Amino-Terminal Ligands Prolong NMDA Receptor-Mediated EPSCs

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The amino-terminal domains of NMDA receptor subunits are important for receptor assembly and desensitization, and incorporate the high-affinity binding sites for zinc and ifenprodil. These amino-terminal ligands are thought of as subunit-specific receptor inhibitors. However, multiple NMDA receptor subtypes contribute to EPSCs at wild-type hippocampal synapses. To understand the action of amino-terminal ligands, we first used cultured hippocampal neurons from N2A and N2B knock-out mice. EPSCs from these neurons have properties that are consistent with N1/N2B and N1/N2A diheteromeric receptors, respectively. As expected, zinc reduced the EPSC peak amplitude from N2B KO neurons, but surprisingly also prolonged the deactivation, resulting in a marked redistribution of charge. Consistent with prolongation of the EPSC, zinc produced a longer latency to first opening of glutamate-bound receptors, which resulted in a decrease in the number of receptors that opened by the peak. Ifenprodil had similar effects on EPSCs from N2A KO neurons. In neurons from wild-type mice, zinc or ifenprodil reduced the EPSC peak, but only zinc caused significant charge redistribution, consistent with a small contribution of N1/N2B diheteromers in these neurons. Our results indicate that ligand binding to amino-terminal domains can alter the behavior of synaptic NMDA receptors under the nonequilibrium conditions of glutamate release during synaptic transmission. By prolonging EPSCs, amino-terminal ligands could markedly affect the computational properties of NMDA receptors and could potentially be exploited for therapeutic purposes.

Introduction

NMDA receptors are composed of four subunits derived from seven genes. One gene, with multiple splice variants, encodes the GluN1 subunit (N1), four genes encode GluN2 subunits (N2A–D) and two genes encode GluN3 subunits (N3A and B). Functional NMDA receptors require two N1 and two N2 subunits per receptor (Furukawa et al., 2005), however receptors containing an N2 and an N3 subunit can also respond to glutamate (Pérez-Otaño et al., 2001). In the rodent hippocampus, N1, N2A and N2B are the predominant subunits expressed in pyramidal and dentate granule neurons, whereas N2C and N2D are limited to subsets of interneurons (Monyer et al., 1994). Likewise, expression of N3A in the hippocampus is limited to the adult (Wong et al., 2002) and N3B is predominantly in motor neurons (Chatterton et al., 2002). Thus, EPSCs in hippocampal neurons result from activation of diheteromeric and triheteromeric (N1/N2A/N2B) receptors (Rauner and Köhr, 2011) and display complicated pharmacology and kinetic behavior. NMDA receptor subunit knock-out (KO) mice (Sakimura et al., 1995; Kutsuwada et al., 1996) provide the opportunity to study a relatively homogenous receptor population (Tovar et al., 2000; Thomas et al., 2006).

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Zinc and ifenprodil have high affinity binding sites in the amino-terminal domains of N2A and N2B, respectively (Paoletti et al., 2000; Perin-Dureau et al., 2002). In the CNS, zinc is present at high concentrations in vesicles at many presynaptic terminals, yet evidence of zinc release is controversial (Kay and Toth, 2008). However, even nanomolar concentrations of zinc are sufficient to affect N2A-containing receptors (Paoletti, 2011). Ifenprodil and related phenylethanolamines have been studied as neuroprotectants (Mony et al., 2009). Zinc and ifenprodil inhibit currents from recombinant N1/N2A and N1/N2B receptors (Williams, 1993; Paoletti et al., 1997), but their efficacy in a mixed population of synaptic NMDA receptors has been less well studied.

We examined the action of zinc or ifenprodil on EPSCs in cultured hippocampal neurons from mice lacking N2A or N2B, and in wild-type neurons. In neurons from N2B KO mice, zinc reduced the EPSC peak amplitude but increased the EPSC duration. Zinc decreased the rate at which channels open after binding glutamate and decreased the probability of channels having opened by the peak of the EPSC (P_o^*). However, the total open probability changed very little because of EPSC prolongation. Ifenprodil had the same qualitative effects in N2A KO neurons. Thus amino-terminal ligands produced a significant redistribution of charge in EPSCs in N2A and N2B KO neurons. In wild-type neurons, zinc or ifenprodil reduced the EPSC peak, but only zinc prolonged the deactivation. Zinc-induced EPSC prolongation in wild-type neurons almost completely compensated for the reduction of the peak, thus the total open probability was nearly unaffected. Therefore, whether amino-terminal ligands act as

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modulators, like zinc, or as inhibitors, like ifenprodil, depends on the mixture of NMDA receptor types at synapses.

