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Allosteric interaction between zinc and glutamate binding domains on NR2A causes desensitization of NMDA receptors

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Fast desensitization is an important regulatory mechanism of neuronal NMDA receptor function. Previous work suggests that fast desensitization of NR1/NR2A receptors is caused by ambient zinc, and that a positive allosteric interaction occurs between the extracellular zinc-binding amino terminal domain and the glutamate-binding domain of NR2A. The relaxation of macroscopic currents in the presence of zinc reflects a shift to a new equilibrium due to increased zinc affinity following the binding of glutamate. Here we demonstrate that this allosteric coupling reflects interactions within the NR2A subunit, and that the affinity of zinc for its binding site is regulated by glutamate binding and not by glycine binding nor by channel pore opening. We fit an explicit model to experimental data over a wide range of parameters, demonstrating that allosteric theory can quantitatively account for the fast zinc-dependent component of desensitization for NR1/NR2A NMDA receptors. We subsequently use this model to evaluate the effects of extracellular zinc on NR1/NR2A excitatory postsynaptic currents (EPSCs) by simulating the response to a brief synaptic-like pulse of glutamate. Modelling results show that zinc at a steady-state concentration of at least 100 nM has a significant effect on the amplitude of NMDA EPSCs but that concurrent release of 10 μ M zinc with synaptic glutamate release has little effect on the amplitude of a single NR1/NR2A NMDA EPSC. These data suggest that while steady-state zinc can regulate the amplitude of synaptic NMDA currents, zinc co-released with glutamate will only have significant impact under conditions of high frequency activity or at concentrations high enough to cause voltage-dependent channel block.

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Temporal integration of synaptic inputs is largely controlled by the time course of NMDA channel currents (Popescu *et al.* 2004; Erreger *et al.* 2005). Alteration of the time course of synaptic NMDA currents has important functional implications because it changes the magnitude and kinetics of calcium influx through NMDA channels, a key determinant of synaptic plasticity. One mechanism by which the time course of NMDA currents can be modulated is desensitization, classically defined as a reduction of NMDA current amplitude in the continuous presence of glutamate. Several forms of NMDA receptor desensitization have been reported (Dingledine *et al.* 1999). The term 'glycine-dependent desensitization' refers to the decrement of NMDA receptor currents that occurs when the glycine concentration is not saturating (Mayer *et al.* 1989; Benveniste *et al.* 1990). The term 'glycine-independent desensitization' has been used to describe desensitization of NMDA receptors

that cannot be prevented by a high concentration of glycine (Sather *et al.* 1990, 1992; Tong & Jahr, 1994; Tong *et al.* 1995), which in some cases may be due to inactivation of NMDA currents caused by an increase in intracellular calcium concentration (Clark *et al.* 1990; Vyklicky *et al.* 1990; Legendre *et al.* 1993; Rosenmund & Westbrook, 1993*a,b*; Krupp *et al.* 1996). More recently, the term 'glycine-independent desensitization' has been used to describe all forms of calcium-independent and glycine-independent desensitization, which are particularly prominent for recombinant NR1/NR2A receptors.

Two kinetic components have been described for glycine-independent desensitization, a fast component with a time constant of \sim 100–200 ms and a slow component with a time constant of \sim 1–2 s (Chen *et al.* 1997; Krupp *et al.* 1998; Villarroel *et al.* 1998; Zheng *et al.* 2001). The fast component of glycine-independent

desensitization was initially suggested to be enhanced by extracellular zinc (Zn^{2+}) (Chen *et al.* 1997). Consistent with this idea, the amino terminal domain (ATD), which contains the high-affinity zinc binding site (Fayyazuddin *et al.* 2000; Low *et al.* 2000; Paoletti *et al.* 2000), had been previously implicated as a structural determinant of desensitization (Krupp *et al.* 1998; Villarroel *et al.* 1998). Zheng *et al.* (2001) subsequently proposed that the time course of this fast desensitization reflects binding of ambient zinc to the extracellular zinc site in the ATD following glutamate binding to the agonist site in the S1S2 domain. This idea was dependent on a hypothetical positive allosteric interaction between the ATD and the glutamate binding site, which implies that Zn^{2+} will bind to NR1/NR2A receptors with a lower affinity in the absence of glutamate, and bind to the receptors with a higher affinity when glutamate is complexed with the NR2A agonist-binding S1S2 domain. Consistent with the allosteric hypothesis, Zn^{2+} binding to the ATD decreases glutamate EC_{50} , presumably through an increase in glutamate affinity (Zheng *et al.* 2001). As a result of this allosteric interaction, the binding of glutamate causes the receptor to switch from a relatively lower affinity to a higher affinity state for zinc binding. Thus, the occupancy of the zinc binding site increases over time following the glutamate-induced shift to the higher affinity state. The increasing occupancy of the Zn^{2+} binding site with time inhibits channel function to create a new form of desensitization.

In the present study, we present two new lines of evidence supporting this hypothesis. First, we show that glutamate binding is both necessary and sufficient to regulate Zn^{2+} affinity, and that zinc affinity is independent of both glycine binding and channel opening. Second, we show that an explicit allosteric model of glutamate and Zn^{2+} interactions can quantitatively describe the amplitude and time course of NMDA currents over a wide range of parameters. We subsequently use this model to explore the potential effects of Zn^{2+} -induced desensitization on the synaptic response time course using simulations of current responses to a brief (~ 1 ms) synaptic pulse of glutamate.

Methods

Transfection of HEK cells

Cells from the human embryonic kidney cell line HEK 293 (CRL 1573; ATCC, Rockville, MD, USA) were maintained at $37^{\circ}C$ and 5% CO_2 and plated on 12 mm diameter glass coverslips as previously described (Zheng *et al.* 1998). Low-confluency cells were transiently transfected by the calcium phosphate method with cDNA encoding NR1-1a (GenBank U11418, U08261; pCIneo vector; hereafter NR1), NR2A (D13211; pCIneo) and

GFP at a ratio of 1:2:0.2 ($0.25 \mu g ml^{-1}$ NR1) for 4–12 h. After transfection, NMDA antagonists ($200 \mu M$ 2-amino-5-phosphonovaleric acid, $2 mM Mg^{2+}$) were added to the culture medium.

Whole-cell patch-clamp recording from HEK 293 cells

Patch-clamp recording in the whole-cell configuration was made with an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA). Recording electrodes ($5\text{--}12 M\Omega$) were filled with (mM): 140 caesium gluconate, 5 Hepes, 4 NaCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 1 ATP, 0.3 GTP and 5 BAPTA (pH 7.4, $23^{\circ}C$). The recording chamber was continually perfused with recording solution composed of (mM): 150 NaCl, 10 Hepes, 1 $CaCl_2$, 3 KCl and 10–20 mannitol (pH 7.4 unless otherwise noted). Glutamate ($100 \mu M$) was applied using a theta glass pipette driven by a piezo-electric translator (Burleigh Instruments, Fishers, NY, USA). We have previously measured the solution exchange time of this system by stepping a cell held at 0 mV in the absence of agonist into a solution in which 150 mM NaCl was replaced by KCl (Mott *et al.* 2001). The time course of the change in the holding current had a 10–90% rise time of 16 ms and could be fitted with a single exponential component with a time constant of 7.3 ms.

Glycine ($50 \mu M$) was present in all solutions except where noted. Data used for analysis were collected within 5–15 min of obtaining the whole-cell patch configuration to minimize time-dependent changes in glycine-independent desensitization. In some experiments, series resistance was corrected off-line (Traynelis, 1998). Correction of series resistance did not alter the time constant for zinc-induced current relaxation.

