

Tyrosine kinase potentiates NMDA receptor currents by reducing tonic zinc inhibition

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Activation of the tyrosine kinase Src potentiates NMDA-receptor currents, which is thought to be necessary for induction of hippocampal long-term potentiation. Although the carboxy(C)-terminal domain of the NR2A subunit contains potential tyrosine phosphorylation sites, the mechanism by which Src modulates synaptic plasticity and NMDA receptor currents is not fully understood. Here we present evidence from NR1 mutants and splice variants that Src potentiates NMDA-receptor currents by reducing the tonic inhibition of receptors composed of NR1 and NR2A subunits by extracellular zinc. Using site-directed mutagenesis, we have identified three C-terminal tyrosine residues of NR2A that are required for Src's modulation of the zinc sensitivity of NMDA receptors. Our data link two modulatory sites of NMDA receptors that were previously thought to be independent.

Recent studies suggest that tyrosine kinases are important for synaptic plasticity¹⁻³. The requirement for Src activation in long-term potentiation (LTP) induction has been attributed to its enhancement of NMDA-receptor currents⁴⁻⁶ because an NMDA-receptor antagonist blocks this effect³. The non-receptor tyrosine kinases Src and Fyn potentiate receptors composed of NR1 and NR2A subunits, but have no apparent effect on receptors composed of NR1 and other NR2 subunits⁵. Furthermore, deletion of the C-terminal domain of NR2A eliminates the potentiation of NR1/NR2A-receptor currents by Src⁵ and impairs synaptic plasticity and contextual memory⁷. Thus, several lines of evidence suggest that a unique tyrosine phosphorylation site on the C-terminal of NR2A may confer sensitivity to Src and be critical for the induction of LTP. However, the mechanisms by which tyrosine kinase potentiates NMDA-receptor function are unknown.

In addition, NR2B subunits are phosphorylated by one of the Src family of tyrosine kinases⁸, but no functional effect of this phosphorylation has been identified. This discrepancy between biochemical and physiological data raises the possibility that either tyrosine phosphorylation of NR2B affects receptor properties other than channel activity, or another property of NR2A may be responsible for the apparent selective potentiation by tyrosine kinase of receptors containing this subunit.

NMDA receptors are allosterically modulated by a variety of endogenous extracellular ions⁹⁻¹³. Zinc, one of these modulators, is accumulated in some nerve terminals in specific brain regions and released during neuronal activity¹⁴. Zinc inhibits NMDA receptors at two independent sites¹⁵⁻¹⁷. A low-affinity zinc site is probably located inside the channel pore, and binding of zinc to this site causes a voltage-dependent inhibition of NMDA-receptor channels. A high-affinity site is likely located outside the channel pore and causes a voltage-independent inhibition. Receptors containing NR2A subunits exhibit far greater affinity for zinc at the extracellular, voltage-independent site than receptors containing

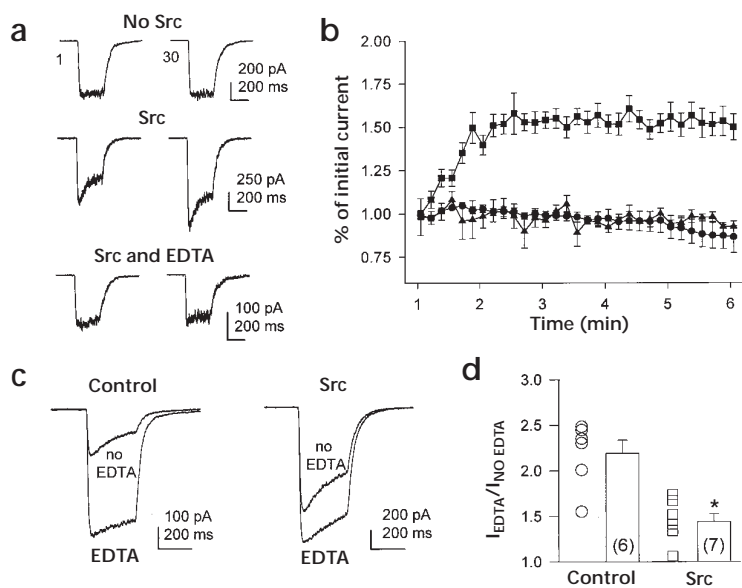
other NR2 subunits¹⁸⁻²¹. This sensitivity to zinc is high enough to allow ambient zinc (either *in vivo*²² or as a contaminant of experimental solutions²³) to tonically inhibit NR1/NR2A receptors. Here we show that Src reduces zinc sensitivity of recombinant NR1/NR2A and NR1/NR2B receptors, thereby relieving tonic inhibition by zinc and potentiating the NMDA-receptor response. Splice variants and mutants of NR1 subunits that have a low apparent affinity for zinc are, as predicted, potentiated to a lesser degree by Src. Conversely, NR1 mutants with higher apparent zinc affinity are potentiated to a greater degree. Using site-directed mutagenesis, we have identified three C-terminal tyrosine residues of NR2A that are required for Src's modulation of NMDA-receptor zinc sensitivity. By showing that Src potentiates NMDA receptors by specifically reducing tonic zinc inhibition, our data link two modulatory sites of NMDA receptors that were previously thought to be independent.