Materials and Methods

Cell culture. Mouse hippocampal neurons were cultured on isolated glial micro-islands. Cultures were prepared as in Tovar et al. (2009). Neonatal wild-type, N2A KO and N2B KO mouse pups were decapitated, the brains were removed and hippocampi were dissected from the brain. Neurons were grown on wild-type glia. Neurons were plated at a density of 25,000 cells/35 mm culture dish. Before plating neurons, glial micro-island dishes were treated with 200 μ M glutamate for 30 min to kill neurons from the previous round of plating. For micro-island glial cultures, cells were plated at 125,000 cells/35 mm dish. Cultures were kept in a tissue culture incubator (37°C and 5% CO₂), in medium that contained Minimum Essential Media with 2 mM Glutamax (Invitrogen), 5% heat-inactivated fetal calf serum (Lonza), 1 ml/L of Mito+ Serum Extender (BD Bioscience) and supplemented with glucose to an added concentration of 21 mM. Cultures were done within 1 d after birth. N2B KO mice were bred from N2B ^{+/−} animals because loss of N2B is lethal. N2B KO mice could be identified by the absence of milk in the abdomen (Kutsuwada et al., 1996). N2A and N2B genotypes were verified using PCR (Tovar et al., 2000; Thomas et al., 2006). Male mice were used for all cell cultures. All mice were in C57BL/6 genetic background. All animals were treated in accord with OHSU and NIH policies on animal care and use.

Solutions, electrophysiology and analysis. Recordings were done using the zinc chelators TPEN (N,N,N',N'-Tetrakis-(2-pyridylmethyl)ethylenediamine; 1 μ M), DTPA (diethylenetriamine-pentaacetic acid; 10 μ M) or the zinc buffer tricine (10 mM), unless otherwise noted. High purity salts and HPLC water were used for external solutions. For tricine-buffered zinc experiments, we used no added zinc as the "0" zinc reference; the peak amplitude of N2B KO EPSCs in DTPA plus tricine were $97.0 \pm 1.5\%$ of EPSCs with tricine alone ($n = 5$), indicating that in our conditions tricine was sufficient as a baseline for zinc dose-inhibition experiments. The free zinc concentration was calculated by the method in Fayyazuddin et al. (2000). The highest zinc concentration we tested was 1 μ M free zinc because at higher concentrations zinc acts as a channel blocker (Legendre and Westbrook, 1990; Paoletti et al., 1997). During recordings, micro-islands were perfused via flow pipes by gravity flow with extracellular solution containing (in mM) 158 NaCl₂; 10 tricine; 2.4 KCl; 10 HEPES; 10 D-glucose; 1.3 CaCl₂ and 0.02–0.05 glycine and no added Mg²⁺; pH was adjusted to 7.4 with NaOH. The pipette solution contained the following (in mM): 140 potassium gluconate; 4 CaCl₂; 8 NaCl; 2 MgCl₂; 10 EGTA; 2 Na₂ATP and 0.2 Na₂GTP. The pH was adjusted to 7.4 with KOH. The osmolality of both solutions was adjusted to 320 mosmol. External solution also contained NBQX (2, 3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide; 2.5 μ M) to block AMPA receptors, R-CPP ((R)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid; 0.05–10 μ M) to block NMDA receptors and SR95531 (10 μ M) to block GABA_A receptors as needed. All antagonists, including MK-801 and ifenprodil, were purchased from Ascent Scientific. Salts were purchased from Sigma-Aldrich or Fluka.

Whole-cell voltage-clamp recordings were done from neurons that were plated on glial micro-island cultures that had been growing for at least 6 d *in vitro* (DIV) and recordings were done in neurons up to 16 DIV. We used single neurons or pairs of neurons for voltage-clamp recordings. Because N2B KO mice die soon after birth (Kutsuwada et al., 1996) cultures from neonatal N2B KO mice provide a method of studying the properties of synaptic NMDA receptors from these neurons (Tovar et al., 2000). EPSCs were evoked by brief depolarizations (to +20 mV for 0.5 ms) to the soma, resulting in an unclamped action potential, followed by postsynaptic currents. This culture system contains principal neurons and interneurons. We could unambiguously identify the neurotransmitter phenotype (glutamate or GABA) of the neurons from which we recorded. Data obtained from postsynaptic neurons that were determined to be GABA-releasing were excluded. All drug applications were done using custom made flowpipes placed within 100 micrometers of the neuron being recorded. This allowed fast solution exchanges

(within 100 ms) across the entire neuron. When MK-801 was used, it was present only for 1–3 EPSC episodes.

Axopatch 1C amplifiers and AxoGraph acquisition software (Axograph X) were used for data acquisition. Series resistance was always < 10 m Ω and was compensated by > 80% using the amplifier circuitry. Data were low-pass filtered at 5 kHz and acquired at 10 kHz. Postsynaptic currents were evoked at low frequency (0.1–0.2 Hz). All recordings were done at room temperature. All data analysis was done with Axograph or Igor Pro. Fits of EPSC deactivations were done using Axograph and were fit with,

$$I(t) = I_f \exp(-t/\tau_f) + I_s \exp(-t/\tau_s) + c,$$

where I_f and I_s are the amplitudes of the fast (τ_f) and slow (τ_s) components of decay and c is an added constant. The fitted duration of the deactivation was typically 8 times the longest time constant to insure that we were reliably measuring the deactivation components. In some cases, this was not done for EPSCs from N2A KO neurons because we did not record a long enough duration. In these cases, the shortest duration was 4.5 times the longest time constant. The charge associated with each component of the bi-exponential decay is proportional to the product of the time constant and the amplitude contribution of that component. Weighted deactivation time constants of decay were calculated with,

