Strychnine-sensitive glycine receptors on pyramidal neurons in layers II/III of the mouse prefrontal cortex are tonically activated

Michael C. Salling1 and Neil L. Harrison1,2
1Department of Anesthesiology, Columbia University Medical Center, New York, New York; 2Department of Pharmacology, Columbia University, New York, New York

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Salling MC, Harrison NL. Strychnine-sensitive glycine receptors on pyramidal neurons in layers II/III of the mouse prefrontal cortex are tonically activated. J Neurophysiol 112: 1169–1178, 2014. First published May 28, 2014; doi:10.1152/jn.00714.2013.—Processing of signals within the cerebral cortex requires integration of synaptic inputs and a coordination between excitatory and inhibitory neurotransmission. In addition to the classic form of synaptic inhibition, another important mechanism that can regulate neuronal excitability is tonic inhibition via sustained activation of receptors by ambient levels of inhibitory neurotransmitter, usually GABA. The purpose of this study was to determine whether this occurs in layer II/III pyramidal neurons (PNs) in the prelimbic region of the mouse medial prefrontal cortex (mPFC). We found that these neurons respond to exogenous GABA and to the α,β-containing GABAA receptor (GABAAR)-selective agonist gaboxadol, consistent with the presence of extrasynaptic GABAAR populations. Spontaneous and miniature synaptic currents were blocked by the GABAAR antagonist gabazine and had fast decay kinetics, consistent with typical synaptic GABAARs. Very few layer II/III neurons showed a baseline current shift in response to gabazine, but almost all showed a current shift (15–25 pA) in response to picrotoxin. In addition to being a noncompetitive antagonist at GABAARs, picrotoxin also blocks homomeric glycine receptors (GlyRs). Application of the GlyR antagonist strychnine caused a modest but consistent shift (~15 pA) in membrane current, without affecting spontaneous synaptic events, consistent with the tonic activation of GlyRs. Further investigation showed that these neurons respond in a concentration-dependent manner to glycine and taurine. Inhibition of glycine transporter 1 (GlyT1) with sarcosine resulted in an inward current and an increase in the strychnine-sensitive current. Our data demonstrate the existence of functional GlyRs in layer II/III of the mPFC and a role for these receptors in tonic inhibition that can have an important influence on mPFC excitability and signal processing.

glycine; GABA; tonic current; prefrontal cortex

THE PREFRONTAL CORTEX (PFC) is a heavily interconnected cortical subregion that plays a prominent role in the organization of behavior. Abnormal PFC activity has been implicated in psychiatric illnesses such as schizophrenia and depression (Elliott et al. 1997; Weinberger et al. 1986; Yoon et al. 2008), and environmental influences like stress and drug addiction (e.g., alcohol, cocaine) can lead to a long-term depression of PFC activity (Abernathy et al. 2010; Goldstein and Volkow 2011). The rodent PFC has a considerably smaller volume relative to brain size than is the case in humans, yet it remains a valuable model to study PFC signaling as it retains many essential features of the primate PFC, including the same cell types and circuitry, while mediating similar behavioral functions like working memory, behavioral flexibility, and attention (Chudasama and Robbins 2006; Seamans et al. 2008; Uylings et al. 2003). A better understanding of PFC connectivity and physiology is essential in providing the context in which prefrontal dysfunction can be understood.

The cortex has a distinct laminar organization that serves to integrate cortical and subcortical signals. In the rodent medial (m)PFC, the conventional view is that the superficial layers (I, II, III) are input layers receiving cortical and mediodorsal thalamic afferents while the deeper layers (V, VI) project to output regions like striatum and thalamus (Krettek and Price 1977; Ongur and Price 2000). Local cortical processing between layers is complex, with a myriad of interneuron subtypes forming distinct connections with dendritic, somatic, and axonal subcompartments of pyramidal neurons (PNs) and other interneurons (DeFelice et al. 2013; Fino et al. 2013; Rudy et al. 2011). Layer II/III (II/III) PNs are known to be sparsely activated and receive a dense inhibitory synaptic input from a rich variety of interneurons including parvalbumin-containing fast-spiking (FS), somatostatin-containing, and neurogliaform interneurons, to provide tight control of neuronal excitability (Olah et al. 2009; Petersen and Crochet 2013).

Neuronal activity in the cortex may also be regulated by tonic inhibition. Tonic inhibition occurs when ambient levels of GABA (originating from synaptic “spillover” or via release from glia) cause sustained activation of a population of highly sensitive receptors located in the peri- or extrasynaptic space (Belletti et al. 2009; Farrant and Nusser 2005). This phenomenon has been described in several brain regions, including the cerebellum (Brickley et al. 1996), hippocampus (Nusser and Mody 2002), thalamus (Cope et al. 2005; Jia et al. 2005), and somatosensory cortex (Salin et al. 1995; Yamada et al. 2007). The magnitude of these tonic currents can be increased by inhibiting GABA transmitters (Semeynov et al. 2003; Vardya et al. 2008), emphasizing the important role of transporters in regulating tonic currents through the local control of neurotransmitter availability.

