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Changes in Agonist Concentration Dependence That Are a Function of Duration of Exposure Suggest *N*-Methyl-D-aspartate Receptor Nonsaturation during Synaptic Stimulation

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ABSTRACT

Evidence suggests that *N*-methyl-D-aspartate receptors (NMDARs) have a relatively high affinity for agonist compared with non-NMDA receptors. Dose-response curves constructed with sustained agonist application suggest that the 50% effective concentration (EC_{50}) for peak glutamate-evoked current at NMDARs is 1 to 10 μM, whereas that of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors is ~500 μM. Given estimates of synaptic cleft glutamate concentration in the millimolar range, it would be expected that NMDARs would be saturated with agonist. However, studies of synaptic NMDAR responses indicate that these receptors may not be saturated during single release events at many synapses. To address this apparent contradiction, we have compared the glutamate dose-response curve for the peak NMDAR current generated by sustained glutamate application with that obtained during brief synaptic-like pulses of agonist. Our results using both recombinant and native NMDARs indicate a marked reduction in glutamate potency with reduced agonist application duration (EC_{50} = 100 to 200 μM with 1 ms application). A kinetic model suggested that the reduction in potency with shorter agonist application duration could be attributed to the relatively slow activation and deactivation rates of the NMDARs. Comparison of room temperature to 37°C indicated that NMDAR activation and deactivation were strongly accelerated by increased temperature. However, at 37°C, we still observed a significant increase in potency with longer agonist application duration. We propose that glutamate has a relatively lower potency at NMDARs than previously thought from agonist application under equilibrium conditions. This lower potency would account for data that shows nonsaturation of NMDARs during synaptic transmission.

One of the assumptions of glutamate receptor synaptic physiology is that *N*-methyl-D-aspartate receptors (NMDARs) have a relatively high affinity for agonist compared with non-NMDA receptors. Previous studies analyzing peak current response have indicated that NMDARs have an EC_{50} value for glutamate in the range of 1 to 10 μM, whereas that of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors is in the range of 500 μM (Patneau and Mayer, 1990; Jonas and Sakmann, 1992; Barbour and Hausser, 1997). Because synaptic cleft glutamate concentrations have been estimated to be in the range of 1 mM (Clements et al., 1992; Holmes, 1995), it has been suggested that NMDARs are saturated during synaptic glutamate release (Frerking and Wilson, 1996). However, recent data indicate that NMDARs may be not saturated. With the use of calcium imaging to visualize responses at single synapses (Mainen et al., 1999), it was shown that the amplitude of the postsynaptic calcium transient was significantly increased with paired stimuli. Studies also indicate that the amplitude of NMDAR-mediated miniature synaptic currents varies in parallel with the amplitude of the AMPAR component when multiple events are observed at a single synapse (Umemiya et al., 1999; McAllister and Stevens, 2000) (Dube and Liu 1999). This phenomenon is presumably caused by variable amounts of glutamate release onto nonsaturated receptors. Moreover, results of experiments in which release probability was altered to change synaptic cleft glutamate concentration indicate that the ratio of NMDAR to AMPAR responsiveness is relatively constant (Perkel and Nicoll, 1993), suggesting that both classes of receptors are not saturated. These results seem difficult to reconcile with the assumption that NMDARs are saturated during synaptic transmission

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ABBREVIATIONS: NMDAR, *N*-methyl-D-aspartate receptor; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDG, *N*-methyl-D-glucamine; HEK, human embryonic kidney; NR1, NMDA receptor subunit 1; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*, *N*,*N*′,*N*′-tetraacetic acid; NMDA, *N*-methyl-D-aspartate; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; TTX, tetrodotoxin.

(Frerking and Wilson, 1996). One possible explanation for this discrepancy is that the conditions used for determining NMDAR dose-response curves were different from those encountered during synaptic transmission. For example, peak current glutamate dose-response curves have been determined using sustained applications of agonist (Patneau and Mayer, 1990). Because NMDARs have relatively slow activation and deactivation time courses compared with AMPARs (Dingledine et al., 1999), a large difference in potency may exist between short, synaptic-like agonist applications and longer pulses. In support of this notion, results of experiments in which glutamate was delivered to single boutons by brief iontophoretic pulses indicate that glutamate potency at NMDARs may be lower than previously thought (Dube and Liu 1999). We have sought to address this question more directly using rapid, synaptic-like perfusion of known glutamate concentrations to determine the glutamate dose-response curve of recombinant and native NMDARs. Our results indicate a marked reduction in glutamate potency for activation of peak NMDAR current when shorter agonist application pulses were used. These data help to explain the apparent nonsaturation of NMDARs during synaptic transmission.