$$\tau_w = (I_f/(I_f + I_s))\tau_f + (I_s/(I_f + I_s))\tau_s,$$

where τ_w is the weighted time constant. Fits to the dose-inhibition data were done using Igor and least-squares fitting routine and were fit with,

$$I_{ATL} = 100 - (a_{max}/(1 + (IC_{50}/[ATL])^{n_H})),$$

where I_{ATL} is the EPSC peak in zinc or ifenprodil, a_{max} is the maximal inhibition, IC_{50} is the concentration at which half maximal reduction occurs, $[ATL]$ is the zinc or ifenprodil concentration and n_H is the Hill coefficient. Ifenprodil dose-inhibition data in wild-type EPSCs were fit with,

$$I_{ifen} = 100 - (100/(1 + (IC_{50}/[ifen])^{n_H})),$$

where I_{ifen} is the current in ifenprodil and $[ifen]$ is the ifenprodil concentration. The results of fits of dose-inhibition curves are reported as mean $\pm$ SD. All other data are reported as mean $\pm$ SEM. Significance levels were set at 0.05.

Results

We examined synaptic NMDA receptors using zinc and ifenprodil, the prototypic ligands for the amino-terminal domains of N2A and N2B, respectively (Paoletti et al., 2000; Perin-Dureau et al., 2002). The ambient contaminating zinc concentration can be higher than the IC_{50} for zinc at recombinant N1/N2A receptors (Paoletti et al., 1997). Consistent with the presence of contaminating zinc, EPSCs in cultured hippocampal neurons from N2B KO were potentiated by the high affinity zinc chelator TPEN (1 μ M; $132.8 \pm 6.4\%$, $n = 9$). We therefore used high purity salts and HPLC water for external solutions, and recordings were done in the zinc buffer tricine (10 mM). As expected, buffered zinc application to N2B KO neurons resulted in a dose-dependent decrease in the EPSC peak with an IC_{50} of 31.4 ± 5.5 nM ($n_H = 0.97 \pm 0.13$; $a_{max} = 76.2 \pm 3.3\%$; Fig. 1A,C). These values are comparable to recombinant N1/N2A receptors (Rachline et al., 2005). EPSCs in these neurons were very fast; the weighted deactivation time constant (23.4 ± 0.4 ms; $n = 20$) was also comparable to recombinant N1/N2A receptors (Vicini et al., 1998). These data suggest that synaptic receptors in this preparation are a homogenous population of N1/N2A diheteromers. The potentiation of N2B KO EPSCs in TPEN predicts a contaminating zinc concentration of 20–30 nM. Therefore, without zinc buffering or chelation, the

Figure 1. Amino-terminal ligands prolong NMDA receptor-mediated EPSCs. **A**, Top, Buffered zinc application (30 and 300 nM free zinc) to N2B KO neurons reduced the EPSC peak (left) and prolonged the deactivation in a dose-dependent manner, shown in the peak-scaled EPSCs (right). The competitive antagonist *R*-CPP (50 nM) reduced the peak (bottom, left) but did not affect the deactivation kinetics in these same cells (bottom left and right). Zinc (**C**) and ifenprodil (**D**) dose-inhibition curves of the N2B KO and N2A KO EPSC peak (open circles) and total charge (closed circles) demonstrating the divergence from each other of these parameters by zinc or ifenprodil. Dashed lines in **C** and **D** are fits to the data. For ifenprodil on N2A KO EPSCs, $IC_{50} = 489 \pm 143$ nM; $n_H = 1.00 \pm 0.21$; $a_{max} = 97.4 \pm 8.5\%$ of control. **E**, *R*-CPP reduced EPSC peak (open circles) and charge (closed circles) to similar degrees in N2B and N2A KO EPSCs. For zinc dose-inhibition experiments, each concentration represents data from 5 to 9 cells. For zinc concentrations of 3 nM and greater, the differences in the means between the EPSC peak and charge have *p* values of 0.002 or less (paired comparisons). For ifenprodil dose-inhibition experiments, each concentration represents data from 3 to 16 cells. For ifenprodil concentrations of 0.3 μ M and greater, the differences in the means between the EPSC peak and charge have *p* values of 0.04 or less (paired comparisons).

contaminating zinc concentration is comparable to the IC_{50} of the N2A amino-terminal domain.

Amino-terminal ligands increase the EPSC duration

Surprisingly, zinc reduction of the EPSC peak in N2B KO neurons was accompanied by a prolongation of the deactivation time course (Fig. 1A, top right) resulting in a relative potentiation of the total charge. For example, 100 nM zinc decreased the EPSC peak to $44.4 \pm 1.4\%$ of control, whereas the total charge was only reduced to $82.9 \pm 2.0\%$ of control ($n = 9$; Fig. 1C). The charge resulting from the EPSC is a record of all the NMDA receptor channel openings after glutamate binding. Therefore, the marked EPSC prolongation by 100 nM zinc reflects only a 17% decrease in the total open probability (the total time NMDA receptors open while glutamate is bound) compared with no added zinc. By comparison, the competitive antagonist *R*-CPP had no effect on the deactivation time course and thus reduced the EPSC peak and charge in parallel (Fig. 1A, bottom; 1E).

