn (Hangamu et al. 2001, 2002), taurine is most probably also the endogenous agonist of glycine receptors on SPn. Therefore we also investigated the effect of taurine on the excitation threshold under perforated-patch conditions. Application of 100 μM taurine caused a small membrane depolarization by 4.5 ± 1.1 mV ($n = 20$) whereas 100 μM taurine also reduced the current amplitude required to elicit an AP from 17.1 ± 2 to 11.1 ± 2.4 pA ($n = 14$; Fig. 5C), without affecting AP threshold (−43.1 ± 1.1 vs. −44.3 ± 1.1 mV). These results suggest that glycine has an excitatory effect on SPn. To further study this issue, we investigated the effect of subthreshold glycine applications on AMPA-receptor–mediated excitatory postsynaptic potentials (AMPA–EPSPs) evoked by electrical stimulation of deeper cortical layers including the subplate. AMPA–EPSPs were isolated by bath application of the NMDA-receptor antagonist (±) CPP (20 μM) and the GABA$_A$-receptor antagonist gabazine (10–20 μM). The combination of subthreshold electrical stimulation and subthreshold glycine application significantly ($P = 0.028$) reduced the stimulation strength required to elicit suprathreshold AMPA–EPSPs from 43 ± 10.8 to 24.3 ± 7.4 V ($n = 6$), corresponding to 56.3 ± 4.9% of the stimulation strength under control conditions (Fig. 5D). In additional five SPn, in which under control conditions maximal stimulation failed to induce APs, a combination of electrical stimulation and subthreshold glycine application was capable of triggering APs.

Glycine receptors are composed of α$_{1–3}$ and β-subunits and are functional as α homomeric or α/β heteromeric receptors (Betz and Laube 2006). To assess the subunit composition of functional glycine receptors on SPn, glycine was applied in the presence of 30 μM picrotoxin, which has been reported to block α homomeric glycine receptors (Pribilla et al. 1992). The glycine-induced inward current was not significantly affected by 30 μM picrotoxin (102.7 ± 2.0%, $n = 6$), indicating that α homomeric receptors do not profoundly contribute to glycine-induced membrane responses (Fig. 4, A and C). In addition, the α$_3$-subunit–specific inhibitor CTB (20 μM) (Rundstrom et al. 1994) had no significant effect on the glycine-induced currents (95.2 ± 3.6%, $n = 8$, Fig. 4, A and C), indicating that the glycine-induced response is not mediated by α$_3$-subunit–containing receptors. In summary, both experiments suggest that functional glycine receptors on SPn have a non-α$_3$-containing heteromeric subtype composition.

**Excitatory action of glycine-induced membrane responses on SPn**

Ligand-gated Cl$^-$ channels like GABA$_A$ or glycine receptors have been found to mediate depolarizing membrane responses in different types of immature neurons, most likely caused by the altered Cl$^-$ homeostasis in these cells (Ben-Ari et al. 1989; Ehrlich et al. 1999; Ito and Cherubini 1991; Kandler and Friauf 1995; Singer et al. 1998; for review see Ben-Ari 2002). To determine whether activation of glycine receptors mediate a de- or hyperpolarizing membrane response in SPn, we performed gramicidin-perforated patch-clamp recordings (Kyrozis and Reichling 1995) from 19 SPn. Under these conditions, focal application of 1 mM glycine induced in all investigated neurons a membrane depolarization of 19.2 ± 1.6 mV ($n = 19$). In 16 of these 19 SPn, the glycine-induced depolarization was sufficient to elicit APs (Fig. 5A). Analysis of the reversal potentials of glycine-induced currents ($E_{Gly}$) revealed values between −25.8 and −46.4 mV and an average reversal potential of −36.7 ± 2 mV (Fig. 5B). No significant difference in $E_{Gly}$ was observed between P0 and P1 (−34.6 ± 3.31 mV; $n = 7$) or between P3 and P4 (−37.3 ± 3.53 mV; $n = 5$).
suggest that glycine and low concentrations of taurine can promote excitation in SPn. It was previously demonstrated that glycine can directly facilitate glutamate release (Turecek and Trussell 2001). To rule out such direct effects of glycine or taurine on glutamate release, we examined the effect of these transmitters on miniature glutamatergic postsynaptic currents (mPSCs). Bath application of 10 \text{mM} glycine has no significant effect on amplitude (8.4 \pm 1 vs. 9.5 \pm 1.6 \text{pA}) and frequency (0.058 \pm 0.019 vs. 0.055 \pm 0.021 \text{Hz}, n = 5) of AMPA-receptor-mediated mPSCs, which were isolated in 0.2 \text{mM} TTX, 3–10 \text{mM} gabazine, and 60 \text{mM} APV. Similar results were observed in the presence of 100 \text{mM} taurine, where neither amplitude (14 \pm 1.1 vs. 13 \pm 0.9 \text{pA}) nor frequency (0.24 \pm 0.06 vs. 0.18 \pm 0.02 \text{Hz}, n = 8) of glutamatergic mPSCs (isolated in 10 \text{mM} GBZ/0.2 \text{mM} TTX) was significantly affected. In summary, these results suggest that neither glycine nor taurine considerably affected glutamate release in synapses at SPn.

