Allosteric interaction between the amino terminal domain and the ligand binding domain of NR2A

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Fast desensitization is an important regulatory mechanism of neuronal NMDA receptor function. Only recombinant NMDA receptors composed of NR1/NR2A exhibit a fast component of desensitization similar to neuronal NMDA receptors. Here we report that the fast desensitization of NR1/NR2A receptors is caused by ambient zinc, and that a positive allosteric interaction occurs between the extracellular zinc-binding site located in the amino terminal domain and the glutamate-binding domain of NR2A. The relaxation of macroscopic currents reflects a shift to a new equilibrium due to increased zinc affinity after binding of glutamate. We also show a similar interaction between the ifenprodil binding site and the glutamate binding site of NR1/NR2B receptors. These data raise the possibility that there is an allosteric interaction between the amino terminal domain and the ligand-binding domain of other glutamate receptors. Our findings may provide insight into how zinc and other extracellular modulators regulate NMDA receptor function.

Glutamate is the most widely used neurotransmitter in the vertebrate central nervous system. The NMDA receptor, a subtype of glutamate receptor exhibiting high calcium permeability, is critical in synaptic plasticity, and is implicated in a variety of neuropathological processes, including seizures and excitotoxicity associated with stroke[1]. Alteration of the time course of macroscopic NMDA currents has important functional implications because it changes the amount of calcium influx. One mechanism by which the time course of macroscopic currents can be modulated is desensitization[2], defined as a reduction of macroscopic NMDA currents in the continuous presence of glutamate. Several forms of desensitization have been reported (for review, see refs. 1, 2). The term 'glycine-dependent desensitization' is used to describe the decrement of NMDA receptor currents that occurs when glycine concentrations are not over-saturating; this is due to a negative allosteric interaction between glutamate binding and glycine binding[3,4]. The term 'glycine-independent desensitization' describes desensitization of NMDA receptors that cannot be prevented by a high concentration of glycine[5–8]. The glycine-independent desensitization described in some earlier works may involve calcium-dependent inactivation. An increase in intracellular calcium, resulting from either activation of NMDA receptors or from activation of other calcium-permeable channels, can reduce NMDA receptor currents[9–13]. 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. Recent progress in molecular biology has begun to make it possible to differentiate various forms of desensitization on a structural basis. Molecular determinants for the glycine-independent desensitization have been mapped to two distinct extracellular domains of NR2 subunits[14,15]. A four-amino-acid domain just upstream of the M1 region ('pre-M1 domain') has been reported to influence the slower component of glycine-independent desensitization ($\tau = 2$ s). A leucine/isoleucine/valine-binding protein (LIVBP)-homologous amino terminal domain ('LIVBP domain') is required for the fast component of glycine-independent desensitization[14,15].

Despite this progress, the kinetics for desensitization of NMDA receptors are not well understood. Several groups have described two components of glycine-independent desensitization of NR1/NR2A receptors[15,16], whereas others observe a single component[14]. The onset of calcium-dependent inactivation is slow in some studies[17,18], but faster in others[19,20]. The underlying basis of these discrepancies seems to be the variable degree of a fast component with a time constant of 200–300 ms. In the present study, we present several lines of evidence to suggest that this fast component of desensitization is caused by ambient zinc. The time course of this fast component reflects binding of ambient zinc to the extracellular zinc site in the amino terminal domain (ATD) after binding of glutamate to the agonist site in the S1/S2 domain, as binding of glutamate increases zinc affinity through an allosteric interaction of the two sites. We show a similar interaction between the ifenprodil and glutamate binding site for receptors comprising NR1/NR2B subunits (see also refs. 21, 22). We propose that such an allosteric interaction 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.

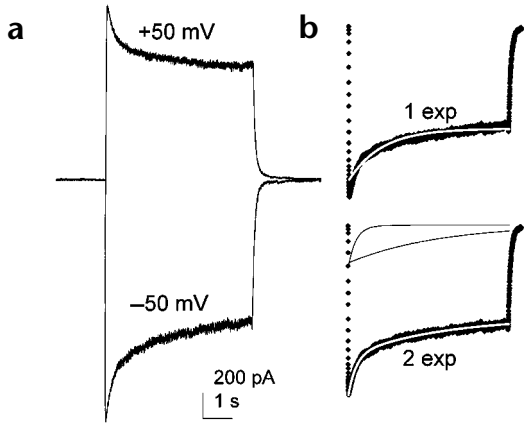

Fig. 1. Desensitization of recombinant NR1/NR2A receptors has two kinetic components. (**a**) Typical current traces recorded from HEK 293 cells expressing NR1/NR2A receptors. A rapid perfusion system applied 100 μM glutamate for 5 s. Glycine (30–60 μM) was present all the time. Ambient zinc in the recording solution was estimated to be 300 nM[24]. Similar desensitization was observed for both the inward (V_h = –50 mV) and outward current (V_h = +50 mV). (**b**) Fitted curves are plotted as smooth lines; data points are shown as dots. The desensitization was poorly fitted with a single exponential component (top, τ = 963 ms; runs test, Z = 12.78). It was fitted with two exponential components (inset, bottom panel, τ_1 = 205 ms, τ_2 = 2233 ms; runs test, Z = 1.499).

RESULTS

EDTA alters desensitization of NR1/NR2A receptors

We investigated the desensitization of recombinant NR1/NR2A receptors under conditions that favor the glycine-independent desensitization. We use the term 'desensitization' to describe the reduction in the amplitude of macroscopic currents during a prolonged application of glutamate. In the internal solution for whole-cell patch-clamp recording, we used a strong calcium buffer (5 mM BAPTA) that prevents calcium-dependent inactivation[17,18]. Glycine (at least 20× median effective concentration (EC_{50}) for NR1/NR2A), a co-agonist for NMDA receptors, was added to all solutions to prevent glycine-dependent desensitization. The degree and time course of desensitization of the inward and outward currents were indistinguishable (**Fig. 1a**). The desensitization of NMDA receptor current could not be fitted with a single exponential component (**Fig. 1b**, top); it was best fitted with two exponential components (**Fig. 1b**, bottom; τ_1 = 0.27 ± 0.04 s, τ_2 = 1.86 ± 0.26 s). On average, χ^2 was also reduced by 2.5 ± 0.6-fold by introducing the second exponential component (n = 9). Our data confirmed the presence of two distinct kinetic components of desensitization of NR1/NR2A receptors, as reported previously[15,19,20]. We also confirmed the previous finding[14,20] that this fast component is not consistently present when the C-terminal of NR1 is truncated (n = 7, data not shown).