EPSCs from N2A KO neurons had a much slower deactivation than EPSCs from N2B KO neurons. The weighted time constant of decay of EPSCs from N2A KO neurons was 308.1 ± 16.0 ms ($n = 20$), consistent with recombinant N1/N2B receptors (Vicini et al., 1998). As with zinc and N2B KO EPSCs, ifenprodil prolonged the EPSC deactivation in N2A KO neurons (Fig. 1B, top; 1D) whereas, *R*-CPP decreased the EPSC peak and the total charge to the same extent (Fig. 1B, bottom; 1E). Thus ifenprodil reduced the peak, but also prolonged EPSCs in N2A KO neurons. Our data demonstrate that occupancy of the amino-terminal binding sites for zinc or ifenprodil increased EPSC duration.

Although zinc or ifenprodil increased EPSC duration, the underlying deactivation kinetics showed some differences. EPSC deactivations in knock-out and wild-type neurons were well fitted by the sum of two exponentials (τ_{fast} and τ_{slow}). In N2B KO neurons, zinc increased the amplitude contribution of the slow component (I_{slow}) in a dose-dependent manner with little effect

Table 1. Effects of amino-terminal ligands on NMDA receptor-mediated EPSCs

	% Cont. amp.	τ_{fast} (ms)	% Fast	τ_{slow} (ms)	% Slow	% Q_{slow}	P_o^*
N2B KO EPSCs							
[zinc], nM							
0	100	21.2 ± 0.8	98.4 ± 0.9	132.4 ± 18.1	3.9 ± 0.4	15.4 ± 2.8	0.5 ± 0.03
10	78.3 ± 2.9	24.9 ± 0.5	89.4 ± 2.2	106.0 ± 5.8	10.1 ± 0.76		
30	62.6 ± 2.1	25.8 ± 1.2	86.0 ± 1.2	127.5 ± 7.5	14.0 ± 0.7		
100	44.4 ± 1.4	26.0 ± 1.2	74.2 ± 1.6	135.3 ± 7.8	24.7 ± 1.0	63.2 ± 0.9	0.25 ± 0.01
300	31.4 ± 1.1	25.1 ± 0.4	68.6 ± 5.0	161.2 ± 22.1	29.3 ± 5.3		
N2A KO EPSCs							
[ifen], μM							
0	100	147.5 ± 11.5	59.1 ± 1.4	600.1 ± 28.6	37.2 ± 1.3	69.0 ± 2.9	0.18 ± 0.02
0.3	55.6 ± 3.3	143.3 ± 11.4	64.4 ± 3.1	726.7 ± 62.8	32.0 ± 2.6		
1	37.8 ± 1.4	183.1 ± 10.3	56.4 ± 0.9	1107 ± 10.7	35.3 ± 0.8	80.2 ± 1.4	0.10 ± 0.01
3	18.2 ± 1.5	189.6 ± 14.9	48.2 ± 1.4	1374.8 ± 141.6	35.9 ± 1.6		
WT EPSCs							
[zinc], nM							
0	100	37.8 ± 1.1	84.1 ± 1.3	284.8 ± 16.1	15.9 ± 1.1	57.3 ± 2.2	0.33 ± 0.02
30	73.6 ± 2.2	40.9 ± 1.1	71.4 ± 1.3	277.3 ± 17.5	25.9 ± 1.2		
100	60.3 ± 1.6	44.6 ± 2.1	67.0 ± 0.9	275.2 ± 14.7	30.8 ± 0.6	73.8 ± 0.8	0.19 ± 0.02
300	55.4 ± 4.1	53.1 ± 0.5	60.6 ± 2.0	279.0 ± 13.1	35.0 ± 2.9		
[ifen], μM							
1	65.8 ± 3.8	54.4 ± 1.7	80.1 ± 2.7	500.8 ± 36.3	15.0 ± 1.6		
3	58.5 ± 5.5	46.2 ± 2.9	85.4 ± 2.0	375.0 ± 108.5	11.3 ± 2.5		

on the time constants (Table 1). Thus the EPSC prolongation by zinc resulted from an increase in the charge associated with the slow component (Q_{slow}). For example, Q_{slow} increased fourfold in 100 nM free zinc compared with no added zinc ($15.4 \pm 2.8\%$ of the total charge in control; $63.2 \pm 0.9\%$ of the total charge in 100 nM free zinc; $n = 7$, paired comparison). In contrast, ifenprodil increased the values of τ_{fast} and τ_{slow} in N2A KO neurons with little change in I_{fast} or I_{slow} (Table 1). Therefore, in N2A KO neurons, prolongation of the EPSC predominantly resulted from a dose-dependent increase in τ_{slow} , rather than I_{slow} , as in N2B KO neurons.

Amino-terminal ligands slow the NMDA receptor channel opening rate

Prolongation of the EPSC by zinc or ifenprodil could reflect an increase in glutamate-bound desensitized channels. The amino-terminal domain of N2A is essential for NMDA receptor desensitization (Krupp et al., 1998) and an interaction between the N2A amino-terminal domain and the agonist binding domain results in desensitization (Erreger and Traynelis, 2005). In the presence of zinc, if more channels directly enter a desensitized state after binding glutamate, this could prolong the deactivation by increasing the first latency, the time required for channels to open for the first time after binding glutamate. The high affinity open channel blocker (+)-MK-801 can be used to test this possibility. MK-801 can be used to tag NMDA receptors that are activated by synaptically released glutamate because MK-801 only binds to open channels and is unlikely to become unblocked during the course of an experiment (Huettnner and Bean, 1988; Tovar and Westbrook, 2002). We used MK-801 to estimate the first latency of NMDA receptor channel opening in the presence and absence of amino-terminal ligands.

