Tonic NMDA receptor-mediated current in prefrontal cortical pyramidal cells and fast-spiking interneurons

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Povysheva NV, Johnson JW. Tonic NMDA receptor-mediated current in prefrontal cortical pyramidal cells and fast-spiking interneurons. J Neurophysiol 107: 2232–2243, 2012. First published January 11, 2012; doi:10.1152/jn.01017.2011.—Tonically activated neuronal currents mediated by N-methyl-D-aspartate receptors (NMDARs) have been hypothesized to contribute to normal neuronal function as well as to neuronal pathology resulting from excessive activation of glutamate receptors (e.g., excitotoxicity). Whereas cortical excitatory cells are very vulnerable to excitotoxic insult, the data regarding resistance of inhibitory cells (or interneurons) are inconsistent. Types of neurons with more pronounced tonic NMDAR current potentially associated with the activation of extrasynaptic NMDARs could be expected to be more vulnerable to excessive activation by glutamate. In this study, we compared tonic activation of NMDARs in excitatory pyramidal cells and inhibitory fast-spiking interneurons in prefrontal cortical slices. We assessed tonic NMDAR current by measuring holding current shift as well as noise reduction following NMDAR blockade after removal of spontaneous glutamate release. In addition, we compared NMDAR miniature excitatory postsynaptic currents (mEPSCs) in both cell types. We have demonstrated for the first time that tonic NMDAR currents are present in inhibitory fast-spiking interneurons. We found that the magnitude of tonic NMDAR current is similar in pyramidal cells and fast-spiking interneurons, and that quantal release of glutamate does not significantly impact tonic NMDAR current.

N-methyl-D-aspartate receptors; tonic current; miniature excitatory postsynaptic currents; bafilomycin

EXCITOTOXICITY IS ASSOCIATED with overactivation of glutamate receptors and is a pathophysiological feature of various disorders of the central nervous system, including Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease, as well as of head trauma and stroke. N-methyl-D-aspartate receptors (NMDARs) are a glutamate receptor subtype strongly associated with excitotoxicity (Brennan et al. 2009; Lafon-Cazal et al. 1993). NMDARs are located in both synaptic and extrasynaptic dendritic regions, and their activation produces phasic NMDAR current through fast neurotransmitter release and tonic current through action of ambient glutamate, respectively. Excessive stimulation of extrasynaptic NMDARs launches cascades that result in production of free radicals, alterations in mitochondrial membrane potential, inhibition of survival pathways, and, eventually, neuronal cell death (Hardingham and Bading 2002; Tu et al. 2010). In contrast, activation of synaptic NMDARs triggers cyclic AMP response element-binding protein-associated antioxidant pathways and is believed to be neuroprotective (Hardingham and Bading 2002; Okamoto et al. 2009; Papadia et al. 2008). Excitotoxicity resulting in death of individual neurons can also lead to impairment of excitation-inhibition balance in brain circuitry, since excitatory and inhibitory neurons are known to be differentially vulnerable to excessive glutamatergic activation (Avignone et al. 2005; Lipton 1999).

Parvalbumin (PV)-positive interneurons can be identified morphologically as basket or chandelier cells (Conde et al. 1994; Gabbott et al. 1997) and are characterized physiologically by their narrow action potentials and nonadapting firing pattern, defining features of the “fast-spiking (FS) phenotype.” Similarly, cells identified as FS usually express PV (Kawaguchi and Kubota 1997; Toledo-Rodriguez et al. 2004; Zaitsev et al. 2005). Thus PV-positive interneurons and FS interneurons typically are considered synonymous. In the cortex, PV-positive/FS interneurons provide the major inhibitory drive to pyramidal cells and control their outputs because of the strategic location of their synapses on the soma, proximal dendrites, and axon initial segment of pyramidal cells (Somogyi et al. 1998).

A number of existing studies demonstrate high vulnerability of pyramidal cells to excitotoxic insult in animal models of ischemia in various brain regions (Freund et al. 1990; Lipton 1999; Papp et al. 2008). At the same time, the data regarding vulnerability of PV-positive/FS interneurons are rather inconsistent. Some of the studies show that the PV-positive/FS interneurons are resistant to excitotoxic insult. Thus, in rats that have undergone forebrain ischemia, the number and distribution of hippocampal PV-positive interneurons did not change, whereas pyramidal cells were severely affected (Papp et al. 2008). PV-positive interneurons did not appear to be damaged as a result of transient ischemia in gerbil somatosensory cortex, and even showed an increase in number in the CA3 and dentate gyrus (Ferrer et al. 1995; Tortosa and Ferrer 1993). Alternatively, a certain amount of degeneration of PV-positive interneurons was observed in the CA1 and the retrotetropezoid nucleus after experimental transient ischemia (Freund et al. 1990).

Although the cause of these potential differences in neuron-type vulnerability is still debated (Avignone et al. 2005; Calabresi et al. 2002; Kang et al. 2003), differential NMDAR expression seems to be a plausible mechanism (Avignone et al. 2005). Indeed, there is evidence showing that NMDAR subunits are expressed at very low levels in hippocampal PV-positive interneurons (Nyiri et al. 2003). PV-positive interneurons were shown to have NMDAR subunit composition different from that of pyramidal cells with prevalence of GluN2A subunit in PV-positive interneurons and GluN2B in pyramidal cells (Kinney et al. 2006; Wang and Gao 2009; Xi et al. 2009).
Excitatory responses onto FS interneurons (shown to be PV positive) have a less substantial NMDAR-mediated component than regular-spiking pyramidal cells (Angulo et al. 1999; Hull et al. 2009; Rotaru et al. 2011) or other types of interneurons (Lu et al. 2007). To our knowledge, there are no studies showing NMDAR activation in PV-positive interneurons being similar to or more substantial than in pyramidal cells.