GABAAR receptors (GABAARs) that are located extrasynaptically have been identified as essential to the generation of tonic inhibition. These receptors are composed primarily of combinations of α4/6-, β2/3-, and δ-subunits (Belletti et al. 2009) or α5-, β2/3-, and γ2-subunits (Belletti et al. 2009). Extrasynaptic receptors have a high affinity for GABA, with a slow desensitization rate, permitting a sustained elevation in

Address for reprint requests and other correspondence: M. C. Salling, Depts. of Anesthesiology and Pharmacology, Columbia Univ. College of Physicians and Surgeons, 630 West 168th St., Rm. 7-422, New York, NY 10032 (e-mail: ms4431@columbia.edu).
chloride conductance even at very low concentrations of GABA (Bai et al. 2001; Brickley et al. 1996; Saxena and Macdonald 1994). In the ventrobasal thalamus, $\alpha_{2}$ $\beta_{6}$-containing GABA$\alpha_{Rs}$ mediate tonic current and control the excitability of relay neurons (Jia et al. 2005, 2008). In the superficial layers of the rodent PFC these GABA$\alpha_{Rs}$ and $\delta$-subunits appear to be highly expressed (Peng et al. 2002; Pirker et al. 2000), yet GABA-mediated tonic inhibition has not been reported (Hoeftgaard-Jensen et al. 2010). Here we test the hypothesis that tonic inhibition occurs in layer II/III of the prelimbic region of the mPFC, and we show that a tonic current mediated by glycine receptors (GlyR$s$), but not GABA$\alpha_{Rs}$, exists in the majority of these neurons.

**METHODS**

Experimental procedures were performed in male C57BL/6J mice (Jackson Laboratories) under the guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee of Columbia University.

**Brain slice preparation.** Mice (25–50 days old) were fully anesthetized with sevoflurane and decapitated into ice-cold (4°C) artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 2.5 KCl, 26 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 2 CaCl$_2$, 2 MgSO$_4$, and 10 glucose. Brains were dissected and sectioned in cold ACSF with a vibrating microtome (Leica VT1000S) into coronal slices (300 $\mu$m) that contained the prelimbic region of the mPFC at between $2.8$ and $1.78$ mm from bregma, according to a mouse brain atlas (Franklin and Paxinos 1997). Slices were then incubated at 32°C in oxygengenated (bubbled with 95% O$_2$-5% CO$_2$) ACSF for ~30–45 min and then moved to room temperature (22–25°C) for at least 45 min before recordings began.

**Slice electrophysiology.** Slices were placed in a submerged chamber and continuously superfused with room-temperature oxygenated ACSF. mPFC neurons were visualized under an upright light microscope (Olympus BX51WI) using infrared and differential interference contrast. PFC cortical layers were identified under a $\times 4$ objective (layer II/III between ~100 and 300 $\mu$m and layer V/VI between 350 and 500 $\mu$m from the pial surface), and PNs were identified under a $\times 40$ objective by their characteristic size and shape. Pipettes (open tip resistance 2–5 $\Omega$M for CsCl and 3–6 $\Omega$M for K-glucuronate solutions) were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) by a pipette puller (Sutter Instrument, Novato, CA) and used for electrophysiological recordings. Data were collected with a Multiclamp 700B amplifier (Axon Instruments, Union City, CA) and Clampex 10.2 Software (Molecular Devices, Sunnyvale, CA) in identified PNs after reaching a >1-G$\Omega$ seal and after minimization of capacitative currents. Data were collected at 10 kHz and low-pass filtered at 2 kHz. For whole cell recordings under current-clamp conditions, a standard intracellular pipette solution was used (in mM: 130 K$^+$-glucuronate, 5 NaCl, 2 MgCl$_2$, 2 HEPES, 0.2 EGTA, 2 ATP-K$^+$, 0.3 GTP-Na$^+$) and data collection was initiated ~5 min after achieving whole cell configuration. Passive membrane properties [input resistance (R$_{in}$), membrane capacitance, time constant] were measured from the resting membrane potential (RMP) with 20-pA command increments (6 steps starting at ~60 pA, 500 ms), and firing properties (amplitude, frequency, accommodation, I/O relationship) were measured with 40-pA command increments (21 steps starting at ~400 pA, 500 ms). To maximize chloride currents, recordings made under voltage-clamp conditions used a high-chloride intracellular solution (in mM: 140 CsCl, 4 NaCl, 1 MgCl$_2$, 10 HEPES, 0.05 EGTA, 2 Mg-ATP, 0.3 GTP-Na$^+$) adjusted to ~295 mOsm by adding sucrose. To allow equilibration, 7 min of recording occurred prior to data acquisition and only cells demonstrating a stable baseline current were used in the experiment. For phasic events, neurons were voltage clamped at ~70 mV, 30-s epochs were collected for analysis of spontaneous postsynaptic currents (sPSCs), and 45-s epochs were collected for analysis of miniature inhibitory postsynaptic currents (mIPSCs) before and after drug application, allowing time for stabilization of the baseline and drug application. Access resistance was monitored for all cells in voltage clamp via 10-mV hyperpolarizing test pulses, and any cell with access resistance that changed >25% from the start of recording or reached >20 M$\Omega$ was omitted from the data analysis.