Materials and Methods

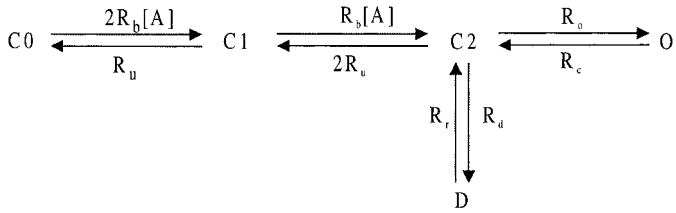
Culture and transfection of HEK 293 cells (American Type Cultire Collection, Manassas, VA) were as described previously (Chen and Okayama, 1987). Cells were passaged once every 2 to 4 days. For calcium phosphate transfection (Chen and Okayama, 1987), cells were plated at a density of 1×10^6 cells/ml in 10-cm culture dishes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ). Cells were transfected with cDNAs encoding NR1A and NR2B [a gift from Dr. S. Nakanishi, Kyoto University, Kyoto, Japan; nomenclature of (Sugihara et al., 1992); also known as NR1A-1a (Dingledine et al., 1999)] or NR2A (from mouse brain, also called ϵ1; a gift from Dr. M. Mishina, University of Tokyo, Tokyo, Japan) at a ratio of 1:1. A total of 10 μg of plasmid cDNA was used for transfection of a 10-cm culture plate. After transfection, 1 mM (+)-2-amino-5-phosphonopentanoic acid (RBI, Natick, MA) was added to the culture media and the cells were transferred onto glass coverslips in 35-mm culture plates (Falcon).

The whole-cell, patch-clamp recording technique and recording solutions for HEK 293 cells were essentially the same as described previously (Chen et al., 1997). Twenty-four to 36 h after the start of transfection, the HEK 293 cells were transferred to the recording chamber on the stage of an inverted microscope (Aviovert 100; Carl Zeiss, Thornburg, NY). Agonist-evoked currents were recorded in the whole-cell mode under voltage clamp ($V_H = -60$ mV). Electrodes with open tip resistances of 1 to 5 MΩ were used. After establishing the whole-cell mode, cells were lifted from the coverslip. Ultra-fast application of agonists was achieved by a piezo-driven θ-tube (Hilgenburg, Malsfeld, Germany) (Chen et al., 1999). Control and agonist solutions were continuously gravity-fed through the two sides of the θ-tube. Solution exchange time (10 to 90%) across the open tip of the recording electrode was 0.2 ms (see Fig. $2b_1$). Solution exchange time across the whole cell was measured at the end of a recording by switching between two different extracellular solutions containing *N*-methyl-D-glucamine (NMDG) versus NaCl; the 10–90% rise- and decay-time was 3.0 to 3.9 ms (see Fig. 1a) (Chen et al., 1999). All experiments with the exception of those in Fig. 4 (performed at 22 and 37°C) were performed at room temperature (~22°C). For experiments performed at 37°C, we used a Warner in-line heater to preheat the solution that perfused the bath. A small thermistor placed near the center of the bath confirmed that it was maintained at 37 ± 1°C. The solution within the agonist application θ-tubes was heated by the surrounding bath. Heating was facilitated by submerging about 3 to 4 mm of the θ-tube within the heated bath. To confirm that the temperature of the θ-tube perfusate was 37 ± 1°C, we monitored the effect of temperature on the resistance of an open electrode tip (simulates a perfused patch of membrane). The electrode resistance dropped with elevated temperature and was calibrated to monitor temperature of the applied perfusate. The extracellular recording solution contained in 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 11 mM glucose, and 10 mM HEPES, titrated to pH 7.35 with NaOH. In all experiments, 50 μM glycine was added to both control and glutamate-containing extracellular solutions. For recordings from HEK 293 cells, the intracellular recording solution contained 145 mM KCl, 10 mM HEPES, 5.5 mM BAPTA, 4 mM MgATP, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, and 2 mM tetraethylammonium, titrated to pH 7.2 with NaOH. The extracellular solution contains no added Mg^{2+} to enhance NMDAR-mediated currents at negative holding potentials. Data suggests that extracellular Mg^{2+} (in the presence of saturating glycine as we have done) does not alter the apparent affinity of glutamate for the NMDAR (Wang and MacDonald, 1995).

Primary cultures of neocortical neurons were prepared as described previously (Mackenzie et al., 1996). Briefly, neurons and glia were dissociated from rat fetuses (gestational day 17 to 18), and maintained at least 2 to 4 weeks to mature. Records of glutamate-activated currents were made from outside-out marco-patches. Electrodes with open tip resistances of 1 to 5 MΩ were used. After formation of a GigaOhm seal and membrane rupture (to form the whole-cell recording configuration), the pipette was removed slowly from the neuron over several minutes. Particularly large patches (easily visible under the microscope at 200×) were abandoned to select smaller patches that permitted better solution exchange times. The pipette solution contained 120 mM $CsMeSO_4$, 4 mM NaCl, 5 mM BAPTA, 1 mM $MgCl_2$, 3 mM MgATP, 0.3 mM GTP-Tris, and 10 mM HEPES, titrated to pH 7.2 with CsOH. The extracellular solution was identical to that used for HEK 293 cell recording, except that we added 20 μM CNQX and 0.5 μM TTX to block AMPARs and voltage-gated sodium channels, respectively.