Results

Src REDUCES TONIC INHIBITION OF NR1/NR2A RECEPTORS

Our hypothesis was that potentiation of NMDA receptor currents by Src may be due to its relief of tonic inhibition by zinc acting at the voltage-independent site, in a manner analogous to polyamine relief of tonic proton inhibition of NMDA receptors¹³. To test this hypothesis, we examined the effects of EDTA, a chelator of transition metals such as zinc, on Src's potentiation of NR1/NR2A receptors. The NMDA receptor currents were recorded with a rapid perfusion system in HEK293 cells transiently transfected with NR1-1a/NR2A receptors. In control experiments, the amplitudes of evoked NMDA-receptor currents did not change significantly over time (Fig. 1a and b). When purified recombinant human c-Src was added to the internal solution, the amplitude of NMDA-receptor currents was gradually increased over a five-minute period (Fig. 1a). On average, the peak of NMDA receptor currents evoked by 100 μ M glutamate and 30 μ M

Fig. 1. Src potentiates NR1/NR2A receptors by reducing tonic zinc inhibition. **(a)** Removal of tonic zinc inhibition of NR1-1a/NR2A receptors occludes the potentiation of these receptors by Src. NMDA-receptor current traces at left (labeled 1) were the first of a series of current traces recorded 60–90 seconds after obtaining the whole-cell configuration. Traces at right (labeled 30) were the last of 30 consecutive traces recorded every ten seconds over a five-minute period. EDTA is added into extracellular solutions to chelate zinc in some experiments, whereas recombinant human c-Src is added into the internal solution of patch pipettes. Under control conditions, no significant rundown of the NMDA-receptor current was observed. Inclusion of c-Src (30 units per ml) resulted in a 50% potentiation. However, this potentiation was not observed in the presence of 10 μ M EDTA. **(b)** Average changes of the NMDA-receptor current amplitudes over the same periods. Error bars are standard error for all panels. Number of cells is indicated in parentheses. \square control (5), \triangle Src (10), \circ Src and EDTA (5). **(c)** The effects of EDTA (10 μ M) on NMDA currents evoked by agonists (100 μ M glutamate, 30 μ M glycine) from representative HEK 293 cells with or without inclusion of Src in the pipette solution ($V_h = -50$ mV). **(d)** The potentiation of whole-cell NR1-1a/NR2A-receptor currents by EDTA in HEK cells is reduced by inclusion of Src in the patch pipette (* $p < 0.01$). Circles are control cells; squares are cells perfused with Src.



glycine was potentiated by $53 \pm 9\%$ ($n = 10$), whereas the peak of currents evoked by 300 μ M glutamate and 100 μ M glycine was potentiated by $53 \pm 10\%$ ($n = 4$). Our data agree with the 40% potentiation of recombinant NR1-3a/NR2A receptor by Src reported previously⁵. As others⁵ and we have used saturating concentrations of glutamate and glycine, it is unlikely that the potentiation of NMDA receptors by Src is due to a change in apparent affinity for glutamate or glycine.

If our hypothesis is correct, complete removal of the tonic inhibition should prevent the potentiation of NR1/NR2A receptor currents by Src. To remove transition-metal contamination, we added a trace amount of EDTA (10 μ M) to all recording solutions. Assuming 100 nM contaminant zinc and 1 mM extracellular Ca^{2+} , the free zinc is less than 0.03 nM, whereas the free Ca^{2+} remains at 0.99 mM. In the presence of EDTA, Src failed to potentiate NR1/NR2A receptors (Fig. 1a and b). In the presence of 10 mM tricine, another metal chelator, Src also failed to potentiate NR1/NR2A receptors ($n = 10$, data not shown). Thus, removal of tonic inhibition of NR1/NR2A receptors by transition metals occludes potentiation by Src.

We used the ratio of the peak NMDA-receptor current in the presence of EDTA (I_{EDTA}) to the peak current in the absence of EDTA ($I_{NO EDTA}$) as a measure of the amount of tonic inhibition of NR1/NR2A by transition metals. A smaller ratio indicates less tonic inhibition. Without Src added to the patch pipettes, $I_{EDTA} / I_{NO EDTA}$ ranged from 1.6 to 2.5 (mean, 2.2 ± 0.1 , $n = 6$, Fig. 1c and d). With Src added to the patch pipettes, $I_{EDTA} / I_{NO EDTA}$ ranged from 1.2 to 1.7 (mean = 1.4 ± 0.1 , $n = 7$). Thus, Src significantly reduced the tonic inhibition of NR1/NR2A receptors by transition metals ($p < 0.005$). Inclusion of the phosphatase inhibitor orthovanadate (500 μ M) with Src results in an $I_{EDTA} / I_{NO EDTA}$ value of 1.18 ± 0.03 ($n = 3$), which is not significantly different from the value with Src alone.

SRC SHIFTS IC_{50} FOR THE HIGH-AFFINITY, ZINC SITE

Zinc is probably the transition metal responsible for the EDTA-sensitive tonic inhibition of NR1/NR2A receptors, although

