Cellular/Molecular

Presynaptic NMDA Receptor Mechanisms for Enhancing Spontaneous Neurotransmitter Release

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NMDA receptors (NMDARs) are required for experience-driven plasticity during formative periods of brain development and are critical for neurotransmission throughout postnatal life. Most NMDAR functions have been ascribed to postsynaptic sites of action, but there is now an appreciation that presynaptic NMDARs (preNMDARs) can modulate neurotransmitter release in many brain regions, including the neocortex. Despite these advances, the cellular mechanisms by which preNMDARs can affect neurotransmitter release are largely unknown. Here we interrogated preNMDAR functions pharmacologically to determine how these receptors promote spontaneous neurotransmitter release in mouse primary visual cortex. Our results provide three new insights into the mechanisms by which preNMDARs can function. First, preNMDARs can enhance spontaneous neurotransmitter release tonically with minimal extracellular Ca^{2+} or with major sources of intracellular Ca^{2+} blocked. Second, lowering extracellular Na^{+} levels reduces the contribution of preNMDARs to spontaneous transmitter release significantly. Third, preNMDAR enhance transmitter release in part through protein kinase C signaling. These data demonstrate that preNMDARs can act through novel pathways to promote neurotransmitter release in the absence of action potentials.

Introduction

NMDA receptors (NMDARs) are critical for a wide range of neural functions, including memory formation, injury responses, and proper wiring of the developing nervous system (Cull-Candy et al., 2001; Pérez-Otaño and Ehlers, 2004; Lau and Zukin, 2007). Not surprisingly, NMDAR dysfunction has been implicated in a number of neurological disorders, including schizophrenia, Alzheimer's disease, epilepsy, ethanol toxicity, pain, depression, and certain neurodevelopmental disorders (Rice and DeLorenzo, 1998; Cull-Candy et al., 2001; Sze et al., 2001; Mueller and Meador-Woodruff, 2004; Coyle, 2006; Fan and Raymond, 2007; Autry et al., 2011). As a consequence, NMDARs are targets for many therapeutic drugs (Kemp and McKernan, 2002; Lipton, 2004; Autry et al., 2011; Filali et al., 2011).

Although most researchers have assumed a postsynaptic role for NMDARs, there is now compelling evidence that NMDARs can be localized presynaptically, where they are well positioned to regulate neurotransmitter release (Hestrin et al., 1990; Aoki et al., 1994; Charton et al., 1999; Corlew et al., 2007; Corlew et al., 2008; Larsen et al., 2011). Indeed, NMDARs can regulate spontaneous and evoked neurotransmitter release in the cortex and hippocampus in a developmental and region-specific manner (Berretta and Jones, 1996; Mameli et al., 2005; Corlew et al., 2007; Brasier and Feldman, 2008; McGuinness et al., 2010; Larsen et al., 2011). Presynaptic NMDARs (preNMDARs) are also critical for the induction of spike timing-dependent long-term depression (Sjöström et al., 2003; Bender et al., 2006; Corlew et al., 2007; Larsen et al., 2011), a candidate plasticity mechanism for refining cortical circuits and receptive field maps (Yao and Dan, 2005). The precise anatomical localization of preNMDARs has been debated (Christie and Jahr, 2008; Corlew et al., 2008; Christie and Jahr, 2009), but recent studies have shown that axonal NMDARs, rather than dendritic or somatic NMDARs on the presynaptic neuron, can increase the probability of evoked neurotransmitter release in the hippocampus (McGuinness et al., 2010; Rossi et al., 2012) and are required for timing-dependent long-term depression in the neocortex (Sjöström et al., 2003; Rodríguez-Moreno et al., 2010; Larsen et al., 2011). In addition to an increased understanding of the anatomical localization of preNMDARs, the molecular composition of preNMDARs is beginning to be elucidated. There is general agreement that cortical preNMDARs contain the GluN2B subunit (Bender et al., 2006; Brasier and Feldman, 2008; Larsen et al., 2011). At least in the developing visual cortex, preNMDARs require the GluN3A subunit to promote spontaneous, action-potential-independent transmitter release (Larsen et al., 2011).

However, despite advances in understanding the roles and molecular composition of preNMDARs, the cellular processes of preNMDAR-mediated release are poorly understood. Here we used a common assay for preNMDAR functions to probe pharmacologically the mechanisms by which these receptors promote

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spontaneous neurotransmitter release. Surprisingly, we found that preNMDARs can function in the virtual absence of extracellular Ca^{2+} in a protein kinase C (PKC)-dependent manner. Furthermore, in normal Ca^{2+} conditions, lowering extracellular Na^+ or inhibiting PKC activity reduces preNMDAR-mediated enhancement of spontaneous transmitter release. These results provide new insights into the mechanisms by which preNMDARs function.

Materials and Methods

Subjects. C57BL/6 mice were purchased from Charles River Laboratories and then bred and maintained at the University of North Carolina. Experiments were conducted between postnatal day 13 (P13) and P18 in mice of either sex. Mice were kept in a 12 h light/dark cycle and were provided food and water *ad libitum*. All experiments were conducted under the animal care guidelines for the University of North Carolina at Chapel Hill.

Cortical slice preparation. Mice were decapitated following anesthetization with pentobarbital Na^+ (40 mg/kg, i.p.). Brains were removed and placed in ice-cold dissection buffer bubbled with 95% O_2 and 5% CO_2. Dissection buffered consisted of the following (in mM): 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 75 sucrose, 10 dextrose, 1.3 ascorbic acid, 7 $MgCl_2$, and 0.5 $CaCl_2$. Next, 300 μM coronal slices of the visual cortex were cut using a vibrating microtome (VT1200S; Leica). Slices were placed for 20 min in a submersion chamber at 35°C containing artificial CSF (ACSF) containing the following (in mM): 124 NaCl, 3 KCl, 1.25 Na_2PO_4, 26 $NaHCO_3$, 1 $MgCl_2$, 2 $CaCl_2$, and 20 D-glucose saturated with 95% O_2 and 5% CO_2 at 315 mOsm and pH 7.25. After the initial 20 min recovery period, slices were allowed to recover for an additional 40 min at room temperature, where they were maintained until use. For electrophysiological recordings, visual cortex slices were placed in a submersion chamber warmed to 30°C and perfused with oxygenated ACSF. Borosilicate glass pipettes had an open tip resistance between 3 and 6 MΩ when filled with an internal solution containing the following (in mM): 20 KCl, 100 (K)gluconate, 10 HEPES, 4 (Mg)ATP, 0.3 (Na)GTP, 10 (Na) phosphocreatine, and 1 MK-801 with pH adjusted to 7.25 and osmolarity adjusted to 300 mOsm with sucrose. All recordings were performed in whole-cell configurations using a patch-clamp amplifier (Multiclamp 700A/B; Molecular Devices) and were acquired and analyzed using pClamp 9.2 or 10.2 software (Molecular Devices).