Currents were sampled at 2 kHz and acquired and analyzed using pCLAMP software and the Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Results are expressed as the mean ± S.E.M. For calculation of EC_{50} values for both experiments and simulations, the amplitude of the glutamate-evoked current was measured at the peak of the response. Peak response EC_{50} was calculated from the equation: $I = I_{max} \times [1 / (1 + (EC_{50}/[A])^n)]$, where I is the measured peak current amplitude, I_{max} is the maximum peak current amplitude (evoked by 1 mM glutamate), [A] is the agonist concentration and n is the Hill coefficient. It should be noted that this equation was derived based on assumptions of equilibrium (i.e., that both drug concentration and receptor binding/channel opening have achieved equilibrium). However, it is commonly used in studies of ligand-gated ion channels to calculate the effective concentration required to achieve 50% of the peak current (peak response EC_{50}), and is accepted as an empirical measure of agonist potency. It does not reflect agonist affinity, nor can the equation be used (in this context) to analyze numbers of, or cooperativity between, binding sites on the receptors.

For simulation of NMDAR activation we adapted previously used kinetic models (Clements and Westbrook, 1991; Hessler et al., 1993; Destexhe et al., 1998) as described below. Because there was no Mg^{2+} in our extracellular recording solutions, Mg^{2+} block of NMDARs was not taken into consideration in the modeling. The NMDAR current was described by the product of maximal conductance (g_{NMDA}), the driving force, and open probability P_o: $I_{NMDA} = g_{NMDA} \times P_o \times (V - E_{NMDA})$. The maximal conductance g_{NMDA} was set to 0.05 nS in all simulations and the reversal potential E_{NMDA} is 0 mV (Destexhe et al., 1998). The open probability P_o, or macroscopically the fraction of NMDA receptors in the open state, is derived



Scheme 1. Kinetic model for NMDAR activation.

from Scheme 1 (Clements and Westbrook, 1991; Hessler et al., 1993; Destexhe et al., 1998).

The model assumes two (independent) glutamate binding sites that must both be occupied for the channel to open (giving 3 different closed states—C0, C1, and C2), one desensitized form of the receptor (D), and an open state (O). In the model, R_b is the binding rate of glutamate to the NMDAR, R_u is the unbinding rate of glutamate, R_o is the opening rate of the double-liganded receptor, R_c is the closing rate, R_d is the densensitization rate, R_r is the resensitization rate, and [A] indicates agonist concentration. The values of these rate constants were adjusted to match the EC_{50} values and 10 to 90% rise times recorded for different NMDAR subtypes with 2-s application of glutamate (see Fig. 1). For simplicity we have assumed that transmitter levels rise and fall instantaneously. Because reports suggest that the levels are approximated by an instantaneous rise, but fall exponentially (Clements, 1996), we are probably overestimating the length of the peak glutamate transient, so we also performed simulations using a double exponential decay of transmitter concentration. We performed our simulation using NEURON (version 4.1.1) (Hines and Carnevale, 1997) on a PII-450 PC, using a 5- to 100-μs time step (depending on the kinetic model used).

Results

In whole-cell patch clamp recordings from HEK 293 cells transfected with NR1A/NR2A- or NR1A/NR2B-type NMDARs, we examined the relationship between agonist application duration and response. Analysis of single current traces recorded from NR1A/NR2B-transfected cells indicated that 2-s application of 30 μM glutamate led to a nearly saturated peak response, whereas shorter 30 μM glutamate pulses (20 ms) resulted in little more than half-maximal activation of the receptors (Fig. 1a). We measured EC_{50} values of 1.3 ± 0.1 μM (n = 4–9) and 7.7 ± 0.8 (n = 6–10) for NR1A/NR2B and NR1A/NR2A, respectively, for peak current responses to sustained (2 s) applications of glutamate (Fig. 1bc). However, when we applied shorter, more synaptic-like pulses of glutamate to the same cells, we observed a marked rightward shift in the peak current dose-response curve. In response to the shortest duration pulses (20 ms) used in these experiments, the glutamate EC_{50} values were 17.8 ± 1.8 μM (n = 3–7) and 24 ± 1.0 (n = 4) for NR1A/NR2B and NR1A/NR2A, respectively (Fig. 1bc). These dose-response curves were significantly different from those observed with longer pulses of glutamate ($p < 0.001$, two-way analysis of variance). It is also interesting to note that for sustained (2 s)