Curve fitting and statistical analysis

The time course of desensitization was fitted with one or two exponential components with custom software NPM (S. F. Traynelis, Emory University) using the equation

$$I(t) = \text{Amplitude}_1(\exp(-\text{Time}/\tau_1)) + \text{Amplitude}_2(\exp(-\text{Time}/\tau_2)) + I_{\text{steady-state}} \quad (1)$$

where τ is the decay time constant. The zinc concentration dependence for the rate of desensitization was used to calculate the zinc binding and unbinding rates using the equation:

$$1/\tau_{\text{on}} = k_{\text{on}}[Zn^{2+}] + k_{\text{off}} \quad (2)$$

where τ_{on} is the time constant for fast desensitization and k_{on} and k_{off} are the zinc binding and unbinding rates, respectively. Concentration–response relationships

for zinc inhibition were fitted with the equation

$$I/I_0 = (1 - I_{Zn}) / \{1 + ([Zn^{2+}] / IC_{50})^n\} + I_{Zn} \quad (3)$$

where I_0 is the whole cell current in the absence of extracellular Zn^{2+} , n is the Hill slope, and I_{Zn} is the residual current at saturating extracellular Zn^{2+} .

An allosteric model was fitted simultaneously to the averaged NR1/NR2A response waveforms obtained under six different recording conditions (ChanneLab, www.synaptosoft.com). Proton concentrations were corrected with an activity coefficient of 0.8. Averaged waveforms were normalized to the largest amplitude, and fitted by comparing the normalized simulated currents and normalized response waveforms at each step of a simplex algorithm using least squares criteria. Simulations were generated using a 5th order Runge-Kutta numerical integration algorithm. All pooled data are expressed as mean \pm S.E.M. Student's unpaired t test was used unless stated otherwise.

Results

Zinc and glutamate binding sites are allosterically coupled

Currents from HEK 293 cells expressing NR1/NR2A NMDA receptors were elicited by rapid application of $100 \mu\text{M}$ glutamate in the continuous presence of $50 \mu\text{M}$ glycine (Fig. 1A, holding potential $V_{\text{hold}} = +50 \text{ mV}$). In the absence of free Zn^{2+} in the external solution (ambient zinc chelated by $10 \mu\text{M}$ EDTA), the current exhibits only a slow form of glycine-independent desensitization. When free Zn^{2+} is in the nanomolar range (free concentration buffered by tricaine), a fast component of desensitization is observed. We have previously proposed that fast desensitization of NR1/NR2A receptors is caused by an allosteric interaction between the high-affinity nanomolar Zn^{2+} binding site on the amino terminal domain and the glutamate binding site (Zheng *et al.* 2001; see Monod *et al.* 1965). A key feature of this hypothesis is the proposed intra-subunit allosteric regulation between the NR2A amino terminal domain and the NR2A S1S2 glutamate binding domain. Figure 1B shows the difference in the IC_{50} values for peak and steady-state currents, which approximate the apparent Zn^{2+} affinity for glutamate-unbound and glutamate-bound receptors, respectively. Figure 1C illustrates the dependence of the desensitization rate on extracellular Zn^{2+} concentration. The linear relationship of the rate of desensitization on the free zinc concentration is consistent with a single zinc binding event being the rate-limiting step in the desensitization process. Based on linear regression of data presented in Fig. 1C, the binding and unbinding rates for zinc are $3.40 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and 1.38 s^{-1} , respectively (see Methods). From these microscopic rate constants, the K_d

for zinc is calculated to be 40 nM , which is in agreement with the zinc IC_{50} determined for the steady-state currents (26 nM ; Fig. 1B).

Because glutamate binding causes both channel opening and the hypothesized switch in zinc affinity, it remains possible that it is channel opening rather than glutamate binding that causes the switch in zinc affinity. To test this hypothesis, a set of experiments parallel to those in Fig. 1 was performed in which glutamate was present continuously and the co-agonist glycine was rapidly applied (Fig. 2). If the intra-subunit allosteric hypothesis is correct, then pre-equilibration with glutamate will shift receptors into the high-affinity state for zinc binding even before glycine application. Glycine application would then lead to channel opening, but no apparent desensitization. On the other hand, if rapid application of glycine causes the same desensitization as rapid application of glutamate, it would suggest that zinc affinity is controlled by the conductance state of the channel pore.

Figure 2 shows that rapid application of glycine causes no desensitization and that the dose-response relationship for zinc inhibition of currents in response to glycine application exhibits high apparent affinity for zinc inhibition. Figure 2A plots the current from one representative cell using the same glutamate application protocol as in Fig. 1. Figure 2B shows the current from an independent representative cell using a protocol of

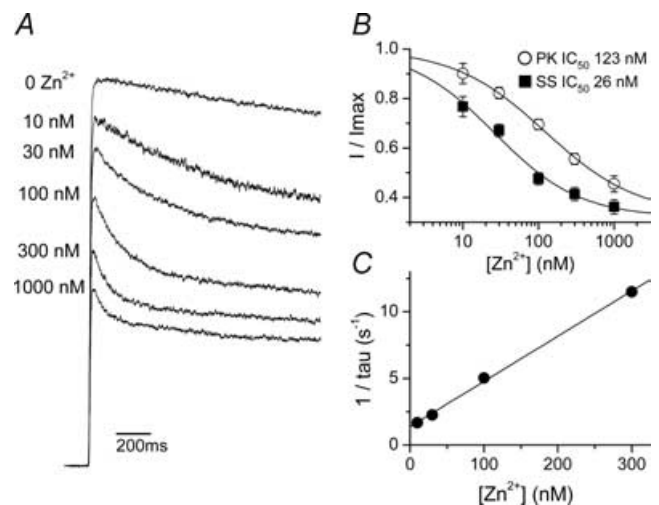


Figure 1. The magnitude and time course of desensitization depend on free zinc concentration

A, mean traces from multiple cells normalized to the 0 nM zinc (EDTA) condition in response to rapid application of $100 \mu\text{M}$ glutamate ($n = 4-14$, $V_{\text{hold}} = +50 \text{ mV}$). B, the steady-state dose-response relationship indicates a higher apparent affinity for zinc than the dose-response relationship for peak currents. (Steady state (SS): IC_{50} 26 nM , I_{Zn} 0.32 , Hill slope 0.77 ; peak (PK): IC_{50} 123 nM , I_{Zn} 0.33 , Hill slope 0.71), where I_{Zn} denotes relative plateau response due to incomplete inhibition at saturating concentrations of Zn^{2+} . C, the time constant for fast desensitization varies linearly with free zinc concentration ($R > 0.99$).

rapid application of 100 μM glycine in the continuous presence of 50 μM glutamate. Note that there is little desensitization of the current, consistent with the receptors already being in a high-affinity state for zinc binding due to pre-equilibration with glutamate before activation of the current by glycine. Figure 2C illustrates the intra-subunit hypothesis that zinc affinity is controlled by the occupancy of the glutamate binding site and not by glycine binding nor channel opening. Consistent with this hypothesis, Fig. 2D shows that the IC_{50} for zinc inhibition of the peak currents in response to glycine application is 29 nM, almost identical to the IC_{50} for zinc inhibition of *steady-state* currents in response to glutamate application (26 nM) and a lower IC_{50} than *peak* currents in response to glutamate application (123 nM, dashed line). The results are consistent with the zinc affinity being controlled by the occupancy of the glutamate binding site, rather than by the glycine binding site or opening of the channel pore. Thus, intra-domain interactions within the NR2A subunit appear to fully mediate the allosteric interactions between Zn^{2+} and glutamate.