Whereas numerous existing studies compare synaptic/phasic NMDAR activation in PV-positive/FS interneurons and other types of neurons, there are no studies addressing potential differences in tonic NMDAR activation between PV-positive/FS interneurons and pyramidal cells that could underlie their differential sensitivity to excitotoxic insult. Although the existence of tonic NMDAR-mediated current was examined previously in pyramidal cells from several brain regions (Angulo et al. 2004; Herman and Jahr 2007; Le Meur et al. 2007; Sah et al. 1989), the presence of tonic NMDAR-mediated current was never measured in PV-positive/FS interneurons.

In this study, we measured tonic NMDAR current in pyramidal cells and PV-positive/FS interneurons in the prefrontal cortex, a brain region that exhibits substantial cell loss associated with excitotoxicity and oxidative stress in a number of neurological disorders (Hof and Morrison 2004; Melrose et al. 2011). Importantly, excitotoxic cell loss affects mostly prefrontal pyramidal cells, whereas PV-positive/FS interneurons seem to be relatively resistant to degeneration (Hof et al. 1991).

METHODS

Experiments were performed on brain slices obtained from 30- to 40-day-old (150–250 g) male Sprague-Dawley rats. All animals were treated as approved by the University of Pittsburgh Institutional Animal Care and Use Committee and in accordance with the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were deeply anesthetized with chloral hydrate and decapitated. The brain was quickly removed and immersed in ice-cold preoxygenated artificial cerebrospinal fluid (ACSF). A tissue block containing the prelimbic cortex in rats was excised for slicing. Coronal slices (400 μm thick) were cut with a vibratome (Leica VT1000S; Leica, Nussloch, Germany). Slices were incubated at 37°C for 0.5–1 h and further stored at room temperature until transferred to a recording chamber perfused with ACSF at 31–32°C. Through all steps of the experiments, ACSF of the following composition was used (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 1 MgSO₄, 2 CaCl₂, 24 NaHCO₃, and 10–20 glucose, pH 7.25–7.3. ACSF was perfused with 95% O₂-5% CO₂ gas mixture.

Electrophysiological Data Analysis

Membrane properties of neurons were analyzed using the Clampfit 10.2 software package (Molecular Devices). To characterize the membrane properties of neurons, hyper- and depolarizing current steps were applied for 500 ms in 5- to 10-pA increments at 0.5 Hz. Input resistance was measured from the slope of a linear regression fit to the voltage-current relation in a voltage range hyperpolarized of resting potential. The membrane time constant was determined by single-exponential fitting to the average voltage responses activated by hyperpolarizing current steps (5–15 pA). A series of depolarizing current steps of gradually increasing amplitude was used to evoke action potentials. Action potential properties were quantified using the first evoked action potential. Peak amplitudes of the action potential and the afterhyperpolarization were measured relative to the action potential threshold. Duration of the action potential was measured at its half-amplitude. Firing frequency was calculated in hertz as a ratio between number of action potentials and current step duration. The adaptation ratio was used to describe spike frequency adaptation in spike trains and was calculated as the ratio between the first and the last interspike interval during a depolarizing current step 60 pA above the rheobase.

Tonic NMDAR current was measured first as the shift in baseline produced by the application of NMDAR blocker AP-5. The baseline current was measured in the Statistics package of Clampfit during five consecutive 0.2-min intervals before the application of AP-5 and 4 min after its application (the time point after AP-5 application when baselines reach plateau in all cells with the exception of 6 pyramidal cells and 5 FS interneurons held at -40 mV). Statistical significance of the holding current shift induced by AP-5 application was assessed first for each individual cell by comparing five values of holding current before and five values after AP-5 application. Only cells with a stable baseline (SD < 1 pA for the 5 baseline measurements) at -55 and -80 mV were included in the analysis. We also performed comparison of population means. Tonic current was estimated as the difference in average baseline values before and after application of the antagonist. Tonic NMDAR current was assessed as the change in
baseline noise produced by AP-5. Baseline noise was measured in the Statistics’ F package of Clampfit as the baseline variance during five consecutive 0.2-min intervals before the application of the AP-5 and 4 min after its application. Tonic current variance was estimated as the difference in average variance values before and after application of AP-5. Miniature EPSCs were analyzed using the MiniAnalysis program (Synaptosoft, Decatur, GA). Peak events were first detected automatically using an amplitude threshold of two times the average RMS noise, which was comparable in pyramidal cells (5.61 ± 0.28 pA) and FS interneurons (5.94 ± 0.32 pA, P > 0.1). The relatively high detection threshold for miniature responses may have resulted in some events being undetected. Therefore, the mean amplitude of the analyzed mEPSCs might represent an overestimation, whereas the frequency could be underestimated. More than 2,000 events in each cell were included in the analysis. Charge transfers of mEPSCs were determined as the area (pA × ms = fC) of each miniature response. NMDAR mEPSC mean current was calculated as the mean NMDAR charge transfer of a single mEPSC multiplied by frequency of events (fC × Hz/1,000 = pA) in each cell (Potapenko et al. 2011).