NR1/NR2A receptors are inhibited in a voltage-independent manner by zinc that acts at a high-affinity extracellular site[1]. The median inhibitory concentration (IC_{50}) of zinc for this high-affinity site[23–25] of NR1/NR2A is 10–80 nM; thus, NR1/NR2A receptors are tonically inhibited by ambient zinc (~300 nM) in the recording solution. Removal of ambient zinc by 10 μM EDTA not only enhanced the peak currents, but also reduced the desensitization of NR1/NR2A (**Fig. 2a** and **b**). The ratio of the steady-state current (I_{ss}) over the peak current (I_{pk}) was increased from 0.47 ± 0.06 to 0.75 ± 0.05 by addition of EDTA (**Fig. 2c**, n = 9), indicating a reduction in desensitization. In the presence of EDTA, the desensitization of NR1/NR2A was best fitted with a single exponential decay (τ = 1.62 ± 0.20 s, **Fig. 2d**). Introducing a second exponential component into curve fitting for desensitization in the presence of EDTA resulted in no significant reduction of the Z value from the runs test or the χ^2 value. These data suggest that the fast component (τ = 0.27 s) of desensitization of NR1/NR2A receptors is selectively abolished by the metal chelator EDTA. Furthermore, these data also suggest that zinc does not accelerate the slow component of desensitization (**Fig. 2d**).

EDTA is also a chelator for calcium, and could potentially reduce the decay of NR1/NR2A currents by disrupting calcium-dependent inactivation. However, we used a low concentration of EDTA (10 μM), which reduced the free extracellular calcium concentration by less than 1%, but reduced the free extracellular zinc concentration to the subpicomolar range (Winmax[26]). Furthermore, we recorded under conditions that prevented the calcium-dependent inactivation. Following removal of extracellular ambient zinc with 10 μM EDTA, we found a similar degree of desensitization in calcium-containing and nominally calcium-free external solution (I_{ss}/I_{pk} = 0.78 ± 0.07, nominally 0 Ca^{2+}; I_{ss}/I_{pk} = 0.76 ± 0.05,

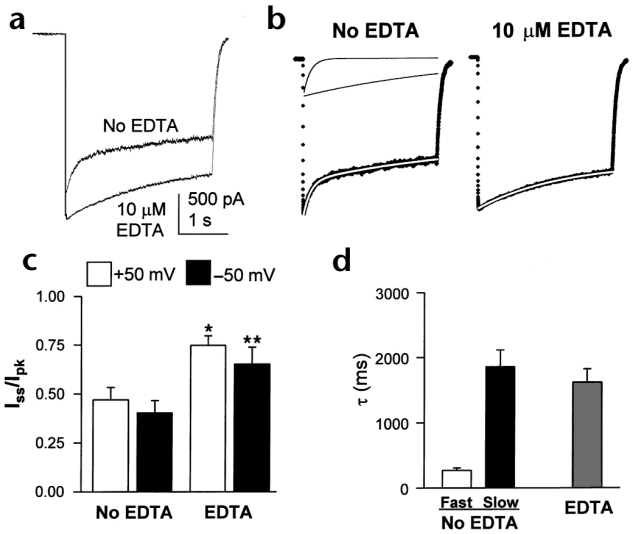

Fig. 2. EDTA abolishes the fast component of NR1/NR2A receptor desensitization. (**a**) Typical current traces in the presence and absence of EDTA (10 μM) were recorded from the same HEK 293 cell expressing NR1/NR2A (V_h = –50 mV; 100 μM glutamate, 3 s). (**b**) Fitted curves were plotted as smooth lines superimposed over the actual data (plotted as dots). Without EDTA, the desensitization was fitted with two exponential functions (τ_1 = 160 ms, τ_2 = 1.85 s). In the presence of EDTA, the desensitization was fitted with a single exponential function (τ = 1.80 s). (**c**) The degree of desensitization was shown as the ratio of the steady state current measured at the end of the glutamate application (3–5 s) over the peak current (*p < 0.01, n = 9; **p < 0.05, n = 9). (**d**) Time constants were obtained through curve fitting of the outward currents recorded at +40 or +50 mV (n = 9). In the absence of EDTA, the desensitization time course was fitted by two exponential components. In the presence of EDTA, the desensitization was fitted satisfactorily with a single exponential component. The time constant for desensitization in EDTA was not significantly different from the time constant for the slow component of desensitization in the presence of ambient zinc (p > 0.1).