Finally, we investigated whether intrinsically released glycine can activate glycine receptors. To exclude the involvement of GABAA receptors, all of these experiments were performed in the continuous presence of 3 \text{mM} gabazine. Application of 30 \text{mM} strychnine had no effect on the holding current (−1.2 \pm 0.9 \text{pA}, n = 7), demonstrating a lack of tonic

**FIG. 5.** Glycinergic responses of SPn in gramicidin perforated patch-clamp recordings. A: focal application of 1 mM glycine (black triangle) induced a membrane depolarization and elicited action potentials (APs). B: voltage dependence of glycine-induced currents. The current–voltage diagram of glycine-induced peak currents demonstrates a linear voltage dependence and reversal potential at about −37 mV. Inset shows typical current traces on focal application of 1 mM glycine (black triangle). C: consecutive recording of membrane potential (black traces) in response to intracellular injection of increasing current intensities (gray traces). Subthreshold glycine applications (black triangles) decreased the threshold current to evoke APs and increased the number of APs elicited by identical current intensities. Similar results were obtained if the pulses were applied in reverse order (C2). The traces shown in C1 and C2 were obtained from the same SPn. D: membrane potential responses of an SPn on focal glycine application (black triangle) and α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA)–receptor-mediated excitatory postsynaptic potentials (EPSPs) elicited by electrical stimulation (white triangles). Whereas focal glycine application (top trace) and AMPA–EPSP (middle trace) were subthreshold, consecutive application of both stimuli induced an AP (bottom trace). E: membrane potential responses on various stimulation currents in the absence (left traces) and presence of taurine (right traces). Taurine induced a small membrane depolarization and decreased the threshold current to elicit APs.
glycinergic currents. However, in the presence of the endogenous glycinergic agonist taurine (100 μM), 30 μM strychnine induced a significant (P = 0.028) shift in the holding current by 4 ± 1.9 pA (n = 7; Fig. 6A). Inhibition of taurine uptake with GES (300 μM) induced an outward current (−13.9 ± 4 pA; n = 9), which was inhibited by 30 μM strychnine (Fig. 6B). Since it had been reported that hypoxosmolar solutions can induce a taurine release in immature neocortical slices (Flint et al. 1998), we focally applied hypoxosmolar solutions to neurons in the vicinity of the recorded SPn, while blocking GABA<sub>A</sub> and ionotropic glutamate receptors with 3 μM gabazine, 60 μM APV, and 10 μM CNQX. In five of nine experiments strychnine-sensitive inward currents with average amplitudes of −60 ± 28.1 pA were evoked by hypoxosmolar solutions (Fig. 6C).

**Discussion**

The results of the present study demonstrate for the first time the functional expression of glycine receptors on SPn. Activation of glycine receptors by glycine, β-alanine, and taurine elicited membrane responses that could be blocked by the selective glycinergic antagonist strychnine, but not by GABA<sub>E</sub> or glutamatergic antagonists. Pharmacological experiments suggest that SPn express functional heteromeric glycine receptors that do not contain α<sub>1</sub>-subunits. The activation of glycine receptors by glycine and taurine induced a membrane depolarization, which mediated excitatory effects. Considering the key role of SPn in immature cortical networks (Dupont et al. 2006; Friauf et al. 1990; Hanganu et al. 2002) and the development of thalamocortical connections (Ghosh and Shatz 1993; Ghosh et al. 1990; McConnell et al. 1989), this glycinergic excitation may influence the properties of early cortical networks and the formation of cortical circuits.

**Functional expression of glycine receptors in SPn**

The application of glycine and the glycinergic agonists taurine and β-alanine evoked membrane responses in all SPn, with a higher affinity for glycine than for taurine and β-alanine. The obvious cross-desensitization between glycine and taurine suggests that both substances act on similar receptors. The affinities for glycine, β-alanine, and taurine observed in the present study were comparable to those in other immature cortical neurons (Okabe et al. 2004), resembling the values found in *Xenopus* oocytes expressing α<sub>2</sub>-subunit–containing glycine receptors (Schmieden et al. 1992). Because glycinergic responses were blocked by the glycine-receptor antagonist strychnine and since the GABA<sub>A</sub>-receptor antagonist gabazine and the NMDA-receptor antagonist APV were without effect, we can conclude that classical strychnine-sensitive glycine receptors in the SPn contribute to the glycine-induced currents observed. The SPn glycine receptors showed a rather low strychnine sensitivity, which is a characteristic property of glycine receptors during early development (Ye 2000). Although β-alanine is known to block GABA transporters expressed in immature neurons (GAT3; Liu et al. 1993), a significant contamination of the membrane currents by altered transporter currents could be excluded because of their low turnover rates, resulting in currents below the resolution of patch-clamp recordings in vertebrate neurons (Masson et al. 1999).