there are several other candidate ions, such as cadmium or copper¹⁸. To directly assess the effect of Src on zinc-induced inhibition of NR1/NR2A-receptor currents, we used tricine-buffered zinc to determine the IC_{50} value of zinc acting at its high-affinity, voltage-independent site on NR1/NR2A receptors¹⁸. In *Xenopus* oocytes expressing NR1-1a/NR2A receptors, tricine-buffered zinc inhibited NMDA-receptor currents with an IC_{50} value of 15 nM for the high-affinity zinc site (data not shown), in agreement with a previous report¹⁸. In HEK293 cells, low concentrations of zinc caused a similar concentration-dependent inhibition (Fig. 2a). With zinc concentrations up to 2.2 μ M, the NR1-1a/NR2A-receptor current still exhibits a linear I-V relationship (Fig. 2b), indicating that up to this concentration, zinc acts exclusively on the voltage-independent, high-affinity site ($n = 3$). The IC_{50} for the high-affinity zinc site of NR1-1a/NR2A in HEK cells is 86 nM (Fig. 2c), which is fourfold higher than the IC_{50} obtained in oocytes¹⁸. We are uncertain about the cause of this fourfold difference. Based on the fitted dose-response curves and $I_{EDTA} / I_{NO EDTA}$ values (2.86 for oocytes, $n = 10$; 2.13 for HEK cells, $n = 6$), we estimate the zinc contamination to be 275 nM for both recording systems, which is in line with a previously reported value (300 nM)²³ and our own measurements (338 nM by VG Plasma Quad 3 ICP-MS mass spectrometer, 330 nM by Jarrell-Ash965 ICP plasma emission spectrometer). Addition of Src increased the IC_{50} for NR1-1a/NR2A receptors in HEK cells to 392 nM, approximately fivefold (Fig. 2c). Based on this IC_{50} value and the estimated zinc contamination level (275 nM), we predict that the potentiation caused by EDTA in the presence of Src should be 42%, which is very close to the actual potentiation observed experimentally ($44 \pm 9\%$).

SRC POTENTIATION CORRELATES WITH ZINC INHIBITION

If Src potentiates NMDA receptors by reducing tonic inhibition by zinc, NMDA receptors that are less sensitive to zinc should also be potentiated to a lesser degree by Src. Recent

studies have identified some splice variants and mutant NR1 receptors with reduced zinc sensitivity²¹. These recombinant receptors were tested in HEK cells to determine the amount of tonic inhibition and Src potentiation for each. The IC₅₀ value for NR1-1a(C744A,C798A)²⁴, co-expressed with NR2A, is 168 nM in oocytes, about eightfold higher than the wild-type NR1-1a/NR2A receptor (Fig. 3a). In HEK cells, this mutant is only slightly inhibited by ambient zinc, as indicated by the I_{EDTA}/I_{No EDTA} ratio of 1.11 ± 0.03 (Fig. 3b). As predicted, NR1-1a(C744A,C798A) is not potentiated by Src in HEK cells (Fig. 3c). NR1-1b, which has a 21-amino-acid insertion in the N-terminal region encoded by alternatively spliced exon 5, also shows reduced zinc sensitivity, with an IC₅₀ value of 198 nM for heteromeric receptors containing NR2A in oocytes (see also refs. 18 and 21). As expected, recombinant NR1-1b/NR2A receptors, with an I_{EDTA}/I_{No EDTA} ratio of 1.08 ± 0.04 in HEK cells, were not potentiated by Src (Fig. 3b and c).

With the NR1-1b and the double cysteine mutant of NR1-1a, we demonstrated that reduction of tonic inhibition indeed reduces the Src potentiation of NMDA receptors. By the same reasoning, if a mutation within NR1 exon 5 could restore zinc sensitivity of the heteromeric receptors to levels comparable to wild-type receptors lacking exon 5, it should also restore the potentiation by Src. Several point mutations within exon 5 are reported to restore zinc sensitivity to various degrees²¹. NR1-1bm207-211, a triple point mutant (K207G, R208G, K211G) in the exon 5 region¹³, exhibits the highest level of zinc sensitivity among all mutant receptors tested, with an IC₅₀ value of 40 nM in oocytes (Fig. 3a). Recombinant NR1-1bm207-211/NR2A receptors are under a significant amount of tonic

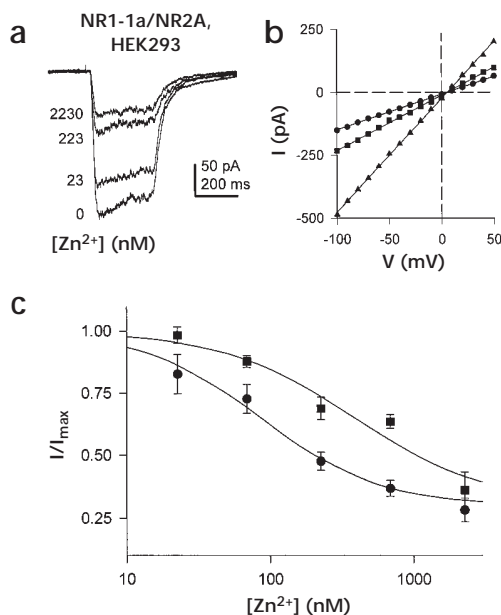


Fig. 2. Src shifts IC₅₀ for the voltage-independent zinc site of NR1/NR2A receptors. (a) Typical current traces showing dose-dependent inhibition of NR1-1a/NR2A receptors by zinc in HEK 293 cells (V_h, -50 mV). Tricine was used to buffer zinc. (b) The I-V relationship of the peak NMDA-receptor currents shows no voltage-dependent inhibition at up to 2 μM buffered zinc. P 2230 nM, L 223 nM, G 0 nM. (c) Src shifts the IC₅₀ for the high-affinity, voltage-independent zinc site of NR1-1a/NR2A receptors. Tricine was used to buffer zinc. Each point on the curve is averaged data from 4–9 cells. The IC₅₀ is 86 nM without Src and 392 nM with Src. P control, L Src.

inhibition as indicated by an I_{EDTA}/I_{No EDTA} ratio of 1.77 ± 0.09 (Fig. 3b). Again, consistent with our hypothesis, the peak current of NR1-1bm207-211/NR2A receptors is potentiated by Src (Fig. 3c). Overall, the amount of tonic inhibition of the NMDA receptors (I_{EDTA}/I_{No EDTA}) is highly correlated (R = 0.997, p < 0.005) with the amount of potentiation by Src of NMDA receptors (Fig. 3d).