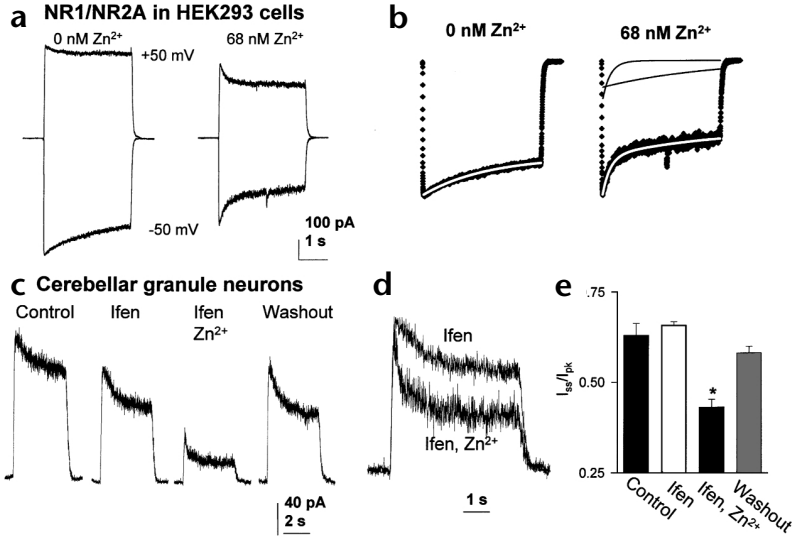

Fig. 3. Tricine-buffered zinc causes fast desensitization of recombinant and neuronal NR1/NR2A receptors. (**a**) NMDA receptor currents were recorded from the same HEK293 cell transiently transfected with NR1/NR2A at both positive and negative holding potentials (+50, –50 mV; 100 μM glutamate, 3 s). Free zinc concentrations are shown. (**b**) Current in tricine-buffered zinc was best fit by two exponential components (0.20 s and 2.8 s), whereas current in the absence of zinc was fit by one component (1.6 s). (**c**) NMDA currents in the same cultured cerebellar granule neuron maintained in 25 mM K^+ (V_h = +50 mV, pH 7.3; 1 mM NMDA, 5 s). Glycine (50 μM) was present at all times. (**d**) Normalized ifenprodil and ifenprodil/Zn^{2+} traces from (**c**). Tricine-buffered zinc caused a current relaxation (τ_{fast} = 221 ± 33 ms from 2 component exponential fits, n = 6) similar to the fast desensitization of recombinant NR1/NR2A receptors. (**e**) The mean I_{ss}/I_{pk} for ifenprodil/Zn^{2+} was significantly different from both ifenprodil and washout (ANOVA with Tukey *post hoc* test, $p < 0.01$, $n = 6$). There was no statistically significant difference among control, ifenprodil and washout conditions ($p > 0.05$).

1.8 mM extracellular Ca^{2+}; n = 3, V_h = –50 mV). Furthermore, with 1.8 mM extracellular calcium, the I_{ss}/I_{pk} for inward currents was similar to the I_{ss}/I_{pk} for outward currents (0.70 ± 0.06, V_h = –50 mV; 0.75 ± 0.06, V_h = 50 mV; $p > 0.1$, n = 7). These data suggest that the reduction of the degree of desensitization by EDTA that we observed is not likely due to alteration of calcium-dependent inactivation of these receptors. Rather, the selective removal of the fast component of glycine-independent desensitization by EDTA suggests that this fast component may be caused by ambient zinc.

Zinc causes fast desensitization of NMDA receptors

To confirm that extracellular zinc can cause the fast desensitization of NR1/NR2A receptors, we used tricine buffer to obtain the desired free zinc concentration as described previously[23,24]. The desensitization of NR1/NR2A receptors in the absence of free zinc was again best fitted with a single exponential decay (**Fig. 3a** and **b**). In the presence of tricine-buffered zinc, an additional fast component of desensitization appeared (**Fig. 3a** and **b**; see also **Fig. 6c**), and the overall degree of desensitization was increased (n = 3–5 for each free zinc concentration tested; **Fig. 5b**). Thus, the EDTA-sensitive fast component of desensitization of recombinant NR1/NR2A receptors can be restored by elevating the extracellular free zinc concentration.

Fast desensitization of NMDA receptors in neurons has been well documented[1,2] and has typically been described as glycine-independent desensitization or calcium-dependent inactivation. To determine whether zinc could also cause fast desensitization of neuronal NMDA receptors, we recorded from cultured cerebellar granule neurons, using perforated patch recording to eliminate dialysis as a confounding variable. Previous analysis of mRNAs shows that the predominant NR2 subunit expressed in these neurons after a week in high potassium media is NR2A[27]. We confirmed that NR2A is the predominant subunit, based on pharmacological properties of NMDA currents. NMDA currents showed little sensitivity to 3 μM ifenprodil (peak current, 85.6 ± 6.8% of control, n = 6; **Fig. 3c**). This concentration was sufficient to block 75 ± 6% of the NMDA current in granule neurons that were maintained in media not supplemented with high potassium (n = 3), and that were presumably expressing predominantly NR2B-containing receptors (data not shown). Granule neurons maintained in high potassium also showed high sensitivity to zinc. The peak current was reduced by 48.6 ± 5.1% in the presence of 100 nM free zinc (tricine-buffered; **Fig. 3c**, n = 6), suggesting the presence of high-affinity voltage-independent zinc inhibition[23,24]. At the same concentration, zinc also caused a fast desensitization of the outward NMDA currents in these granule neurons (**Fig. 3c–e**) that was similar to the fast desensitization of recombinant NR1/NR2A receptors. Furthermore, the enhanced desensitization was reversible upon washout of zinc (**Fig. 3c** and **e**). Taken together, our data suggest that zinc causes fast desensitization of neuronal NMDA receptors when NR2A is the predominant NR2 subunit.

Zinc acts at the extracellular site in the ATD

Previous studies have identified the amino terminal domain of NR2A as the location for the high-affinity zinc site that is responsible for the voltage-independent inhibition by zinc[28–31]. If the fast component of desensitization is caused by zinc acting at this site, it should be disrupted by mutations that disrupt the voltage-independent zinc inhibition of NR1/NR2A receptors. We selected two histidine mutations (H44G and H128A) that greatly reduce the zinc affinity[28–30], and we tested the effects of these two mutations on the desensitization of NR1/NR2A receptors. Both histidine point mutations significantly altered the desensitization of NR1/NR2A receptors in the presence of ambient zinc (**Fig. 4a**). The degree of desensitization of NR1/NR2A(H44G) receptors or NR1/NR2A(H128A) receptors was significantly less than the desensitization of wild-type NR1/NR2A receptors in the presence of ambient zinc, but was identical to the desensitization of wild-type receptors in the presence of 10 μM EDTA (**Fig. 4b**). The onset of desensitization of mutant receptors was best fitted with a single exponential component, with a time constant comparable to that observed in the presence of EDTA for the wild-type NR1/NR2A receptors (**Fig. 4c**). The elimination of the fast component of desensitization by mutations that disrupt high-affinity zinc inhibition supports our hypothesis that binding of zinc to the high-affinity site in the amino terminal domain causes fast desensitization. Henceforth, we refer to the fast desensitization caused by zinc binding to the high-affinity site in the amino terminal domain as 'zinc-induced desensitization.'