As shown in Figure 2A, MK-801 decreased the EPSC amplitude, accelerated the deactivation and reduced the total charge transfer of neurons from N2B KO mice. These effects of MK-801 were dose-dependent (Fig. 2B,C) and result from a reduction in the channel mean open time (Rosenmund et al., 1993). However, the acceleration of the EPSC decay saturated at concentrations

above 10 μM MK-801 (Fig. 2C) because the ability to block at higher MK-801 concentrations is limited by the rate of channel opening (Jahr, 1992). Therefore the EPSC in 25 μM MK-801 (Fig. 2C, inset) reflects the time course of channel opening after binding synaptically released glutamate. In MK-801 the ratio of charge at the time of the EPSC peak (without MK-801) to the total charge gives the probability of channels having opened by the time of the peak (P_o^*). We tested the validity of this approach with a subsaturating concentration of R-CPP, a competitive antagonist that is not predicted to affect opening kinetics (Fig. 2D). R-CPP (50 nM) had little effect on the time at 60% charge transfer (14.1 ± 0.5 ms for control; 15.5 ± 0.6 ms in R-CPP) or the P_o^* (0.52 ± 0.03 for control; 0.50 ± 0.02 in R-CPP; $n = 6$, paired comparison; Fig. 2E,F). Likewise, R-CPP did not interfere with the ability of MK-801 to block open channels (Fig. 2D, arrowheads) because MK-801 reduced the EPSC peak to a similar extent in the absence of R-CPP ($29.3 \pm 2.8\%$ of control) or in 50 nM R-CPP ($36.0 \pm 2.4\%$ of control).

In contrast, 100 nM free zinc increased the time at 60% charge transfer from 14.2 ± 0.8 ms to 40.3 ± 2.6 ms ($p < 0.001$), resulting in a decrease of the P_o^* from 0.5 ± 0.03 to 0.25 ± 0.01 ($p < 0.0005$; $n = 5$, paired comparisons; Fig. 3E,F). As shown with the superimposed, peak-scaled EPSCs in Figure 3C, channels opened much more slowly in the presence in 100 nM free zinc. There was no difference in the ability of 25 μM MK-801 to reduce the EPSC peak ($35.3 \pm 0.3\%$ of control in no added zinc; $33.3 \pm 1.1\%$ of control in 100 nM free zinc), indicating that this concentration of zinc did not interfere with the ability of MK-801 to directly block the channel. Thus binding of zinc to the amino-terminal domain of N2A delayed the time it takes for NMDA channels to open for the first time after glutamate binding.

The effect of ifenprodil on N2A KO EPSCs was qualitatively similar to that of zinc on EPSCs in N2B KO neurons. In N2A KO neurons (Fig. 3D), ifenprodil (1 μM) increased the time at 60% charge transfer in 25 μM MK-801 from 137.8 ± 8.9 ms to 338.7 ± 9.8 ms ($p < 0.02$), and decreased P_o^* from 0.18 ± 0.02 to 0.10 ± 0.01 ($p < 0.02$; $n = 5$; paired comparison; Fig. 3E,F). Ifenprodil did not inhibit the ability of MK-801 to block open channels

Figure 2. MK-801 reveals the opening kinetics of synaptic NMDA receptors. **A**, EPSCs from an N2B KO neuron in control (black trace) and in 10 μM MK-801 (red trace) showing the reduction of the peak amplitude and the acceleration of the deactivation (top) and the charge transfer (bottom). Note that MK-801 decreases the time at 60% charge transfer compared with control (dashed lines). MK-801 decreases the EPSC peak and total charge in a dose-dependent manner (**B**) but the time at 60% charge transfer does not decrease at concentrations above 10 μM MK-801 (**C**). The time at 60% charge transfer was reduced from 25.3 ± 1.1 ms ($n = 9$) to 13.8 ± 0.8 ms in 10 μM MK-801 ($n = 9$) and 25 μM MK-801 caused no further decrease (13.0 ± 0.9 ms, $n = 7$). The inset shows peak-scaled EPSCs from the same neuron in two MK-801 concentrations. **D**, EPSCs from N2B KO neurons in control $\pm$ MK-801 (left) or R-CPP $\pm$ MK-801 (middle), showing that R-CPP does not prevent MK-801 from reducing the EPSC amplitude (see arrowheads). R-CPP did not prolong the EPSC as shown by the superimposed traces (right). **E**, R-CPP had very little effect on the time at 60% charge transfer or the P_o^* (**F**). Horizontal red bars in **E** and **F** indicate the means for each condition indicated. P_o^* is the ratio of the charge in MK-801 at the time of the control EPSC peak (double arrowheads in **A**) to the total charge.