**Biocytin visualization.** To identify neurons, recordings were performed with a modification of the current-clamp procedure and slices were cut at 150-$\mu$m thickness to enable better visualization of 0.5% biocytin (Invitrogen, Carlsbad, CA), which was added to the pipette solution. After measurement of passive membrane and firing properties, depolarizing current (+200 pA) was injected at 2 Hz for 15 min. After recording, slices were incubated in ACSF at room temperature for 45 min and then placed in 4% PFA for 1 h. Slices were then processed with the Vectastain ABC kit (Vector Labs, Burlington, CA) and reacted with the chromogen diamobenzidine. Filled cells were then examined under a low-power microscope, and images of labeled cells were collected with imaging software (HC Image) on a light microscope (Olympus BX51WI).

**Pharmacology.** Drug solutions were applied to the slice by bath perfusion. Stock solutions were made by dissolving drugs in ACSF, adjusting pH to 7.4, and freezing. On the day of recording, drug stock solutions were diluted to yield the appropriate concentration. Glycine, taurine, strychnine, picrotoxin, and sarcosine were purchased from Sigma (St. Louis, MO). Gabazine, gaboxadol ([4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridine-3-ol hydrochloride (THIP)], tetrodotoxin (TTX), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo/[f]quinoline-2,3-dione (NBQX), and d(-)-2-amino-5-phosphonopentanoic acid (AP5) were purchased from Tocris (Bristol, UK).

**Data analysis.** Off-line analyses of current-clamp and voltage-clamp data were performed with Clampfit 10.3.02 software. For changes in baseline current, 0.75- to 1.5-s epochs before and after experimental drug applications that contained minimal spontaneous events were selected and all-points histograms were created. These data were fitted with a single Gaussian curve. Responses to concentrations of GABA and glycine were normalized to a common agonist concentration in each cell (1 mM for GABA, 3 mM for glycine), analyzed with a nonlinear regression and fit to the Hill equation $I = I_{max} \times [\text{agonist}]^n/[\text{agonist}]^n + EC_{50}^n$, where $I$ is the peak current, $I_{max}$ is the maximal whole cell current amplitude, [agonist] is the agonist concentration eliciting a half-maximal current response, and $n$ is the Hill coefficient. Mini Analysis software (Synaptosoft, Decatur, GA) was used to detect synaptic events >18 pA in amplitude 4 times root mean square noise for all cells and 30 pA·ms in area. Amplitude, frequency, rise time (10–90%), and decay time were measured and compared before and after drug application. Graphing and statistical analyses were performed with GraphPad Prism software. Where appropriate, Student’s paired $t$-test and ANOVA were used to test for statistical significance ($P < 0.05$). Data are expressed as means ± SE.

**RESULTS**

Characterization of pyramidal neurons in mouse prefrontal cortex. Many electrophysiological studies in the mouse PFC have focused on PNs in layer V/VI. In the present study, we recorded from a large number of neurons in layer II/III and measured their passive and active membrane properties using pipettes filled with K-glucuronate-based intracellular solutions. A subset of layer II/III cells identified by their large size and triangular cell body shape were filled with biocytin (Fig. 1A) and confirmed to be LII/III PNs of the prelimbic region of the...
mPFC on the basis of morphological and electrophysiological properties. PFC PNs have specific intrinsic and firing properties that distinguish them from GABAergic interneurons, the other principal neuronal subtype within layer II/III (Amatrudo et al. 2012; Kawaguchi 1993). In total, identified LII/III PNs \((n = 30)\) had a RMP of \(-67.9 \pm 1\) mV, \(I_R = 156 \pm 9\) \(\Omega\), membrane time constant \(\tau_m = 28 \pm 2\) ms, membrane capacitance \(C_m = 176 \pm 7\) pF, rheobase = 111 \(\pm 10\) pA, and firing threshold = \(-38 \pm 1\) mV (Table 1), all of which are consistent with previous reports for PNs in this area (Amatrudo et al. 2012; Kawaguchi 1993). When prolonged depolarizing current injections were made at RMP, the LII/III neurons fired in a pattern characteristic of PNs, including pronounced accommodation of firing rate. Accommodation index \((AI) = 0.5 \pm 0.02\) was expressed as the ratio of the interspike frequency for the last two spikes versus the first two spikes (Fig. 1E, a and b) during a 500-ms, 240-pA command. A pronounced “sag” in the electrotonic potential \((+3.0 \pm 0.5\) mV) was observed in response to larger hyperpolarizing currents. This was expressed as the voltage difference between the peak and the steady state during a 500-ms, \(-400\)-pA current (Fig. 1Ed). Accommodation was defined as the peak voltage difference between RMP and the overshoot following \(-400\)-pA current (Fig. 1Ec). Morphological analysis of LII/III neurons successfully filled with biocytin and retrieved after processing the slices \((n = 8)\) revealed extensive apical dendrites that extended into layer I and cell bodies located in layer II/III of the prelimbic region of the PFC (Franklin and Paxinos 2008). We conclude that we have been able to identify neurons in layers II/III with the morphological and electrical signatures typical of PNs (accommodation, voltage sag).