The glycinergic currents recorded in SPn showed a significant desensitization. The slow time constant of this desensitization is in the range observed for α<sub>2</sub>-subunit–containing glycine receptors (Kungel and Friauf 1997). Estimation of the reversal potentials, as well as the reduction of GABA<sub>E</sub> currents in the presence of glycine, revealed that part of the desensitization was caused by an attenuation of the Cl<sup>−</sup> gradient. In SPn, the time constant of desensitization was similar for all three glycinergic agonists tested. In contrast, in Cajal-
Retzius cells or cortical plate neurons, a significantly slower desensitization was observed for taurine (Okabe et al. 2004). Since the kinetics of glycine-receptor desensitization depend on agonist concentration (Gisselmann et al. 2002), this finding may indicate that the effective taurine concentration at the receptor site may vary between SPn, Cajal-Retzius cells, and cortical plate neurons. A more effective taurine uptake or a denser extracellular matrix in the cortical plate and marginal zone may explain different taurine concentrations and glycine-receptor desensitization kinetics. In any case, reducing the taurine concentration attenuated the desensitization in SPn. At a taurine concentration of 100 μM no significant desensitization was observed, suggesting that taurine is capable of inducing stable tonic currents in SPn.

Pharmacological evidences suggest the functional expression of α/β heteromeric glycine receptors, which did not contain α1-subunits, in SPn. The lack of inhibition by 30 μM picrotoxin, which at this concentration predominantly inhibits homomeric α-receptors (Kungel and Friauf 1997; Pribilla et al. 1992), argues against the expression of homomeric α receptors in SPn. The α1-subunit-specific inhibitor CTB was also without effect on the glycine responses, demonstrating that α1-subunit-containing glycine receptors did not significantly contribute to the observed glycineric effects. The pharmacological properties of glycine receptors on SPn were similar to those of receptors found in other cell types of the developing cerebral cortex (Kilb et al. 2002; Okabe et al. 2004) and other parts of the immature CNS (Ye 2000). Since previous studies demonstrate the predominant expression of α2 and β glycine-receptor subunits in all layers of the immature cortex (Flint et al. 1998; Fujita et al. 1991; Malosio et al. 1991; Okabe et al. 2004; Sato et al. 1991), glycine receptors in SPn most probably have mainly an α2/β heteromeric composition. Glycine, taurine, and β-alanine are capable of activating α2/β heteromeric glycine receptors (Farroni and McCool 2004).

Functional consequences of glycine-receptor activation

Although glycine evokes hyperpolarizing membrane responses in the adult nervous system (Curtis et al. 1967; Vannier and Triller 1997), glycine receptors mediate a depolarizing action in immature neurons (Ehrlich et al. 1999; Flint et al. 1998; Ito and Cherubini 1991; Kandler and Friauf 1995; Kilb et al. 2002; Singer et al. 1998; Yoshida et al. 2004). Since activation of GABA_A receptors depolarizes SPn (Hangasu et al. 2002), we predicted that glycineric responses in this cell type would also have a depolarizing effect. Indeed, depolarizing membrane responses were induced by glycine-receptor activation in all investigated SPn. The depolarizing action of glycine most probably reflects an increased intracellular Cl^− concentration in immature cortical neurons (Cherubini et al. 1991; Ehrlich et al. 1999; Owens et al. 1996; Rivera et al. 1999; Shimizu-Okabe et al. 2002), although a contribution of HC^O_3^− fluxes has been suggested in fetal motoneurons (Kulik et al. 2000; for review see Payne et al. 2003). From the reversal potential of glycine-induced currents in SPn (−36.7 ± 2 mV) an intracellular Cl^− concentration of 34.3 ± 2.7 mM can be estimated, which is higher than the passive Cl^− distribution (∼15 mM at a membrane potential of −60 mV). Thus an active uptake mechanism for Cl^− ions must exist in SPn. There is strong evidence that the Na^+−K^+/2Cl^− transporter (isoform NKCC-1) mediates this active Cl^− accumulation in immature cortical neurons (Achilles et al. 2007; Sun and Murrai 1999; Yamada et al. 2004), although an NKCC-1-independent regulation of intracellular Cl^− has been observed in other brain regions (Balakrishnan et al. 2003; Titz et al. 2003).

