# Zinc effects on NMDA receptor gating kinetics

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## Supplementary Methods

Rat GluN1-1a (NR1-1a, U08261) and GluN2A (NR2A, M91561) were sub-cloned into pcDNA3.1(+). Receptors with decreased sensitivity to voltage-dependent block were produced by substituting glycine for the GluN2A asparagine at position 615 (including signal peptide) (1-2). We refer to the resulting construct GluN2A(N615G) as N/G. In all experiments, we co-expressed green fluorescent protein to aid the visual identification of transfected cells. All cDNAs were checked periodically by full-length sequencing before transfections into human embryonic kidney (HEK293) cells (ATCC CRL-1573). Cells were maintained in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% pen-strep cocktail at 37°C in a 5% $CO_2$ atmosphere; passed when reaching 85–95% confluence; and passages 22–35 were used for transfections. Transfections were done with FuGENE 6 or the calcium-phosphate method by incubating exponentially growing cells for 2 hrs with ~1 μg cDNA per 35-mm dish (3-4). Following treatment, cells were maintained 24–48 hours in DMEM supplemented with 2 mM $Mg^{2+}$ to prevent NMDA receptor-mediated cell death. Several hours before recording whole-cell currents, cells were re-plated at low density to obtain cultures with numerous solitary cells.

*Whole-cell currents* were recorded with electrodes pulled from borosilicate glass capillaries (BF150-86-10, Sutter Instrument Company, Novato, CA) that were fire-polished to a resistance of 3–6 MΩ when filled with intracellular solution (mM): 135 CsF, 33 CsOH, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES and 11 EGTA, adjusted to pH 7.4 with CsOH and clamped at -70 mV. Clamped cells were perfused using a lightly pressurized system (BPS-8, ALA Scientific Instruments, Westbury, NY) with extracellular solutions containing (mM): 150 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 0.1 glycine and 1 glutamate, in 10 HEPBS ($pK_a$, 8.3) adjusted to pH 8 (NaOH). Chelators, 0.01 EDTA or 10 tricine, or tricine-buffered $Zn^{2+}$ were also included as specified. Free $Zn^{2+}$ concentrations in 10 mM tricine-buffered solutions were calculated using Maxchelator software (www.stanford.edu/~cpatton/maxc.html) using a binding constant of $10^{-5}$ M as previously reported and adjusted for our conditions: 150 mM NaCl, pH 8 (6). 67 nM free $Zn^{2+}$ was prepared by adding 200 μM $ZnCl_2$ and 10 mM tricine into the working extracellular solution. Currents were amplified and low-pass filtered at 2 kHz (Axopatch 200B; 4-pole Bessel), sampled at 5 kHz (Digidata, 1322A) and written into digital files with pClamp 10 acquisition software (Molecular Devices, Sunnyvale, CA). Whole-cell traces were analyzed in OriginPro 7.0. Dose-response curve (**Fig 1C**) was obtained by fitting the exponential decay equation $y = y_0 + A_1e^{-x/t}{}_1 + A_2e^{-x/t}{}_2$ to means of steady-state current values obtained from 4–10 cells.

*Excised patch currents* were recorded with pipette (intracellular) solution containing (mM): 135 CsF, 33 CsOH, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES and 11 EGTA, adjusted to pH 7.4 (CsOH) and clamped at -100 mV. Upon reaching the outside-out configuration the patch was perfused with (extracellular) solutions containing (mM): 150 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 0.1 glycine, 10 tricine, 10 HEPBS, pH 8.0. Zinc and glutamate were added as indicated. Two extracellular solutions were applied simultaneously through a glass theta tube (2.0 mm diameter, Harvard Apparatus, Holliston, MA). A lightly pressurized perfusion system maintained the flow rate at 200 μl $min^{-1}$ and helped to form a sharp interface between the two streams. The recording pipette containing

the excised membrane patch was positioned within one stream close to the interface and was moved across into the second stream and back to the initial position using a piezoelectric translator (Burleigh LSS-3100/3200). Open-tip potential measurements done at the end of each experiment showed that the 10–90% exchange occurred within 0.15–0.25 ms. Each barrel of the theta tube was connected to several solutions through a micro manifold controlled with pinch valves (VC 6, Warner Instruments, Hamden, CT). Recorded currents were low-pass filtered at 5 kHz (Axopatch 200B; 4-pole Bessel), sampled at 50 kHz (Digidata, 1440A, Molecular Devices) and written into digital files with pClamp 10.2 software. Traces were analyzed in Clampfit 10.2 and further with OriginPro8.