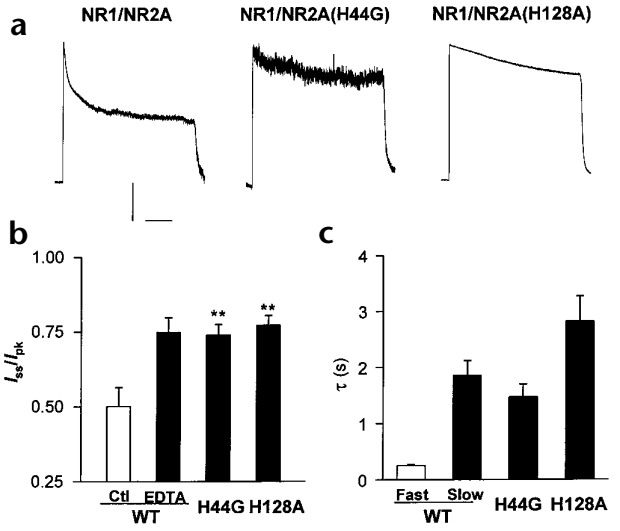

Fig. 4. Histidine point mutations in the amino terminal domain of NR2A abolish the fast desensitization of NR1/NR2A receptors. (**a**) Sample traces of wild-type and mutant NR1/NR2A receptor currents recorded from HEK293 cells in the presence of ambient zinc (V_h = +50 mV; 100 μM glutamate, 5 s). The fast component of desensitization was absent for NR1/NR2A(H44G) and NR1/NR2A(H128A). Horizontal scale bar, 1 s; vertical scale bar, 500, 160 and 800 pA for wild type, H44G and H128A, respectively. The I_{ss}/I_{pk} (**b**) and time constants (**c**) for wild-type (n = 7), H44G (n = 5) and H128A (n = 5) receptors were determined for the outward currents (V_h = +50 mV; $^{**}p < 0.05$, ANOVA with Tukey *post hoc* test).

The zinc and glutamate sites are allosterically coupled

How could zinc act at an extracellular site to cause desensitization of NR1/NR2A receptors? One possible mechanism is that the fast desensitization of NR1/NR2A receptors is caused by an allosteric interaction between the zinc and glutamate binding sites. Our hypothesis is that zinc binds to NR1/NR2A receptors with a low affinity in the absence of glutamate, and binds to the receptors with a high affinity in the presence of glutamate. By definition, allosteric interaction[32] dictates that glutamate binds NR1/NR2A receptors with higher affinity in the presence of zinc. Because the zinc-bound/glutamate-bound state is more stable than either the zinc-bound state or the glutamate-bound state, more receptors will gradually enter this state after a concentration jump into glutamate as the system relaxes to a new equilibrium. This re-equilibration of zinc will result in the time-dependent reduction of whole-cell NR1/NR2A receptor current.

We compared the zinc sensitivity for the peak and steady-state currents of NR1/NR2A receptors (**Fig. 5a**). If the zinc and glutamate binding sites are allosterically coupled, the steady-state current will be more sensitive to zinc than the peak current. To minimize the amount of the slow zinc-independent desensitization, we applied glutamate for only 400–500 ms. We chose this short protocol because separation of the two components (the fast zinc-induced component and the slow zinc-independent component) by curve fitting would fail at very low and very high concentrations of free zinc. With this short protocol, whole-cell currents do not reach steady state at lower free zinc concentrations. Subsequently, the zinc IC_{50} value determined for steady state currents may be overestimated, and thus, the degree of possible allosteric interaction between the zinc and glutamate binding sites could be underestimated. Given these limitations, we still observed a 2.5-fold difference in the zinc IC_{50} for the peak and steady-state currents. The proposed allosteric model implies reciprocity in that there should be a similar magnitude difference in the affinity of glutamate for the zinc-bound versus the zinc-unbound receptor. Consistent with previous reports[28–30], the glutamate EC_{50} was 4.4 μM in the presence of EDTA, and 2.35 μM in the presence of 1 μM zinc as measured in oocytes (n = 15, data not shown). The IC_{50} for the peak currents is not a direct measurement for the zinc affinity of glutamate-unbound receptors, as the peak is determined by the net balance of two processes: the rate of fully liganded receptors going into the open state, and the rate of the fully liganded receptors going into the zinc-bound, high-affinity state. Therefore, the IC_{50} for peak currents is partially influenced by the higher-affinity state, and would be an overestimation for the true zinc affinity of glutamate-unbound receptors. The true degree of the allosteric interaction