C-TERMINAL TYROSINES ARE CRITICAL FOR SRC'S ACTION

To locate the tyrosine residues critical for modulation of zinc sensitivity by Src, we made a series of point mutations in the C-terminal region of NR2A (Fig. 4a). The amino-acid sequence of the C-terminal region contains a consensus phosphorylation site²⁵ for Src around tyrosine residue 1267 (Fig. 4a). Thus, a point mutation of Y1267 to phenylalanine was constructed.

Fig. 3. Potentiation of NMDA-receptor currents by Src correlates with the amount of tonic inhibition of NR1/NR2A receptors by zinc. (a) Dose-response curves for the high-affinity zinc inhibition of heteromeric NR1/NR2A receptors in oocytes (NR1-1a, n = 14; NR1-1b, n = 12; NR1-1a(C744A,C798A), n = 8; NR1-1bm207-211, n = 19). As previously reported²¹, a splice variant with exon 5 (such as NR1-1b) exhibits an IC₅₀ nearly tenfold higher than a splice variant without exon 5 (such as NR1-1a). Mutation of two cysteine residues of NR1-1a(C744A,C798A)²¹ also shifts the IC₅₀ for zinc by approximately tenfold. A triple mutation in exon 5 (NR1-1bm207-211: K207G, R208G, K211G)²¹ nullifies the effects of exon 5 on zinc inhibition. L 1bm209-211, G 1b, P 1a, H 1aC744,798A (b) The ratio of the current in the presence of 10 μM EDTA to that in the absence of EDTA is a measurement of tonic inhibition of NMDA-receptor currents by trace amounts of zinc in HEK 293 cells. The amount of tonic inhibition by zinc is consistent with the IC₅₀ values obtained in oocytes. (c) Effects of Src on current amplitudes of NR1/NR2A in HEK293 cells. Note that NR1-1a and NR1-1bm207-211 are potentiated by Src, whereas NR1-1b and NR1-1aC744,798A are not. P 1a/2A (10), P 1aC744,798A/2A (5), L 1b/2A (9), L 1bm207-211/2A (6). (d) Correlation between the tonic inhibition by zinc and potentiation by Src in HEK cells measured six minutes after obtaining the whole-cell configuration (correlation factor, R = 0.997, p < 0.005).

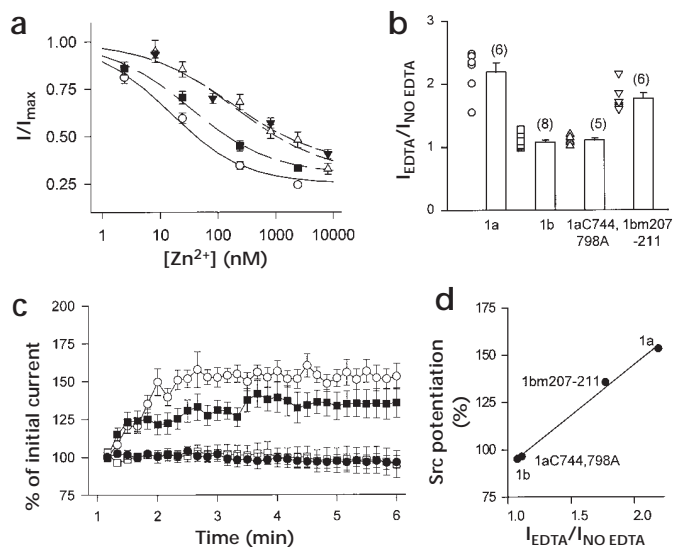
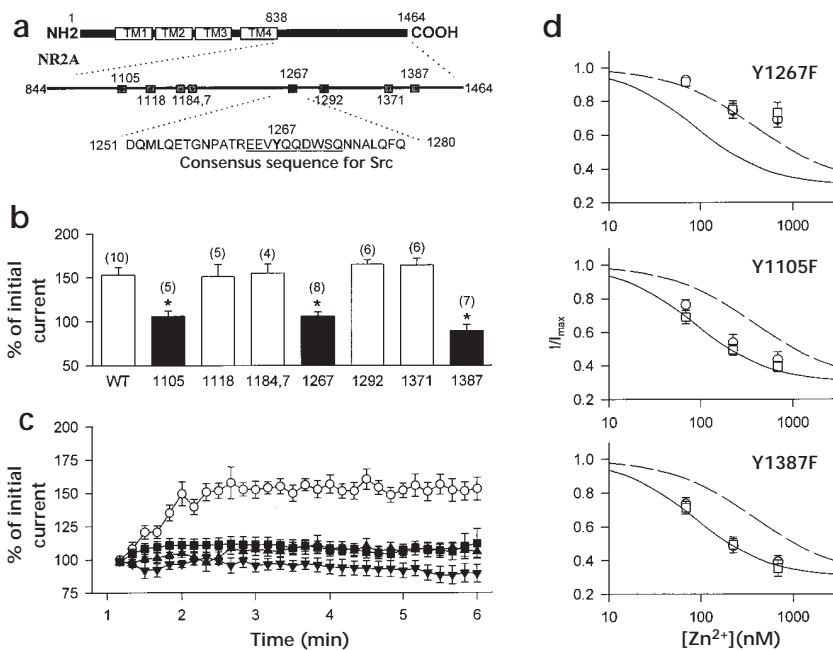