(peak EPSC block: 26.1 ± 2.6 in control, 29.2 ± 2.1 in 1 μM ifenprodil). Thus occupancy by zinc or ifenprodil of the amino-terminal domains of N2A or N2B-containing NMDA receptors in KO neurons has qualitatively similar consequences on receptor gating.

Amino-terminal ligands and wild-type EPSCs

We examined the action of amino-terminal ligands on synaptic NMDA receptors in wild-type neurons because wild-type EPSCs can be comprised of three NMDA receptor types (N1/N2A; N1/N2B and N1/N2A/N2B; Sheng et al., 1994; Rauner and Köhr, 2011). Zinc decreased the wild-type EPSC peak amplitude and prolonged deactivation (Fig. 4*A,B*). The dose-inhibition curve was fit with a single binding site isotherm with an IC_{50} of 32.5 ± 9.7 nM ($n_H = 0.86 \pm 0.17$; $a_{\text{max}} = 54.0 \pm 3.9\%$). Although typically considered an inhibitor, zinc potentiated the charge in wild-type neurons such that at higher zinc concentrations the slower deactivation almost completely compensated for the charge reduction resulting from the decrease of the EPSC peak (Fig. 4*A*, bottom; 4*B*). Thus zinc, at concentrations below 300 nM, almost completely redistributed the charge transfer resulting from activation of synaptic NMDA receptors in wild-type neurons (Fig. 4*A*, bottom). Consistent with this result,

300 nM zinc reduced the total time that wild-type NMDA receptors spend in the open state by only 8%. We examined whether zinc affected the opening kinetics of wild-type synaptic NMDA receptors. Zinc (100 nM free) increased the time at 60% charge transfer in MK-801 (25 μM) from 22.6 ± 2.6 ms to 47.0 ± 4.9 ms ($p < 0.0005$) reflecting a reduction of P_o^* from 0.33 ± 0.02 to 0.20 ± 0.02 ($p < 0.0005$; $n = 7$; paired comparisons; Fig. 4*E–G*). In contrast, ifenprodil reduced the EPSC peak ($\text{IC}_{50} = 3.0 \pm 0.9$ μM , $n_H = 0.91 \pm 0.2$) but did not potentiate the charge (Fig. 4*C,D*) in EPSCs from wild-type neurons. Therefore, with the complement of synaptic NMDA receptor in these wild-type neurons, only occupancy of the N2A amino-terminal domain resulted in significant modulation of the EPSC.

Discussion

The effects of zinc and ifenprodil on recombinant NMDA receptors have been well studied although most of this work has been done under steady-state conditions, where agonist and amino-terminal ligands are at equilibrium, and has focused on mechanisms involved in inhibition of the peak current. We focused on the action of amino-terminal ligands during EPSCs when neurotransmitter is present only briefly. Nonequilibrium conditions, the predominant form of activation of synaptic receptors, em-

Figure 3. Amino-terminal ligands increase the first latency of synaptic NMDA receptors. **A**, EPSCs from an N2B KO neuron in control (black trace) and in 25 μ M MK-801 (red trace) compared with **(B)** EPSCs from the same neuron in 100 nM free zinc (black trace) and 100 nM free zinc plus 25 μ M MK-801 (red trace). **C**, Superimposed EPSCs (from **A** and **B**) in 25 μ M MK-801 $\pm$ 100 nM free zinc demonstrating the increase in the time required for MK-801 to block channels in the presence of zinc (gray trace) compared with no added zinc (black trace). Arrowheads above the traces indicate the time at 60% charge transfer for no added zinc (black) and 100 nM free zinc (gray). **D**, superimposed EPSCs from N2A KO neurons in 25 μ M MK-801 alone (black trace) or with 1 μ M ifenprodil (gray trace) indicating that the ability of MK-801 to block channels is slowed by ifenprodil. Arrowheads above the traces indicate the time at 60% charge transfer in MK-801 alone (black) or MK-801 plus ifenprodil (gray). **E**, The increase in the time at 60% charge in N2B KO neurons and N2A KO neurons for zinc and ifenprodil, respectively. **F**, The decrease in the P_o^* in N2B KO EPSCs and N2A KO EPSCs caused by zinc and ifenprodil, respectively. Horizontal red bars in **E** and **F** represent the means for each condition indicated. In **E** and **F**, open circles are data from N2A KO neurons; closed circles are data from N2B KO neurons.

phasize the kinetic behavior of glutamate-bound channels. Our work demonstrates that NMDA receptor channel kinetic behavior can be profoundly altered by occupancy of the amino-terminal binding sites of N2A and N2B subunits.