PNs were then recorded from layers V/VI to compare membrane and firing properties between layers (Table 1). These neurons showed a slightly hyperpolarized RMP of \(-69.5 \pm 1\) mV. Student’s \(t\)-test revealed several significant differences in PNs between layers, where layer V/VI PNs had higher \(I_R (102 \pm 9\) MΩ, \(P < 0.001)\), \(\tau_m (16 \pm 1\) ms, \(P < 0.001)\), and rheobase \((155 \pm 14\) pA, \(P < 0.05)\) while \(C_m\) was similar \((170 \pm 13\) pF). Additionally, sag was significantly greater in layer V/VI \((sag = 4.0 \pm 0.5, P < 0.05)\). It should be noted that when recorded at room temperature (Amatrudo et al. 2012; Kawaguchi 1993), cortical PNs have higher \(I_R\) and lower rheobase than at more physiological temperatures without effects on RMP and \(I_h\) (Day et al. 2005; Thuault et al. 2013). For a detailed description of the effects of recording temperature on cortical PNs, see Hedrick and Waters (2012).

**Synaptic events.** First, we recorded spontaneous synaptic events in layer II/III of the mPFC, using a CsCl-based intracellular solution. We found that gabazine \((20\) μM, \(n = 6)\) blocked 98% of all spontaneous transient events (Fig. 2A) recorded under voltage clamp at \(-70\) mV (Fig. 2B), which indicated that the majority of synaptic events were sIPSCs mediated by GABA acting at GABA\(_A\)Rs. The GlyR antagonist strychnine \((1\) μM) did not cause any significant changes in frequency (Fig. 2) or amplitude of the sIPSCs. Next, we isolated mIPSCs by blocking sodium channels with TTX and AMPA and NMDA receptor-mediated currents with NBQX \((10\) μM) and n-AP5 \((50\) μM). The properties of these mIPSCs are summarized in Table 2 and indicate that essentially all of these events are fast, with rise times \(\sim 2\) ms and decay time constant lasting \(\sim 4–5\) ms. After application of strychnine we found that amplitude and frequency of mIPSCs were un-

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**Table 1. Passive and active membrane properties of layer II/III and V/VI PNs located in the prelimbic region of the PFC**

<table>
<thead>
<tr>
<th>Layer (II/III)</th>
<th>Layer V/VI</th>
<th>(P) value</th>
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<tbody>
<tr>
<td>RMP, mV</td>
<td>(-67.9 \pm 1)</td>
<td>(-69.5 \pm 1)</td>
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<tr>
<td>Input resistance, (\Omega)</td>
<td>156 (\pm 9)</td>
<td>102 (\pm 9)</td>
</tr>
<tr>
<td>Membrane capacitance, pF</td>
<td>176 (\pm 7)</td>
<td>170 (\pm 13)</td>
</tr>
<tr>
<td>(\tau_m), ms</td>
<td>28 (\pm 2)</td>
<td>16 (\pm 1)</td>
</tr>
<tr>
<td>Rheobase, pA</td>
<td>111 (\pm 10)</td>
<td>155 (\pm 14)</td>
</tr>
<tr>
<td>Sag, mV</td>
<td>2.7 (\pm 0.4)</td>
<td>4.0 (\pm 0.5)</td>
</tr>
<tr>
<td>ADP, mV</td>
<td>3.2 (\pm 0.4)</td>
<td>3.5 (\pm 0.4)</td>
</tr>
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</table>

*Values are means \(\pm\) SE; \(n = 30\) for each group. PN, pyramidal neuron; PFC, prefrontal cortex; RMP, resting membrane potential; \(\tau_m\), membrane time constant; ADP, afterdepolarization. \(P\) values, unpaired \(t\)-test; significant values are in boldface.*