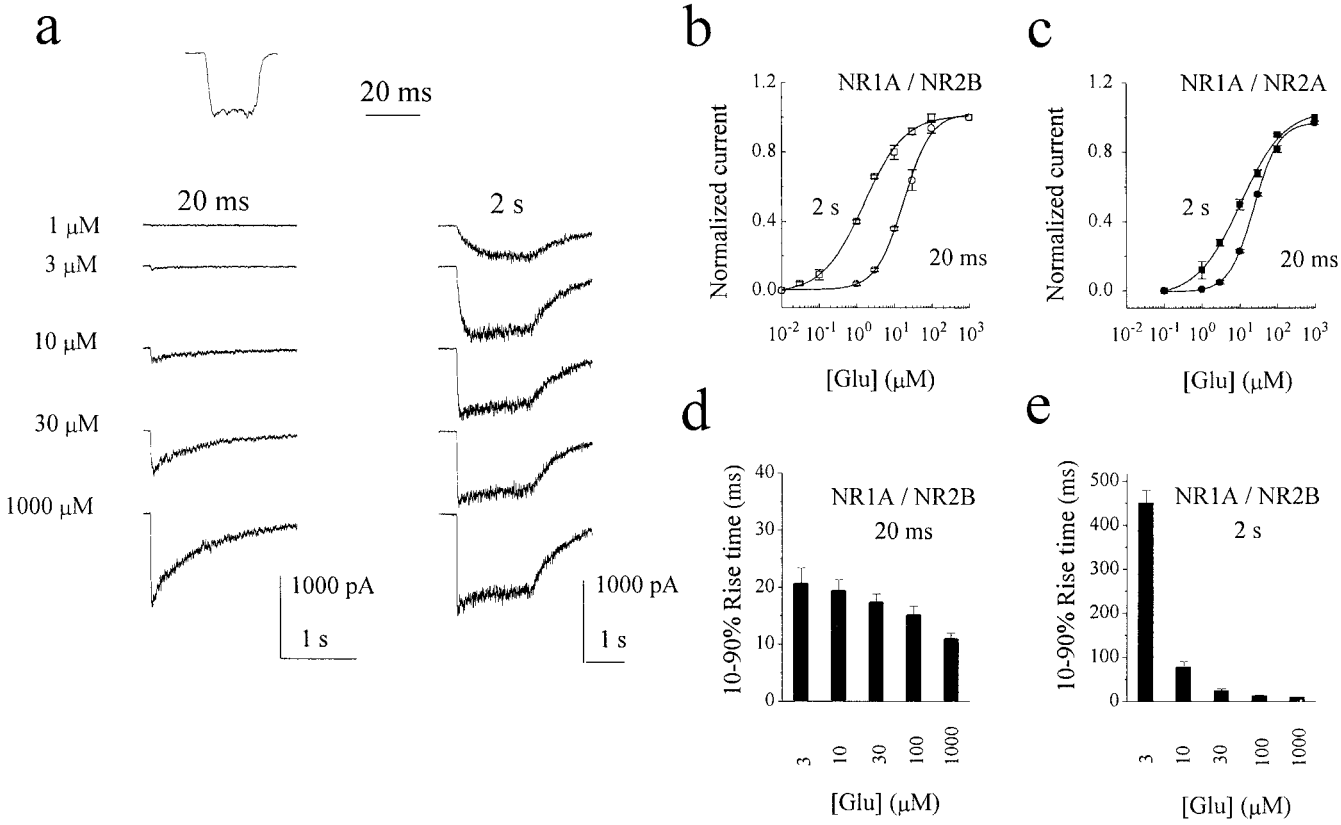


Fig. 1. NMDA receptor-mediated peak current responses are dependent on agonist application duration. a, glutamate-evoked current responses recorded in whole-cell configuration from a representative HEK 293 cell transiently transfected with NR1A/NR2B-subtype of NMDA receptor. A representative trace of the whole cell current recorded by switching between NMDG- and NaCl-containing solutions in the continuous presence of glutamate, reflecting solution exchange time, is shown above the records of glutamate activated currents. 20 ms and 2 s, glutamate application duration. Holding potential V_H = −60 mV. b and c, glutamate peak current dose-responses for NR1A/NR2B (b) and NR1A/NR2A (c) subtypes. Squares and circles represent dose-responses with application duration of 2 s and 20 ms, respectively. n = 4 to 7 different cells for each point. d and e, Bar graphs show the 10 to 90% rise time for NR1A/NR2B-mediated peak currents.

agonist pulses, the rise-time to peak of NMDAR current is a steep function of agonist concentration (Patneau and Mayer, 1990; Clements and Westbrook, 1991). This result is expected because the rise time to peak is (inversely) proportional to the on-rate for binding and channel opening rate, and the former increases with increasing agonist concentration (see *Materials and Methods*). Therefore, at low agonist concentrations, the 10 to 90% rise time to peak is markedly longer in response to a glutamate application of 2 s compared with one of 20 ms (Fig. 1, d and e), whereas the rise time is not different for the two different agonist application durations at the highest glutamate concentrations (6.8 ± 0.5 ms and 7.4 ± 0.2 ms for 2 s and 20 ms, respectively; $p > 0.2$, unpaired t test, $n = 6$).