Current-clamp recordings. Action potentials were evoked either by external stimulation or by direct current injection into the postsynaptic cell. For extracellular-evoked experiments, layer (L) 2/3 pyramidal cells were recorded while L4 axons were stimulated every 30 s with a two-conductor cluster electrode (FHC). Current was injected to maintain a −70 mV resting potential if necessary. Action potentials were evoked for 5 min before a 10 min TTX (200 nM) application. To examine the effect of TTX on postsynaptic spike firing, current (300 pA, 400 ms) was injected before and after a 10 min TTX application.

Voltage-clamp recordings. Miniature EPSCs (mEPSCs) were recorded in the presence of the $GABA_A$ receptor antagonist picrotoxin (50 μM) and the voltage-gated Na^+ channel blocker TTX (200 nM). Neurons were recorded while holding the postsynaptic neuron at −80 mV; only cells with $R_{series} < 30$ MΩ and having <20% change in R_{input}, R_{series}, and $I_{holding}$ were included in analysis. The combination of strong hyperpolarization and inclusion of MK-801 in the postsynaptic pipette nearly abolishes postsynaptic NMDAR activity (Corlew et al., 2007). To measure the effects of preNMDARs on synaptic function, we measured mEPSC amplitude and frequency before and after bath application of 50 μM D-APV (Fig. 1*A*). All statistical comparisons were made between segments of the baseline period (last 5–10 min) and the APV application period (last 5–10 min of application). Greater than 50 mEPSCs were analyzed for each comparison period in a single experiment (Turrigiano et al., 1998). Interleaved control experiments were performed for the same duration but without APV application to verify that the pharmacological agents did not affect the stability of the electrophysiological recordings (data not shown for most experiments). mEPSCs were detected using an automatic template detection program and verified manually (pCLAMP; Molecular Devices; Clements and Bekkers, 1997).

Pharmacological agents. D-APV, TTX, and okadaic acid were purchased from Ascent Scientific. Picrotoxin, thapsigargin, dantrolene, and cantharadin were purchased from Sigma-Aldrich. 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H7), KT5720, and GF 109203X (GFX) were purchased from Tocris Bioscience. Cyclopiazonic acid (CPA) and EGTA were purchased from EMD and Fisher Scientific, respectively.

Statistics. Data are reported as mean ± SEM unless noted otherwise, and statistical significance was assessed using two-tailed paired or unpaired Student's *t* tests with significance placed at $p < 0.05$. For comparison of multiple groups, a one-way ANOVA was used; a Tukey–Kramer multiple-comparisons test was used for the *post hoc* analyses; statistical significance was defined as $p < 0.05$.

Results

PreNMDARs can enhance neurotransmitter release with minimal Ca^{2+} requirements

Recent studies demonstrated that NMDA application can evoke axonal Ca^{2+} responses (Lin et al., 2010; McGuinness et al., 2010; Buchanan et al., 2012), consistent with findings that preNMDARs can promote neurotransmitter release via Ca^{2+}-dependent mechanisms (Berretta and Jones, 1996; Cochilla and Alford, 1999; Woodhall et al., 2001; Mameli et al., 2005). Therefore, we decided to determine the signaling cascades downstream of preNMDAR activation by first clarifying the requirements for Ca^{2+}. To determine the mechanisms of preNMDAR-mediated transmitter release, we examined the effects of NMDAR blockade on spontaneous release in the presence of TTX (200 nM). In 9 of 9 experiments, a 5 min application of this TTX concentration effectively blocked spike formation in L2/3 neurons in response to direct current injection or L4 extracellular stimulation (Fig. 1*A*). In addition to performing experiments in the presence of TTX, in subsequent experiments, we first blocked postsynaptic NMDARs by including the NMDAR antagonist MK-801 in the recording pipette and by strongly hyperpolarizing the postsynaptic neuron (Corlew et al., 2007). We then determined the role of preNMDARs in spontaneous transmitter release by examining how bath application of the competitive NMDAR antagonist D-APV (50 μM) altered postsynaptic AMPAR-mediated mEPSCs. As demonstrated previously under these control conditions (Corlew et al., 2007; Brasier and Feldman, 2008; Corlew et al., 2008; Larsen et al., 2011), bath application of APV reduced mEPSC frequency but not amplitude (Fig. 1*B*), indicating that preNMDARs tonically facilitate glutamate release. To determine the extent to which preNMDARs require Ca^{2+} to promote neurotransmitter release, we examined the effects of APV on spontaneous transmitter release in neurons recorded in ACSF lacking external Ca^{2+} and with EGTA (3 mM) to further buffer residual Ca^{2+}. These low Ca^{2+} conditions profoundly reduced mEPSC frequency compared with controls with normal ACSF ($p < 0.05$; note different axes and average baseline values in Fig. 1B_3 and Fig. 1C_2). However, even in the nominally zero Ca^{2+} ACSF, APV application reduced mEPSC frequency ($p < 0.05$), but not amplitude, compared with baseline (Fig. 1*C*). mEPSC frequency returned to baseline levels after APV was removed, indicating that this decrease was specific to APV application and was not a general rundown of cellular responses (Fig. 1C_2). Moreover, the normalized reduction in mEPSC frequency by APV in nominally zero Ca^{2+} ACSF was not statistically different ($p = 0.82$) than the normalized reduction in mEPSC frequency by APV in normal Ca^{2+} ACSF (Fig. 1C_3). These data suggest that preNMDARs are able to promote spontaneous neurotransmitter release with minimal Ca^{2+} entry from extracellular sources.