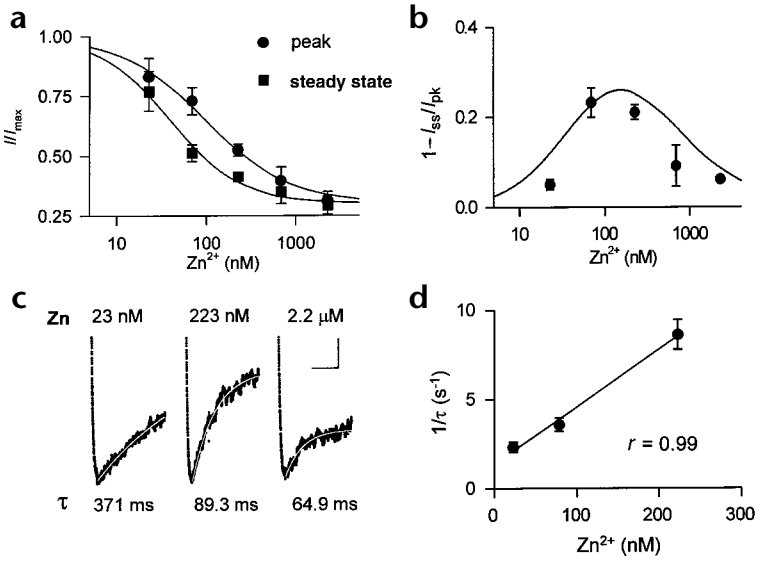

Fig. 5. The degree and the time course of the zinc-induced fast desensitization of NR1/NR2A receptors depend on extracellular free zinc concentration. (**a**) A rapid perfusion system applied a 400-ms pulse of 100 μM glutamate. Tricine buffer was used to achieve the desired free zinc concentrations. The maximal currents for the peak and steady state were defined as the peak and steady-state currents in nominally zinc-free solutions (10 mM tricine without added zinc). For the peak currents, the zinc IC_{50} was 97 nM. For the steady-state currents, the zinc IC_{50} was 37 nM. The Hill slopes were 0.92 and 1.09 for the peak and steady-state currents, respectively (n = 4–7). (**b**) The predicted dose–response curve for the degree of zinc-dependent desensitization (smooth curve) was in agreement with data measured using a 400–500 ms glutamate pulse (circles, n = 5–9 for each free zinc concentration). (**c**) Normalized sample current traces recorded in the same cell in the presence of free extracellular zinc were shown on an expanded time scale. Scale bar, 100 ms; 15, 12 and 9 pA for currents recorded in the presence of 23, 223 and 2230 nM zinc, respectively). Tricine buffer was used to achieve desired free zinc concentrations. (**d**) The time constant of the fast desensitization depended on the free zinc concentration. A short glutamate pulse was used to minimize slow glycine-independent desensitization. The onset of desensitization was then fitted with a single exponential component (n = 3–12 for each concentration of free zinc tested; r, correlation efficiency).

is likely to be greater than the 2.5-fold difference indicated by the differences in zinc IC_{50} for the peak and steady-state currents.

If the underlying mechanism for the zinc-induced desensitization is an allosteric interaction that occurs when glutamate binds to the S1/S2 domain of NR2A and increases affinity for zinc, then one would predict that the degree of zinc-induced desensitization would be less at very low and very high concentrations of free zinc. At very low concentrations, there is not enough free zinc to substantially alter the occupancy of the high affinity zinc site in the amino terminal domain. At very high concentrations, zinc would saturate all binding sites even before the application of glutamate, reducing the impact of enhancement of zinc affinity by glutamate. Thus, the predicted dose–response curve for the degree of zinc-induced desensitization would be bell-shaped (**Fig. 5b**, **Eq. 5**). This predicted curve is in general agreement with the degree of desensitization measured at various concentrations of free zinc (**Fig. 5b**).

If the onset of the fast desensitization of NR1/NR2A receptors reflects zinc binding, then the time constant for this component should be dependent on the free zinc concentration, and from the concentration dependence of the time constant, we can calculate the dissociation constant (K_d) for zinc. Indeed, the time constant showed strong dependence on the free zinc concentration ($p < 0.001$; **Fig. 5c** and **d**). Based on the linear regression of data presented in Fig. 5d (**Eq. 6**), the k_{on} and k_{off} rates for zinc are 3.22×10^7/M/s and 1.35/s, respectively (see Methods). From these microscopic rate constants, we calculate that the K_d for zinc is 42 nM, which is in agreement with the zinc IC_{50} determined for the steady-state currents (37 nM; **Fig. 5a**).

Zinc-induced desensitization is pH-dependent

Zinc, acting at the high-affinity site in the amino terminal domain of NR2A, inhibits NR1/NR2A receptors by enhancing tonic proton inhibition[28,29]. Such a mechanism provides accurate prediction for the pH-dependency of the residual currents observed when the high-affinity zinc site is saturated[29]. The residual currents are smaller at lower pH values, at which more free protons are available to inhibit the zinc-bound receptors. As our hypothesis implies that the amount of residual current during full occupation of the zinc binding site is pivotal in determining the degree of zinc-induced desensitization (**Eq. 5**), the degree of zinc-induced desensitization should be pH-dependent in the same manner. At alkaline pH values, the degree of zinc-induced desensitization would be greatly diminished, because the lack of free protons prevents zinc/glutamate-bound receptors from being inhibited. At lower pH values, the increased free proton concentration leads to reduced residual currents, and therefore greater degree of zinc-induced desensitization.

Indeed, the degree of zinc-induced desensitization showed the predicted pH-dependency. The zinc dose–response curve for steady-state currents in HEK293 cells (**Fig. 6a**) showed that the residual current in the presence of saturating zinc concentration was smaller at more acidic pH values. The degree of desensitization predicted using these residual current values (**Fig. 6b** and **c**) was in agreement with the observed values.

Ifenprodil produces desensitization of NR1/NR2B

Our data suggest that the amino terminal domain and the glutamate-binding domain of NR2A interact, causing the fast desensitization of NR1/NR2A receptor currents in the presence of submaximal levels of zinc. Because homologous amino terminal domains are present in all glutamate receptor subunits, it is possible that the allosteric interaction between the amino terminal domain and glutamate-binding domain is a general form of regulation of glutamate receptor function. Data from chimeric receptors and point mutations suggest that the ifenprodil binding site might be located in the amino terminal domain of NR2B[33,34]. Furthermore, zinc inhibition of NR2A-containing receptors and ifenprodil inhibition of NR2B-containing receptors share the same mechanism, enhancement of tonic proton inhibition at physiological pH[22,28,29]. By drawing analogy to the effects of zinc on NR2A, one would predict that submaximal ifenprodil should produce desensitization of macroscopic NR1/NR2B receptor currents. Thus, we examined the effects of submaximal concentrations of ifenprodil on the relaxation of whole-cell currents of NR1/NR2B receptors.