Fig. 4. Point mutations of tyrosine residues in NR2A C-terminal block Src's potentiation of NR1/NR2A currents and relief of zinc inhibition. **(a)** A diagram showing mutations of C-terminal tyrosine residues of NR2A subunit. Note that Y1267 is part of a putative consensus phosphorylation site for Src. Other tyrosine residues with high surface probability³⁷ were also mutated as indicated. **(b)** Summary bar graph showing the effects of nine point mutations of tyrosine residues to phenylalanine on the Src's potentiation of whole-cell NR1/NR2A-receptor currents measured six minutes after obtaining the whole-cell configuration (* $p < 0.01$ when compared to the wild-type NR2A by ANOVA and Newman-Keuls post hoc test). For all panels, number of cells is shown in parentheses. **(c)** Point mutation of Y1105F, Y1267F or Y1387F blocks Src's potentiation of whole-cell NR1/NR2A-receptor currents. \square wt (10), \square Y1105F (5), \square Y1267F (8), \square Y1387F (7). **(d)** Zinc sensitivity of heteromeric receptors containing three mutant NR2A subunits. Each data point is the average from 4–7 cells. Solid lines are fitted zinc curves for wild-type NR1/NR2A receptor without Src; dashed lines are the curves with Src. Note that Y1267F has lower affinity for zinc and that Src failed to relieve the zinc inhibition further. Although Y1105F and Y1387F have normal zinc affinity, Src failed to relieve zinc inhibition. \square control, \square Src.



Additional tyrosine residues with high surface probability in the neighboring region were also mutated. Point mutation Y1267F blocked the potentiation by Src (Fig. 4b and c). In addition, mutants Y1105F and Y1387F also abolished Src potentiation (Fig. 4b and c). In contrast, mutations of several other tyrosine residues in the same region had no detectable effects on Src-induced potentiation (Fig. 4b). Consistent with our hypothesis, Src failed to change the zinc sensitivity of receptors with any one of the three tyrosine mutations that eliminated Src potentiation of NMDA receptor currents (Fig. 4d). The zinc-inhibition curves for receptors with two of these mutations (Y1105F and Y1387F) were identical to the zinc-inhibition curve for the wild-type receptor. In these cases, the only effect of the tyrosine mutation was to prevent the Src-induced reduction in zinc sensitivity. The third mutation (Y1267F) reduced zinc sensitivity of the receptor in the absence of Src, which precluded further reduction of zinc sensitivity by Src. The IC_{50} for zinc of NR1-1a/NR2A(Y1267F) in oocytes was 92 nM ($n = 13$, data not shown), which is approximately fivefold higher than the IC_{50} for the wild-type NR1/NR2A. The amount of maximal inhibition was not altered (data not shown). A similar shift of IC_{50} for zinc was also observed in HEK cells (Fig. 4d). In contrast, the EC_{50} values for glutamate ($n = 7$) and glycine ($n = 7$) and the IC_{50} value for proton inhibition ($n = 8$) for NR1-1a/NR2A(Y1267F) were not significantly different from those of the wild-type receptors (data not shown). These data suggest that the Y1267F mutation results in a receptor that behaves like one that is fully potentiated by Src. We have also constructed double tyrosine mutations NR2A(Y1105F,Y1387F) and NR2A(Y1267F,1387F). There was no detectable difference between NR2A(Y1105F,1387F) and the corresponding single tyrosine mutants (Y1105F and Y1387F) (Table 1). NR2A(Y1267F,Y1387F) behaved like Y1267F, with a reduced apparent zinc affinity and lack of modulation by Src (Table 1).

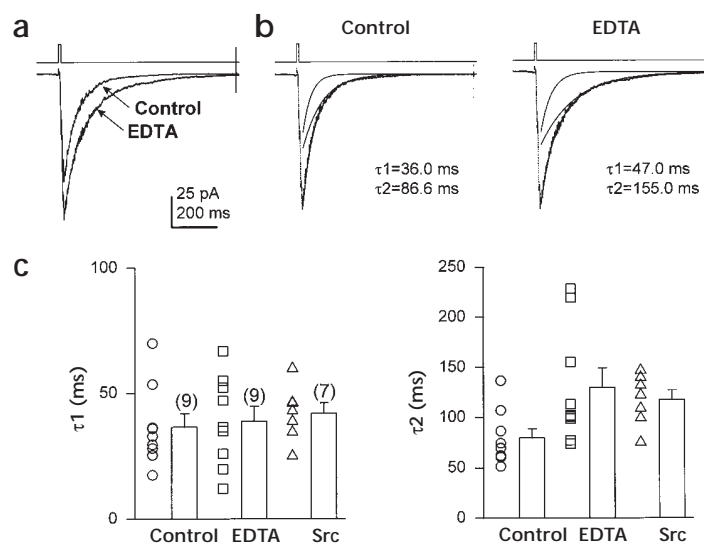
EDTA AND SRC INCREASE THE CURRENT DECAY TIME CONSTANT
Published evidence conflicts about the mechanism by which Src potentiates NMDA receptors. It has been reported that Src increases the open probability, open time, burst length, cluster length and supercluster length of NMDA channels⁶. Although the relaxation time constant of macroscopic current may be influenced by the first latency of NMDA channels²⁶, an increase in the duration of individual channel activation by Src should increase the relaxation time constant. However, no change of the relaxation time constant is reported when the macroscopic current amplitude is potentiated⁵. In our whole-cell recording, the decay of NR1/NR2A current could not be fitted with a single exponential component. To circumvent the possibility that the slower, second component reflects slow removal of the agonists due to turbulence around the whole cell, we investigated the decay time constant on NMDA-recep-