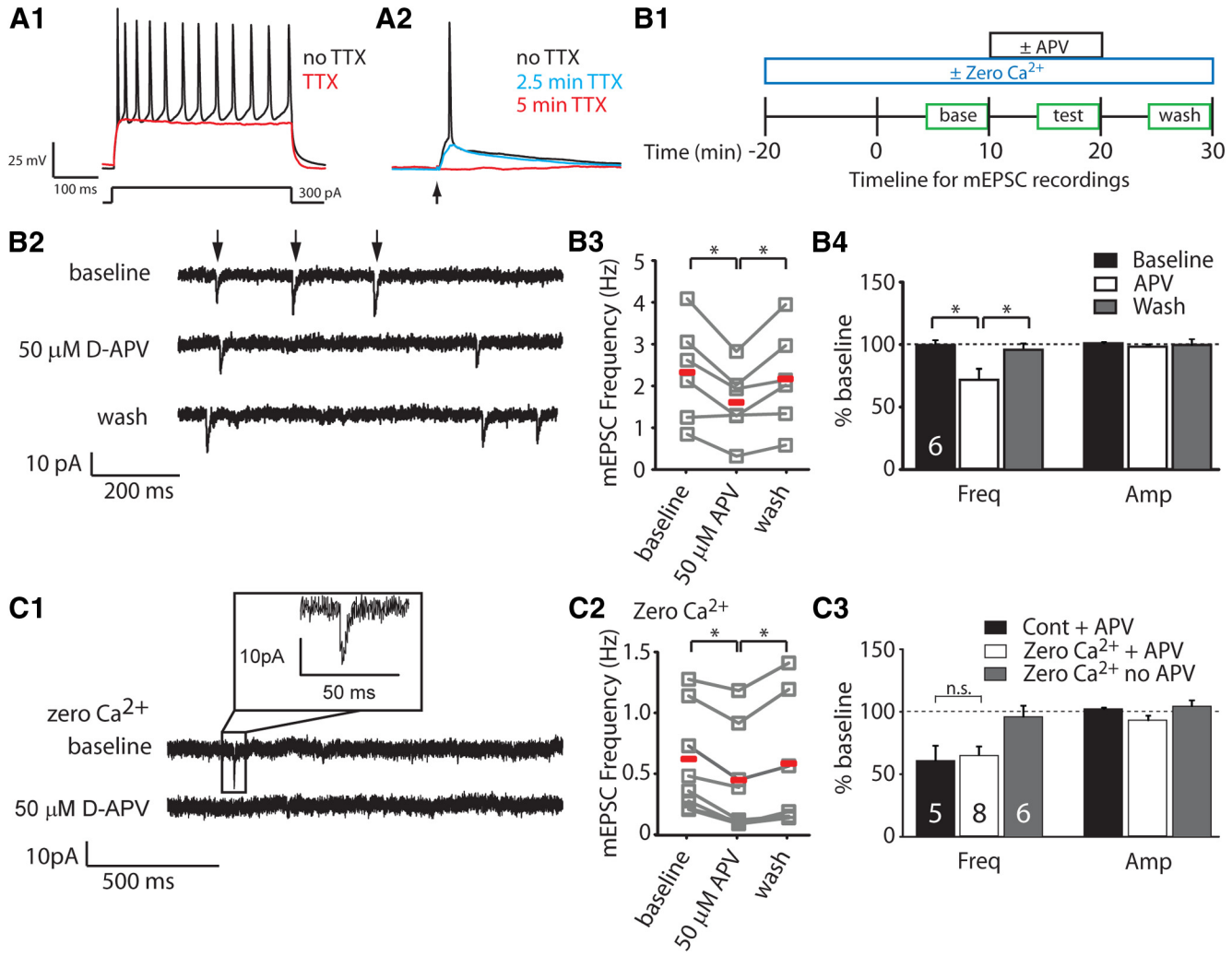

Figure 1. PreNMDARs can tonically promote neurotransmitter release with minimal Ca^{2+} requirements. ***A*$_1$**, Example trace of the response of a layer (L) 2/3 pyramidal neuron to a 300 pA current pulse. After TTX (200 nM) was washed onto the slice for 5 min, no action potentials were observed. ***A*$_2$**, Example trace measuring the response in a L2/3 pyramidal neuron to extracellular stimulation of L4. Within 5 min of TTX application, a stimulation that previously could elicit a postsynaptic spike, failed to elicit a detectable EPSP. ***B*$_1$**, Schematic of the experimental protocol used to delineate the signaling pathways downstream of preNMDAR activation (see Materials and Methods and Results sections for details). ***B*$_2$**, Example whole-cell recording depicting mEPSCs (arrows) from a L2/3 pyramidal cell in a visual cortex slice from a P15 mouse. ***B*$_3$**, Scatterplot of mEPSC frequency from individual recordings in normal ACSF (baseline) demonstrating that D-APV (50 μM) antagonism of preNMDARs reversibly reduces mEPSC frequency in L2/3 pyramidal neurons. Group means (depicted by red bar) and SDs are as follows: baseline, 2.31 ± 1.2; APV, 1.59 ± 0.86; and wash, 2.14 ± 1.19. ***B*$_4$**, Bar chart depicting the effects of APV application on the normalized mEPSC frequency and amplitude. ***C*$_1$**, Example whole-cell recording from an L2/3 pyramidal cell recorded in ACSF with nominally zero Ca^{2+}. ***C*$_2$**, APV reversibly decreases mEPSC frequency in neurons recorded in nominally zero Ca^{2+} [paired *t* test; $t(8) = 6.73, p < 0.001$]. Group means (depicted by red bar) and SD are as follows: baseline, 0.63 ± 0.43; APV, 0.47 ± 0.42; and wash, 0.59 ± 0.55. ***C*$_3$**, Bar graph displaying the normalized and averaged effect of APV treatment on the normalized mEPSC frequency and amplitude in experiments performed in control conditions with normal Ca^{2+} versus zero Ca^{2+} conditions. No statistical differences in APV effects were observed (unpaired *t* tests; frequency: $t_{(12)} = 0.62, p = 0.82$; amplitude: $t_{(12)} = 1.57, p = 0.14$). In control experiments, no changes in mEPSC frequency or amplitude were observed in neurons recorded in zero Ca^{2+} over the same time course but in the absence of APV treatment (paired *t* tests; frequency: $t_{(4)} = 0.37, p = 0.73$; amplitude: $t_{(4)} = 1.66, p = 0.17$)]. Asterisk denotes significant differences from baseline. Error bars represent SEM.