Morphological Data Analysis

During recordings, neurons were filled with the fluorescent dyes Alexa 488 or 568 (0.075%; Molecular Probes) added to the recording pipette solution as previously described (Povyshcheva et al. 2006). Whole cell recordings were maintained for at least 30 min to ensure extensive cell labeling by the dyes. Slices were fixed in ice-cold 4% paraformaldehyde for at least 72 h, then transferred into an antifreeze solution (ethylene glycol and glycerol in 0.1 M phosphate buffer), and stored in the freezer. Neurons were reconstructed three-dimensionally using an Olympus Fluoview BX61 confocal microscope (Olympus America, Melville, NY) with FITC and Cy3 filters. Images were acquired with Fluoview software (Olympus America).

Statistical Analysis

Two-tailed t-tests and paired t-tests were used for group comparisons in most cases. Unless otherwise noted, values are means ± SE. Statistical tests were performed using Excel (Microsoft, Redmond, WA).

RESULTS

Identification of Pyramidal Cells and FS Interneurons

Whole cell recordings were performed on visually and electrophysiologically identified FS interneurons and pyramidal cells in layer 2–3 of rat medial prefrontal cortex. FS interneurons were recognized physiologically according to previously described criteria (Kawaguchi 1995). These cells had a relatively low input resistance (153 ± 6.5 MΩ) and fast membrane time constant (6.3 ± 0.4 ms), and they did not typically show significant sag on their responses to hyperpolarizing current steps (Fig. 1, A, D, and E). In pyramidal cells, the input resistance was slightly lower (122.3 ± 13.3 MΩ) and membrane time constant was significantly longer (18.8 ± 1.1 ms) compared with FS interneurons (Fig. 1, D and E). FS interneurons displayed action potentials with a duration (0.75 ± 0.06 ms at half-amplitude) that was about 2.5 times shorter than in pyramidal cells (Fig. 1C), followed by a relatively deep afterhyperpolarization (18.5 ± 0.05 mV). With suprathreshold current intensities, FS interneurons demonstrated a high-frequency firing pattern without substantial spike frequency adaptation (adaptation ratio = 0.92 ± 0.03). All pyramidal cells exhibited a regular-spiking firing pattern (Kawaguchi 1995) (Fig. 1A). Unlike FS interneurons, pyramidal cells displayed firing with substantial adaptation in spike trains evoked by depolarizing current injection (adaptation ratio = 0.42 ± 0.03).

Morphology was recovered for 9 FS interneurons and 15 pyramidal cells in rat prefrontal slices (Fig. 1G). The FS interneurons had multipolar aspiny dendrites and axonal arbors spreading in predominantly the horizontal direction, features characteristic of the basket cells described previously in rat cortex (Kawaguchi 1995). Thus all pyramidal cells and FS interneurons identified in this study were clearly distinguished by their firing patterns (Fig. 1, A and F), action potential properties (Fig. 1, B and C), membrane time constants (Fig. 1, A and D), and morphologies (Fig. 1G).

Assessment of NMDAR-Mediated Tonic Current Through AP-5-Dependent Shifts in Holding Currents of Pyramidal Cells and FS Interneurons

Although tonic NMDAR current was measured previously in neocortical (Sah et al. 1989) and hippocampal (Herman and Jahr 2007; Le Meur et al. 2007) pyramidal cells, its presence was never examined in FS interneurons. In this study, we measured tonic NMDAR current in FS interneurons as well as in pyramidal cells utilizing two different approaches.

First, tonic activation of NMDAR was assessed as the shift in holding current at +40 mV and at near-rest potentials of −80 and −55 mV produced by application of the NMDAR blocker AP-5 (50 μM). These holding potentials were chosen because they permit evaluation of the voltage dependence of NMDARs due to block by external Mg2+ over a wide range of potentials. NMDAR inhibition by Mg2+ differs considerably at these three potentials. Experiments were performed in the presence of AMPAR, GABA_A, and voltage-gated Na+ channel blockers as well as the NMDAR coagonist glycine.

Whereas at negative potentials baseline was relatively stable in the absence of pharmacological manipulations, at a holding potential of +40 mV there was a slight but steady inward shift of holding current during the course of the experiment. However, this shift was observed in 6 of 12 pyramidal cells and in 5 of 9 FS interneurons. To minimize contamination of NMDAR tonic current measurements, the inward shift in holding current was measured before application of AP-5, extrapolated, and subtracted from the AP-5-induced shift of holding current. To perform the extrapolation, holding current was measured at five time points before AP-5 application, and a linear regression was used to project holding current shift at a time point of 5 min after the AP-5 application. Importantly, the linear regression extrapolation of the shift coincided with the actual shift of the baseline observed when AP-5 was not applied (Fig. 2, A and B).