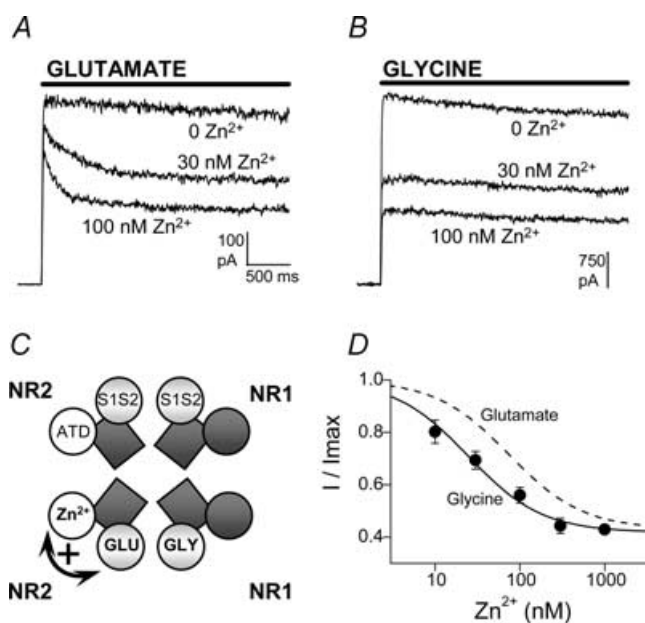


Figure 2. Zinc affinity is controlled by glutamate binding, not glycine binding nor channel opening

A, rapid application of glutamate in the continuous presence of glycine causes zinc-dependent fast desensitization. *B*, rapid application of glycine in the continuous presence of glutamate *does not* cause a zinc-dependent fast desensitization. *C*, NMDA receptors are thought to be tetramers composed of 2 NR1 and 2 NR2 subunits. Each subunit contains both an agonist binding domain (S1S2) and a modulatory amino terminal domain (ATD). A positive allosteric interaction between the glutamate binding (S1S2) domain and Zn^{2+} binding (ATD) domain on NR2A is hypothesized. *D*, rapid application of glycine to cells pre-equilibrated with glutamate reveals that they are already in a high-affinity state for zinc inhibition (peak IC_{50} 29 nM, $n = 8-16$). The peak dose-response curve for glutamate application (Fig. 1B) is shown for comparison (dashed line).

An allosteric model accounts for the zinc and pH dependence of desensitization

In order to assess the zinc-induced desensitization in the context of the putative connection between zinc inhibition and regulation of NMDA receptors by protons, we recorded glutamate-induced whole cell currents over a range of Zn^{2+} concentrations and proton concentrations (pH). Figure 3 illustrates the pH dependence of zinc-dependent desensitization, with more desensitization observed when the proton concentration is elevated (lower pH). Multiple lines of evidence have thus far suggested that Zn^{2+} inhibition is mediated through an increase in sensitivity to tonic proton inhibition at physiological pH (Choi & Lipton, 1999; Low *et al.* 2000; Zheng *et al.* 2001). We therefore hypothesize that a positive allosteric interaction also occurs between Zn^{2+} binding and proton binding, although the compact nature of proton binding sites may create special properties for such an interaction (see Discussion). Figure 4 shows the simplest representation of this dual allosteric regulation between Zn^{2+} -glutamate, and Zn^{2+} -protons within a single glutamate-binding subunit, in which thermodynamic balance has been maintained. In this model, we have simplified all potential pre-gating steps (Popescu *et al.* 2004; Erreger *et al.* 2005) to a single closed-open transition (Lester & Jahr, 1992). This allows us to focus attention within the complex model on the dual intra-subunit allosteric interactions while still adequately describing macroscopic current and open probability.

To quantitatively test this model, the time course of currents over a range of zinc concentrations and pH values

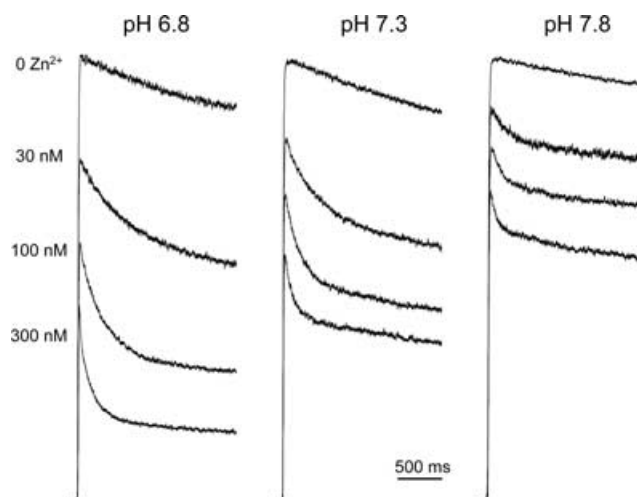


Figure 3. The magnitude of desensitization depends on free zinc concentration and on proton concentration (pH)

Mean current responses to rapid application of glutamate in the presence of 0, 30, 100, or 300 nM Zn^{2+} ($n = 7-13$ cells). Currents are normalized to the 0 nM Zn^{2+} (EDTA) condition for each pH, and plotted on same scale even though acidic pH reduced the current.

were recorded, and composite average traces obtained across multiple patches. These data were simultaneously fitted by this model (see Methods), which embodied the concepts of dual positive allosteric interactions between Zn^{2+} and glutamate as well as Zn^{2+} and protons (Fig. 4). Our goal in evaluating this model was to test whether we could predict the time course and amplitude of responses over a wide range of conditions by incorporating two ideas into the model: (1) glutamate binding increases the affinity for zinc, and (2) zinc alters protonation rates resulting in greater proton affinity. This model assumes that Zn^{2+} -bound receptors can open, consistent with the significant plateau currents observed for Zn^{2+} inhibition curves at saturating Zn^{2+} concentrations (e.g. Williams, 1996; Paoletti *et al.* 1997; Low *et al.* 2000). By contrast, we assume protonated channels do not open, as suggested by Banke *et al.* (2005). Intuitively, this is a reasonable assumption since the concentration–response relationship for proton inhibition is complete, with no current detectable at saturating proton concentrations (Low *et al.* 2000). Moreover, known structural determinants for proton inhibition lie in the same region of the receptor

hypothesized to be critical for channel gating, so it seems likely that protonation directly impacts the gating process (Low *et al.* 2003; Banke *et al.* 2005; Hu & Zheng, 2005). The model contains a slow desensitized state to account for the gradual decrement of the current even in the absence of Zn^{2+} .

Before evaluating the ability of this model to predict the response time course that we recorded, we placed several simplifying constraints on the model. First, we assumed a proton association rate for all steps of $1.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (Banke *et al.* 2005). We set the closing rate to 269 s^{-1} , which is the reciprocal of the mean open time previously reported in outside-out patches (3.72 ms from Erreger *et al.* 2005). We then set the opening rate to a value (560 s^{-1}) that gave an open probability of 0.5 at pH 7.3 (Erreger *et al.* 2005). The glutamate dissociation rate (80 s^{-1}) was selected to give a relaxation time course after a brief application of agonist with a time constant of 32 ms (Erreger *et al.* 2005). The glutamate binding rate was set to a value ($8.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) that gave a glutamate EC_{50} value of $4.4 \mu\text{M}$ at steady state (Zheng *et al.* 2001).