PreNMDAR-mediated enhancement of spontaneous transmitter release does not require Ca^{2+} release from intracellular stores

Recent work has suggested that preNMDARs function through Ca^{2+}-induced Ca^{2+} release (Rossi et al., 2012). Therefore, we next investigated whether intracellular Ca^{2+} stores were required for preNMDAR-mediated release of glutamate (Fig. 2) using pharmacological agents that disrupt release of Ca^{2+} from intracellular stores and that have been shown previously to affect spontaneous release (Simkus and Stricker, 2002; Collin et al., 2005). For all experiments, we first determined whether the pharmacological agent being used affected spontaneous release. Some drugs affected the baseline frequency of mEPSCs, however, we were still able to determine whether APV could modulate the drug-modified baseline. To determine the consequences of the SERCA sarco/endoplasmic reticulum Ca^{2+}-ATPase pump antagonist, cyclopiazonic acid, (CPA; 30 μM), we first bathed visual cortex slices in CPA for at least 1 h (thereby depleting and inhibiting release of Ca^{2+} from these intracellular stores) (Simkus and Stricker, 2002). Despite blocking release from SERCA-sensitive intracellular stores, we still found that APV application significantly reduced mEPSC frequency ($p < 0.05$) without modifying amplitude ($p = 0.12$; Fig. 2*B*). Further, the normalized reduction in mEPSC frequency was not significantly different from slices incubated in normal ACSF ($p = 0.45$). To confirm these results, we next investigated the influence of another SERCA inhibitor, thapsigargin (4 μM), compared with control conditions (Fig. 2*C*). As with CPA, visual cortex slices were exposed to thapsigargin for

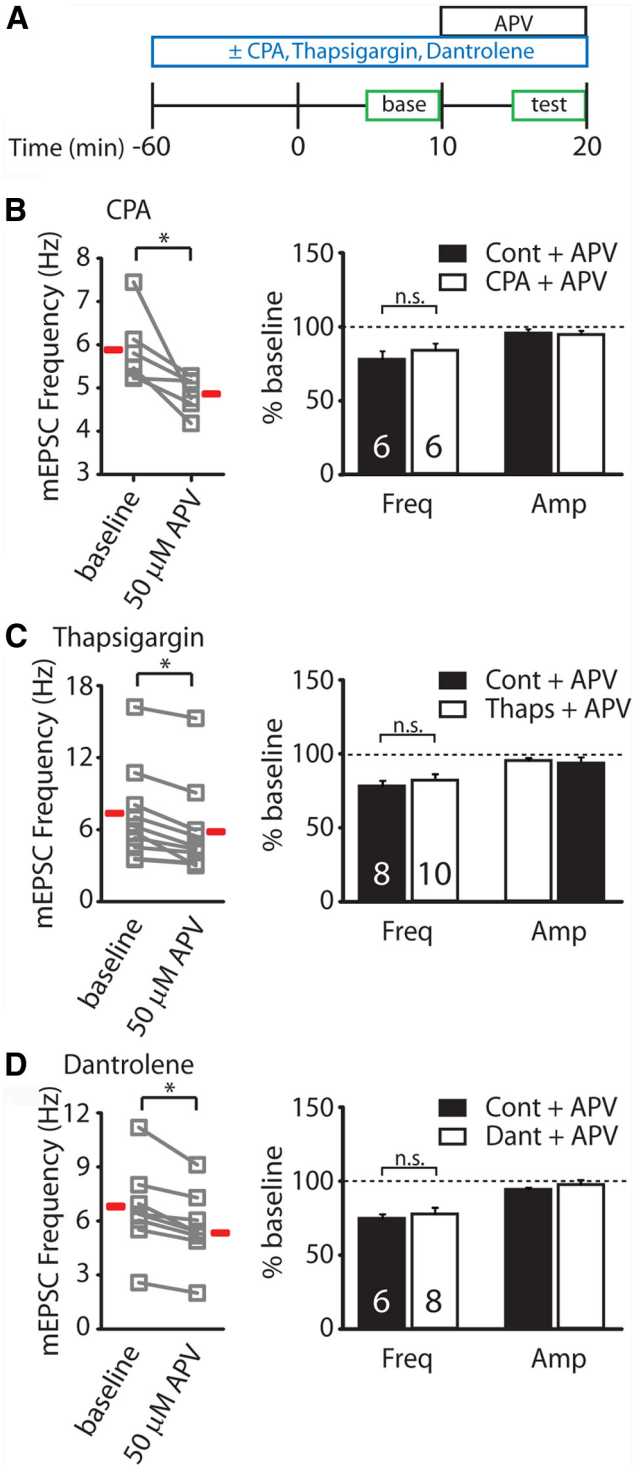

Figure 2. Calcium release from intracellular stores is not required to promote tonic preNMDAR-mediated enhancement of neurotransmitter release. ***A***, Schematic of the experimental protocol. ***B–D***, Scatterplots (left) indicate a significant reduction in mEPSC frequency for experiments performed in the presence of the SERCA inhibitors (CPA and thapsigargin) or a ryanodine antagonist (dantrolene). Group means (depicted by red bar) and SDs are as follows: CPA baseline, 5.88 ± 0.84; CPA APV, 4.8 ± 0.42; thapsigargin baseline, 7.15 ± 3.91; thapsigargin APV, 5.8 ± 3.82; dantrolene baseline, 6.52 ± 2.32; and dantrolene APV, 5.29 ± 2.20. Paired *t* tests for all groups demonstrated that APV effectively reduced mEPSC frequency (CPA: $t_{(5)} = 2.75, p < 0.05$; thapsigargin: $t_{(9)} = 5.01, p < 0.001$; dantrolene: $t_{(7)} = 4.84, p < 0.01$). Bar graphs (right) display the normalized and averaged changes in mEPSC frequency and amplitude by APV treatment in neurons recorded in the presence of CPA, thapsigargin, dantrolene, or their interleaved controls (Cont). CPA, thapsigargin, and dantrolene treatments did not significantly affect the APV-induced reduction in mEPSC frequency compared with control

at least 1 h before recording. In the presence of thapsigargin, APV application reduced mEPSC frequency ($p < 0.05$) without affecting amplitude ($p = 0.27$). Further, the effects of APV on mEPSC frequency under control and thapsigargin-treated slices were statistically indistinguishable ($p = 0.15$). These data suggest that preNMDARs do not require activation of SERCA-mediated Ca^{2+} stores to promote spontaneous transmitter release.

