Distinct gating modes determine the biphasic relaxation of NMDA receptor currents

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Supplementary Information

Supplementary Methods

Recombinant receptor expression Naïve HEK293 cells or the tSA201 sub-clone were co-transfected with plasmids encoding rat NR1-1a, rat NR2A and GFP (1:1:2) using the calcium phosphate method[1]. The precipitate was removed after 2 hr and replaced with fresh medium containing 2 mM $MgCl_2$. Currents were recorded 24-48 hr later.

Primary neuronal cultures Cortical neuronal cultures were prepared from Sprague-Dawley rat embryos (E18) with minor modifications from previously described methods[2]. Briefly, the frontal cortical area was dissected, and cells were dissociated using trypsin and trituration through a Pasteur pipette. The neurons were plated in 35 mm dishes coated with poly-lysine in MEM with 10% fetal bovine serum and 10% horse serum at a density of $1x10^5$ cells/ml. When neurons attached to the dish, the medium was changed to Neurobasal with B27 supplement (Invitrogen, GI). Twenty-four hours later, the cultures were treated with cytosine-β-D-arabinofuranoside (10 mM) to prevent non-neuronal cell proliferation. Neurons were maintained in culture for up to ~ 4 weeks and were used for single-channel recordings.

Electrophysiology All currents were recorded at room temperature. For the excised-patch experiments, the pipette solution was (in mM): 135 CsF, 33 CsOH, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, and 11 EGTA, adjusted to pH 7.4 (CsOH). Cells were constantly superfused with control solution (in mM): 150 NaCl, 2.5 KCl, 1 $CaCl_2$, and 0.1 glycine in 10 HEPBS buffer (N-(2-Hydroxyethyl)piperazine-N'(4-butanesulfonic acid, pKa=8.3) adjusted to pH 8 (NaOH).

Glutamate (1 mM) was applied in external solution with theta pipettes mounted on a piezoelectric bimorph as described[3]. Patches were positioned near the interface of the solutions flowing from adjacent barrels, and the interface was moved by applying voltage across the bimorph. The solution exchange estimated from open-tip potentials occurred within 0.1 to 0.2 ms. Glutamate-evoked currents were recorded with an EPC9 amplifier (HEKA), low-pass filtered (3-8 kHz, 4-pole Bessel) and digitally sampled at 25 kHz. Membrane potential was –100 mV.

For the cell-attached experiments, the pipette solution was (in mM): 150 NaCl, 2.5 KCl, 1 EDTA, 1 glutamate and 0.1 glycine in 10 HEPBS adjusted to pH 8 (NaOH). For neuronal recordings, the recording pipette also contained CNQX (20 mM) and bicuculline (10 mM). These conditions were chosen to ensure that single-channel records reflect exclusively receptor transitions associated with intrinsic gating[4] and facilitate detection of possible differences in MOT due to modal behavior. Inclusion of EDTA (1mM) reduced the concentration of free divalent cations to minimize magnesium block, allosteric inhibition by zinc and calcium-dependent desensitization; whereas very low proton concentration (pH 8) prevented proton inhibition (IC_{50} = 7.3)[5].

Currents from on-cell patches were amplified and low-pass filtered (10 kHz, 4-pole Bessel) with an Axopatch200B amplifier and digitized at 20 or 40 kHz directly onto a PC hard-drive with the QUB acquisition software (www.qub.buffalo.edu). The pipette potential was +100 mV (estimated membrane potential –120 to –140 mV).

Kinetic analyses For single-channel analysis, only records from patches containing a single active channel (no double openings) were selected. Idealization was done using the segmentation k-means (SKM) algorithm. Cell-attached data were subjected to digital low-pass filtering at 12 kHz before analysis. Kinetic modeling of the idealized intervals was done using

the maximum interval log likelihood (MIL) algorithm after imposing a conservative dead time of 0.15 ms.