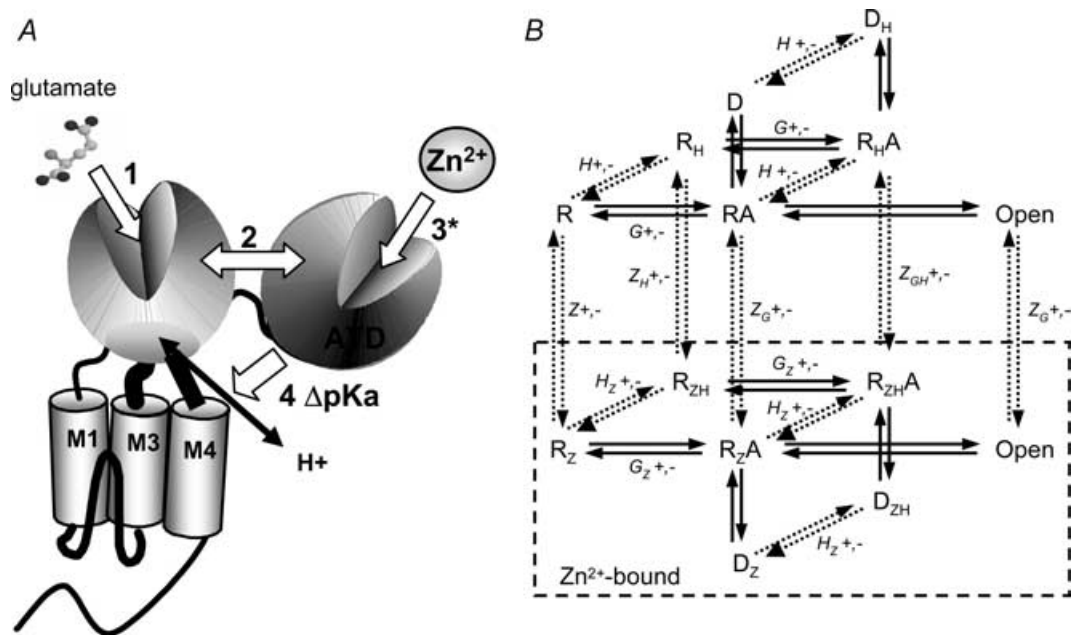


Figure 4. Hypothesized model of the molecular events contributing to zinc-dependent fast desensitization of NR2A-containing NMDA receptors

A, the conceptual model of the hypothesized sequence of events shown has four steps. (1) Glutamate binds to the S1S2 domain. (2) Glutamate binding leads to allosteric changes in the amino terminal domain that alter zinc affinity. (3) As the system relaxes into a new equilibrium, the occupancy of the zinc binding site increases, which is the rate-limiting step (indicated by *). (4) Subsequent conformational changes of the receptor enhance binding of protons to the pH-sensitive gating elements, reducing channel open probability. **B**, explicit kinetic model for Zn^{2+} and proton regulation of a single subunit used to fit data, which formalizes the ideas illustrated in panel A. A, agonist (glutamate); Z, zinc; H, proton; D, slow zinc-independent desensitized state. '+' rates indicate binding and '-' rates indicate unbinding. The zinc affinity is increased following glutamate binding. The zinc-bound channels have a higher proton affinity quantified as a higher pK_a ($\log[\text{H}^+ \text{ on}/\text{H}^+ \text{ off}]$). Protonated channels are not capable of opening (Banke *et al.* 2005).

We subsequently fitted current responses recorded in the presence of EDTA at pH 6.7, 7.3 and 7.8 with the model varying only desensitization rates and proton dissociation rates. Once these rate constants were obtained, the slow desensitization rates were fixed in subsequent evaluation of the model in the presence of Zn^{2+} . By implementing these constraints, assumptions and thermodynamic balance in loops, we reduced the full model to only five free parameters – the ‘on’ and ‘off’ rates for Zn^{2+} binding to receptors that lack glutamate, the ‘on’ and ‘off’ rates for Zn^{2+} binding to receptors that have bound glutamate, and the ‘off’ rate for protons from Zn^{2+} -bound receptors.

We subsequently fitted a set of six composite averaged current response waveforms with this model, varying these five parameters using a least-squares criteria and a simplex algorithm (Fig. 5A). Figure 5A–C shows how this straightforward model with only five free parameters can predict the amplitude and time course of Zn^{2+} inhibition over a wide range of pH values and Zn^{2+} concentrations. Table 1 summarizes the rate constants for this fit. Despite several simplifying assumptions and constraints, the model successfully predicts the relative amplitudes and time courses for conditions across the full range of parameters for both Zn^{2+} and proton concentration. In addition, the model also predicts the effect of Zn^{2+} on the glutamate EC_{50} . The glutamate dissociation rate was

set to give an EC_{50} for steady-state currents of $4.4 \mu\text{M}$ (Zheng *et al.* 2001). The fitted model predicted an EC_{50} for Zn^{2+} -bound receptors of $1.8 \mu\text{M}$, similar to the value ($2.3 \mu\text{M}$) previously observed in the presence of saturating Zn^{2+} (Zheng *et al.* 2001). In addition, the IC_{50} value for Zn^{2+} inhibition of peak and steady-state currents was 141 and 64 nM, respectively, similar to previously determined experimental values (123 and 26 nM, see Fig. 1). Thus, to a first approximation, we can account for the main features of this data set by the dual positive allosteric interactions between Zn^{2+} and both proton and glutamate binding. We also refitted the data with a series of models in which the Zn^{2+} -bound and Zn^{2+} -unbound open states were uncoupled or had independent closing and/or opening rates (data not shown). In all cases, similar fits to the data were obtained, confirming that this allosteric representation of the interactions between Zn^{2+} and glutamate is relatively insensitive to the nature of the representation of the gating steps in the model.

Figure 5D illustrates one of the main hypotheses encapsulated in the model: the regulation of proton affinity by Zn^{2+} binding. The model was used to simulate the fraction of receptors in the protonated state at steady state in the presence of 1 mM glutamate as a function of Zn^{2+} concentration. The pK_a is the pH at which half of the proton binding sites are occupied. Zn^{2+} increases

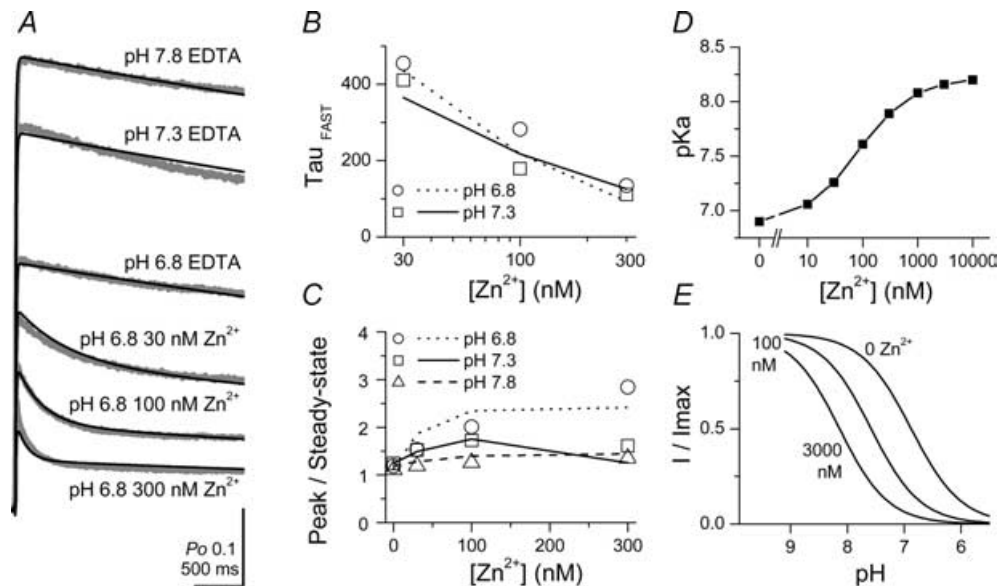


Figure 5. A simplified kinetic model can account for the pH and zinc concentration dependence of desensitization

A, mean experimental data are shown as grey lines and model fit predictions as black lines. Rate constants from the best fitted model were determined by simultaneously fitting the 6 curves in A, and are listed in Table 1. B, the best fitted model was used to predict the desensitization time constant (ms) (lines) for comparison to experimental values (symbols; $n = 7-36$). C, comparison of model predictions (lines) to data (symbols; $n = 7-36$) for the peak to steady-state current ratio across a range of Zn^{2+} and proton concentrations. D, model predictions of the proton sensor pK_a (the pH at which half of the receptors are protonated) are plotted as a function of Zn^{2+} concentration. E, concentration–effect relationships predicted by the model for proton inhibition of steady-state currents in the presence of 1 mM glutamate and the indicated Zn^{2+} concentration.