Table 1. Inhibition of wild-type versus mutant NR1/NR2A receptors by tricine-buffered zinc (223 nM) in HEK293 cells

Internal Solution	No Src	Src
Wild-Type NR2A	0.48 ± 0.04 (5)	0.69 ± 0.05 (6)*
Y1105F	0.54 ± 0.05 (7)	0.50 ± 0.03 (5)
Y1267F	0.74 ± 0.04 (4)*	0.75 ± 0.05 (4)*
Y1387F	0.49 ± 0.04 (5)	0.49 ± 0.05 (6)
Y1105F,1387F	0.44 ± 0.09 (4)	0.45 ± 0.07 (5)
Y1267F,1387F	0.79 ± 0.03 (3)*	0.82 ± 0.04 (4)*

Data presented in the table are I/I_{max} . I_{max} is the current recorded under nominally zinc-free conditions (in the presence of 10 mM tricine without added zinc). Number of observations is indicated in parentheses. *significant difference ($p < 0.05$) compared to wild type by ANOVA and Newman-Keuls post-hoc test.

Fig. 5. EDTA and Src increase the time constant of the slower component of NMDA currents. **(a)** Typical current responses from an outside-out patch expressing NR1/NR2A receptors. A brief glutamate-concentration jump (10 ms, to 100 μM) was applied to the patches. Saturating glycine (10–30 μM) was present in all solutions. Traces shown are averaged from 25 consecutive responses for control and 20 responses for EDTA. The top traces in (a) and (b) show the glutamate application time. **(b)** The decay of NMDA-receptor-mediated currents was fitted with two exponential components (smooth lines). The raw current trace is scaled to the same size to illustrate the change of relaxation time constant. **(c)** EDTA and Src increased the time constant of the slower exponential component significantly ($p < 0.05$, paired t -test for EDTA, unpaired t -test for Src), whereas the time constant for the faster exponential component is not changed. \square control, \blacksquare EDTA, \triangle Src.



tor currents in outside-out patches exposed to brief pulses (10 ms) of glutamate (with submillisecond solution-exchange rates at the open tip²⁷). Under such conditions, two exponential components were still needed to fit the decay of the macroscopic NMDA receptor currents. The two averaged time constants were 36.6 ± 5.3 and 79.8 ± 8.9 ms without EDTA and Src ($n = 9$). Inclusion of EDTA increased the peak of macroscopic NR1/NR2A currents recorded from outside-out patches by $63 \pm 30\%$ ($n = 9$, $p < 0.05$, paired t -test), which is consistent with our whole-cell data as well as a previous report that Src increases open probability⁶. In addition, EDTA significantly increased the slower relaxation time constant to 130 ± 19 ms, whereas the faster relaxation time constant remained unchanged with a mean of 38.9 ± 6.0 (Fig. 5). Interestingly, Src induced a similar selective change in the slower relaxation time constant. The averaged time constants in the presence of Src were 42.0 ± 4.1 and 117.7 ± 9.5 ms ($n = 7$). Because the amount of calcium influx during an EPSC is determined by the charge integral ($Q = \sum_i A_i \tau_i$) of the NMDA current, the resulting increase of synaptic calcium influx could be amplified by as much as 2.3-fold by Src. These observations indicate that Src and EDTA change the NMDA-receptor channel kinetics in similar ways, consistent with our hypothesis that Src potentiates NMDA receptors by relieving tonic zinc inhibition.

SRC REDUCES ZINC SENSITIVITY OF NR1/NR2B RECEPTORS

Is the reduction of zinc sensitivity by Src unique for receptors containing NR2A subunits, or is it a more generalized regulatory

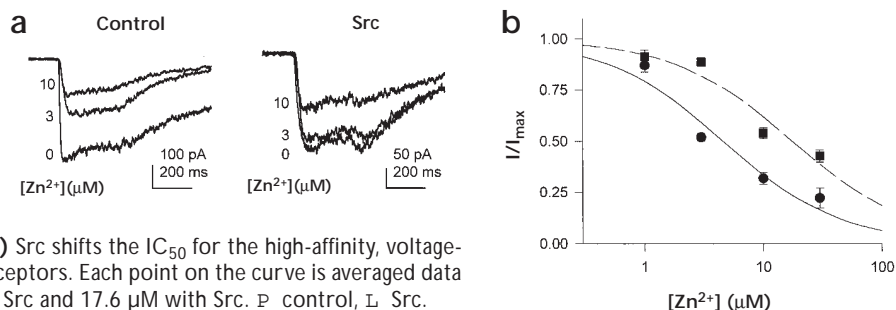
mechanism for other NMDA-receptor subunits? To address this question, we examined the effects of Src on zinc sensitivity of recombinant NR1-1a/NR2B receptors. Zinc inhibits NR1/NR2B receptors with an IC_{50} value of 4.6 μM under control conditions in HEK293 cells (Fig. 6a and b). In the presence of c-Src (30 units per ml), the IC_{50} was shifted to 17.6 μM . Thus, NMDA receptors comprised of NR2A or NR2B subunits are modulated by tyrosine kinase Src in a similar way, through a reduction of zinc sensitivity.