Under control conditions, there is only minimal desensitization for NR1/NR2B receptors with an averaged I_{ss}/I_{pk} of 0.90 ± 0.02 ($n = 6$, **Fig.7a** and **b**). Ifenprodil (270 nM) induced a time-dependent relaxation in current responses. As a result of this time-dependent relaxation, the I_{ss}/I_{pk} was significantly reduced (**Fig. 7b**). Previous studies have shown that a point mutation in the amino terminal domain of NR2B, E201R, reduces ifenprodil binding[33,34]. The desensitization caused by ifenprodil was abolished by the same point mutation (**Fig.7c** and **d**). These data are con-

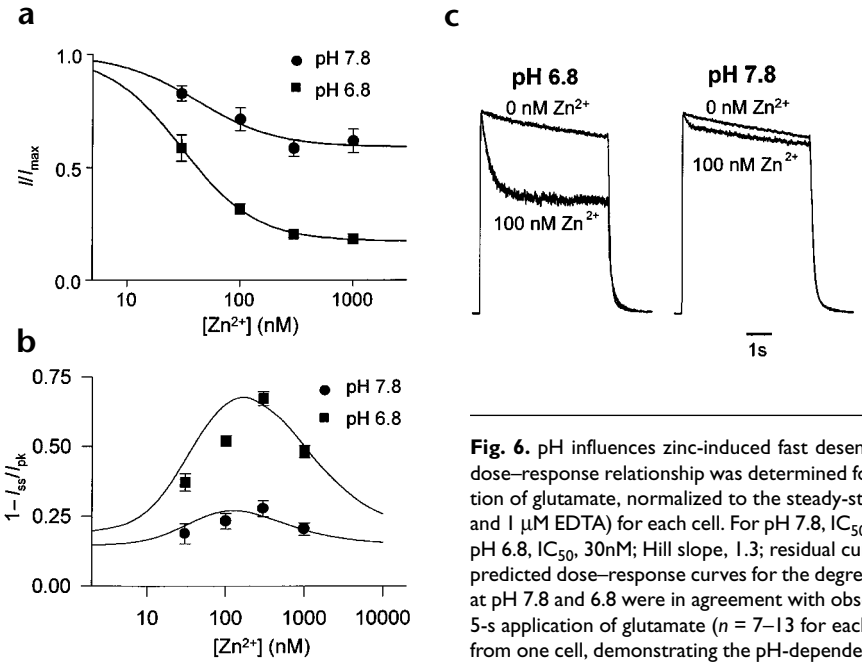

Fig. 6. pH influences zinc-induced fast desensitization of NR1/NR2A receptors. (**a**) The zinc dose–response relationship was determined for steady-state current at the end of a 5-s application of glutamate, normalized to the steady-state current in the absence of zinc (10 mM tricine and 1 μM EDTA) for each cell. For pH 7.8, IC_{50}, 41 nM; Hill slope, 1.2; residual current, 0.59. For pH 6.8, IC_{50}, 30nM; Hill slope, 1.3; residual current, 0.17 (n = 7–13 for each condition). (**b**) The predicted dose–response curves for the degree of zinc-induced desensitization (smooth curves) at pH 7.8 and 6.8 were in agreement with observed data (circles and squares) measured using a 5-s application of glutamate (n = 7–13 for each free zinc concentration). (**c**) Normalized traces from one cell, demonstrating the pH-dependency of zinc-induced desensitization.

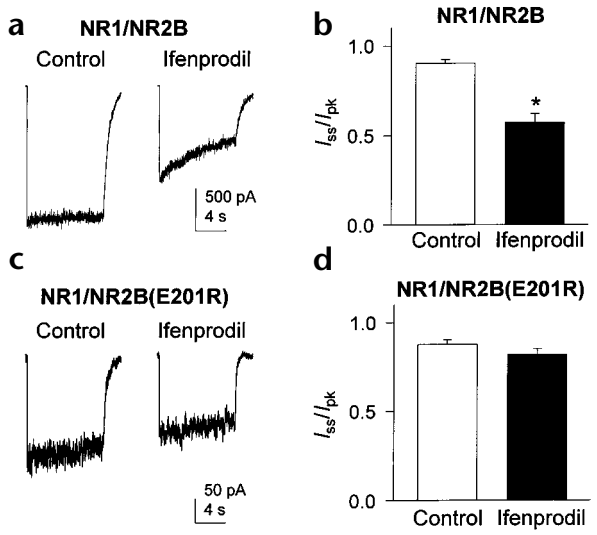

Fig. 7. Ifenprodil produces desensitization of NR1/NR2B receptors. (**a**) Typical current traces in the presence and absence of ifenprodil from a HEK 293 cell expressing NR1/NR2B. A rapid perfusion system applied a 10-s pulse of 100 μM glutamate. Glycine (20 μM) and ifenprodil (270 nM) were added to both the glutamate and wash solution (V_h = –50 mV). (**b**) The degree of desensitization was determined by the ratio of the steady state current measured at the end of a long glutamate pulse (10 s) to the peak current. Ifenprodil caused desensitization of NR1/NR2B receptors (*p < 0.01, n = 6). (**c**) Sample traces of mutant NR1/NR2B(E201R). Recording conditions were identical to that for the wild-type NR1/NR2B receptors. Ifenprodil (270 nM) had no effect on desensitization. (**d**) Desensitization of NR1/NR2B(E201R) receptors was identical in the presence and absence of ifenprodil (p > 0.01, n = 4).

sistent with the presence of an allosteric interaction between the amino terminal domain and the glutamate-binding domain of NR2B (see also refs. 21, 22) that is analogous to the allosteric interaction between the amino terminal domain and the glutamate-binding domain of NR2A.