**Fig. 1.** A: schematic illustrating location of whole cell recordings in layer II/III region of prelimbic cortex (dashed line). B and C: pipettes were directed at layer II/III (×4, B), and pyramidal neurons (PNs) were identified by their triangular shape with infrared-differential interference contrast (IR-DIC) optics (×40, C). D: biocytin-filled neuron confirming PN morphology in recorded cell. E: current-clamp recording of firing pattern of filled neuron in D at resting membrane potential \((\sim 68\) mV) showing single action potential after 80-pA stimulus (black spike) and multiple action potentials after 380-pA stimulus (gray spikes). Accommodation (index measured from \(ab\)) that is characteristic of cortical PNs is present. Additionally, other properties of PNs including sag (\(d\)) and an afterdepolarization (\(c\)) are present in this neuron. Active and passive membrane properties of layer (L)II/III PNs are summarized in Table 1.
changed, although we noted a small change in decay time of mIPSCs (Table 2; $P < 0.05$, $t$-test).

**LII/III PN pharmacology: responses to GABA and THIP.** To further study the presence and sensitivity of GABA$_A$Rs on PNs, exogenous GABA (1 $\mu$M–3 mM) was applied to PNs under voltage-clamp conditions after stabilization of baseline recording current. Responses to GABA were then normalized to the response to 1 mM GABA ($n = 7$). A paired $t$-test revealed a significant inward current following application of 10 $\mu$M GABA ($-40 \pm 13$ pA, $P < 0.05$) and above (Fig. 3A). A concentration-response curve was generated (Fig. 3B) and fitted to the data with the Hill equation, yielding a GABA EC$_{50}$ of 353 $\mu$M and Hill slope = 1.43.

We also investigated whether $\delta$-containing GABA$_A$Rs were present with the selective agonist THIP. We observed a modest but significant response ($-14 \pm 4$ pA, $P < 0.05$, paired $t$-test) to a low concentration of THIP (0.3 $\mu$M, $n = 5$) that selectively activates $\delta$-containing GABA$_A$Rs. Application of 1.0 $\mu$M THIP ($n = 9$) elicited a larger response ($-56 \pm 7$ pA, $P < 0.005$, paired-$t$-test) (Fig. 3A). Responses to THIP were fully blocked by the GABA$_A$R antagonist gabazine (20 $\mu$M) (Fig. 4). These results suggest that $\delta$-containing GABA$_A$Rs are likely present on LII/III PNs and that they can be fully inhibited by gabazine.

**Picrotoxin- and strychnine-sensitive tonic inhibitory current.** The presence of a tonic inhibitory current is common in forebrain regions and can be routinely demonstrated by applying a GABA$_A$ receptor (GABA$_A$R) antagonist and measuring an outward (positive) shift in baseline current under conditions where internal chloride is high and tonic currents are inwardly directed at $-70$ mV (e.g., Jia et al. 2009). To investigate the presence of a GABAergic tonic current in mPFC LII/III PNs, we applied gabazine (20 $\mu$M) at a concentration that has previously been shown to block GABA-mediated tonic inhibition in thalamic relay neurons (Jia et al. 2009). We did not observe a significant reproducible change in baseline current ($+3.0 \pm 3$ pA, $n = 13$, only 4 of 13 neurons with a shift > 6 pA), despite a near-total block of synaptic events by gabazine (Fig. 5A), indicating the absence of a GABA-mediated tonic current in these neurons. Some labs report that higher concentrations of gabazine are needed to demonstrate a tonic current (Bai et al. 2001). Increasing the concentration of gabazine (50 $\mu$M) also did not alter the results in our hands ($+3.9 \pm 6$ pA, $n = 7$, 3 of 7 neurons with a shift > 6 pA). In our next series of experiments, we applied picrotoxin (PTX, 100 $\mu$M), a compound that blocks the chloride channel of open GABA$_A$Rs. After application of PTX, we routinely observed a large positive shift in baseline current ($+21 \pm 4$ pA, $P < 0.005$, $n = 9$); eight of nine neurons had a shift > 6 pA (Fig. 5, B and D). In addition to GABA$_A$Rs, PTX is known to inhibit other ligand-gated chloride channels, including homomeric GlyRs (Pribilla et al. 1992; Yoon et al. 2008). We subsequently found that application of strychnine (1 $\mu$M), a selective GlyR antagonist, caused a reproducible (9 of 12 neurons > 6 pA) and significant shift ($15 \pm 3$ pA, $P < 0.005$, $n = 12$) in baseline holding current (Fig. 5, C and D). From these results, we conclude that LII/III neurons have a strychnine- and PTX-sensitive tonic current.