Initially, all intervals were fitted with kinetic schemes having increasing numbers of closed and open states to determine the minimum number of states in each conductance class. An arbitrary threshold of >20 LL units per added state was used to settle on a best fitting model. Files selected for analysis contained between 50,000 and 600,000 events and resulted in LL/event in the 4.3 to 4.5 range. This analysis helped calculate the t_{crit} with which to define clusters and bursts[6]. Cluster lengths and mean open durations (MOTs) were estimated with SKM. For each file, we segregated clusters by their respective MOTs into one, two or three populations according to the observed open durations distributions in file analyzed. When clusters with MOTs within 1 s.d. of their population mean occurred consecutively in a record, the entire period containing channels gating with similar MOTs (clusters and the intervening gaps) was extracted into a separate file and used further to determine mode-specific gating and desensitization rate constants with MIL.

Macroscopic currents (whether measured, averaged from single-channel traces or simulated) were analyzed by fitting the declining phase of the trace by mono- or bi- exponential functions with Igor, Origin or QuB software. All values reported are given as mean ± s.e.m. unless otherwise stated.

Statistical analyses Significance of differences was assessed with the two-tailed Student's t-test assuming unequal variance and using $P < 0.05$ as threshold for significance.

Simulations Single-channel and macroscopic currents were simulated with the QuB and Channelab software. To simulate transmitter pulses, the glutamate concentration was stepped from zero to 1 mM for 1 ms. Prior to pulse application, all channels occupied the resting unbound state B_1 of the indicated kinetic model.

Supplementary Table 1. Kinetic parameters of macroscopic responses from NR1/NR2A receptors in excised patches

Deactivation				Desensitization					Recovery	
n	t_f (ms)	t_s (ms)	slow (%)	n	t_f (ms)	t_s (ms)	slow (%)	$I_{steady\text{-}state}/I_{peak}$ (%)	n	t (ms)
21	31.4 ± 1.3	110 ± 6.1	23.1 ± 1.9	5	129 ± 45	514 ± 189	50.5 ± 9.0	10.6 ± 2.8	5	2,240 ± 254
				6	-	617 ± 86	100	23.2 ± 2.6		

Values for deactivation were obtained from bi-exponential fits to the decays of currents evoked by 1-ms applications of 1 mM glutamate. Values for desensitization are from mono- or bi-exponential fits (plus a steady-state component) to the decays of currents evoked by 4-s applications of 1 mM glutamate. Recovery time constants were obtained from mono-exponential fits to the peak amplitudes of the second in a pair of 2-s applications of 1 mM glutamate, after expressing this amplitude as a fraction of the peak amplitude of the current evoked by the corresponding first application. All solutions contained 0.1 mM glycine. Values are mean ± s.e.m.

Supplementary Results

As for native receptors in attached patches (**Fig. 2**), activity recorded from NR1/NR2A residing in excised patches during continuous exposure to saturating concentrations of glutamate and glycine occurs in long clusters (**Supplementary Fig. 1a**). As we reported before for cell-attached patches under similar conditions[4], shut-time distributions contained four components (**Supplementary Fig. 1b**). In each patch, the long desensitization gaps gave rise to a single exponential component in the shut-time distribution. The mean time constant and relative area for this component were 1,580 ± 180 ms and 2.6% ± 0.4% (n=5).

To quantify the gating behavior within clusters in each patch, we used the fits to the distribution of all shut times to define a t_{crit} value that best separated the longest and next longest shut-time components[6] (**Supplementary Fig. 1b**). This allowed us to detach individual clusters from each record and to analyze the gating behavior during these periods when the channel escaped desensitization. For each cluster, we measured the mean open durations, the mean duration of bursts (defined with a t_{crit} value of 2.5 ms), and the fraction of time that the channel was open during each cluster (P_{open}). In each of the 5 patches examined, this analysis revealed clear evidence of modal gating. This is illustrated by plotting mean burst duration for 118 consecutive clusters from a portion of one of the records (**Supplementary Fig. 1c**). The mean burst duration for most clusters was about 5 ms, indicative of L-mode gating (MOT, 2.8 ms). Occasionally, we observed clusters that consisted of bursts with much longer mean durations. Such clusters tended to occur in runs, typically consisting of 2 or 3 consecutive clusters that exhibited MOTs (6.8 ms), burst durations (15 to 30 ms), and P_{open} values (0.45 to 0.65) characteristic of M-mode gating. Although infrequently, we also observed clusters with mean burst durations (50 to 100 ms) and P_{open} values (0.85 to 0.92) indicative of H-mode gating (**Supplementary Fig. 1d**).