Finally, to test for a requirement of Ca^{2+} release from ryanodine-sensitive stores, we incubated visual cortex slices in the ryanodine antagonist dantrolene (10 μM) for at least 1 h before recordings (Fig. 2*D*). Under these conditions, APV application still reduced mEPSC frequency ($p < 0.05$) without affecting mEPSC amplitude ($p = 0.78$). Further, dantrolene-treated and control slices responded similarly to APV treatment ($p = 0.43$). These data indicate that preNMDARs can promote spontaneous transmitter release in a manner that can be independent of Ca^{2+} release from intracellular stores.

PreNMDARs require Na^+-dependent depolarization to enhance tonic transmitter release

We were surprised that preNMDARs could continue to promote presynaptic neurotransmitter release in nominally zero Ca^{2+} conditions. We therefore sought to determine additional mechanisms that could contribute to preNMDAR-mediated transmitter release. Because preNMDARs can spontaneously promote neurotransmitter release in the absence of action potentials and spontaneous preNMDAR activity in the visual cortex requires GluN3A-containing NMDARs (Larsen et al., 2011), which are known to have reduced Ca^{2+} permeability (Das et al., 1998; Pérez-Otaño et al., 2001; Matsuda et al., 2002; Sasaki et al., 2002; Tong et al., 2008), we hypothesized that Na^+-dependent depolarization may serve as an important contributor to preNMDAR-mediated release. Depolarization can influence presynaptic release directly by influencing voltage-gated Ca^{2+} channels or indirectly through the activation of intracellular signaling cascades (Leenders and Sheng, 2005). To determine the influence of depolarization, we replaced either 50% (final concentration of 62 mM) or 75% (final concentration of 31 mM) of the extracellular NaCl with the impermeable monovalent cation *N*-methyl-D-glucamine (Fig. 3). We could not completely remove Na^+ from the extracellular solution because Na^+ flow through AMPA receptors is required to measure our primary readout, AMPAR-mediated currents. As expected, lowering NaCl to 31 mM significantly reduced mEPSC frequency and amplitude in baseline conditions ($p < 0.05$) through a large reduction in the driving force. This large reduction (31 mM) in extracellular sodium was sufficient to block the reduction in mEPSC frequency by APV ($p = 0.93$, paired *t* test; Fig. 3*B*). However, reducing extracellular NaCl to 62 mM was not sufficient to block the APV-mediated reduction in mEPSC frequency ($p < 0.05$; paired *t* test). In addition, an ANOVA comparing the APV-induced change in normalized mEPSC frequency in the low 31 mM Na^+ group, the 62 mM Na^+ group, and the 124 mM Na^+ group (controls) yielded a significant main effect ($p < 0.05$). *Post hoc* tests to probe this significant difference indicated that the 31 mM Na^+ group

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experiments performed in normal ACSF (CPA: $t_{(10)} = 0.78, p = 0.45$; thapsigargin: $t_{(16)} = 1.41, p = 0.15$; dantrolene: $t_{(12)} = 0.88, p = 0.43$), nor did these treatments affect the lack of APV influence on mEPSC amplitude (CPA: $t_{(10)} = 0.20, p = 0.85$; thapsigargin: $t_{(16)} = 0.50, p = 0.20$; dantrolene: $t_{(12)} = 0.93, p = 0.62$). Asterisk denotes significant differences from baseline. Error bars represent SEM.