Table 1. Fitting results of an allosteric model to experimental data

Transition	Label Fig. 5	Rate	Units	K_d (nM)	Constraints
Glutamate on, glutamate on (H)	G+	8.4×10^6	$M^{-1} s^{-1}$	9524	Erreger <i>et al.</i> (2005), fixed
Glutamate on (Z), glutamate on (Z,H)	G _Z +	8.4×10^6	$M^{-1} s^{-1}$	2024	Fixed, equal to glutamate on
Glutamate off, glutamate off (H)	G-	80	s^{-1}		Erreger <i>et al.</i> (2005), fixed
Glutamate off (Z), glutamate off (Z,H)	G _Z -	17	s^{-1}		Microscopic reversibility
Zinc on	Z+	1.5×10^7	$M^{-1} s^{-1}$	1811	Free fitted parameter
Zinc on (G)	Z _G +	3.8×10^7	$M^{-1} s^{-1}$	368	Free fitted parameter
Zinc on (H)	Z _H +	1.5×10^7	$M^{-1} s^{-1}$	87	Fixed, equal to zinc on
Zinc on (G,H)	Z _{GH} +	3.8×10^7	$M^{-1} s^{-1}$	18	Fixed, equal to zinc on (G)
Zinc off	Z-	27	s^{-1}		Free fitted parameter
Zinc off (G)	Z _G -	14	s^{-1}		Free fitted parameter
Zinc off (H)	Z _H -	1.3	s^{-1}		Microscopic reversibility
Zinc off (G,H)	Z _{GH} -	0.68	s^{-1}		Microscopic reversibility
Proton on, proton on (G)	H+	1.4×10^9	$M^{-1} s^{-1}$	55	Banke <i>et al.</i> (2005), fixed
Proton on (Z), proton on (Z,G)	H _Z +	1.4×10^9	$M^{-1} s^{-1}$	3	Fixed, equal to proton on
Proton off, proton off (G)	H-	77	s^{-1}		Banke <i>et al.</i> (2005), fixed
Proton off (Z), proton off (Z,G)	H _Z -	3.8	s^{-1}		Free fitted parameter
Desensitization	—	0.12	s^{-1}		Fixed from recordings in EDTA
Desensitization recovery	—	0.02	s^{-1}		Fixed from recordings in EDTA
Opening rate	—	560	s^{-1}		$P_o = 0.5$, Erreger <i>et al.</i> (2005)
Closing rate	—	269	s^{-1}		Reciprocal of mean open time

See Fig. 4 for a description of the model and Fig. 5 for the comparison of the model fits and predictions to experimental data. P_o , open probability.

sensitivity to protons, which is manifested as a leftward shift in the functional proton concentration–response curve (Fig. 5E). Therefore the model accurately describes the established Zn^{2+} -induced shift in the proton concentration–response relationship (Choi & Lipton, 1999; Low *et al.* 2000).

Sensitivity of simulated synaptic currents to Zn^{2+} inhibition

The complex nature of the effects of extracellular Zn^{2+} on NMDA receptor currents (as well as uncertainty about the magnitude and time course of Zn^{2+} concentrations at synapses) make prediction of the effects of Zn^{2+} on synaptic currents difficult. However, our elucidation of the rate constants describing Zn^{2+} binding, glutamate binding, as well as the allosteric interaction between Zn^{2+} and glutamate binding provides a tool that can be used to explore the conditions under which Zn^{2+} might have meaningful effects on synaptic NMDA receptor function. We initially estimated the sensitivity of synaptic responses (excitatory postsynaptic currents, EPSCs) to low steady-state concentrations of Zn^{2+} by driving the allosteric model described above with a synaptic glutamate concentration waveform similar to that described for hippocampal neurones, with a peak concentration of 1.1 mM and an exponential decay time of 1.2 ms (Clements *et al.* 1992). We evaluated the sensitivity of a single pulse of

glutamate to different steady-state levels of Zn^{2+} at pH 7.3. Figure 6A confirms that NMDA receptors activated by brief synaptic-like stimuli can be inhibited by extracellular Zn^{2+} in a similar fashion to the inhibition observed for currents in response to a prolonged application of glutamate (Fig. 1). Note that the fast desensitization apparent during prolonged glutamate application (Fig. 1) does not occur under this protocol of a single brief glutamate exposure. Moreover, the occupancy of the zinc binding site remains almost constant as the ~1 ms synaptic pulse of glutamate is insufficient in duration to significantly influence zinc binding. Nonetheless, exposure to steady-state zinc in the nanomolar range inhibits the amplitude of the EPSC. Figure 6B demonstrates the concentration-dependent nature of this inhibition.

We next investigated how Zn^{2+} would influence EPSC amplitude if it were released simultaneously with glutamate at a synapse. To accomplish this, we simulated coincident release of glutamate (1.1 mM, τ 1.2 ms) and a constant amount of Zn^{2+} such that there is a sustained, step elevation of Zn^{2+} concentration. We chose to evaluate a step concentration change in Zn^{2+} since it would set an upper limit on the possible effects of Zn^{2+} co-released with glutamate, and because the time course of Zn^{2+} concentration changes in the synaptic cleft is unknown. Figure 6C demonstrates that 300 nM Zn^{2+} co-released with glutamate would have no impact on the amplitude of the synaptic current because the occupancy of the Zn^{2+} binding site does not become significant until well

after the peak of the current. Figure 6D illustrates the relative insensitivity of an individual synaptic current to co-release of Zn^{2+} over a wide concentration range. Zn^{2+} dose-response data for peak current, total charge transfer, and decay time constant are plotted in Fig. 7. The IC_{50} value for Zn^{2+} inhibition of a simulated synaptic NR1/NR2A current (160 nM, Fig. 7A) is similar to the low affinity inhibition observed for peak whole-cell currents in response to rapid application of glutamate (123 nM, Fig. 1). The sensitivity of the charge transfer (estimated

under voltage clamp) is somewhat shallower than that of the peak response because the Zn^{2+} binding increases glutamate affinity and modestly slows the deactivation time course. Figure 7B demonstrates that zinc co-released with glutamate does not appreciably alter EPSC properties. These simulation results suggest that steady-state zinc has a much stronger effect on a single synaptic NMDA current than zinc co-released with glutamate. While all simulations in Figs 6 and 7 were performed at pH 7.3, a similar insensitivity to co-released zinc is predicted by