When individual clusters were sorted by gating mode, plots of mean cluster duration as a function of MOT revealed a positive linear correlation between MOT and cluster duration (**Supplementary Fig. 1e**). The number of openings per cluster also increased linearly for the three modes, being smallest for the L mode and largest for the H mode. Thus L, M, and H mode clusters became progressively longer both because the openings within them were longer and because they contained more openings. Although we observed occasionally long L-mode clusters, their low frequency and low P_{open} suggested that such clusters contribute only minimally to the decay of ensemble currents.

Previously we showed that modal gating during clusters of steady-state activity can be accounted for by a relatively simple reaction mechanism (**Fig. 3a**) where fully occupied channels transit through three consecutive closed states before opening[4, 7]. We further showed that the L, M, and H modes evident in cell-attached recordings differ primarily in the mean duration of channel openings, as well as the rate constants in and out of C_3, the closed state that immediately precedes the open state. To further test our hypothesis that modal gating accounts for the biphasic nature of macroscopic deactivation, we used hidden Markov models to estimate rate constants for this reaction mechanism using our steady-state data from excised patches (**Supplementary Fig. 1a**). The rate constants for glutamate binding to states B_1 and B_2 were constrained to values found in our previous work (K_D = 3 μM)[7].

Portions of the records consisting entirely of L- and M-mode gating (on the basis of MOT, mean burst length, and P_{open}) were analyzed separately, allowing us to determine and compare the microscopic gating and desensitization rate constants obtained for each gating mode. Because we consistently distinguished only a single component of desensitization gaps in the shut-time distributions, we included a single desensitization state for both L and M-mode models. Based on our observation that desensitized events were less frequent when channel openings were long, we limited our analysis to kinetic models where the desensitized state was appended to a closed state (C_1, C_2 or C_3). Connecting the desensitized state, D, to C_3 gave

poor fits to the single-channel data, and log-likelihood values were consistently largest when D was connected to C_1.

The results from analyses of both types of patches were in excellent agreement regarding the two key features of L- and M-mode gating. First, the two gating modes differ in the stability of the composite open state, as reflected in the values of the rate constant for transition from O to C_3. Second, for L-mode gating the value of the rate constant for C_3 to C_2 transitions is larger than the corresponding value for C_3 to O transitions, whereas the converse is true for M-mode gating. These differences shift the relative occupancies of the C states toward C_3 for the M mode, resulting in fewer sojourns in C_1 and increasing the open probability of M-mode clusters (relative to L-mode clusters).

The rate constants for the L- and M-mode reaction mechanisms in **Fig. 3a** reproduced the experimental differences we observed under steady-state conditions in MOT, burst length, P_{open}, and cluster duration for L- and M-mode clusters (by simulating and analyzing steady-state data). The rate constants for transitions between the L- and M-mode gating mechanisms (0.05 s^{-1} and 0.5 s^{-1}) were estimated from analysis of the steady-state single-channel data. In these records, L-mode clusters outnumbered M-mode clusters approximately 10 to 1 and, on average, channels remained in the M gating mode for approximately 2 s.

Simulations of ensemble currents were run with 1,000 channels. The low frequency with which mode switching occurred makes it highly unlikely that channels switch modes during activations elicited by a single 1 ms pulse of glutamate. For simplicity, we therefore mixed the ensemble currents generated independently for each reaction mechanism, assuming that at the time the pulse was applied, the ratio of L-mode to M-mode channels was 10 to 1. As shown in **Fig. 3d,** this resulted in a response that decayed bi-exponentially with time constants and relative amplitudes for the two components of deactivation that are very close to our experimental values. This was also true for simulations of desensitization. In addition, the

reaction mechanism reproduced the experimental results for the two-pulse experiments (**Fig. 3e**), and gave EC_{50} values for macroscopic currents of 2 to 3 μM.