Kinetics of amino-terminal ligand-bound synaptic NMDA receptors

The modulation of EPSCs by amino-terminal ligands differs from either *R*-CPP or MK-801 in that the peak current is reduced whereas the duration is increased. Generally, for ligand-gated

Figure 4. Amino-terminal ligands and wild-type NMDA receptor-mediated EPSCs. **A**, Zinc application to wild-type EPSCs decreased the EPSC peak (top) and prolonged the deactivation, as indicated by the charge transfer (bottom) in control (black trace) and 300 nM zinc (red trace). Inset in **A**, top, is the same EPSC but peak-scaled to show the EPSC prolongation. **B**, Zinc dose-inhibition curve on wild-type EPSCs demonstrates that the EPSC peak (open circles) and charge (closed circles) were reduced to different degrees. The dashed line is the fit to the data. The gray line in **B**, shown for comparison, is the fit to the zinc dose-inhibition data from N2B KO neuron EPSCs. For zinc concentrations of 10 nM and greater, the differences in the means between the EPSC peak and charge have *p* values of 0.03 or less (paired comparisons). **C**, Ifenprodil decreased the wild-type EPSC peak and charge to similar degrees. Inset in **C**, top, is the same EPSC but peak-scaled demonstrating similar deactivation in control and ifenprodil. **D**, Dose-inhibition curve on wild-type EPSCs demonstrates that reduction of the EPSC peak (open circles) and charge (closed circles) are reduced to the same degree for all ifenprodil concentrations tested. The dashed line is the fit to the data. The gray line is the fit to the ifenprodil dose-inhibition data from N2A KO EPSCs, shown for comparison. **E**, Zinc significantly slowed the rate at which wild-type NMDA receptors opened following glutamate binding as shown by the superimposed EPSCs in MK-801 (25 μ M) in control (black trace) or in 100 nM free zinc (gray trace). The arrowheads above the traces indicate the time at 60% charge transfer for control (black) and 100 nM free zinc (gray) conditions. Free zinc (100 nM) increases the time at 60% charge transfer (**F**) and decreases P_o^* (**G**) in wild-type neurons. Horizontal red lines in **E** and **F** represent the means for the indicated conditions. For the zinc dose-inhibition curve, each concentration represents data from 5 to 9 cells. For the ifenprodil dose-inhibition curve, each concentration represents data from 5 to 13 cells.

channels, drug-receptor interactions either prevent gating transitions or shift transitions between kinetic states. For example, receptors bound by competitive antagonists like *R*-CPP are unable to open so these drugs decrease the channel P_o to 0 and leave unliganded channels unaffected. On the other hand, the high affinity open channel blocker MK-801 shortens the channel mean open time, resulting in NMDA receptor-mediated EPSCs of shorter duration. The divergence between the effects on EPSC peak and charge by zinc or ifenprodil requires that significant changes in the opening kinetics compensate for the reduction of charge that arises from decreasing the EPSC peak. Indeed, zinc and ifenprodil slowed the rate at which channels first opened (by 2.8-fold and 2.4-fold, respectively) as measured by the time at 60% charge transfer. This resulted in significant reductions (50% for zinc; 44% for ifenprodil) in the number of channels that open by the EPSC peak. Thus, slowing the entry of NMDA receptors into the open state (longer first latency) contributes to the EPSC prolongation.

Amino terminal ligands may also prolong the EPSC by altering entry and exit from agonist-bound states, either by affecting glutamate unbinding or increasing the probability of transitions into bound, nonconducting (desensitized) states. For example, chelation of ambient zinc decreased the glutamate dissociation rate in recombinant N1/N2A receptors (Paoletti et al., 1997) and ifenprodil increased the apparent glutamate affinity and dissociation rate in recombinant N1/N2B receptors (Kew et al., 1996). Agonist unbinding rate generally correlates with the rate of deactivation (Lester and Jahr, 1992; Jones and Westbrook, 1995). In addition, a delay in glutamate unbinding can result from longer dwell time in states, such as a desensitized state, from which agonist cannot unbind. Consistent with this idea, zinc increased the mean closed durations of agonist-bound recombinant N1/N2A receptors in single-channel recording (Amico-Ruvio et al., 2011).

An “enhanced desensitization” mechanism is an attractive possibility for amino-terminal ligands because transitions through a desensitized state preclude agonist unbinding and increase delayed channel openings. Desensitization can prolong deactivation of GABA_A receptors by this mechanism (Jones and Westbrook, 1995). Our proposal almost certainly does not capture the full repertoire of modulation by amino-terminal ligands. Amino-terminal ligands can also potentiate proton inhibition (Mott et al., 1998), decrease mean open times (Legendre and Westbrook, 1991; Erreger and Traynelis, 2008), or decrease stability of the open state (Amico-Ruvio et al., 2011). However, these additional mechanisms cannot explain the prolongation of the EPSC observed here.

Modulation of wild-type EPSCs by amino-terminal ligands

The upregulation of N2A as neurons mature means that N2A and N2B are expressed in wild-type neurons and thus diheteromeric (N1/N2A; N1/N2B) and triheteromeric (N1/N2A/N2B) receptors could contribute to NMDA-receptor-mediated EPSCs (Sheng et al., 1994; Rauner and Köhr, 2011; although, see Al-Hallaq et al., 2007). In fact EPSC deactivation kinetics in our experiments were slower than in N2B knock-out neurons, but faster than in N2A knock-out neurons (Table 1). Therefore, interpretations regarding the effects of amino-terminal ligands on wild-type EPSCs are complicated by the contribution of receptor heterogeneity and by the relatively unknown properties of triheteromeric receptors. Based on the differences in P_o^* between diheteromeric receptors (Table 1; see also Chen et al., 1999), there would be 2.5 times as many N1/N2B receptors as N1/N2A recep-

tors at synapses if N1/N2A and N1/N2B receptors contribute equally to the amplitude of wild-type EPSCs.