Discussion

Recombinant NR1/NR2A receptors have been used extensively as a model system to investigate the underlying mechanism of desensitization of NMDA receptors, because NR1/NR2A receptor and NMDA receptor forms of desensitization are thought to closely resemble one another. In particular, only receptors comprising NR1/NR2A subunits exhibit a fast onset of glycine-independent desensitization similar to that of neuronal NMDA receptors. In this study, we demonstrated by four lines of evidence that this fast component of the glycine-independent desensitization of NR1/NR2A is likely caused by extracellular ambient zinc, and the time course of the relaxation of macroscopic currents reflects zinc binding to the extracellular high-affinity site. First, removal of ambient zinc by the metal chelator EDTA selectively abolishes this fast component without any effect on the slow component of glycine-independent desensitization. Second, tricine-buffered zinc reproduces the fast glycine-independent desensitization of NR1/NR2A. Third, mutations that disrupt the voltage-independent zinc inhibition also abolish the fast desensitization. Fourth, the K_d for zinc determined from kinetic analysis of the concentration dependence of the onset time constants of the fast desensitization is identical to the zinc IC_{50} for the amplitudes of steady-state currents. The last point is perhaps the strongest evidence in support of our hypothesis. Thus, the fast component of the glycine-independent desensitization of NR1/NR2A receptors (τ = 0.3 s) is mechanistically distinct from the slow component (τ = 1.8 s). It requires not only the presence of the agonist, but also the presence of an allosteric modulator, that is, zinc. Our results further extend the model for desensitization of neurotransmitter receptors. In addition to the fast glycine-dependent desensitization of NMDA receptors that is caused by an allosteric interaction between the agonist (glutamate) and co-agonist (glycine) binding sites[3,4], our data suggest that desensitization could also be caused by an allosteric interaction between the binding sites for the agonist and an allosteric modulator.

This zinc-induced desensitization has caused some confusion in the literature. Because the onset kinetics depend on the free zinc concentration, variations in the amount of ambient zinc would make this fast component variable and at times difficult to separate from the slow component of glycine-independent desensitization. For example, a single component with a time constant of 700 ms has been observed under nominally calcium-free conditions[16]. This time constant is significantly faster than the slower component of glycine-independent desensitization (t = 1.8 s), suggesting that they may have observed a mixture of the zinc-dependent component and the slow glycine-independent component. The prominent fast desensitization described as 'calcium-dependent inactivation' in the presence of 5 mM intracellular EGTA has been reported to disappear in the presence of extracellular EDTA (1 mM)[20], which is used to remove extracellular free calcium, but also removes ambient zinc. C-terminal truncation of NR1 may alter zinc or pH-sensitivity of NR1/NR2A receptors (F.Z., unpublished data). Subtle changes in zinc or pH sensitivity could alter the degree and kinetics of zinc-induced desensitization, making it more difficult to detect through curve fitting[14].

The zinc-induced desensitization of NR1/NR2A receptors could be described as a defined sequence of events involving two specific structural domains of NR2A, the ligand binding domain and the amino terminal domain. Previous studies have

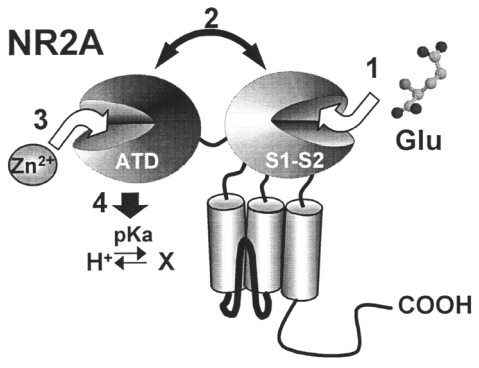

Fig. 8. A model for an allosteric interaction between the amino terminal domain and S1/S2 domain of NR2. We hypothesize that the desensitization caused by zinc results from the following events. (1) Glutamate binds to the S1-S2 domain of NR2A. (2) Glutamate binding leads to allosteric changes in the amino terminal domain that alter zinc affinity. (3) As the system relaxes into a new equilibrium, the occupancy of the zinc binding site increases in a time-dependent manner. (4) Zinc binding to the amino terminal domain of NR2A causes conformational changes of the receptor that enhance binding of protons to the pH-sensitive gating elements[28,29], shifting more receptors into closed states[35].

proposed two functional roles for the amino terminal domain of NR2A: involvement in the fast component of desensitization of NR1/NR2A receptors[14,15] and location of the high-affinity extracellular zinc site[28–31]. Our data demonstrate that involvement of the amino terminal domain in the fast desensitization is the functional consequence of the zinc-binding site in the amino terminal of NR2A. Although the amino terminal domain and the glutamate-binding domain of NR2A (that is, S1/S2 domain) are modular, they interact to produce use-dependent regulation of NMDA receptor function (**Fig. 8**). Specifically, we propose that the zinc-induced desensitization is due to the following molecular events. First, glutamate binds to the S1/S2 domain of NR2A. Second, glutamate binding leads to allosteric changes of the amino terminal domain of NR2A, increasing the affinity of the zinc binding site. 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 amino terminal domain of NR2A causes conformational changes of the receptor that enhance binding of protons to the pH sensitive gating elements[28,29], shifting more receptors into closed states[35]. 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 that is likely zinc binding, as glutamate binding[36] and protonation[35] both occur more rapidly than the 270-ms time constant measured for the fast component of desensitization. A similar sequence of events may occur for ifenprodil-induced desensitization of NR2B.

It is possible that our finding of an allosteric interaction between the amino terminal domain and the glutamate-binding domain of NR2A and NR2B is subunit-specific. However, it may well extend to other members of glutamate receptor family. The amino terminal domain of glutamate receptor subunits beyond NR2A and NR2B may contain a binding site for other extracellular regulators, and such regulatory sites could be allosterically coupled to the agonist-binding site in the S1/S2 domain. At present time, this hypothesis could not be tested for different subtypes of glutamate receptors because little is known about whether ligands exist for the amino terminal domain of various glutamate receptor subunits. However, the multiplicity of common mechanistic features in regulation of receptor function by the amino terminal domain of NR2A and NR2B make it unlikely that the allosteric interaction between the amino terminal domain and the agonist-binding domain reported here is incidental. Rather, it seems to be a fundamental part of the regulation of NMDA receptor function.