Quantification of the tonic NMDAR current at the three different holding potentials revealed that its amplitude did not differ significantly between pyramidal cells and FS interneurons (Fig. 2, C–E). In accordance with the voltage dependence of NMDAR-mediated current associated with Mg2+ block, maximum tonic current was observed at the holding potential of +40 mV in pyramidal cells (n = 12) and FS interneurons (n = 9). It is worth noting that amplitude of tonic NMDAR current in pyramidal cells in our study was comparable to that previously reported by Le Meur et al. (2007) for CA1 pyramidal cells at a holding potential of +40 mV. Tonic NMDAR current at negative holding potentials of −55 and −80 mV was measured as an AP-5-dependent...
outward shift of the holding current (Fig. 2D) and had a relatively small yet statistically significant amplitude for each individual cell ($P < 0.001$, see METHODS) (Fig. 2E). Significance of AP-5-induced change of holding current was also estimated for the population means at $-80$ mV ($P < 0.01$, $n = 6$ for pyramidal cells; $P < 0.001$, $n = 6$ for FS interneurons; see METHODS). Similar to tonic current at the $+40$-mV holding potential, tonic NMDAR current was not different in pyramidal cells and FS interneurons at $-55$-mV ($n = 8$ and 7) and at $-80$-mV ($n = 6$ and 6) holding potentials (Fig. 2E).

We next addressed the potential effect of tonic NMDAR current on spiking behavior of pyramidal cells and FS interneurons. Depolarizing current pulses were used to induce action potentials. Action potential firing frequency was estimated before and after inhibition by AP-5 of NMDAR current (Le Meur et al. 2007; Sah et al. 1989) at two levels of stimulating current: low intensity (10 pA above rheobase current) and high intensity (60 pA above rheobase current). At both current intensities, AP-5 did not change firing frequency in pyramidal cells ($n = 4$) and FS interneurons ($n = 4$) (Fig. 2F). Although these results suggest that tonic NMDAR current does not regulate responses to steady current injections under these experimental conditions, it may play important roles under other circumstances (see DISCUSSION).

**Assessment of NMDAR-Mediated Tonic Current After Blockade of Action Potential-Independent Glutamate Neurotransmission**

Spontaneous action potential-independent release of glutamate can affect measurements of tonic NMDAR current by increasing ambient glutamate concentration, as well as by...
introducing NMDAR mEPSCs to the baseline current. Thus our next goal was to assess tonic NMDAR current in pyramidal cells and FS interneurons in the absence of glutamate neurotransmission. In this study, tonic NMDAR current was assessed using two approaches: first, as the AP-5-dependent shift in holding current, and second, as the AP-5-dependent reduction in background current noise (Ascher et al. 1988; Cull-Candy and Usowicz 1989). NMDAR-associated background noise could be substantially contaminated by NMDAR mEPSCs; thus, for proper assessment of tonic NMDAR current as an AP-5-dependent noise reduction, it was critical to eliminate NMDAR mEPSCs that could potentially be present in both pyramidal cells and FS interneurons. To investigate the potential impact of spontaneous action potential-independent vesicular release of glutamate on our measurements of tonic current, we first characterized NMDAR mEPSCs in pyramidal cells and FS interneurons (Fig. 3). After that, tonic current was evaluated using both holding current and current noise measurements in the absence of spontaneous action potential-independent vesicular release of glutamate (Fig. 4).

**NMDAR mediated mEPSCs in pyramidal cells and FS interneurons.** NMDAR mEPSCs have been demonstrated in physiological Mg$^{2+}$ at negative membrane potentials in pyramidal cells from the somatosensory cortex (Espinosa and Kavalali 2009) and could contribute significantly both to the AP-5-induced change of holding current and to baseline noise. Thus our next step was to determine whether NMDAR mEPSCs are detectable in both prefrontal pyramidal cells and FS interneurons. In physiological Mg$^{2+}$ and AMPAR antagonists, individual NMDAR mEPSCs are almost impossible to detect at negative voltages because they are hardly distinguishable from baseline noise (McBain and Dingledine 1992). Although NMDAR mEPSCs can be detected at +40 mV, signal-to-noise ratio still limits their detection. Therefore, we quantified NMDAR mEPSC properties by averaging mEPSCs at +55 mV in the absence of AMPAR antagonist, both before and during AP-5 (50 μM) application. We chose this approach because at −55 mV, the AMPAR component of mEPSCs is pronounced, the NMDAR component is only partially inhibited by Mg$^{2+}$, and mEPSCs are large and easily detected. A limitation of this approach is that potential mEPSCs from silent synapses (containing exclusively NMDARs) would go undetected. However, silent synapses are much less common in the 30- to 40-day-old animals used here than early in development (Gonzalez-Burgos et al. 2008; Losi et al. 2002; Malinow and Malenka 2002).

We found that AP-5 (50 μM) produced a decrease in charge transfer mediated by mEPSCs in all the cells tested, and digital subtraction of averaged mEPSC (average mEPSC before AP-5 minus average mEPSC after AP-5) revealed an NMDAR component in both cell types (Fig. 3, B–D). Importantly, miniature NMDAR EPSCs had larger average charge transfer in pyramidal cells than in interneurons (Fig. 3, C and D), which is in accord with several previous studies showing that the NMDAR component of synaptic responses is smaller in FS interneurons than in regular-spiking pyramidal cells at negative potentials (Hull et al. 2009; Rotaru et al. 2011). However, to make a proper assessment of the contribution of NMDAR mEPSCs to the AP-5-dependent shift in holding current, it is...
necessary to take into consideration not only the magnitude of NMDAR mEPSCs but also their frequency. We found that the estimated frequency of mEPSCs was larger in FS interneurons than in pyramidal cells (Fig. 3, A and E). Based on these data, the smaller NMDAR component of FS interneuron mEPSCs could potentially be compensated for by the higher mEPSC frequency in FS interneurons than in pyramidal cells. Indeed, assuming that the contribution of silent synapses is negligible and that mEPSCs are similarly detectable in both cell types, the estimated mean current contributed by NMDAR mEPSCs (mean NMDAR-mediated charge per mEPSC multiplied by frequency of mEPSCs, calculated separately for each cell) was comparable in pyramidal cells and FS interneurons (Fig. 3F).