Interestingly, zinc and ifenprodil differed in their action on wild-type EPSCs from diheteromeric receptors in knock-out neurons in that only zinc prolonged the deactivation. In N2B KO EPSCs, increasing the free zinc concentration increased the percentage contribution of τ_{slow} to deactivation whereas ifenprodil increased τ_{fast} and τ_{slow} with minimal effects on the amplitude contribution of each component. The values in Table 1 can be used to estimate the impact of diheteromeric receptors on the behavior of wild-type EPSCs. For example, in N2A KO EPSCs, τ_{slow} contributes 37% in control and 7% in 3 μ M ifenprodil, relative to control. If N1/N2B receptors contribute 25% to the wild-type EPSC amplitude, the contribution from τ_{slow} for this population would decrease from 9% in control to 1.5% in 3 μ M ifenprodil. In contrast, if N1/N2A receptors contribute 25% to the wild-type EPSC amplitude, then τ_{slow} in those diheteromers would increase from 1% in control to 6% in 100 nM zinc. The increase contributed by τ_{slow} in N1/N2A diheteromers could explain why zinc increased the wild-type EPSC duration, but ifenprodil did not. However, a low amplitude ifenprodil-induced slow component contributed by N1/N2B diheteromers may be difficult to resolve in our recording conditions. Finally, it is possible that the EPSC contribution from triheteromeric N1/N2A/N2B receptors is prolonged by zinc but not by ifenprodil. The unknown kinetic properties of these receptors currently prevent us from answering this question.

Modulation of wild-type EPSCs by amino-terminal ligands is likely to depend on the relative contribution of different NMDA receptor types to the EPSC. The zinc sensitivity of wild-type EPSCs can be used to estimate the relative contribution of diheteromeric and triheteromeric receptors. If N1/N2A and N1/N2A/N2B receptors are the only zinc-sensitive receptors, then the maximal reduction by zinc in wild-type neurons will range from 17% (for exclusively N1/N2A/N2B receptors) to 76% (for exclusively N1/N2A receptors). The wild-type sensitivity can be calculated from the simple relationship:

$$a_{\text{wild-type}} = (a_{\text{N1/N2A}})(C_{\text{N1/N2A}}) + (a_{\text{N1/N2A/N2B}})(C_{\text{N1/N2A/N2B}}),$$

where $a_{\text{wild-type}}$ is the maximum reduction of the wild-type EPSC peak, $a_{\text{N1/N2A}}$ is the maximum zinc reduction of N1/N2A receptors, $C_{\text{N1/N2A}}$ is the contribution of N1/N2A receptors to the EPSC amplitude, $a_{\text{N1/N2A/N2B}}$ is the maximum zinc reduction of N1/N2A/N2B receptors and $C_{\text{N1/N2A/N2B}}$ is the contribution of N1/N2A/N2B receptors to the EPSC amplitude. We used a_{max} from the zinc dose-inhibition curve of N2B KO EPSCs (76%) for $a_{\text{N1/N2A}}$. The maximal reduction of wild-type EPSCs by zinc was 54% and $a_{\text{N1/N2A/N2B}}$ ranges from 17 to 38% (Hatton and Paoletti, 2005). Therefore, the contribution from N1/N2A/N2B receptors to wild-type EPSCs would be 36–58%. These values may be overestimates because N1/N2B receptors are also sensitive to zinc albeit with much lower affinity (Rachline et al., 2005). However, the deactivation kinetics and P_o^* from wild-type EPSCs are consistent with a major, and possibly majority, contribution to the EPSC from receptors other than diheteromers (Gray et al., 2011; Rauner and Köhr, 2011). We cannot determine from our data whether different NMDA receptor types are present in the same postsynaptic density, although there can be selective distribution

of receptor types opposite high release probability presynaptic release sites (Chavis and Westbrook, 2001) for example.

Functional implications

The amino-terminal domains of NMDA receptor subunits are not essential for channel function (Madry et al., 2007), consistent with a modulatory role. The structure of glutamate receptor amino-terminal domains is highly homologous to the clam-shell structure of bacterial periplasmic binding proteins (Paoletti, 2011), but the degree of movement and the sites of ligand binding vary between different subunits (Mayer, 2011). Thus it is interesting that zinc and ifenprodil have related, but distinct, actions on NMDA channel gating in our experiments, consistent with differences in ligand-receptor interactions (Karakas et al., 2009, 2011). The redistribution of charge by amino-terminal ligands has important implications for the actions of NMDA receptors. In terms of modulation of NMDA receptors for clinical purposes, the clear distinction between the effects of zinc and ifenprodil highlights that occupancy of the zinc binding site could be more promising. The role of NMDA receptors in short-term plasticity and neuronal injury relies largely on the total charge (calcium entry) associated with NMDA receptor activation. However, the slow time course of NMDA-receptor-mediated EPSCs has distinct computational consequences on single synapses (Schoppa and Westbrook, 1999) or more global processes such as working memory (Wang, 2001). It is in this latter context that amino-terminal ligands and the domains to which they bind may have a previously unappreciated role.

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