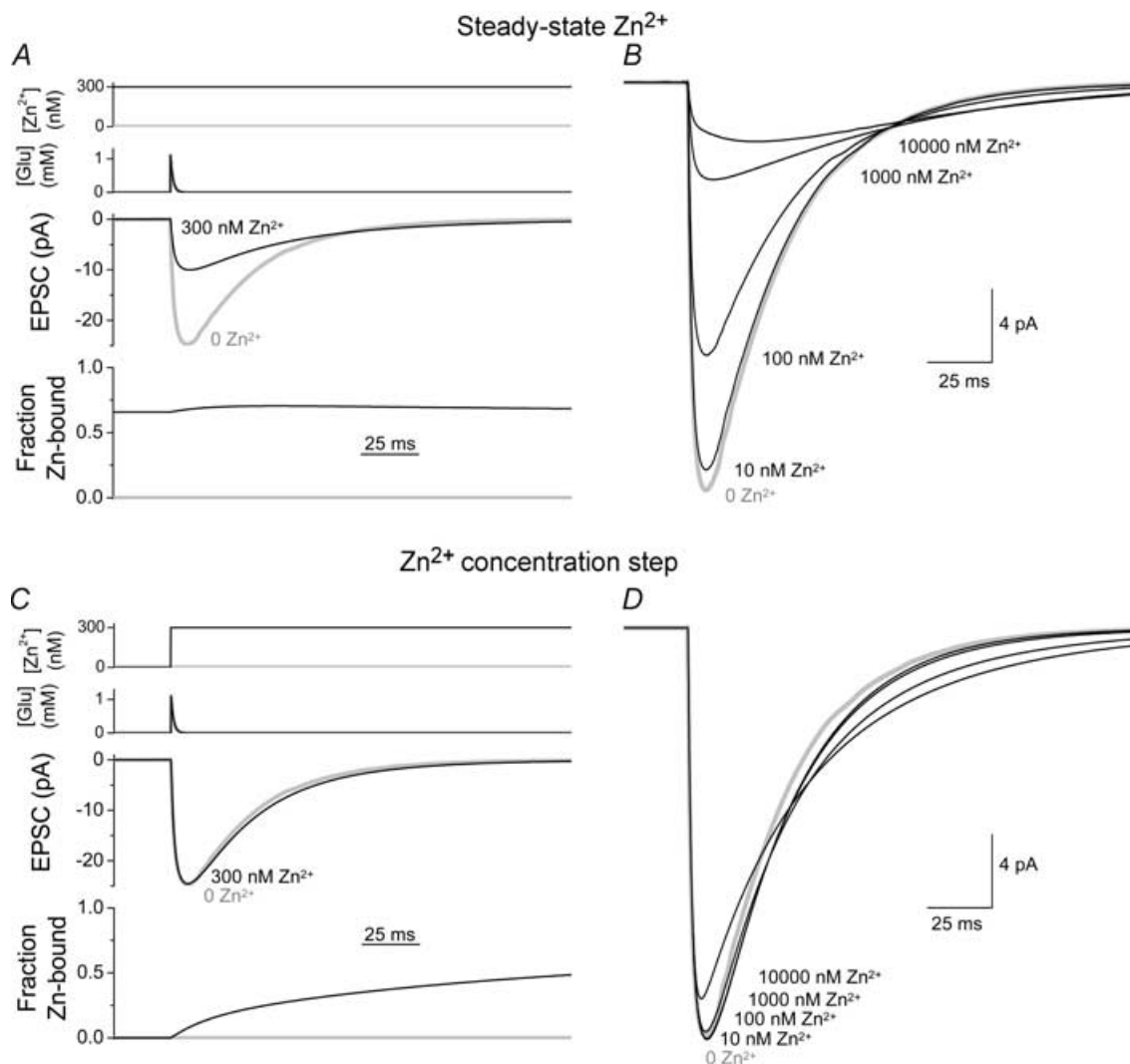


Figure 6. Simulated synaptic responses to a brief pulse of glutamate are more sensitive to steady-state Zn^{2+} than to Zn^{2+} co-released with glutamate

A, simulated current responses under voltage clamp of 20 channels are plotted in response to a 1.1 mM pulse of glutamate with an exponentially decaying time course ($\tau = 1.2$ ms; Clements *et al.* 1992) in the continuous presence of 300 nM Zn^{2+} ($V_{hold} = -60$ mV, pH 7.3). The time course for the probability of occupancy of the zinc binding site is plotted below the simulated EPSC. B, the inhibition of simulated synaptic currents is dependent on the concentration of Zn^{2+} at steady state. C, simulated current responses to a brief pulse of glutamate along with a concurrent step from 0 to 300 nM Zn^{2+} . The occupancy of the zinc binding site is slower than the EPSC time course preventing inhibition by co-released Zn^{2+} . D, simulated synaptic currents are insensitive to co-released Zn^{2+} over a wide concentration range.

simulations at lower pH. For example, at pH 6.8, $1 \mu\text{M}$ Zn^{2+} co-released with glutamate reduces the current by only 5.1%.

In order to evaluate the frequency dependence of zinc inhibition, we subsequently drove the same allosteric model presented above with a variable length train of synaptic glutamate pulses over a frequency range of 0.5–100 Hz (Fig. 8A). Figure 8B summarizes the effects of extracellular Zn^{2+} on total charge transfer associated with responses at different frequencies. As expected, these simulations show that increasing the synaptic stimulus frequency can cause more inhibition of charge transfer by Zn^{2+} due to the Zn^{2+} -induced desensitization. This reflects, as described above, increasing occupancy of the glutamate binding site, the consequent allosteric coupling of glutamate binding to Zn^{2+} binding, and the resulting relaxation to a new equilibrium as Zn^{2+} binds to its site at a higher affinity. Interestingly, the IC_{50} value for the responses at the end of a 1 s, 100 Hz train (136 nM, Fig. 8D steady state) is slightly lower than that associated with inhibition of a single pulse (160 nM, Fig. 7A), but quite similar to that associated with inhibition of a whole-cell current recorded in response to continuous application of glutamate (123 nM, Fig. 1A and B). These results suggest that any condition (such as high frequency stimulation) that promotes increased occupancy of the glutamate binding site will enhance Zn^{2+} inhibition by engaging the allosteric mechanism described here. These data suggest that the concentration–effect relationship for Zn^{2+} inhibition of synaptically evoked NMDA receptor responses resembles that for the peak current in our

concentration-jump experiments. Thus, the conclusions drawn from this study of recombinant currents appear directly applicable to the synaptic situation of a brief glutamate time course.

Discussion

The NR2A subunit is expressed widely in the central nervous system. Recombinant NR1/NR2A receptors have been used extensively as a model system to investigate the underlying mechanisms of desensitization of native NMDA receptors (Krupp *et al.* 2002; Ren *et al.* 2003; Chen *et al.* 2004). The main finding of this study is that fast glycine-independent desensitization of NR1/NR2A is mediated by an allosteric interaction between the glutamate and zinc binding site, which are both known to reside within the NR2A subunit. Activation of NMDA receptor currents by rapid application of glycine in the continued presence of glutamate does not result in this form of desensitization, demonstrating that the zinc affinity is specifically regulated by glutamate binding, not by glycine binding and not by opening of the ion channel pore.