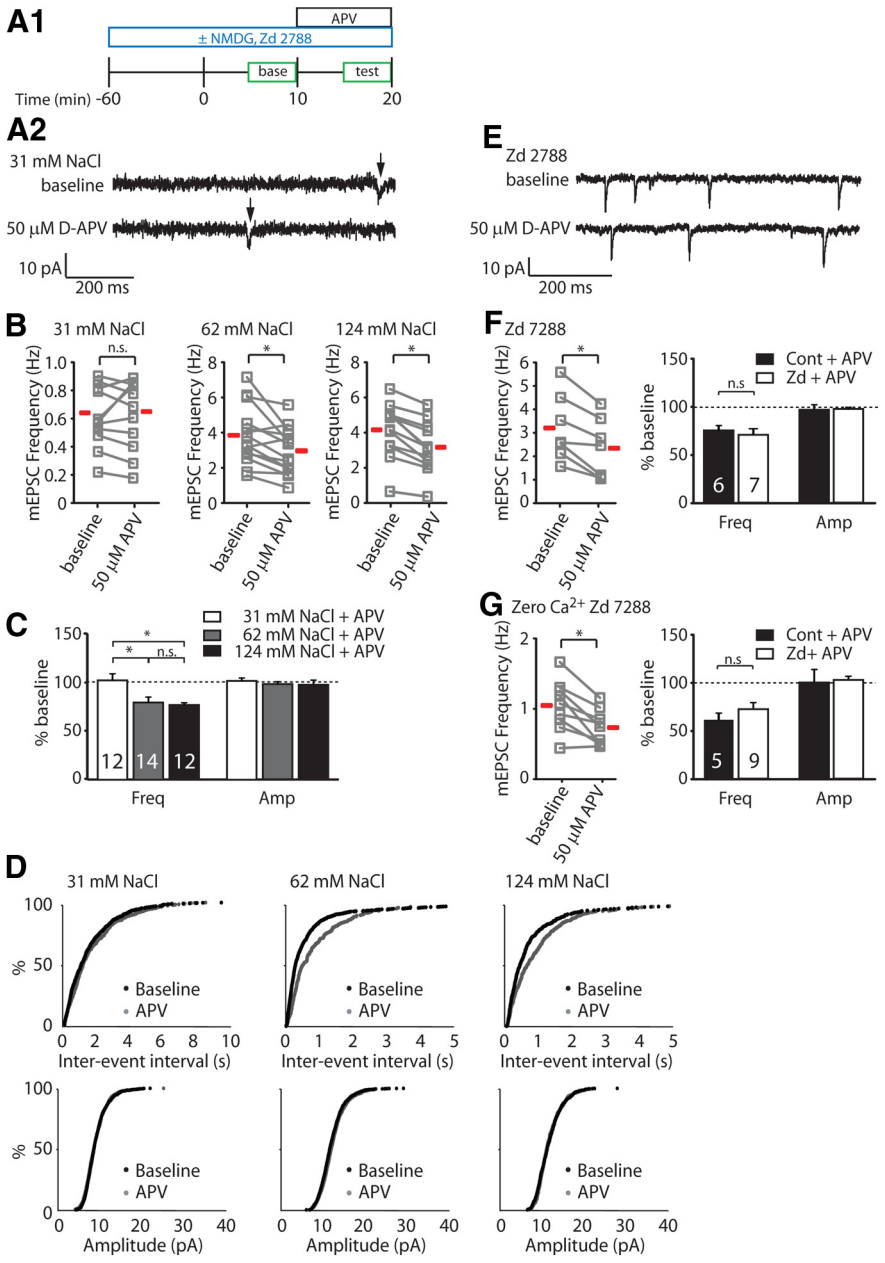

Figure 3. Partial replacement of Na^+ with *N*-methyl-D-glucamine blocks the tonic activity of preNMDARs. ***A*$_1$**, Schematic depicting the experimental protocol used to test the contributions of Na^+ for preNMDAR function. ***A*$_2$**, Representative voltage-clamp recordings from an experiment performed in low Na^+ (31 mM). ***B***, Scatterplots indicating mEPSC frequency for experiments performed in 31 and 62 mM Na^+ during the baseline period and during APV application. APV application failed to consistently reduce mEPSC frequency in neurons recorded in in 31 mM extracellular Na^+ ($t_{(11)} = 0.81$, $p = 0.2$), but did reduce mEPSC frequency in 62 mM Na^+ ($t_{(13)} = 2.03$, $p < 0.01$). Group means (depicted by red bar) and SDs are as follows: 31 mM baseline, 0.64 ± 0.24; 31 mM APV, 0.64 ± 0.26; 62 mM baseline, 3.92 ± 1.67; 62 mM APV, 3.30 ± 1.45; 124 mM baseline, 4.01 ± 1.50; and 124 mM APV, 3.04 ± 1.38. ***C***, Bar graph displaying the normalized and averaged mEPSC frequency and amplitude in neurons recorded in 31 mM extracellular NaCl, 62 mM extracellular NaCl, and in control 124 mM extracellular NaCl. The 62 mM and the 124 mM NaCl groups were significantly more sensitive to APV wash compared with the 31 mM group as indicated by an ANOVA ($F_{(2,35)} = 6.78$, $p < 0.004$) and subsequent *post hoc* tests ($p < 0.05$). No significant differences in mESPC amplitude were observed (ANOVA; $F_{(2,35)} = 0.51$, $p = 0.60$). Asterisks denote significant differences. ***D***, Average cumulative probability distributions of mEPSC frequency and amplitude under baseline conditions or during APV application. APV application shortened interevent intervals in the presence of 62 and 124 mM NaCl, but not 31 mM NaCl, and had no effect on amplitude, regardless of NaCl concentration. For cumulative probability plots, the same number of events were analyzed for each neuron within a condition so that interneuron differences in frequency would not skew the analysis. ***E***, Representative voltage-clamp recordings from an experiment performed in Zd 7288. ***F*–*G***, Scatterplot (left) indicates a significant reduction in mEPSC frequency for experiments performed with or without external Ca^{2+} in the presence of the Zd 7288, an HCN inhibitor. Paired *t* tests demonstrate that APV reduced mEPSC frequency in either condition (normal ACSF: $t_{(6)} = 4.50$, $p < 0.01$; zero Ca^{2+} ACSF: $t_{(9)} = 3.33$, $p < 0.01$). Group means (depicted by red bar) and SDs are as follows: Zd 7288 baseline, 3.20 ± 1.40; Zd 7288 APV, 2.35 ± 1.27; zero Ca^{2+} Zd 7288 baseline, 1.03 ± 0.35; and zero Ca^{2+} Zd 7288 APV, 0.72 ± 0.24. Bar graphs (right) display the normalized and averaged changes in mEPSC frequency and amplitude by APV treatment in neurons recorded in the presence of Zd 7288 or the interleaved controls. Zd 7288 did not significantly affect the APV-induced reduction in mEPSC frequency compared with control experiments performed in normal ACSF ($t_{(11)} = 0.56$, $p = 0.23$) or zero Ca^{2+} ACSF ($t_{(13)} = 1.37$, $p = 0.19$), nor did these treatments affect the lack of APV influence on mEPSC amplitude (normal ACSF: $t_{(11)} = 1.35$, $p = 0.21$; zero Ca^{2+} ACSF: $t_{(13)} = 0.15$, $p = 0.88$). Asterisk denote significant differences from baseline. Error bars represent SEM.

showed less APV-induced modification of normalized mEPSC frequency compared with either the 62 mM Na^+ group or the 124 mM Na^+ group (control). Normalized mEPSC amplitude was not modified by APV application, because ANOVA failed to detect a significant difference between the various Na^+ treatments ($p = 0.60$).

Presynaptic HCN channels have been shown to significantly affect presynaptic release at excitatory synapses (Huang et al., 2011). Reduction of the Na^+ driving force could also block modulation of neurotransmitter release by HCN channels and potentially confound the interpretation of our APV results. To exclude the possibility that HCN channels contribute to preNMDAR function, we sought to determine whether APV could modify presynaptic release in the presence of the HCN channel inhibitor Zd 7288. In the presence (Fig. 3*F*) or absence (Fig. 3*G*) of extracellular Ca^{2+}, Zd 7288 failed to block the reduction in mEPSC frequency compared with control solutions ($p < 0.05$), suggesting that HCN channels do not contribute to mEPSC frequency in a preNMDAR-dependent manner. These data suggest that Na^+-dependent processes are important for preNMDAR-mediated transmitter release, but only modest extracellular Na^+ levels are needed. However, these data do not determine whether Na^+ flow depolarizes the presynaptic terminal directly, thus affecting transmitter release, or if Na^+-induced depolarization begins a signaling cascade that is critical toward enhancing presynaptic function.