Discussion

Taken together, these data suggest that Src potentiates recombinant NR1/NR2A-receptor function by reducing tonic inhibition of these receptors by extracellular zinc. Furthermore, Src also reduces zinc sensitivity of NR1/NR2B receptors. Thus, reduction of zinc inhibition by Src could be a widely utilized mechanism for the modulation of NMDA receptor function in the brain. Our data are consistent with biochemical observations that both NR2A and NR2B are phosphorylated by the Src family of tyrosine kinases^{8,28} because they show that receptors comprised of either the NR2A or NR2B subunit are modulated functionally by Src. The selective potentiation of NR1/NR2A receptors by Src in HEK293 cells is due to the high zinc sensitivity of NR1/NR2A receptors and the ambient zinc contamination in the recording solutions that causes them to be tonically inhibited. By contrast, receptors comprised of NR1/NR2B are not potentiated by Src in HEK cells because their relatively lower sensitivity to zinc prevents tonic inhibition.

Zinc is actively accumulated in certain nerve terminals in a region-specific manner and can be released into synaptic clefts at

Fig. 6. Src reduces zinc sensitivity of NR1/NR2B receptors. **(a)** The effects of zinc (3 and 10 μM) on NR1/NR2B-receptor currents evoked by agonists (100 μM glutamate, 30 μM glycine) from representative HEK 293 cells with or without inclusion of Src in the patch pipette solution (V_h , -50 mV). **(b)** Src shifts the IC_{50} for the high-affinity, voltage-independent zinc site of NR1/NR2B receptors. Each point on the curve is averaged data from 3–5 cells. The IC_{50} is 4.6 without Src and 17.6 μM with Src. \square control, \blacksquare Src.



concentrations up to 0.1 mM¹⁴. The reported ambient zinc concentration in the brain varies from 150 nM to more than 2 μ M²². Assuming ambient zinc levels of 1 μ M, more than 70% of NR1/NR2A receptors would be inhibited, compared to 25% of NR1/NR2B receptors. The native NMDA receptors in the brain most likely include both NR2A and NR2B subunits. If, as seems likely, ambient zinc concentrations are sufficient (in excess of 1 μ M²²) to tonically inhibit NMDA receptors containing NR2A, then such receptors might serve as marginally functional receptors whose full function can be restored through activation of tyrosine kinases such as Src²⁹. If ambient zinc concentrations are insufficient to cause significant tonic inhibition, Src could still alter the responsiveness of NMDA receptors to synaptically released zinc.

The C-terminal region of NR2A is critical for modulation by Src. A C-terminal deletion mutant of NR2A is not potentiated by Src in HEK293 cells. Mutant mice expressing NR2A with the C-terminal truncation have impaired synaptic plasticity. We have identified three tyrosine residues in the C-terminal region of NR2A that are critical for the modulation of NR1/NR2A receptors by Src. The Y1105 and Y1387 residues function as predicted for potential tyrosine phosphorylation sites, which is that their mutation to phenylalanine causes no apparent changes by itself but blocks the reduction of zinc affinity by Src. Because both these tyrosine residues are conserved between NR2A and NR2B, it is tempting to speculate that phosphorylation of the corresponding tyrosine residues in NR2B is responsible for reducing the apparent zinc affinity of NR1/NR2B receptors. The function of the third tyrosine residue, Y1267, is peculiar. Mutation of Y1267 to phenylalanine results in a receptor with reduced zinc affinity. This tyrosine residue and the small stretch of peptide surrounding it are unique to NR2A. We do not know the mechanism behind this change and can only speculate at this point. One possibility is that this residue is phosphorylated constitutively and may be involved in interactions with the SH2 domain of Src or other proteins. The C-terminal domains of NMDA receptor subunits are larger in size than those of other receptor subunits and may be involved in complex interactions with other proteins in the postsynaptic density and cytoskeleton. One example of such an interaction is the reduction of NMDA-receptor activity by direct binding of calmodulin to NR1 subunits, which inactivates NMDA receptors³⁰. Little is known about the possible protein-protein interactions of the C-terminal regions of NR2 subunits other than their interaction with the PSD95 family of proteins. Although the mechanism by which mutation of Y1267 reduces zinc sensitivity of NR1/NR2A receptors needs to be explored, our data do show that its lack of tonic inhibition due to reduced zinc sensitivity occludes Src potentiation, supporting our hypothesis that Src potentiates NMDA-receptor function by reducing tonic zinc inhibition.

From a structural perspective, our results suggest that tyrosine phosphorylation and/or Src binding on the cytosolic C-terminal domain of NR2A alter the apparent affinity of an extracellular zinc binding site. Although structural information will ultimately be required to understand the interaction between Src and the zinc site, several possible explanations deserve mention. Conformational constraints imposed by Src binding and/or tyrosine phosphorylation (the two events may occur separately) may specifically reduce the affinity of zinc by altering the geometry of the coordination site. Alternatively, Src may change other aspects of the NMDA-channel properties that perturb the binding of zinc. Several modulators or mutations²¹ that modify zinc sensitivity have previously been described. For example, expression of NR1 alternative exon 5, which encodes a highly charged

surface loop, seems to reduce zinc sensitivity in a manner that can be mimicked by extracellular polyamines. In addition, mutations of acidic residues in the NR1 subunit that reduce proton sensitivity similarly reduce zinc sensitivity. One possible explanation for these (and by analogy Src's) effects is that they may be due to reduced electronegativity of the electron-donating residues that form the zinc coordination site, which would reduce the ability of these residues to coordinate zinc. Such an effect could occur by inductive electron withdrawal through portions of the polypeptide chain secondary to Src binding or phosphorylation. Alternatively, Src could change the channel properties in such a way that occupation of the zinc site is less likely to force closure of an open channel, requiring a greater probability of zinc occupancy to inhibit the channel, which might appear as a lower IC₅₀. In either case, the effects of Src and tyrosine phosphorylation need to be transmitted from intracellularly accessible residues to extracellular residues. Clearly additional functional as well as structural information will be required to ultimately understand Src's effects on zinc modulation.