To confirm these findings for native receptors, we recorded NMDAR-mediated currents evoked by glutamate (in the presence of CNQX to block AMPARs) from cultured cortical neurons in the outside-out patch mode. In recordings done in the absence of extracellular magnesium we also observed a significantly lower EC_{50} value for the peak current response with longer pulses of glutamate. Although 2 s pulses of glutamate resulted in an EC_{50} value < 10 μM, short synaptic-like pulses of glutamate (1 ms in duration) showed an EC_{50} value of ~ 100 μM (Fig. 2a and c2). The rise times of the currents associated with native NMDARs were also inversely proportional to agonist concentration for 2-s applications, as was observed for the recombinant receptors (Fig. 2, b_3 and c_1). For short pulses of agonist (1 ms), the rise times for different glutamate concentrations were very similar (Fig. 2, b_2 and c_1). Analysis of deactivation time course in response to short agonist pulses (Vicini et al., 1998; Chen et al., 1999), as well as ifenprodil sensitivity (Williams, 1993), indicated that individual neurons probably expressed receptors composed of NR1A/NR2A and NR1A/NR2B (Fig. 2a and data not shown). It is possible that some of the endogenous NMDA receptors are heterotrimeric (NR1A/2A/2B) (Dingledine et al., 1999). Functional studies indicate that the properties of such heterotrimeric receptors fall between NR1A/NR2A and NR1A/NR2B (Chen et al., 1997; Vicini et al. 1998). Therefore, we predict that the agonist duration-dependence of the EC_{50} value will be similar for the heterotrimeric receptor.

One explanation for the apparent discrepancy in the NMDAR peak response EC_{50} value for short pulses versus sustained pulses of glutamate is that the slow binding rate for agonist at low concentrations results in relatively few doubly liganded receptors and therefore low channel activation afterng short pulses of low agonist concentrations. In contrast, when longer pulses of these low agonist concentrations are given, NMDARs can accumulate in the doubly liganded state, because of the very slow unbinding rates (Lester and Jahr, 1992), so that the binding rate is no longer limiting. Using existing models of NMDARs (Clements et al., 1992; Destexhe et al., 1998) and the Neuron simulator (Hines and Carnevale, 1997), we were able to simulate currents attributed to NR1A/NR2A or NR1A/NR2B receptors (Fig. 3a). To simulate the dose-response curves and time courses of the recombinant receptor peak current responses (to 2 s glutamate application; Fig. 1a) we adjusted the kinetic parameters of the model within limits set by previous modeling and empirical studies (Lester et al., 1990; Clements and Westbrook, 1991; Clements et al., 1992; Edmonds and Colquhoun, 1992; Lester and Jahr, 1992; Destexhe et al., 1998). Our model was able to closely reproduce the rise time and shape of the recombinant NMDAR responses (compare Fig. 1a with Fig. 3a). Without readjusting the model parameters, we then varied the application duration for comparison with our experimental data. The model was able to accurately reproduce the dose-response relationships for both