Methods

Transfection of HEK cells. HEK 293 cells (CRL 1573; ATCC, Rockville, Maryland) were maintained at 37° and 5% CO_2, as described previously[24]. Low-confluency cells were transfected by the calcium phosphate precipitation method[37], with a mixture containing NR1-1a, NR2A (or NR2B) and GFP[38] plasmids (1, 2 and 0.3 μg per 12-mm diameter coverslip, respectively). After transfection, NMDA antagonists (100–200 μM AP5, 2 mM Mg^{2+}, 5–10 mM kynurenic acid) were added to the culture medium.

Buffered zinc solutions. The tricine-buffered zinc solutions used to obtain the zinc dose–response curves were prepared according to the empirically established binding constant 10^{-5} M as described previously[23,24].

Whole-cell patch-clamp recording from HEK 293 cells. Patch-clamp recording in the whole-cell configuration[39] was made with an Axopatch 200B amplifier (Axon Instruments, Union City, California) or a PC501A amplifier (Warner, Hamden, Connecticut). Recording electrodes (5–12 MΩ) were filled with 140 mM Cs-gluconate, 5 mM HEPES, 4 mM NaCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM ATP, 0.3 mM GTP and 5 mM BAPTA (pH 7.4, 23°C). The recording chamber was continually perfused with recording solution composed of 150 mM NaCl, 10 mM HEPES, 1 mM $CaCl_2$, 3 mM KCl and 10–20 mM mannitol (pH 7.4 unless otherwise noted). Glutamate (100 μM) was applied using a multibarrel pipette driven by a piezo-electric bimorph[40] or a nanostepper (SF77B, Warner) with an exchange time of 0.5 ms and 2–8 ms, respectively. Glycine (30–60 μM) was present all the time. Data used for analysis were collected within 5–15 min after initial break-in to minimize time-dependent change of the glycine-independent desensitization. In some experiments, series resistance was corrected off-line[41]. Correction of series resistance did not alter the zinc-induced current relaxation.

Whole-cell perforated-patch recording from cerebellar granule cells. Cerebella from 4- to 7-day postnatal Sprague–Dawley rats were isolated, passed through a 210 μM nylon mesh and plated onto glass coverslips coated with 5 μg/mL poly-D-lysine (approved by Institutional Animal Care and Use Committee of Emory University). Cultures were maintained for 6–8 days at 37°C and 5% CO_2 in DMEM supplemented with L-glutamine (0.2 mM), pyruvate (0.1 mM), penicillin/streptomycin (100 units/mL), 10% fetal bovine serum and 25 mM KCl. Recording electrodes (5–9 MΩ) were filled with the same solution used for HEK293 cell recording with 25 μg/mL gramicidin (Sigma, St. Louis, Missouri). It took 20–30 min to achieve acceptable perforation with series resistances ranging from 15 to 40 MΩ. In the continued presence of glycine (50 μM), NMDA (1 mM) was applied by local perfusion through a capillary tube (1.1 mm inner diameter) positioned near the cell. The solution flow was driven by gravity (flow rate, 1–5 ml/min) and controlled by solenoid valves (Lee, Westbrook, Connecticut). Series resistance correction was not applied given the low amplitude of typical currents (~100 pA).

Curve fitting. The time course of desensitization was fitted with one or two exponential components with NPM (S.F. Traynelis, Emory Univ.) using the following equation.

$$A(t) = A_0 + \sum_n A_n \exp(-t/\tau_n) \quad (1)$$

Here, A_0 is the offset; A_n and τ_n are the amplitudes and time constants for each exponential components.

The zinc IC_{50} was determined by fitting the dose–response curve to the following equation.

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

Here, a and n are the residual and the Hill slope, respectively.

For a given free zinc concentration, the following equations apply.

$$I_{ss} = I_{max}((1-a)/(1 + ([Zn^{2+}]/IC_{50,ss})^n{}_{ss}) + a) \quad (3)$$

$$I_{peak} = I_{max}((1-a)/(1 + ([Zn^{2+}]/IC_{50,peak})^n{}_{peak}) + a) \quad (4)$$

Therefore, the degree of zinc-dependent desensitization, $1 - I_{ss}/I_{pk}$, can be calculated by the following equation.

$$1 - I_{ss}/I_{pk} = 1 - ((1-a)/(1 + ([Zn^{2+}]/IC_{50,ss})^n{}_{ss}) + a)/((1-a)/(1 + ([Zn^{2+}]/IC_{50,peak})^n{}_{peak}) + a) \quad (5)$$

To predict the desensitization for Fig. 6b, an additional offset term was added to Eq. 5 to account for the slow desensitization observed in the absence of zinc. This empirically determined value was 0.20 ± 0.02 (n = 30) for pH 6.8 and 0.14 ± 0.02 (n = 36) for pH 7.8.

Assuming that zinc binds with the extracellular domain of NMDA receptor at a single site, the onset of zinc binding could be described by the following equation.

$$1/\tau_{on} = k_{on}[Zn^{2+}] + k_{off} \quad (6)$$

Here, k_{on} and k_{off} are the association and dissociation rate constants, respectively. These two rate constants could be estimated based on linear regression of Fig. 5d.

Statistics. All pooled data are expressed as mean ± s.e.m. Unpaired Student's *t*-test was used unless stated otherwise. Quality of fits with one or two exponential components was assessed using χ^2 and runs test[42]. The critical *Z* value was 1.96 for the random distribution (that is, symmetrical distribution above and below the fitted curve; $n > 100$, $\alpha = 0.05$). If the *Z* from runs test was less than the critical *Z* value, the fit was considered as good. If the *Z* was greater than the critical *Z* value, the fit was rejected.

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