**Glycine and taurine.** The presence of a tonic strychnine-sensitive current in PFC LII/III neurons was surprising, as there have been few reports on the existence of functional GlyRs in the cortex using slice electrophysiology. In our next experiment, we set out to characterize LII/III PN sensitivity to endogenous ligands of GlyRs, beginning with glycine itself (0.01–10 mM). Bath-applied glycine elicited significant responses at a concentration of 100 $\mu$M ($P < 0.05$, $n = 7$) and EC$_{50}$ of 447 $\mu$M, Hill slope = 2.56, and very large responses (>1.5 nA) at the highest concentrations tested (3 and 10 mM).

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<th>Table 2. Characteristics of synaptic events (sIPSCs and mIPSCs) summarized for neurons before and after application of 1 $\mu$M strychnine</th>
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<td><strong>Events (cells)</strong></td>
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<td>-------------------</td>
</tr>
<tr>
<td>sIPSC</td>
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<tr>
<td>ACSF</td>
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<td>STRY</td>
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<tr>
<td>mIPSC</td>
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<tr>
<td>ACSF</td>
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<td>STRY</td>
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</table>

Values are mean ± SE characteristics of synaptic events [spontaneous (sIPSC) and miniature (mIPSC) inhibitory postsynaptic currents] summarized for neurons before (ACSF) and after application of 1 $\mu$M strychnine (STRY). Significant changes in mIPSC decay time ($P < 0.05$) are in boldface.

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Fig. 3. *A*: representative traces recorded under voltage-clamp conditions using high-chloride internal solution demonstrating prefrontal cortex (PFC) LII/III PN response to 3, 30, and 300 μM GABA. Significant inward current was observed at concentrations ≥ 10 μM, −40 ± 13 pA, *P* < 0.05. *B*: concentration-response curve of GABA as normalized to 1 mM GABA. EC50 = 353 μM; Hill slope = 1.43.

(Fig. 6). Application of taurine (0.1–10 mM) also induced measurable current at 100 μM (*P* < 0.05, *n* = 7) but was less than at glycine at several concentrations (100 μM–10 mM). All responses to glycine and taurine were normalized to the response to 3 mM glycine, established at the end of each recording after a washout period (>5 min) (Fig. 6B).

We then elicited responses (112 ± 24 pA) to an ∼EC20 concentration of glycine (200 μM) recorded in the absence and presence of strychnine (1 μM) to determine whether these glycine-activated currents are mediated by strychnine-sensitive GlyRs. Strychnine applications blocked the response to glycine (Fig. 7A) and generated a positive shift in the baseline current (+22 ± 9 pA) (Fig. 7B). Glycine is known to be an essential coagonist at the NMDA receptor (NMDAR). To examine the contribution of NMDARs, next measured the response to 200 μM glycine before and after inhibition of NMDARs with p-AP5. We did not observe any significant difference of the 200 μM glycine response in the presence of p-AP5 (Fig. 7C).

We next sought to examine the possible role of glycine transporters (GlyTs) in regulating the availability of glycine in the slice. We found that application of sarcosine, a potent inhibitor of GlyT1, caused a significant inward current (−63.5 ± 13 pA) at 500 μM (*P* < 0.001, *t*-test), which could be largely blocked (∼75%) by strychnine (1 μM) (Fig. 8). In addition, GlyT1 inhibition increased the magnitude of the strychnine-sensitive current by ∼30 pA, from ∼15 pA to 45 pA (Fig. 8B).

**DISCUSSION**

**Characterization of pyramidal neurons in mouse prefrontal cortex.** Our investigation of passive and active membrane properties in layers II/III and layers V/VI of mouse PFC identified several layer-specific characteristics. PNs in layers II/III had higher I0, longer τm, and decreased rheobase, providing evidence that they are more excitable than PNs located in layer V/VI. Additionally, we found that both layers exhibit a measurable sag and afterdepolarization in response to hyperpolarizing stimuli consistent with the presence of I0. Layer V/VI PNs showed a larger sag on average, consistent with findings that HCN1 channels predominate in layer V/VI PNs of PFC, where they are important for establishing the RMP and for the generation of persistent firing (Thuault et al. 2013).

**Synaptic GABA_{A,R} signaling.** Spontaneous synaptic GABA-mediated transmission was observed in LII/III PNs. Under conditions of high internal chloride, we observed frequent (∼7 Hz) spontaneous gabazine-sensitive currents averaging ∼40 pA in amplitude. Measurements of mIPSCs show rise times of ∼2 ms and decay times of ∼5 ms. The relatively rapid timescale of these events indicates that they were likely medi-
ated by $\alpha_1\beta_2\gamma_2$-GABA$_A$Rs, as in cerebellar Purkinje neurons and elsewhere in the brain (Vicini et al. 2001). Regional expression analysis supports the presence of $\alpha_1\beta_2\gamma_2$-GABA$_A$Rs in layer LII/III of the frontal cortex (Fritschy and Mohler 1995; Pirker et al. 2000), and sIPSCs recorded in cortical LII/III PNs of $\alpha_1$ knockout mice (Bosman et al. 2002) are significantly slower in time course.