References

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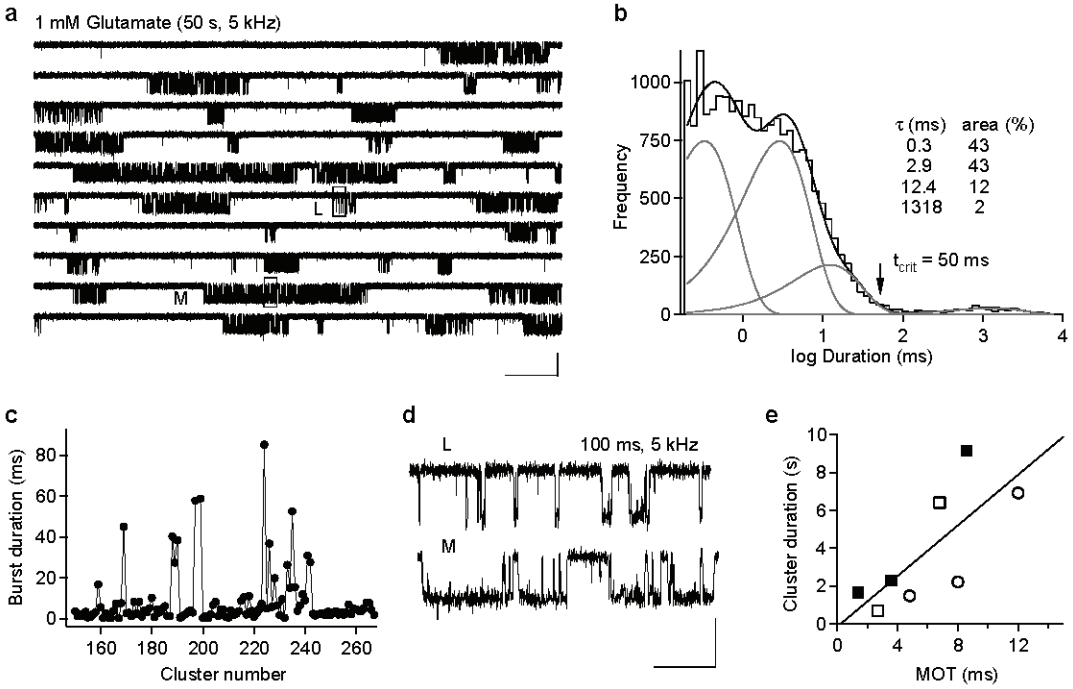
Supplementary Figure 1



Supplementary Figure 1 Gating kinetics of a single NR1/NR2A channel in excised patches during continuous exposure to agonist **a**, Consecutive 5 s records of channel activity recorded from an outside-out patch containing one NR1/NR2A channel. Channel openings during continuous exposure to 1 mM glutamate occur in clusters that are separated by long shut periods (gaps) indicative of channel desensitization. Scale bars are 5 pA and 500 ms. **b**,The distribution of shut times from the entire record was fitted (black line) with four exponential components (gray lines). The time constants and relative areas of the four components are indicated. The fit was used to calculate a duration (t_{crit}) that best separated the third and fourth components (arrow). Clusters were defined as a series of consecutive openings interrupted by closures that were shorter than this t_{crit} value. **c**, Plot of mean burst duration for 118 consecutive individual clusters from the patch illustrated in panel **a**. Bursts were defined as a series of openings separated by closings shorter than 2.5 ms. A total of 482 clusters were recorded in this patch. The number of bursts per cluster was fitted well by a single geometric component that gave a mean value of 6.5 bursts per cluster. Note that the mean duration of bursts in individual clusters varies markedly and that clusters with similar burst duration tend to bunch together. **d**, Examples of bursts of openings during L-mode (top) and M-mode (bottom) clusters. The 100 ms traces are from the boxed portions of the clusters in panel **a** marked L and M. Scale bars are 5 pA and 20 ms. **e**, Clusters recorded in three different cell-attached patches (filled squares, open squares, open circles) were sorted into groups based on the mean duration of openings within each cluster (MOT). The mean duration of the clusters in each group is plotted against the average MOT values for the clusters in each group. The line (slope = 0.71) is the fit to the entire data set.