Assessment of NMDAR tonic current in bafilomycin-treated pyramidal cells and FS interneurons. To assess tonic NMDAR current using both holding current and current noise measurements in the absence of spontaneous action potential-independent vesicular release of glutamate, bafilomycin was used. This drug inhibits the vacuolar-type ATPase (H^+-ATPase) and thus prevents uptake of both glutamate and GABA into synaptic vesicles (Drose and Altendorf 1997). When presynaptic vesicles are depleted of neurotransmitter, synaptic activity is terminated, although vesicular exocytosis appears to be preserved (Ertunc et al. 2007; Zhou et al. 2000).

To eliminate synaptic activity in neurons, brain slices were incubated in 4 μM bafilomycin A1 for at least 3.5 h at 34°C (as described in Le Meur et al. 2007). As expected, bafilomycin eliminated all spontaneous EPSCs (Fig. 4Aa), although it did not affect electrophysiological membrane properties of neurons (spike amplitude, duration and threshold, input resistance; data not shown) (also see Araque et al. 2000). In addition, duration of recordings was not affected by bafilomycin pretreatment. At resting membrane potential in physiological Mg^2+1, spontaneous EPSCs result mostly from activation of AMPARs with a very small NMDAR contribution (see Fig. 3, B and C). Given that AMPARs have a lower affinity for glutamate than NMDARs (Choi et al. 2003, 2000; Putney and Mayer 1990; Renger et al. 2001), it was important to establish that the level of vesicular glutamate was also insufficient to activate NMDARs. Miniature NMDAR EPSCs that could be observed at a holding potential of +40 mV in the presence of AMPAR, GABA_A, and voltage-gated Na^+ channel blockers were completely abolished after incubation of slices with bafilomycin (Fig. 4Ab).

To assess tonic NMDAR current in the absence of spontaneous synaptic activity, we measured first the noise reduction induced by AP-5 application, and second, the shift in holding current after AP-5 application. We made both measurements at holding potentials of +40 and −55 mV; measurements at −80 mV were not included because at −80 mV, NMDAR blockade resulted in a very small shift of holding current.

Both pyramidal cells and FS interneurons incubated in bafilomycin exhibited significantly less baseline noise at a holding potential of +40 mV (80% and 77% reduction in population variances, P < 0.001) than the cells recorded in bafilomycin-free solution (Fig. 4B), demonstrating the substantial contribution of NMDAR mEPSCs to noise measurements. In the absence of synaptic events, we assessed tonic NMDAR activation in pyramidal cells and FS interneurons by measuring baseline noise variance before and after AP-5 application (Fig. 4, C and D). In accord with the existence of tonic NMDAR current, AP-5 reduced background noise in both cell types at holding potentials of +40 and −55 mV. Noise reduction at −55 mV was substantially less than at +40 mV but still significant (P < 0.01). The observation that tonic NMDAR current noise reduction showed an almost sixfold difference.
between the potentials of +40 and −55 mV corresponds well to the voltage dependence of NMDAR-mediated current. Comparison of tonic NMDAR current noise reduction in pyramidal cells and FS interneurons showed no difference between these two cell types (Fig. 4, C and D), thus confirming that tonic NMDAR activation is comparable in pyramidal cells and FS interneurons.

It was shown previously that in hippocampal pyramidal cells, bafilomycin does not affect tonic NMDAR current measured as a shift in holding current, which indicates that most ambient glutamate has a nonvesicular origin (Le Meur et al. 2007). At a holding potential of +40 mV, both bafilomycin-treated pyramidal cells and FS interneurons demonstrated significant tonic NMDAR current measured as an AP-5-associated shift in holding current (the correction for baseline drift at +40 mV demonstrated in Fig. 2, A–C, also was applied to measurements in bafilomycin). The amplitude of this shift was indistinguishable between cell types (Fig. 4, E and F). Tonic NMDAR current of a much lesser amplitude than that at +40 mV but still significant (P < 0.01) was observed in pyramidal cells and FS interneurons at a holding potential of −55 mV. Importantly, at holding potentials of +40 and at −55 mV, the AP-5-dependent shift in holding current was comparable in the presence and in the absence of bafilomycin in both cell types (Figs. 2E and 4F). Thus these data suggest that blockade of action potential-independent vesicular release of glutamate in prefrontal cortical slices does not have a significant effect on tonic NMDAR current in pyramidal cells and FS interneurons either by changing the level of ambient glutamate or by producing NMDAR mEPSC that could potentially contaminate measurements of the AP-5-dependent shift in holding current.