Finally, by demonstrating that potentiation of the NMDA-receptor current by Src reduces the apparent zinc affinity of NMDA receptors, we link two modulatory sites of NMDA receptors that have been previously thought to be independent. Our data provide further support for the convergence of different NMDA receptor modulatory systems that has become evident recently^{13,21,24,31}.

Methods

SITE-DIRECTED MUTAGENESIS. All of the cDNAs used in this study except a mutant of NR1-1b and NR2B (pCDNAI/amp, Invitrogen) were subcloned into pCIneo vector (Promega). Site-directed mutagenesis was done with *Pfu* DNA polymerase (Stratagene) to linearly replicate the parental strand with desired mismatch incorporated into the primer. Methylated parental DNA template was then degraded with Dpn I. The nicked double-stranded mutant DNA was transformed into *E. coli*. The nicks in the plasmid were repaired after transformation. Colonies were selected by screening for a silent mutation that introduces a new restriction site. The mutations were verified by didoxy sequencing through both strands in the region of the mutation.

TRANSFECTION OF HEK CELLS. HEK293 cells (CRL 1573; American Type Culture Collection, Rockville, Maryland) were maintained at 37°C and 5% CO₂ in DMEM supplemented with L-glutamine (200 μ M), sodium pyruvate (100 μ M), penicillin/streptomycin (100 units per ml), and 10% fetal bovine serum. Low-confluence cells were transfected by calcium-phosphate precipitation³². Cells were cotransfected with a mixture containing NR1, NR2 and green fluorescent protein³³ plasmids (1, 2, and 0.3 μ g per 12 mm diameter coverslip, respectively). After transfection, 200 μ M D-AP5 was added to the culture medium.

EXPRESSION OF NMDA RECEPTORS IN XENOPUS OOCYTES. cRNA was synthesized from linearized template cDNA according to manufacturer's specification (Ambion). The quality of synthesized cRNA was assessed by gel electrophoresis and quantified by spectroscopy and gel electrophoresis. Preparation of oocytes and injection of cDNAs coding for wild-type and mutant NMDA receptors were performed as described²¹.

BUFFERED ZINC SOLUTIONS. The tricine-buffered zinc solutions used to obtain the zinc dose-response curves were prepared according to the empirically established binding constant¹⁸ 10⁻⁵ M, by adding into 10 mM tricine (pKa, 8.15) the following concentrations of zinc (in μ M): 0.26, 0.78, 2.6, 7.8, 26, 77.5, and 254. The corresponding estimated concentrations of free zinc for HEK recording at pH 7.4 were calculated with WINMAXC³⁴ and BAD³⁵, and were (in nM) 2.3, 6.88, 22.3, 68.8, 223, 688, 2230, respectively.

VOLTAGE-CLAMP RECORDINGS FROM XENOPUS OOCYTES. Two-electrode voltage-clamp recordings were made as described²¹. Briefly, oocytes were perfused with a solution comprised of (in mM) 90 NaCl, 1 KCl, 10

HEPES and 0.5 BaCl₂ at pH 7.4, and held under voltage clamp at -30 to -40 mV. Electrodes were filled with 300 mM KCl and had resistance of 1–10 MΩ. Solution exchange was computer controlled through an eight-valve manifold.

WHOLE-CELL AND OUTSIDE-OUT RECORDINGS FROM HEK293 CELLS. Patch-clamp recording in the whole-cell configuration and outside-out patch recording were done as described³⁶ with Axopatch 200A or 200B amplifier. Recording electrodes (5–12 MΩ) were filled with (in mM) 140 Cs-gluconate, 5 HEPES, 4 NaCl, 2 MgCl₂, 0.5 CaCl₂, 1 ATP, 0.3 GTP and 5 BAPTA (pH 7.4). The recording chamber was continually perfused with recording solution composed of (in mM) 150 NaCl, 10 HEPES, 1.0 CaCl₂, 3 KCl and 20 mM mannitol. Glutamate (20–100 μM) and glycine (10–30 μM) were applied using a multibarrel pipette driven by a piezo-electric bimorph (exchange time under 0.5 ms) as described²⁷. In some experiments, recombinant human c-Src (Upstate Biotech.) was added into internal solution to a final concentration of 5–30 units per ml.

DATA ANALYSIS AND STATISTICS. All pooled data were expressed as mean ± standard error. Statistical comparisons were done with unpaired Student's *t*-test unless otherwise stated. To obtain IC₅₀ values, pooled zinc inhibition data were fitted with least-squares criterion (SigmaPlot) to equation $I/I_{\max} = (1-a)/(1+([Zn^{2+}]/IC_{50})^n) + a$ where *n* is the Hill slope and *a* is the residual response. (*n* and *a* were not constrained.) If the fitting algorithm returned a value less than 0.05 for *a* (our estimated limit for detection), we fixed *a* to 0 and refit the zinc inhibition data to equation $I/I_{\max} = 1/(1+([Zn^{2+}]/IC_{50})^n)$ (as in Fig. 6).

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