Lack of tonic GABA$_A$R current in LII/III PNs. Tonic inhibition is widespread but by no means ubiquitous in the brain. For tonic inhibition to occur, several requirements must be satisfied. First, there needs to be sufficient expression of extrasynaptic receptor subtypes with high ligand affinity and slow desensitization rates in order to sustain chloride conductance. Second, an inhibitory neurotransmitter needs to be present at sufficiently high levels to activate these receptors. Expression analyses have demonstrated the presence of GABA$_A$R subunits $\alpha_4$ and $\delta$ in the outer layers of the PFC (Peng et al. 2002; Pirker et al. 2000). GABA$_A$ $\alpha_4\beta\delta$ receptors have been shown to have a higher sensitivity to GABA (activation at 100 nM) compared with receptor subtypes associated with synaptic receptors (e.g., $\alpha_1\beta_2\gamma_2$ GABA$_A$Rs, activation at 3 $\mu$M) (Jia et al. 2005).

Fig. 5. A and B: representative recordings under voltage-clamp conditions showing that application of 20 $\mu$M gabazine (GBZ) did not detect a tonic GABA current; however, application of 100 $\mu$M picrotoxin (PTX) did, causing a significant positive shift from baseline current. C: a similar positive shift was revealed after application of 1 $\mu$M strychnine. Results are summarized in D (**) $P < 0.005$, (***) $P < 0.001$).

**Table 1**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Current Shift from Baseline (pA)</th>
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<tr>
<td>GBZ (20 $\mu$M)</td>
<td>-10 pA</td>
</tr>
<tr>
<td>PTX (100 $\mu$M)</td>
<td>-25 pA</td>
</tr>
<tr>
<td>STRY (1 $\mu$M)</td>
<td>-30 pA</td>
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**Fig. 6. A**, top: representative recordings under voltage-clamp conditions showing the response to exogenously applied glycine (30 $\mu$M–3 mM). **Bottom:** taurine application (100 $\mu$M–10 mM) causes a similar inward current at higher concentrations. **B:** glycine and taurine applications are shown on concentration-response curve normalized to 3 mM glycine for each neuron, where glycine EC$_{50}$ = 447 $\mu$M and Hill slope = 2.56.

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Determining neurotransmitter concentrations in brain slices is obviously difficult, but we can infer that ambient GABA concentrations in slices of the mouse PFC must be very low, since although THIP (a selective, highly efficacious agonist at \( \alpha_4\delta \)-containing GABA\(_A\)Rs) can elicit a significant current at 0.3 and 1.0 \( \mu \text{M} \), there is little to no gabazine-sensitive tonic current, perhaps \(<100 \text{ nM} \). The lack of a tonic GABA current here is consistent with previous reports in LII/III PFC neurons in rodents (Drasbek and Jensen 2006; Hoestgaard-Jensen et al. 2010). Variations in brain levels of GABA are presumed to be a result of regional differences in the subtypes and expression levels of the GABA transporters. Tonic inhibition revealed by gabazine has been identified in cortical LII/III PNs, but following extensive blockade of GABA transporters with NO-711 (Vardya et al. 2008). In reports investigating layer V PFC neurons, PTX has been shown to block glycine further showed that a large population of GlyRs identified in the CA1 region after strychnine application (Zhang et al. 2008b). In hippocampal slices, a tonic GlyR current was demonstrated the presence of tonic GlyRs by applying strychnine, and GlyR has been reported in several brain regions, including the PFC (Jonsson et al. 2009; Malosio et al. 1991).

In the hippocampus functional, strychnine-sensitive GlyRs have been identified and are believed to be homomeric assemblies of \( \alpha \)-subunits because of their sensitivity to PTX (Chattipakorn and McMahon 2002, 2003; Zhang et al. 2008b) as well as cyclothiazide, a proposed \( \alpha_2 \)-GlyR antagonist (Zhang et al. 2008b). In hippocampal slices, a tonic GlyR current was identified in the CA1 region after strychnine application (Zhang et al. 2008a). These data support the idea that \( \alpha \)-homomeric GlyRs mediate tonic inhibition, analogous to the role that \( \delta \)-containing GABA\(_A\)Rs play in tonic GABA inhibition.