A hypothetical model for allosteric regulation of NR2A-containing NMDA receptors

Our working hypothesis is that the semi-autonomous amino terminal domain (ATD) and the glutamate-binding domain of NR2A (S1S2 domain) interact to produce use-dependent regulation of NMDA receptor function (Fig. 4). This hypothesis is supported by

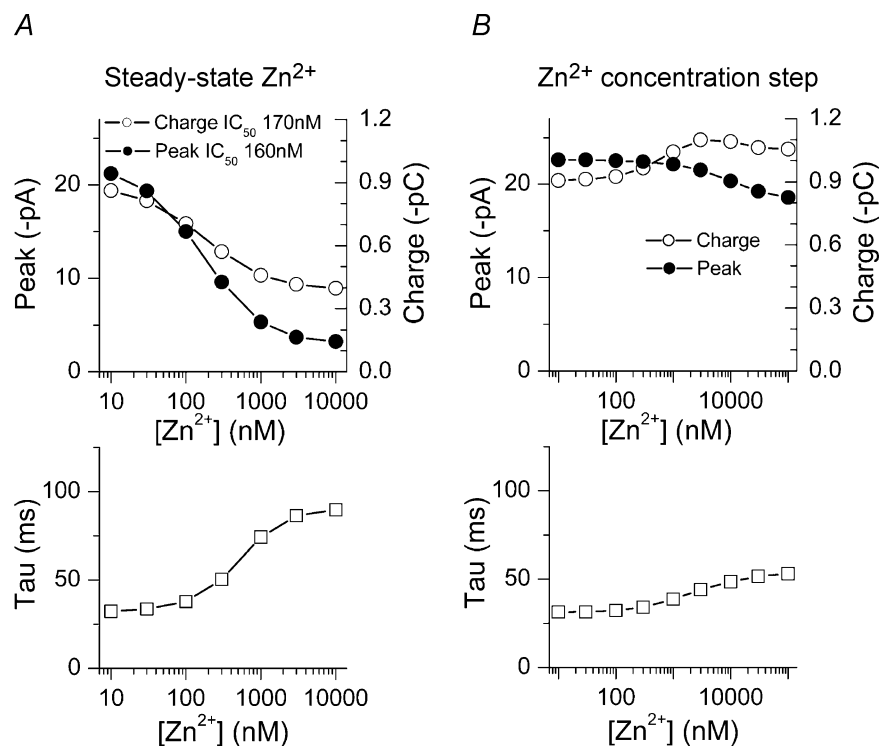


Figure 7. The concentration dependence of simulated synaptic currents to steady-state Zn^{2+} or Zn^{2+} co-released with glutamate

A, steady-state Zn^{2+} reduces both the peak current and the total charge transfer in a concentration-dependent manner. Steady-state Zn^{2+} modestly slows the time constant describing current deactivation by slowing glutamate unbinding. **B**, the amplitude of the current in response to a brief synaptic-like pulse of glutamate is relatively insensitive to Zn^{2+} co-applied with glutamate.

our demonstration that a simple allosteric model of glutamate and Zn^{2+} binding can quantitatively account for the magnitude and time course of zinc-induced desensitization. Moreover, incorporation into this model of the idea that Zn^{2+} binding changes the pK_a of a residue(s) that controls gating can further account for the pH dependence of Zn^{2+} -induced desensitization (Zheng *et al.* 2001). The model represents the following sequence of events. First, glutamate binds to the S1S2 domain of NR2A. Second, the glutamate-binding S1S2 domain and the zinc-binding ATD interact in a manner that shifts the zinc binding site from a low to a high-affinity state following glutamate binding. Third, as the system relaxes into a new equilibrium, the occupancy of the zinc binding site increases in a time-dependent manner. Fourth, zinc binding to the ATD of NR2A causes conformational

changes within the receptor that enhance the binding of protons to the pH-sensitive gating elements (Low *et al.* 2003), thereby reducing channel open probability (Traynelis & Cull-Candy, 1991; Banke *et al.* 2005). The enhancement of proton binding is sufficient to quantitatively account for the degree of inhibition by zinc (Fig. 5; Low *et al.* 2000; Zheng *et al.* 2001). In this model, the zinc-induced desensitization results from re-equilibration of zinc binding to the NMDA receptors and subsequent enhancement of tonic proton inhibition. The time course of zinc-induced desensitization represents the rate-limiting step, which is zinc binding, since glutamate binding and protonation both occur more rapidly than the time course measured for the fast component of desensitization (e.g. Lester & Jahr, 1991; Erreger *et al.* 2005; Banke *et al.* 2005).

The hypothetical model proposed here has a number of strengths and weaknesses. In terms of strengths, the model can reproduce the main features of the data by only incorporating a positive allosteric interaction between semi-autonomous domains that bind glutamate (S1S2) and Zn^{2+} (amino terminal domain), a positive allosteric interaction between Zn^{2+} binding and protonation rates, and thermodynamic balance. Moreover, the model can account for the data over a wide range of conditions (10-fold changes in Zn^{2+} and proton concentration). This quantitative representation of the working hypothesis strengthens the conclusions by demonstrating the compatibility of the hypothesis with data. The model also contains several weaknesses. For example, the Zn^{2+} affinity in simulations, which was previously reported to be enhanced at alkaline pH (Low *et al.* 2000), is modestly decreased in the model at alkaline pH. This may reflect a separate pH sensitivity of the histidine residues that chelate Zn^{2+} (Low *et al.* 2000), which is not incorporated into the model. In addition, the principle of thermodynamic balance as implemented in the model may not fully apply to protonation of an individual residue for the following reason. Binding of Zn^{2+} may alter the interaction of the ATD with a proton-sensing region of the receptor through a large number of van der Waals contacts, hydrogen bonds, and ionic or electrostatic interactions. It is likely that only a subset of these interactions is necessary to shift the pK_a of a key ionizable residue that is intimately involved in gating. This enhancement of proton inhibition probably involves alteration in the pK_a of the proton sensor through alteration in either the partial charge or hydrogen-bonded network around an ionizable residue. Therefore, it seems an oversimplification to expect protonation of a single residue to mediate the full complement of reverse effects on the full set of protein-protein contacts. The complexity of this issue will only be resolved as gating elements are determined at the atomic level, and the structural basis for pH sensitivity of gating becomes understood.

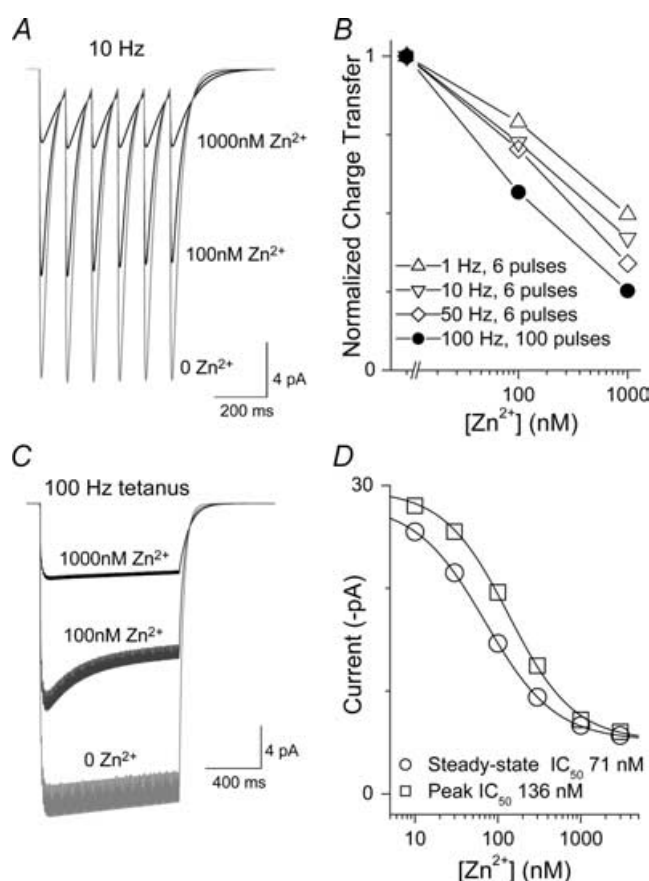


Figure 8. Simulated responses demonstrate the frequency dependence of inhibition of synaptic currents by steady-state Zn^{2+}

A, simulations were driven with 6 brief synaptic-like pulses of glutamate at a variable interstimulus interval. *B*, the total charge transfer quantified under different Zn^{2+} concentrations. *C*, simulations were driven at a frequency of 100 Hz under different Zn^{2+} concentrations. *D*, the peak dose-response curve quantifies the maximal current during the train of stimuli and the steady-state dose-response curve quantifies the current at the end of the 1 s train of stimuli.