Influence of kinase and phosphatase pathways on preNMDAR-mediated transmitter release

Having established a role for Na^+ influx in preNMDAR function, which likely works in addition to the established role for Ca^{2+}-mediated effects (Lin et al., 2010; McGuinness et al., 2010), we then began to investigate downstream signaling cascades. Because the phosphoryla-

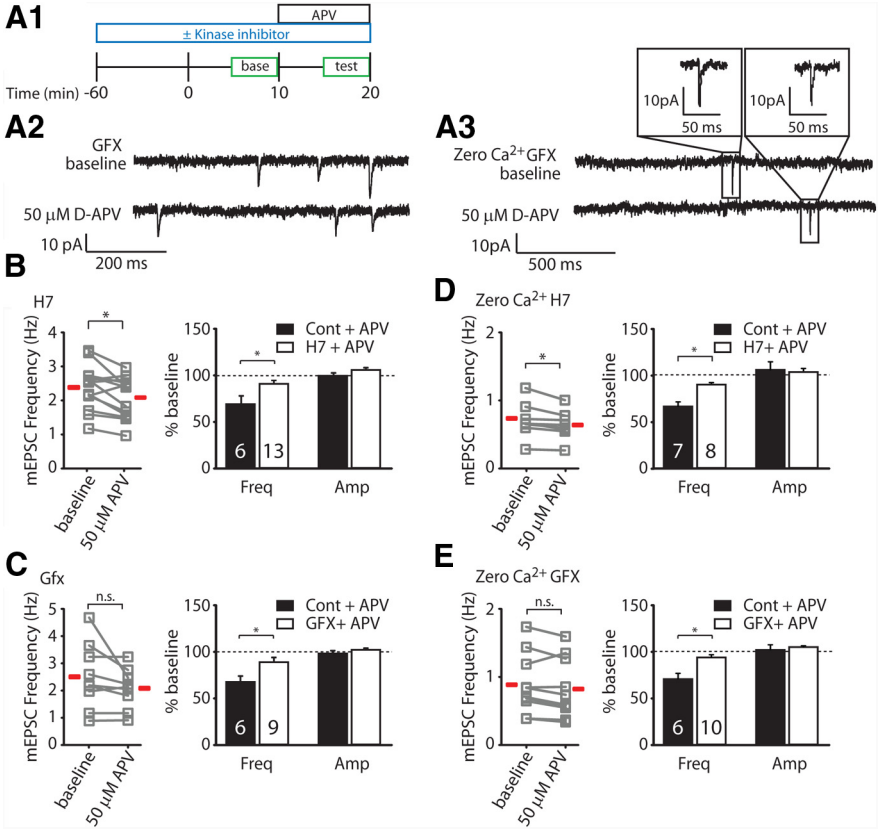

Figure 4. Role of kinase activity in tonic preNMDAR activity. ***A*****1**, Schematic showing the experimental protocol used to test the requirement of kinase activity for preNMDAR function. All inhibitors were bath applied for at least 1 h before experimentation. ***A*****2–3**, Representative voltage-clamp recordings from an experiment performed in the presence of the PKC inhibitor GFX in normal or zero Ca^{2+} ACSF. ***B***, ***C***, Scatterplots (left) indicate significant reductions after APV application for experiments performed in the broad kinase inhibitor H7 (paired *t* test: $t_{(12)} = 2.91$, $p < 0.01$) and the PKC inhibitor GFX (paired *t* test: $t_{(8)} = 1.72$, $p < 0.05$). Group means (depicted by red bar) and SDs are as follows: H7 baseline, 2.45 ± 0.67; H7 APV, 2.15 ± 0.62; GFX baseline, 2.53 ± 1.21; and GFX APV, 2.10 ± 0.74. Bar graphs (right) display the normalized mEPSC frequency for groups incubated and tested in H7 and GFX that were significantly different from the normalized frequency of groups incubated and tested in normal ACSF (unpaired *t* tests: H7: $t_{(17)} = 2.62$, $p < 0.02$; GFX: $t_{(13)} = 0.45$, $p < 0.03$). No statistical differences in normalized mEPSC amplitude were observed between groups tested in kinase inhibitors and normal ACSF (unpaired *t* tests; H7: $t_{(17)} = 0.87$, $p = 0.47$; 10 μM GFX: $t_{(13)} = 1.07$, $p = 0.25$). Asterisks denote significant differences. ***D***, ***E***, Scatterplots (left) indicate significant reductions after APV application for experiments performed in zero Ca^{2+} ACSF in the presence of H7 (paired *t* test: $t_{(7)} = 5.63$, $p < 0.001$) and GFX (paired *t* test: $t_{(9)} = 2.74$, $p < 0.05$). Group means (depicted by red bar) and SDs are as follows: zero Ca^{2+} H7 baseline, 0.55 ± 0.25; zero Ca^{2+} H7 APV, 0.48 ± 0.21; zero Ca^{2+} GFX baseline, 0.89 ± 0.43; and zero Ca^{2+} GFX APV, 0.83 ± 0.43. Bar graphs (right) display the normalized mEPSC frequency for groups incubated and tested in H7/zero Ca^{2+} ACSF and GFX/zero Ca^{2+} ACSF that were significantly different from the normalized frequency of groups incubated and tested in zero Ca^{2+} ACSF (unpaired *t* tests: H7: $t_{(13)} = 4.63$, $p < 0.001$; GFX: $t_{(14)} = 3.87$, $p < 0.01$). No statistical differences in normalized mEPSC amplitude were observed between groups tested in kinase inhibitors and zero Ca^{2+} ACSF (unpaired *t* tests; H7: $t_{(13)} = 0.55$, $p = 0.59$; GFX: $t_{(14)} = 0.35$, $p = 0.73$). Asterisks denote significant differences. Error bars represent SEM.

tion state of presynaptic proteins has a profound influence upon presynaptic efficacy (Leenders and Sheng, 2005), we investigated whether phosphatase or kinase activation was required for preNMDAR-dependent transmitter release.