In the present study, in sets of experiments both with and without bafilomycin, tonic NMDAR current was measured in the presence of TTX, which eliminates spontaneous firing in slices. These results leave open the question whether the level of ambient glutamate can be elevated by action potential-dependent release of glutamate. To address this issue, we measured the AP-5-dependent shift in the holding current at +40 mV also in the absence of TTX. The data obtained in the absence of TTX were not significantly different from those recorded in the presence of TTX (pyramidal cells: −53.8 ± 6.3 pA, n = 4 vs. −56.3 ± 6.4 pA, n = 12; FS interneurons: −49.7 ± 6.9 pA, n = 3 vs. −48.8 ± 4.0 pA, n = 9). Thus glutamate release resulting from spontaneous firing does not seem to elevate ambient glutamate concentration enough to cause a significant increase in tonic NMDAR current.

**DISCUSSION**

In this study, we assessed tonic NMDAR current in pyramidal cells and FS interneurons using two different approaches. First, tonic NMDAR current magnitude was evalu-
ated as the shift in holding current following NMDAR antagonist bath application. Second, tonic NMDAR current was assessed as the difference in baseline noise produced by NMDAR antagonist application. Our data unequivocally show that the magnitude of tonic NMDAR-mediated current is comparable in pyramidal cells and FS interneurons. Thus the amount of tonic NMDAR current does not define potential differences in excitotoxic vulnerability in pyramidal cells and FS interneurons.

Assessment of Tonic NMDAR Current: Methodological Caveats

Two alternative approaches were used to assess tonic NMDAR current in pyramidal cells and FS interneurons. First, it was assessed as the shift in holding current resulting from AP-5 application. Second, AP-5-associated change in a background noise was quantified. Both of these approaches have caveats. When the cells were depolarized to +40 mV, we waited until holding current became relatively stable for at least 5 min, and only after that was AP-5 bath-applied. Yet, in some cells, we observed a slight steady drift in the holding current that could potentially artifactually add to the effects of AP-5. This drift may result from incomplete blockade of K+ channels by Cs+ or from current through slowly inactivating Cs+-insensitive channel. To compensate for this drift, we used linear extrapolation of the initial measured drift in holding current to estimate the magnitude of the drift when holding current was measured in AP-5. The extrapolated drift was subtracted from the current change after the AP-5 application. The accuracy of the compensation was verified in control experiments in which no AP-5 was applied. Thus we compensated for the NMDAR-related shift in holding current.

Evaluating changes in tonic current based exclusively on changes in current variance can lead to inaccuracies because ion channel properties (most importantly, single-channel conductance) influence current variance (Glykys and Mody 2007). Given that NMDAR subunits (and, accordingly, single-channel conductances) associated with the tonic NMDAR current can potentially differ between pyramidal cells and FS interneurons, equivalent noise reductions following AP-5 application might not reflect equivalent reductions in tonic NMDAR current for the two cell types. Yet, the main reported difference in NMDAR subunit expression between pyramidal cells and FS interneurons is the prevalence of GluN2B in the former and of GluN2A in the latter. Because GluN2A and GluN2B subunits have comparable single-channel conductances (Stern et al. 1992), the similar AP-5 associated noise reductions we observed should reflect similar changes in tonic NMDAR current in both cell types. The similar voltage dependencies of NMDAR tonic currents in pyramidal cells and FS interneurons (Fig. 2E) also supports expression of NMDARs with similar single-channel conductance, since NMDAR single-channel conductance and strength of Mg2+ inhibition are correlated (Cull-Candy and Leszkiewicz 2004).

Incomplete space clamp in neurons with elaborated dendritic trees can result in several potential problems with estimation of tonic current. Space clamp insufficiency limits control of the voltage of distant dendrites, leading to underestimation of the voltage dependence of NMDAR responses, and causes filtering of membrane noise, leading to underestimation of variance. Finally, only a portion of current from electrotonically distant sites can be recorded, leading to underestimation of tonic current. Space clamp errors are compounded by the possibility that the errors may affect pyramidal cells and FS interneurons differently. The dendritic tree is larger in pyramidal cells than in FS interneurons (basket and chandelier cells), resulting in greater electrotonic distance from the soma to synapses and differential voltage control in the two cell types (Rall 1967; Seglev and London 1999). Presumed worse voltage control in pyramidal cells might allow greater depolarization of postsynaptic sites (resulting from block of K+ channels by Cs+ in the recording pipette) and a more substantial loss of Mg2+ block at −55 and −80 mV. Thus it is possible, for example, that the difference in average NMDAR-mediated charge transfer in pyramidal cells and FS interneurons (Fig. 3C) is exaggerated by differences in electrotonic structure of the neurons. Importantly, the aforementioned space clamp problem can be mitigated by the presence of Cs+ in recording pipette, which by blocking K+ ion channels makes neurons electrotonically more compact; yet it provides only partial alleviation of the problem (Williams and Mitchell 2008). Despite the multiple consequences of space clamp errors, our consistent observation of powerful voltage dependence of NMDAR tonic current and current noise in Mg2+ (Figs. 2E and 4, D and F) suggests that space clamp errors did not severely limit the accuracy of our measurements.