*Single-channel currents* were recorded with the cell-attached patch-clamp technique (5). Recording electrodes were pulled in two stages with a vertical puller (PC-10, Narishige International USA, East Meadow, NY) from borosilicate glass capillaries (BF150-86-10, Sutter Instrument Company, Novato, CA), and were fire-polished to a resistance of 15–25 MΩ when filled with extracellular solution containing (mM): 150 NaCl, 2.5 KCl, 10 HEPBS, saturating physiological agonists 1 glutamate and 0.1 glycine, adjusted to pH 8 with NaOH. In some cases, the extracellular solution also contained 1 EDTA or 10 tricine, as specified. Channel openings were recorded as sodium influxes after applying either +100 mV or +40 mV through the recording pipette. Currents were amplified and low-pass filtered at 10 kHz (Axopatch 200B; 4-pole Bessel), sampled at 20 kHz (PCI-6229, M Series card, National Instruments, Austin, TX) and written into digital files with QuB acquisition software (www.qub.buffalo.edu, University at Buffalo, Buffalo, NY).

*Processing and analyses* were done on records selected to have only one active channel and that required minimum processing, as described in detail previously (4). Idealization was done with the segmental-k-means (SKM) algorithm in QuB on digitally low-pass filtered data at 12 kHz after imposing 0.15 ms as the dead-time. Modeling was done with the maximum-interval-likelihood (MIL) algorithm by building multi-state models of increasing complexity and fitting these to idealized traces using a log likelihood threshold of > 10 units per added state. The records analyzed had between $9.5 \times 10^3$ and $7.5 \times 10^5$ events and were all best fit by models having 5 closed states and 2–4 open states. To select the best fitting arrangement of states, we limited the search to models that had a sequence of at least two closed states preceding open states, based on previously published results (7), and vicinal open states (8). For simplicity we considered initially only loop-less models and compared the best fitting model with a cyclic model that represents a reaction mechanism based on independent subunits (9). Based on this analysis, we found that a model containing a core 3C2O gating sequence and two branching desensitized states describes all the considered records with maximal log likelihood values.

Using this model we calculated time constants and areas for exponential components, and rate constants for all transitions postulated in the model. These values along with other kinetic parameters were tabulated and reported as means ± SE. Student's *t* tests, two-tailed assuming equal variance, were used to evaluate statistical significance of differences (*P*-values < 0.05). Changes were expressed as: fold-change = experimental/control; or % change = ((experimental/control) - 1) x 100.

*Free-energy profiles* were constructed using the rate constants in each model and the relationship $\Delta\Delta G_0 = -k_B T \ln K_{eq}$, where $k_B$ is the Boltzmann constant, T is the absolute temperature, and $K_{eq}$ is the equilibrium constant of the transition considered. Barrier heights are of arbitrary magnitude and are represented as $E_n = \Delta G^0_n + k_B T(10 - \ln k_{+n})$.

*Simulations* of ensemble responses to square jumps into 1 mM glutamate were simulated as the sum of time-dependent open state occupancies and analyzed in a manner similar to experimental macroscopic traces.

**Supplementary Methods References**


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**Supplementary Tables**

Table S1: Single-channel parameters of NMDA receptors

Table S2: Effects of N/G mutation on closed components

Table S3: Effects of N/G mutation on open components

Table S4: Effects of zinc on single-channel parameters of NMDA receptors

**Table S1: Single-channel parameters of NMDA receptors**

| receptor | EDTA | n | duration (min) | events | amp (pA) | $P_o$ | MCT (ms) | MOT (ms) |
|---|---|---|---|---|---|---|---|---|
| | | | | +100 mV | | | | |
| WT | - | 8 | 195 | 1,739,121 | 8.5 ± 0.3 | 0.10 ± 0.02*† | 22.8 ± 9.0* | 1.3 ± 0.1*† |
| | + | 12 | 509 | 3,305,107 | 8.5 ± 0.4 | 0.65 ± 0.04† | 6.1 ± 0.9† | 11.7 ± 0.6† |
| N/G | - | 7 | 186 | 710,284 | 9.9 ± 1.0 | 0.22 ± 0.05*† | 26.0 ± 4.0*† | 6.1 ± 0.6*† |
| | + | 10 | 185 | 844,112 | 9.6 ± 0.7 | 0.36 ± 0.04* | 15.8 ± 1.9* | 8.1 ± 0.6* |
| | | | | +40 mV | | | | |
| WT | - | 6 | 157 | 833,799 | 3.6 ± 0.2*† | 0.22 ± 0.02*† | 16.2 ± 2.8* | 4.2 ± 0.3*† |
| | + | 8 | 299 | 2,151,155 | 5.8 ± 0.4*† | 0.55 ± 0.04‡ | 7.5 ± 1.2‡ | 8.9 ± 0.3† |
| N/G | - | 10 | 243 | 995,614 | 5.4 ± 0.5*† | 0.21 ± 0.02*† | 23.3 ± 2.1*‡ | 6.0 ± 0.7*‡ |
| | + | 6 | 233 | 1,402,754 | 5.7 ± 0.6*† | 0.44 ± 0.06* | 12.4 ± 2.5* | 8.5 ± 0.8* |