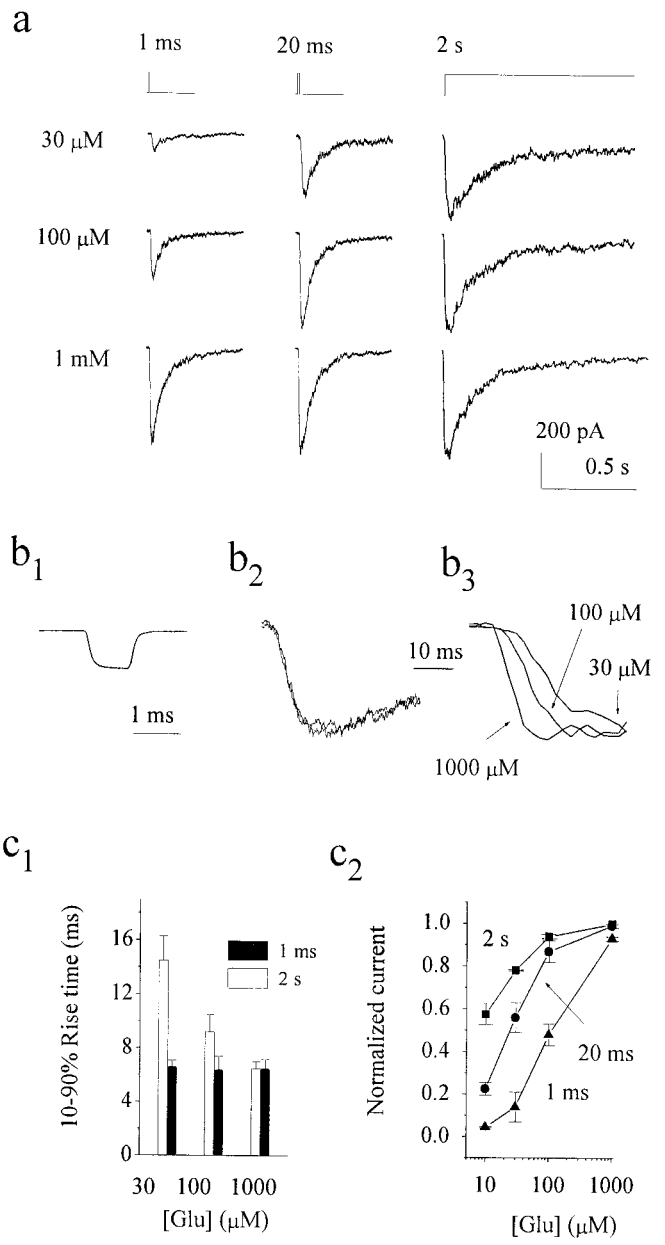


Fig. 2. Native neuronal NMDA receptor-mediated peak current responses are also dependent on agonist application duration. Outside-out patches were excised from cultured cortical neurons after 18 to 28 days in culture. a, current traces recorded from the same patch in response to different glutamate concentrations applied for different durations. 20 μM CNQX and 0.5 μM TTX were included in the recording solutions. b_1, open tip response indicating solution exchange time recorded after patch rupture (10–90% rise time and ~0.2-ms decay time). b_2 and b_3, overplotted traces normalized to peak amplitude showing the rise time of currents activated by either 1-ms (b_2) or 2-s (b_3) application of glutamate, at concentrations of 30 μM, 100 μM, and 1 mM. c_1, rise times (10–90%) for current responses to different glutamate concentrations applied for different durations. c_2, glutamate peak current dose-response for different glutamate application durations. Squares, circles, and triangles represent 2-s, 20-ms, and 1-ms application durations, respectively. $n = 2$ to 8 different cells for each point.

NR1A/NR2A and NR1A/NR2B (Figs. 1, b & c, and 3, b & c) with application durations of 2 s and 20 ms. For these NMDAR models, we assumed rectangular pulses of agonist (instantaneous rise and decay), in contrast to the slower exchange times actually observed (see Figs. 1a and 2a) due to limitations of our perfusion apparatus. However, simulations using ramped glutamate levels that approximated the limitations imposed by the perfusion apparatus produced identical results (data not shown). The peak response EC_{50} values for simulations of NR1A/NR2A were 7.3 and 17 μM for 2 s and 20 ms application durations, respectively; and for NR1A/NR2B were 0.9 and 13 μM for 2s and 20 ms, respectively. With an application duration of 1 ms, the models predicted that peak response EC_{50} values for NR1A/NR2A and NR1A/NR2B were 251 and 241 μM (Fig. 3bc; Table 1). It is interesting to note that the alteration in EC_{50} value observed with changes in agonist pulse duration was not specific to the parameter set we had chosen, because substitution of parameters from six different published models (Lester et al., 1990; Clements and Westbrook, 1991; Clements et al., 1992; Edmonds and Colquhoun, 1992; Lester and Jahr, 1992; Destexhe et al., 1998) yielded similar results (Table 1).

Adjustment of parameter values suggests that the relatively slow unbinding and opening rates of NMDARs [compared with AMPARs (Vyklicky et al., 1991)] make the major contribution to the slow rise time of glutamate-evoked responses and the increasing peak response EC_{50} value with decreasing agonist application duration. Consistent with this idea, previous studies on AMPA receptors (that have faster kinetics) have reported peak response EC_{50} values for short (synaptic-like) agonist pulses that were similar to those reported for longer pulses (Hestrin, 1992; Jonas and Sakmann, 1992). Because the NMDAR channel opening rate is faster than the desensitization rate (in some cases 20–75-fold higher, see Table 1), we assume that under normal conditions, desensitization will not play a significant role in the observed shift in peak current EC_{50} value seen with decreasing agonist application duration. In fact, our simulation results indicate that increasing the desensitization rate by 10-fold had no significant effect on the glutamate EC_{50} value for both short and long pulses of agonist (data not shown).