In layer II/III of the PFC, using PTX (100 \( \mu \text{M} \)), we found that we could elicit a positive shift in baseline current. In dissociated PFC neurons, PTX has been shown to block glycine currents (Lu and Ye 2011). We subsequently demonstrated the presence of tonic GlyRs by applying strychnine, which elicited a significant shift (\(~15 \text{ pA} \)) in baseline current in the absence of an effect on the frequency of GABA-mediated sIPSCs or isolated mIPSCs. Applications of exogenous glycine further showed that a large population of GlyRs is present in PFC LII/III PNs and can generate large inward currents in response to exogenous glycine, which could be
fully blocked by strychnine. Previous reports have shown slightly higher sensitivity of exogenous glycine on hippocampal PNs from 3- to 4-wk-old rats (EC₅₀ = 270 µM) and on PFC PNs from periadolescent rats (EC₅₀ = 117 µM) and that these currents could be blocked by strychnine and PTX (Chattipakorn and McMahon 2002; Lu and Ye 2011). The measurements made in PFC PNs were from freshly dissociated neurons, and differences in glycine sensitivity are likely due to GlyTs actively taking up glycine. Importantly, application of sarcosine (500 µM), a GlyT1 antagonist, elicited a 64-pA inward current that could be blocked by strychnine. GlyT1s are primarily located on astrocytes in the cortex (Kunz et al. 2012), and we conclude that astrocytic GlyT1 plays an important role in regulating the magnitude of tonic GlyR inhibition in the mouse PFC.

A recent report in the mouse lateral orbital frontal cortex (LOFC) is consistent with our findings and has implicated extrasynaptic GlyRs as targets of ethanol modulation (Badanich et al. 2013). Specifically, these authors found that high concentrations of ethanol (66 mM) shift the holding current of deep-layer PNs of the LOFC and that this could be blocked by PTX and strychnine (1 µM) but not by the GABAA Rs antagonist bicuculline. In addition, ethanol, a known positive allosteric modulator of GlyRs, decreased neuronal excitability, an effect that was blocked by the application of strychnine but not bicuculline. They conclude that strychnine-sensitive GlyRs modulate ethanol’s effects on deep-layer LOFC neurons. Unlike our findings in LII/III PFC, they did not detect the presence of a strychnine-sensitive tonic current in LOFC. This may be due to a reduced availability of ambient glycine or other GlyR ligands in this subregion.

Endogenous ligand responsible for tonic GlyR activation. Our evidence supports the expression of functional, extrasynaptic GlyRs, but the source of the endogenous ligand for GlyRs in the PFC remains unclear. In vivo microdialysis has shown that 10 µM of extracellular glycine exists in the extracellular space of the hippocampus (Horio et al. 2011). Additionally, glutamatergic synapses (where concentrations of glycine have been estimated to reach 1 mM) may be a second source of glycine due to synaptic spillover (Vandenberg and Aubrey 2001), although it is believed that GlyTs limit glycine spillover 100- to 1,000-fold (Harsing and Matysus 2013). In addition to glycine, the GlyR partial agonists taurine and β-alanine are known to be abundant in the brain (Dahchour et al. 2000; Murakami and Furuse 2010), and we show here that exogenous taurine can cause an inward current, supporting its role as a GlyR agonist on cortical PNs. In early postnatal cortical PNs, taurine has been shown to mediate tonic nonsynaptic GlyR activation (Flint et al. 1998). In hippocampal PNs, the inhibition of taurine and β-alanine transporters by guanidinoethanesulfonic acid can generate a GABA-independent, strychnine-sensitive current of 15 pA (Mori et al. 2002). Our finding that sarcosine, which selectively inhibits GlyT1, increased the measurable strychnine-sensitive tonic current by ~30 pA indicates that glycine is a major mediator of the strychnine-sensitive current; however, it seems reasonable to propose that sufficient levels of multiple endogenous GlyR ligands can arise to tonically activate GlyRs in the forebrain.

Physiological and functional significance. In the PFC, LII/III PNs are uniquely positioned to integrate several inputs from cortical and subcortical structures. We have shown that inhibition of these neurons is more diverse than previously realized, including a novel role for tonic GlyR activation. Tonic inhibitory conductances of this scale have previously been shown to inhibit neuronal activity (Jia et al. 2008). This novel mechanism may contribute to the sparse action potentials observed in LII/III neurons around resting potential and permit increased fidelity of neuronal output, a proposed tuning mechanism involved in attention and working memory in the PFC (Rao et al. 2000). As GlyR activation increases, we would expect increased fidelity at lower levels of activation and subsequent silencing of PFC PNs at high concentrations of GlyR ligands or positive GlyR modulation.

Summary. We investigated excitability and inhibitory currents in neurons of layer II/III of the prelimbic region of the mouse PFC and found distinct roles for GABA and glycine. Our results indicate that GABA A Rs mediate primarily synaptic inhibitory currents and strychnine-sensitive GlyRs mediate primarily tonic currents on LII/III PNs. These neurons exhibit large currents after the application of glycine and taurine, suggesting the existence of functional GlyRs. These findings reveal a novel form of inhibition in the PFC that may regulate neuronal excitability and contribute to the balance of excitation and inhibition that is critical to maintaining proper cortical function.


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