*, indicates significant difference compared to WT, +EDTA, +100 mV ($P < 0.05$)
†, indicates significant difference compared to N/G, +EDTA, +100 mV ($P < 0.05$)

## Table S2: Effects of N/G mutation on closed components

| receptor | EDTA | n | $\tau_{E1}$ (ms) | $a_{E1}$ (%) | $\tau_{E2}$ (ms) | $a_{E2}$ (%) | $\tau_{E3}$ (ms) | $a_{E3}$ (%) | $\tau_{E4}$ (ms) | $a_{E4}$ (%) | $\tau_{E5}$ (ms) | $a_{E5}$ (%) | $\tau_{block}$ (ms) | $a_{block}$ (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| +100 mV | | | | | | | | | | | | | | |
| WT | - | 8 | 0.54 ± 0.05*‡ | 20 ± 3* | 6.2 ± 1.1*‡ | 30 ± 5*‡ | 14.8 ± 3.0*‡ | 17 ± 6 | 36.3 ± 5.9‡ | 4.4 ± 0.9*‡ | 2,080 ± 412‡ | 0.74 ± 0.28*‡ | 1.2 ± 0.1*‡ | 35 ± 1*‡ |
| | + | 12 | 0.23 ± 0.01‡ | 36 ± 2‡ | 1.9 ± 0.1‡ | 49 ± 2 | 4.8 ± 0.2‡ | 14 ± 1‡ | 28.4 ± 1.6‡ | 0.52 ± 0.08‡ | 2,600 ± 207 | 0.15 ± 0.02‡ | | |
| N/G | - | 7 | 0.38 ± 0.04* | 17 ± 2* | 4.5 ± 0.5* | 52 ± 3 | 13.5 ± 1.6*‡ | 28 ± 3* | 87.4 ± 16.3*‡ | 2.2 ± 0.5* | 2,774 ± 426 | 0.63 ± 0.10*‡ | | |
| | + | 10 | 0.31 ± 0.03* | 20 ± 2* | 3.5 ± 0.3* | 49 ± 4 | 9.3 ± 0.7* | 26 ± 4* | 51.0 ± 8.6* | 4.3 ± 2.1* | 2,719 ± 401 | 0.38 ± 0.04* | | |
| +40 mV | | | | | | | | | | | | | | |
| WT | - | 6 | 0.94 ± 0.25*‡ | 30 ± 4‡ | 4.5 ± 0.4* | 49 ± 2 | 12.9 ± 1.7*‡ | 19 ± 3 | 99.0 ± 41.6* | 1.3 ± 0.5* | 2,810 ± 240 | 0.41 ± 0.13* | | |
| | + | 8 | 0.28 ± 0.04 | 30 ± 4‡ | 2.3 ± 0.3‡ | 45 ± 5 | 5.9 ± 0.3‡ | 24 ± 4* | 65.9 ± 27.5* | 1.1 ± 0.4 | 2,613 ± 334 | 0.19 ± 0.06‡ | | |
| N/G | - | 10 | 0.39 ± 0.02* | 16 ± 1* | 3.7 ± 0.1* | 58 ± 4 | 10.2 ± 0.6* | 24 ± 4* | 160.3 ± 84.5* | 2.2 ± 0.7* | 2,863 ± 366 | 0.66 ± 0.09*‡ | | |
| | + | 6 | 0.37 ± 0.05* | 19 ± 1* | 2.7 ± 0.2* | 55 ± 2 | 7.8 ± 0.9* | 25 ± 3* | 54.4 ± 23.2 | 0.61 ± 0.10 | 2,683 ± 392 | 0.33 ± 0.09* | | |
| % change N/G *vs.* WT +EDTA, +100 mV | | | *+35%* | *-44%* | *+84%* | | *+94%* | *+86%* | *+80%* | *+727%* | | *+153%* | | |