Other shortcomings of the model are the simplification of the binding scheme to a single subunit, when it is clear that there are probably two NR2A subunits with unknown functional coupling. We have neglected any potential cooperativity between Zn²⁺-bound ATDs (Hatton & Paoletti, 2005). Finally, we have hypothesized that Zn²⁺-bound receptors can open on the basis that saturating Zn²⁺ concentrations can leave a pH-sensitive plateau current (Williams, 1996; Paoletti *et al.* 1997; Low *et al.* 2000). Yet we know little about the properties of the Zn²⁺-bound receptor, and thus the gating representation is an oversimplification. However, sensitivity analysis of the gating steps in the model show that the model can still reproduce all features of Zn²⁺ regulation of the receptor if one were to alter the closing rate of the Zn²⁺-bound open state, alter the opening rate of the Zn²⁺-bound closed state, or alter the Zn²⁺ sensitivity of this state (data not shown). That is, the model is insensitive to the kinetics of Zn²⁺-bound channel opening and does not require Zn²⁺ binding to the open state. One notable caveat to the interpretation of the simulations generated using the model is that we have assumed that pH is constant, although it is known that protons are released at synapses and can lower the pH of the cleft under some conditions (Krishtal *et al.* 1987; DeVries, 2001).

Allosteric regulation of other glutamate receptors

Whereas our data were obtained from a pure NR1/NR2A subunit population, there is strong evidence for incorporation of more than one NR2 subunit in an individual native NMDA receptor complex (Chazot *et al.* 1994; Sheng *et al.* 1994). A recent study with heterotrimeric NR1/NR2A/NR2B receptors has suggested that an individual NR2A ATD can bind Zn²⁺ with high affinity but will only induce a partial inhibition compared to having Zn²⁺ bind to both NR2 ATDs in the receptor-channel complex (Hatton & Paoletti, 2005). We hypothesize that this incomplete inhibition reflects a smaller Zn²⁺-induced shift in proton binding rates (and thus proton IC₅₀) compared to receptors that have heterodimeric NR1/NR2A subunit composition.

It is possible that the allosteric interaction described here might exist for other members of the glutamate receptor family and may be involved in the regulation of glutamate receptor function by endogenous modulators and compounds used therapeutically. A similar cooperativity has been suggested between the ifenprodil and glutamate binding site for receptors comprising NR1/NR2B subunits (Kew & Kemp, 1998; Zheng *et al.* 2001). The binding of phenylethanolamines (typified by ifenprodil) to the ATD of NR2B has also been demonstrated to enhance proton sensitivity in much the same way as zinc binding to the ATD of NR2A is hypothesized to enhance proton inhibition (Pahk & Williams, 1997; Mott *et al.* 1998; Zheng *et al.*

2001; Perin-Dureau *et al.* 2002). Similarly, a low affinity Zn²⁺ binding site has been proposed to also exist within the ATD of the NR2B subunit (Rachline *et al.* 2005). The amino terminal domain of other ionotropic glutamate receptor subunits may contain binding sites for other extracellular regulators, and such regulatory sites could be allosterically coupled to the agonist-binding site in the S1S2 domain. The allosteric interaction described here within the NR2A subunit may be a fundamental principle of the regulation of glutamate receptor function that could represent a target for the development of subunit-specific modulators.

Effects of Zn²⁺ on synaptically activated NMDA receptors

Our simulations clearly show that steady-state levels of Zn²⁺ can alter the response to synaptic stimuli by occupying the Zn²⁺ binding site in its lower affinity state before agonist binding. The model presented in Fig. 4 and Table 1 also allows us to evaluate the effect of Zn²⁺ released from synaptic vesicles, which has been a topic under intense investigation (Kay, 2003; Qian & Noebels, 2005; reviewed in detail by Frederickson *et al.* 2005). Although a consensus has yet to emerge on the concentration range and time course of synaptically released Zn²⁺ that may exist within the cleft at various synapses, our direct estimation of Zn²⁺-NR2A association rates allows the evaluation of whether synaptically released Zn²⁺ is capable of altering the synaptic NMDA channel response. To do this we used a step change in Zn²⁺ concentration coincident with simulated glutamate release since there is no information about the rate of clearance of Zn²⁺ from the synaptic cleft. Although such an event is unlikely to occur at synapses, it provides an opportunity to explore the limits at which Zn²⁺ could potentially alter the NMDA receptor response time course. The simulations demonstrate a relative insensitivity of individual synaptic currents to co-release of Zn²⁺. These results make intuitive sense given that the activation (10–90% rise time ~7 ms) and relaxation of NR2A-mediated currents (τ ~30 ms) occur more rapidly than Zn²⁺ binding (Fig. 6). At the higher range of concentrations simulated for synaptic release of zinc (> 10 μ M) binding proceeds rapidly. However, in this situation the subsequent re-equilibration of protonation becomes rate-limiting due to the Zn²⁺-induced slowing of proton unbinding, and thus activation still lags behind the rise time of the EPSC. This effect minimizes Zn²⁺-induced inhibition at the peak of the response. Although the peak amplitude of a single synaptic event appears insensitive to co-released Zn²⁺, it remains likely that co-released Zn²⁺ would have substantially greater effects on the response to high frequency stimulation, with the magnitude of the effect approaching that observed with steady-state levels of Zn²⁺ (Fig. 8). One caveat associated with the simulations of co-released Zn²⁺ is that low-affinity voltage-dependent

channel block by Zn^{2+} becomes a significant factor at concentrations $> 10 \mu M$ (Williams, 1996; Paoletti *et al.* 1997). This inhibition is not represented in our model, and may result in greater inhibition of EPSCs by Zn^{2+} than predicted by our modelling of high-affinity voltage-independent inhibition.

Our data suggest that NR1/NR2A receptors exhibit sensitivity to Zn^{2+} in the nanomolar range but only under conditions of steady-state Zn^{2+} or high frequency activity. Low frequency co-release of Zn^{2+} with glutamate is predicted to have a minimal direct impact on NR1/NR2A NMDA receptor synaptic currents, although synaptically released Zn^{2+} could be important in setting the steady-state levels. Whereas it is often suggested that NR2A-containing receptors may be tonically inhibited by Zn^{2+} due to the high-affinity binding site, we propose that not only the concentration but also the time course of both Zn^{2+} and glutamate release are critical in determining the effects of Zn^{2+} on synaptic NMDA receptors. While the IC_{50} value for high-affinity inhibition of NR1/NR2A NMDA channels in sustained glutamate is ~ 30 nM, our simulations suggest that the IC_{50} for steady-state inhibition of synaptic NR2A-containing NMDA receptors is 160 nM Zn^{2+} , implying that synaptic NMDA currents may not necessarily be under tonic inhibition by Zn^{2+} . While the magnitude of both basal and activity-dependent Zn^{2+} concentrations at the synapse remains the subject of active investigation and debate, the current study places limits on the concentration and time course of Zn^{2+} required for inhibition of NR1/NR2A NMDA receptors and provides a framework in which to interpret new data on the magnitude and time course of Zn^{2+} at the synaptic cleft.

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**Allosteric interaction between zinc and glutamate binding domains on NR2A
causes desensitization of NMDA receptors**

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