According to Le Meur et al. (2007), tonic NMDAR current in CA1 pyramidal cells is activated by ambient glutamate predominantly of a nonvesicular origin. This conclusion was based on data showing the same shift in holding current resulted from NMDAR blockade in control conditions and in the presence of bafilomycin. We also observed no significant difference between the AP-5-dependent shift in holding current in control conditions and in bafilomycin, although we observed a tendency for a smaller shift in bafilomycin. It is possible that the tendency reflects a slightly greater contribution of action potential-independent vesicular release of glutamate to the AP-5-dependent shift in holding current in prefrontal cortex than in the hippocampal CA1 region. Nevertheless, the majority of ambient extracellular glutamate in both cortical and hippocampal slices does not appear to originate from release of synaptic vesicles. Le Meur et al. (2007) observed that inhibition of the astrocytic enzyme responsible for conversion of glutamate to glutamine caused an increase in tonic NMDAR current in hippocampal pyramidal neurons. On the basis of this observation, they suggested that ambient glutamate is mostly of glial origin (Le Meur et al. 2007). It is possible that, similarly, glial cells are the principal source of ambient glutamate in cortical slices. Various possible mechanisms by which glial cells may release glutamate have been reviewed (Cavelier et al. 2005).

Our measurements of tonic NMDAR current through either AP-5-dependent shift in holding current or change in baseline noise depend on the amount of ambient glutamate in slices. Baseline glutamate concentration was estimated to be very low in slices (around 25 nM), yet high enough to activate tonic NMDAR current (Herman and Jahr 2007). It is possible that the glutamate concentration could be influenced by the quality of brain slices, potentially augmenting the variability of the results presented here. However, based on neuronal input resistance, resting potential, action potential amplitude, and
duration of experiments, our slices were of high quality. In addition, the relatively low cell-to-cell variability of tonic NMDAR current amplitude suggests that a minority of low-quality slices did not skew the results.

Another potential caveat of the present study could be associated with the age of the experimental animals: how relevant are our data obtained in adolescent rats to excitotoxic insults to the adult brain? Numerous studies have shown that NMDAR expression changes during development. Yet, the exact time course of these changes is still under debate. Based on existing data, it seems that NMDAR expression in most cells reaches a plateau relatively early in postnatal development. The most critical changes in the function and expression of NMDARs take place during the first 3 postnatal weeks (e.g., Williams et al. 1993). In rat somatosensory cortex, silent thalamocortical synapses disappear by postnatal day 8–9 (Isaac et al. 1997). NMDAR properties do not change from adolescent to adult rats in nucleus accumbens (Kasanetz and Manzoni 2009). In rat hippocampus, the AMPAR/NMDAR ratio increases rapidly during the first postnatal week, with little change during the second week (Zhu and Malinow 2002). In rat olfactory cortex, the AMPAR/NMDAR ratio reaches the steady-state level by the fourth postnatal week (Franks and Isaacson 2005). Although the NMDAR/AMPAR ratio as well as ifenprodil sensitivity is higher in prefrontal pyramidal neurons from juvenile than from adolescent monkey brain, there is no difference between adolescent and adult neurons (Gonzalez-Burgos et al. 2008). In rat brain, by postnatal day 30 the amount of NR2A and NR2B proteins reaches their adult levels. Tyrosine phosphorylation of NR2B reaches its steady-state level by the second postnatal week (Jin et al. 1997). In contrast, some of the properties of NMDAR responses in prefrontal fast-spiking interneurons differ not only between juvenile and adolescent rat but also between adolescent and adult animals (Wang and Gao 2009). Nevertheless, early postnatal developmental periods are the most sensitive periods for synaptic formation of FS/PV-positive interneurons, and insults during these periods are used to mimic numerous pathological conditions (Belforte et al. 2010; Powell et al. 2012; Thomsen et al. 2010). Thus our data obtained from adolescent/young adult animals (postnatal days 30–40) should provide useful predictions of NMDAR signaling in the adult brain.

**NMDAR-Associated Differences and Similarities in FS Interneurons and Pyramidal Cells**

In this study, we showed that tonic NMDAR current assessed using two different approaches, AP-5-associated shift of baseline current (Figs. 2 and 4) and AP-5-associated reduction of baseline noise (Fig. 4), was comparable in pyramidal cells and FS interneurons. In addition, the similar voltage dependence of tonic current in pyramidal cells and FS interneurons (Fig. 2E) suggests that there is not a substantial difference in GluN2A/B vs. C/D subunit expression between the two cell types. Our measurements of tonic NMDAR current in pyramidal cells at +40 mV are in accord with the measurements performed previously in hippocampal pyramidal cells (Le Meur et al. 2007). In this study, for the first time, tonic NMDAR current was measured in FS/PV interneurons. It was shown previously that NMDAR mEPSCs could be recorded in neocortical pyramidal cells in physiological Mg2+ (Espinosa and Kavalali 2009). In this study, NMDAR mEPSCs were compared in the two cell types in 1 mM Mg2+. We found that the average charge transfer per NMDAR mEPSC was less in FS interneurons than in pyramidal cells (Fig. 3, B and C) but that mEPSC frequency was higher in FS interneurons. As a result, the mean current contributed by NMDAR mEPSCs was comparable in the two cell types (Fig. 3F). Previously, similar differences in charge per event for spontaneous NMDAR EPSCs between the pyramidal cells and FS interneurons were demonstrated for events recorded in the mouse prefrontal cortex in the absence of Mg2+ (Rotaru et al. 2011).