*, indicates significant difference compared to WT, +EDTA +100 mV ($P < 0.05$, Student's *t* test)
‡, indicates significant difference compared to N/G, +EDTA +100 mV ($P < 0.05$, Student's *t* test)

## Table S3: Effects of N/G mutation on open components

| receptor | EDTA | n | $\tau_{fast}$ (ms) | $a_{fast}$ (%) | $\tau_{low}$ (ms) | $a_{low}$ (%) | $\tau_{med}$ (ms) | $a_{med}$ (%) | $\tau_{high}$ (ms) | $a_{high}$ (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| +100 mV | | | | | | | | | | |
| WT | - | 8 | | | 1.3 ± 0.1*‡ | 100*‡ | | | | |
| | + | 12 | 0.32 ± 0.03 | 3.0 ± 0.3‡ | 4.8 ± 0.5 | 30 ± 5 | 10.7 ± 0.8 | 56 ± 3 | 21.3 ± 1.5 | 30 ± 5‡ |
| N/G | - | 7 | 0.30 ± 0.05 | 8.5 ± 1.1* | 3.9 ± 0.3 | 48 ± 8 | 9.3 ± 1.0 | 42 ± 6* | 18.0 ± 1.0 | 5.5 ± 4.5* |
| | + | 10 | 0.34 ± 0.09 | 5.9 ± 0.8* | 4.2 ± 0.2 | 47 ± 6 | 10.7 ± 0.7 | 53 ± 7 | 24.3 ± 2.8 | 6.0 ± 2.2* |
| +40 mV | | | | | | | | | | |
| WT | - | 6 | 1.0 ± 0.2*‡ | 9.2 ± 5.2* | 3.2 ± 0.3*‡ | 69 ± 7*‡ | 6.2 ± 0.3*‡ | 36 ± 4* | 11.5 ± 0.8*‡ | 5.1 ± 1.9* |
| | + | 8 | 0.49 ± 0.19 | 5.2 ± 1.4 | 4.6 ± 0.3 | 42 ± 5 | 12.3 ± 1.2 | 50 ± 7 | 37.0 ± 6.6* | 3.9 ± 1.4* |
| N/G | - | 10 | 0.65 ± 0.13* | 6.2 ± 1.1* | 3.9 ± 0.4 | 57 ± 7* | 8.9 ± 0.7 | 35 ± 7* | 28.5 ± 4.7 | 29 ± 5* |
| | + | 6 | 0.51 ± 0.07* | 4.7 ± 0.7* | 4.0 ± 0.3 | 51 ± 5* | 12.8 ± 1.2 | 42 ± 5* | 33.5 ± 5.1* | 5.0 ± 2.7* |
| % change N/G *vs.* WT +EDTA, +100 mV | | | | *+97%* | | | | | | *-80%* |

*, indicates significant difference compared to WT, +EDTA +100 mV ($P < 0.05$, Student's *t* test)
‡, indicates significant difference compared to N/G, +EDTA +100 mV ($P < 0.05$, Student's *t* test)

**Table S4: Effects of zinc on single-channel parameters of NMDA receptors**

| receptor | $Zn^{2+}$ | n | duration (min) | events | amp (pA) | $P_o$ | MCT (ms) | MOT (ms) |
|---|---|---|---|---|---|---|---|---|
| N/G | - | 6 | 139 | 565,592 | 5.5 ± 0.4 | 0.31 ± 0.07 | 21.0 ± 4.0 | 7.8 ± 0.6 |
| | + | 9 | 284 | 750,356 | 5.0 ± 0.4 | 0.10 ± 0.02* | 63.1 ± 12.0* | 4.9 ± 0.4* |
| | | | | | | *-68%* | *+200%* | *-37%* |
| WT | - | 6 | 269 | 1,300,633 | 4.7 ± 0.4 | 0.14 ± 0.03 | 22.9 ± 2.8 | 2.7 ± 0.4 |
| | + | 7 | 210 | 573,841 | 5.5 ± 0.6 | 0.03 ± 0.01† | 43.3 ± 5.6† | 1.2 ± 0.1† |
| | | | | | | *-79%* | *+89%* | *-56%* |

*, $P < 0.05$, as compared to $Zn^{2+}$-free N/G
†, $P < 0.05$, as compared to $Zn^{2+}$-free WT