Depolarizing membrane responses mediated by ligand-gated Cl^− channels do not elicit excitatory responses per se, but may also mediate an inhibitory action due to shunting of membrane currents (Lamsa et al. 2000; Staley and Mody 1992). In fact, it has been demonstrated in the immature cortex that the glycine-induced membrane depolarization mediates shunting inhibition in postnatal Cajal-Retzius cells (Kilb et al. 2002). However, in the present study glycine application elicited APs in the majority of SPn. In addition, subthreshold glycine-induced depolarizations reduced the injection currents required to evoke APs. The generation of APs by AMPA–EPSPs was also facilitated by simultaneous application of glycine in SPn. However, since no glycineric synaptic currents had been observed in SPn (Hangasu et al. 2001), this stimulation paradigm does not mimic the effect of physiological glycine-receptor activation. Because taurine is the endogenous agonist of glycine receptors in the immature cortex (Flint et al. 1998) and is acting mainly via glycine receptors in the immature cortex (Yoshida et al. 2004), it is also highly significant that low concentrations of this agonist also reduced the thresholds to elicit APs by current injection or synaptic activation. In summary, these results demonstrate a robust excitatory effect of glycine receptor activation in this cell type, which may contribute to the maintenance of adequate activity levels in SPn to facilitate neuronal information processing in the immature cortex. Whether an excitatory effect of SPn will increase the activity level within the cortex cannot be predicted from our experiments due to the heterogeneity of subplate neurons (Antonini and Shatz 1990) and possible implication of inhibitory interneurons. It was previously demonstrated at corticostriatal and hippocampal synapses that taurine can induce long-lasting enhancements of synaptic efficiency (Sergeeva et al. 2003). However, since this phenomenon is pronounced in adults and much smaller in animals between the second and fourth postnatal weeks (Chepkova et al. 2002) it may not occur in the early postnatal neocortex.

Because a synaptic activation of glycine receptors does not occur under physiological conditions in SPn, as suggested by the complete suppression of synaptic activity in the presence of GABA_A and glutamate receptor blockers (Hangasu et al. 2001, 2002), glycine receptors are most probably activated by nonsynaptic processes. One possibility is the tonic activation of these receptors by the presumably endogenous neurotransmitter taurine, which can be released by nonsynaptic processes (Flint et al. 1998). In contrast to this publication, an inhibition of glycine receptors with strychnine had no effect on holding currents, indicating the lack of tonic glycine-receptor–mediated currents in our slice preparations. Although we cannot rule out that experimental differences like the different pipette solutions, temperature, or solution exchange rates contribute to this diverse findings, the lower density of SPn, compared with that of the cortical plate neurons investigated by Flint et al. (1998), may result in lower extracellular taurine levels. However, the conditions used in both studies did not exactly reproduce the in vivo situation, where extracellular neurotransmitter levels are sufficient to maintain tonic activation of
receptors (Chadderton et al. 2004). Indeed, addition of low taurine concentrations to the bathing solution uncovered a strychnine-sensitive tonic current. The observation that this current did not desensitize demonstrates that taurine at a physiological concentration, which is probably <100 μM (Andiné et al. 1991), can indeed induce stable tonic currents. The strychnine-sensitive currents induced by the taurine uptake blocker GES indicate that functional taurine transporters are present in the immature cortex and suggest that these transporters may contribute to the regulation of extracellular taurine concentrations. Application of hypoxosmolar solution also induced a strychnine-sensitive current. However, because the induction of these currents required rather low osmolarities (Flint et al. 1998), this process is most probably not directly involved in taurine release under physiological conditions.

Our results demonstrate that activation of glycine receptors with taurine, even at concentrations that could not elicit action potentials by themselves, had an excitatory influence on SPn. This excitatory effect of taurine on SPn influences the neuronal activity of this important neuronal population but can subsequently also modulate neuronal activity in downstream neocortical networks (Dupont et al. 2006; Friauf and Shatz 1994; Anderson and Price 2002; Antonini and Shatz 1990; Ghosh and Shatz 1994; Kanold et al. 2003). Its excitatory effect on SPn may therefore contribute to the crucial role of taurine in neuronal development (Maar et al. 1995; Palackal et al. 1986; Sturman et al. 1994).

ACKNOWLEDGMENTS

We thank B. Krumm for excellent technical assistance, Dr. Till Opatz (Institute of Organic Chemistry, Univ. of Mainz) for the CTB, and G. A. O’Sullivan for help with a previous version of the manuscript.

GRANTS

This work was supported by Deutsche Forschungsgemeinschaft (DFG) Grant KI 835/2 to W. Kilb and Japan Society for the Promotion of Science–DFG Cooperative Research Grant and Grant-in-Aid 18021015 from the Ministry of Education, Science, Sports, Culture and Technology of Japan to A. Fukuda.

REFERENCES