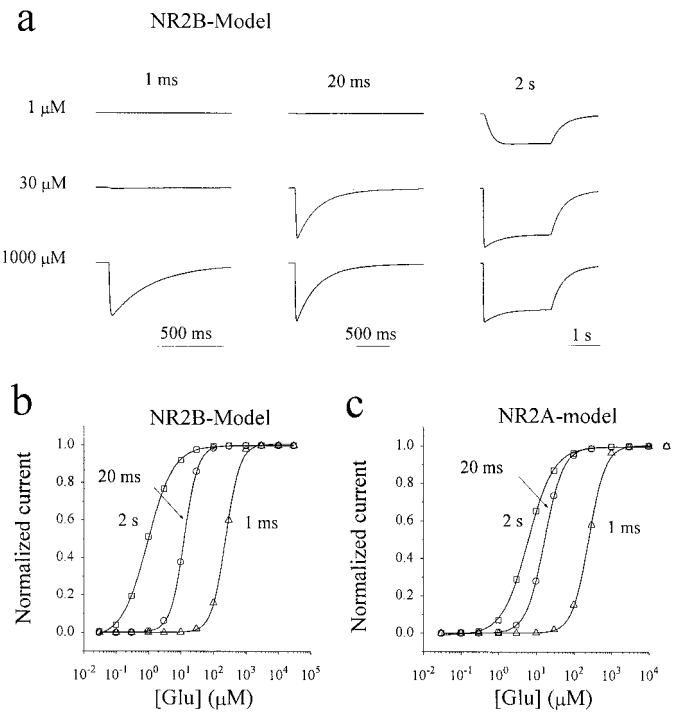


Fig. 3. Model of NMDA receptor activation in response to glutamate predicts that the peak current dose-response is application duration-dependent. In our kinetic scheme for NMDAR activation (see *Materials and Methods*), parameters used for binding (Rb × [A]), unbinding (Ru), desensitizing (Rd), resensitizing (Rr), opening (Ro), and closing (Rc) were modified to ensure that the basic biophysical properties, including activation, deactivation, and dose-response relationships, obtained with 2-s glutamate application duration matched that of the experimental data (NR1A/NR2B-mediated currents). a, representative current traces generated by a NR1A/NR2B receptor model. The model was based on existing kinetic models of NMDARs (Clements et al., 1992; Destexhe et al., 1998). b and c, glutamate peak current dose-response for NR1A/NR2B (b) and NR1A/NR2A (c) model receptors with glutamate application duration of 2 s (□), 20 ms (○) and 1 ms (△), respectively. Rates used are shown in Table 1.

Data suggest that the time course of glutamate within the synaptic cleft is affected greatly by diffusional barriers and clearance mechanisms (Clements, 1996). Taking this information into account suggests a glutamate transient exhibiting an (approximately) instantaneous peak and a double exponential decay with components having 0.1 ms and 2.1 ms τ values (initial amplitudes of 2.7 mM and 0.4 mM) (Clements, 1996). Using these parameters and our NR1A/NR2A model parameters, we have calculated NMDAR occupancy and compared the results with those obtained by modeling synaptic cleft glutamate levels as a 1-ms square pulse or by adjusting the time course and peak glutamate concentrations for the double exponentially decaying pulse (Table 2). We find that if peak glutamate levels are in the 1.1 mM range and transmitter clearance is accelerated, NMDARs will not be saturated. Furthermore, we have examined temperature dependence by scaling all rate constants by the NMDAR Q_{10} (McLarnon and Curry, 1990). As expected, this resulted in faster NMDAR activation and deactivation (data not shown). This analysis indicated that 37°C temperature would diminish the difference between EC_{50} values for different pulse durations of glutamate. The EC_{50} values for 2s, 20 ms, and 1 ms rectangular pulses estimated from the model at 37°C were 7.5, 8.1, and 70.6 μM (determined using NR1A/NR2A kinetic parameters; Table 1).

Consistent with the modeling data, actual records of glutamate-evoked currents from outside-out patches indicated that at 37°C (as compared with ~22°C) the activation and deactivation of the currents were considerably accelerated (Fig. 4a and b); the 10 to 90% rise time was decreased from 9.4 ± 0.3 (n = 4) to 2.4 ± 0.5 ms (n = 8) and the 10 to 90% decay time decreased from 408 ± 87 ms (n = 5) to 35 ± 9 ms (n = 8) (Fig. 4, c and d). We also observed that at 37°C, the occupancy of NMDARs was increased during short agonist pulses compared with room temperature. For example, when 20 ms and 2 s pulses of 10 μM glutamate (at 37°C) were compared, we observed no significant difference in receptor occupancy (Fig. 4e; 0.53 ± 0.04 and 0.57 ± 0.04, n = 4, respectively $P > 0.05$ paired t test) in contrast to the room temperature data (Fig. 2, c_2). This data agreed well with our model that predicted occupancy of 0.58 and 0.59 at 37°C for 20 ms and 2 s pulses of agonist (Fig. 4f). Although the effect of agonist application duration on receptor occupancy is re-

duced at 37°C, our model predicts that for brief synaptically mediated pulses of glutamate the receptor would be not necessarily be saturated (Table 2; Fig. 4f). Consistent with this, we found that shorter applications of 10 μM glutamate to outside-out patches led to a significantly lower peak current amplitude ($P < 0.05$, paired t test) than that observed with 20 ms or 2 s application (Fig. 4, e; 1–5 ms application response was 20 ± 4% of the 2 s pulse, $n = 3$ patches at 1 ms and 1 at 5 ms). Thus the duration dependence of the agonist potency we observe at room temperature is also observed at 37°C (albeit at reduced levels).