Differences in NMDAR activation and expression between pyramidal cells and FS/PV interneurons were reported previously in a number of studies. It was demonstrated that NMDAR subunits are expressed at low levels in PV-positive interneurons in the hippocampus (Nyiri et al. 2003). Electrophysiological studies similarly demonstrate considerably less synaptic NMDAR activation in FS interneurons than in pyramidal cells (Angulo et al. 1999; Hull et al. 2009; Rotaru et al. 2011). In addition, Rotaru et al. (2011) showed that, unlike in pyramidal cells, NMDARs have a small impact on excitatory postsynaptic potential (EPSP)-spike coupling and summation of excitatory responses in FS interneurons. It was accordingly concluded that EPSP-spike coupling in FS interneurons depends predominantly on NMDAR-independent coincident detection, whereas in pyramidal cells EPSP summation and EPSP-spike coupling were consistent with NMDAR-dependent temporal integration. However, several reports suggest that NMDAR activation of FS interneurons plays an important role early in development (Belforte et al. 2010) as well as in circuitry functioning in the adult brain (Homayoun and Moghaddam 2007; Mann and Mody 2010).

Other differences in NMDAR signaling in pyramidal cells and FS interneurons include different NMDAR subunit composition, with a prevalence of GluN2A subunits in PV-positive interneurons and of GluN2B subunits in pyramidal cells (Kinney et al. 2006; Wang and Gao 2009; Xi et al. 2009). In addition, in rat prefrontal cortex, FS interneurons and pyramidal cells are differentially affected by NMDAR channel blockers in vivo (Homayoun and Moghaddam 2007) and in vitro (Wang and Gao 2010).

**Tonic NMDAR Activation in Normal and Pathological Brain**

Tonic NMDAR current was shown to affect excitability of pyramidal cells: blocking the tonic current reduced action potential firing frequency and functional coupling between dendritic and somatic compartments of CA1 neurons (Sah et al. 1989). However, Le Meur et al. (2007) failed to observe a change in action potential discharge of CA1 neurons with NMDAR blockade. Similarly, in the present study, application of AP-5 did not result in reduction of firing frequency in either pyramidal cells or FS interneurons stimulated by depolarizing current pulses at resting membrane potential (Supplementary Fig. S1). (Supplemental data for this article is available online at the Journal of Neurophysiology website.) It is nevertheless possible that, under other conditions, tonic NMDAR current plays an important role in brain circuitry function. For example, Mann and Mody (2010) suggested that tonic NMDAR current in interneurons may contribute to generation of gamma oscillations in hippocampal circuitry. They demonstrated that...
exogenous application of NMDA, which they hypothesized predominantly produced an increase in tonic NMDAR current, increased the frequency of cholinergically induced oscillations in the juvenile hippocampal CA3 region. The data of Mann and Mody (2010) also suggest that the significance of tonic NMDAR current may differ in PV/FS interneurons and pyramidal cells. Numerous studies have shown that PV-containing FS basket cells are responsible for oscillations in the gamma frequency range in the hippocampus (Buzsaki and Draguhn 2004; Gulyas et al. 2010), as well as in the neocortex (Sohal et al. 2009). Sohal et al. (2009) took advantage of the optogenetic approach to decrease activity of PV-positive interneurons in a selective manner, which resulted in suppression of gamma oscillations in the neocortex of anesthetized mice. Because gamma oscillations are one of the hallmark activities of the prefrontal cortex (Benchenane et al. 2011), tonic NMDAR current in FS/PV interneurons may be of especially high significance to prefrontal function.

There also is increasing evidence suggesting involvement of tonic NMDAR current in various brain pathologies. Thus tonic NMDAR current could be involved in the pathophysiology of Alzheimer’s disease, since it was shown that amyloid β-oligomers preferentially activate extrasynaptic GluN2B-containing receptors [which potentially mediate tonic NMDAR current (Le Meur et al. 2007)] and consequently facilitate long-term depression (Li et al. 2009). Recently, it was shown that soluble amyloid β-oligomers decrease neuronal glutamate reuptake and induce glutamate “spillover” to extrasynaptic NMDAR sites (Li et al. 2011). It was shown previously that tonic NMDAR activation resulting from reduction of extracellular Mg2+ impaired long-term potentiation in hippocampal slices (Frankiewicz and Parsons 1999). In addition, some of the beneficial effects of the neuroprotective drug memantine are thought to be based on its ability to block extrasynaptic (presumably responsible for tonic current) versus synaptic NMDARs, the effect shown in a recent study by Xia et al. (2010) in hippocampal cultured neurons.

Alternatively, some studies have shown that tonic NMDAR current can have a neuroprotective effect. Kambe et al. (2010) showed that prior tonic activation of NMDAR induced tolerance to the excitotoxicity mediated by NMDAR through a mechanism related to calpain-induced downregulation of NMDARs in rat striatal neurons. An inverse relationship between epileptogenic activity and amount of tonic NMDAR current was demonstrated in organotypic hippocampal slices where chronic treatment with AP-5 resulted in an increase in seizures and decrease in tonic current (Bausch et al. 2010).

Thus the role of tonic NMDAR current in the normal and pathological brain is not well understood. Its assessment in excitatory and inhibitory neurons can shed a new light on its role in brain circuitry.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: N.V.P. and J.W.J. conception and design of research; N.V.P. performed experiments; N.V.P. and J.W.J. analyzed data; N.V.P. and J.W.J. interpreted results of experiments; N.V.P. prepared figures; N.V.P. drafted manuscript; N.V.P. and J.W.J. edited and revised manuscript; N.V.P. and J.W.J. approved final version of manuscript.

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