We found no evidence that blocking phosphatase activity with cantharidin (10 μM) or okadaic acid (1 μM) could affect preNMDAR-mediated enhancement of transmitter release (data not shown), so we sought to determine the role of kinase activity on this phenomenon using a broad-spectrum kinase inhibitor, H7. After incubating our slices in H7 (10 μM) for at least 1 h (Fig. 4*B*), we observed a significant reduction in mEPSC frequency from baseline in response to APV ($p < 0.05$), with no change in mEPSC amplitude ($p = 0.35$). However, when the normalized reduction in mEPSC frequency by APV was compared between the control group and the H7 group, we observed a significant difference ($p < 0.05$). These findings suggest that kinase activity is at least partially required for preNMDARs to influence presynaptic release. Because H7 is a broad kinase inhibitor, we next sought to determine which specific kinases influenced preNMDAR activity by incubating the slices in KT5720 (1 μM), a PKA inhibitor. APV significantly reduced mEPSC frequency in the presence of KT5720 (data not shown; $p < 0.05$). No changes in mEPSC amplitude were observed ($p = 0.54$). When we compared the normalized reduction in mEPSC frequency with a control group given APV in normal ACSF, no significant difference was observed ($p = 0.95$), suggesting that PKA activity was not required for preNMDARs to affect presynaptic transmitter release. We next investigated the role of PKC by bathing our slices in GFX (10 μM), a specific inhibitor of PKC. APV failed to reduce mEPSC frequency in the presence of GFX ($p > 0.05$; Fig. 4*C*), with no change in mEPSC amplitude ($p = 0.68$). In addition, slices incubated with GFX were significantly less sensitive to changes in normalized mEPSC frequency during the APV wash compared with the ACSF controls ($p < 0.05$). No change in mEPSC amplitude was measured ($p = 0.53$). These data suggest that PKC activity is partially required for preNMDAR function. We next sought to determine whether these mechanisms contribute to preNMDAR function in the modified zero Ca^{2+} ACSF. In nominally zero Ca^{2+}, H7 and GFX still blocked the effect of APV on mEPSC frequency without affecting mEPSC amplitude [(H7: normalized mEPSC frequency, $p < 0.05$; normalized mEPSC amplitude, $p = 0.69$) and (GFX: normalized mEPSC frequency, $p < 0.05$; normalized mESPC amplitude, $p = 0.73$)] compared with zero Ca^{2+} ACSF controls (Fig. 4*D*,*E*). This suggests that key mechanisms for preNMDAR-mediated enhancement of neurotransmitter release function in the presence or absence of Ca^{2+}.

Discussion

The goal of this study was to help to elucidate the cellular mechanisms that operate downstream of preNMDAR activation to enhance neurotransmitter release. Our evidence indicates that the preNMDAR-dependent signaling pathways responsible for enhancing presynaptic neurotransmitter release can operate in nominally zero extracellular Ca^{2+}. Further, we also show that release of Ca^{2+} from intracellular stores is not required. Our data demonstrate that preNMDARs can function with minimal Ca^{2+} requirements and may even be able to function in a Ca^{2+}-independent manner. There is also good evidence that preNMDARs may normally promote transmitter release through Ca^{2+}-dependent mechanisms (Berretta and Jones, 1996; Cochilla

and Alford, 1999; Woodhall et al., 2001; Mameli et al., 2005; McGuinness et al., 2010; Buchanan et al., 2012). These data suggest that there may be two mechanisms underlying preNMDAR-influenced vesicular release: one that requires normal levels of extracellular Ca^{2+} and another that can affect release in a largely Ca^{2+}-independent manner. Although we were surprised to see that preNMDARs could continue to promote spontaneous transmitter release with minimal Ca^{2+} requirements, this observation is consistent with a recent finding from our laboratory indicating that preNMDARs in the visual cortex are triheteromeric NMDARs with low Ca^{2+} permeability (Larsen et al., 2011). Specifically, tonic preNMDAR activity appears to require GluN1, GluN2B, and GluN3A subunits (Brasier and Feldman, 2008; Larsen et al., 2011). These GluN3A-containing NMDARs have interesting properties that make them ideally suited for a role in presynaptic release. First, these NMDARs are relatively insensitive to magnesium block near resting membrane potentials, allowing them to function tonically in a non-Hebbian manner. Second, these receptors have relatively low Ca^{2+} permeability, consistent with our finding that extracellular Ca^{2+} may not be required for tonic preNMDAR enhancement of transmitter release. We predict that NMDAR blockade by APV would not affect mEPSC frequency in low Ca^{2+} solutions in visual cortical slices from GluN3A knock-out mice, because the APV effect on mEPSC frequency is lost in normal ACSF in these mice (Larsen et al., 2011).

Although both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms support preNMDAR enhancement of transmitter release, there is also an important contribution of Na^+ influx to preNMDAR-mediated transmitter release. The most parsimonious explanation is that Na^+ influx provides depolarization of the presynaptic terminal, which in turn activates a voltage-sensitive signaling cascade that affects presynaptic release. However, we cannot exclude the possibility that Na^+ is serving as the second messenger to mediate these effects. Nor can we rule out a role of Na^+/Ca^{2+} exchange and subsequent effects upon voltage-gated Ca^{2+} channels (Korn and Horn, 1989), or that Na^+ could serve directly to facilitate Ca^{2+} entry through preNMDARs. Further, we cannot rule out other, less parsimonious interpretations of the reduced Na^+ experiments. Finally, presynaptic HCN channels have been shown to modulate presynaptic release at glutamatergic synapses (Huang et al., 2011). Although these channels are active at rest and would be affected by a low Na^+ solution, we found no evidence for their involvement in preNMDAR-mediated spontaneous release.

The signaling cascades involved in preNMDAR enhancement of transmitter release are largely unknown. Our data begin to provide some insights because we demonstrate that blockade of phosphatase activity fails to prevent preNMDAR enhancement of spontaneous release. However, we did determine that kinase activity, specifically PKC activity, helps to promote preNMDAR transmitter release. It is difficult to determine whether PKC involvement is direct or indirect—is PKC activated directly by preNMDAR-mediated depolarization or by downstream signaling cascades? PKC activity can facilitate transmitter release in both a Ca^{2+}-dependent and a Ca^{2+}-independent manners (Koponen et al., 1999; Saitoh et al., 2001; Scholze et al., 2002), which is consistent with our observations that the Ca^{2+} requirements necessary to support preNMDAR-transmitter release can be minimal.

Despite progress in determining the subunit composition, localization, and function of preNMDARs (Brasier and Feldman, 2008; Christie and Jahr, 2008; Corlew et al., 2008; Larsen et al., 2011), there is still much to learn about how preNMDAR activity promotes tonic transmitter release. We have begun to elucidate this process by showing that tonic activation of preNMDARs enhances transmitter release through a depolarization-dependent mechanism. Although preNMDARs enhance transmitter release through Ca^{2+}-dependent mechanisms (Berretta and Jones, 1996; Cochilla and Alford, 1999; Woodhall et al., 2001; Mameli et al., 2005; McGuinness et al., 2010; Buchanan et al., 2012), our work shows that preNMDARs can continue to enhance transmitter release with a minimal requirement for Ca^{2+} and that preNMDARs enhance transmitter release in part through a PKC-dependent pathway. These insights suggest that preNMDARs are capable of promoting transmitter release via a noncanonical signaling pathway that is largely independent of a direct link to Ca^{2+}-dependent release machinery, as well as through a more traditional Ca^{2+}-dependent pathway.

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