Discussion

Our data with native NMDARs indicate that the peak response EC_{50} value for short synaptic-like pulses of glutamate is in the 100 μM range. Simulation results suggest that the EC_{50} value might be even higher (~250 μM; Fig. 3, b and c). Our measured value suggests that during physiological glutamate release at central mammalian synapses up to 90% of NMDARs may be saturated if the peak glutamate concentration were 1 mM (Holmes, 1995). However, recent data (Somogyi et al., 1998; Racca et al., 2000) indicate that the distribution of NMDARs and AMPARs within a synapse may not be equivalent and thus both receptor classes may not experience the same concentration of transmitter. Given that NMDARs can be clustered within a synapse, that release rate itself may be regulated (Choi et al., 2000), and given that transporters rapidly buffer glutamate (Diamond and Jahr, 1997), it is possible that NMDARs may not experience 1 mM peak glutamate concentration. Therefore, if the synaptic glutamate concentration were less than 1 mM or transmitter clearance was faster than previously estimated, NMDARs may not be fully occupied with agonist during synaptic release, consistent with reports that synaptic receptors are not saturated (Dube and Liu 1999; Mainen et al., 1999; Umemiya et al., 1999; McAllister and Stevens, 2000).

TABLE 1

Comparison of duration dependent changes in NMDAR EC_{50} observed with our model parameters and other published parameters.

EC_{50} values calculated using our NR1A/N2A and NR1A/NR2B kinetic parameters. Comparison with the following published kinetic parameters. EC_{50} values for 2-s and 1-ms glutamate pulses are shown (our model below uses room temperature kinetic parameters, most of the published parameters below are also apparently based on room temperature data).

Model	Rb	Ru	Rd	Rr	Ro	Rc	EC_{50}	
							2 s	1 ms
	$\mu M^{-1}\ ms^{-1}$			ms^{-1}			μM	
NR1A/NR2A	0.005	0.025	0.002	0.001	0.15	0.15	7.3	251
NR1A/NR2B	0.005	0.003	0.001	0.0016	0.08	0.08	0.9	241
Dextexhe et al. (1998)	0.005	0.0129	0.0084	0.0068	0.0465	0.0738	4.8	247
Hessler et al. (1993)	0.005	0.0095	0.016	0.013	0.025	0.059	3.6	246
Lester et al. (1993)	0.005	0.005	0.0051	0.00095	0.104	0.285	3.4	244
Clements et al. (1992)	0.005	0.0095	0.016	0.013	0.025	0.059	3.6	246
Edmonds and Colquhoun (1992)	0.005	0.0047	0.0084	0.0018	0.0465	0.0916	2.6	243
Lester and Jahr (1992)	0.005	0.0067	0.0152	0.0094	0.0838	0.0838	2.6	243
Clements and Westbrook (1991)	0.005	0.005	0.004	0.0003	0.01	0.322	3.6	246

TABLE 2

Comparison of receptor occupancy obtained with different transmitter release models at two simulated temperatures

The Q_{10} of NMDARs was set at 2.5 based on previous work (McLarnon and Curry, 1990). The time course of the 'square' pulse with a linear ramp-up and down was set according to the limitations of our perfusion system. The time course and amplitudes for the first two double exponential decay pulses were set as described in Clements (1996). The time course and amplitudes for the last six double exponential decay pulses were adjusted to examine the effect of altered transmitter peak amplitude and faster glutamate clearance. Receptor occupancy was calculated from the ratio of the peak current obtained under the conditions described below to the maximal current obtained (saturating dose, long application). The NMDAR model used below is based on the NR1A/NR2A parameters from Table 1.

Transmitter Release Model		Description of Transmitter Pulse	Temp.[d] (°C)	Receptor Occupancy
1 mM 1-ms pulse	Constant, [a]'Square'	0.2 ms ramp-up +0.8 ms +0.2 ms ramp-down	22	78%
1 mM 1-ms pulse	Constant, 'Square'	0.2 ms ramp-up +0.8 ms +0.2 ms ramp-down	37	93%
Double-exp-decay pulse	High,[b] slow[c]	A_1 = 2.7 mM; τ_1 = 0.1 ms A_2 = 0.41 mM; τ_2 = 2.1 ms	22	84%
Double-exp-decay pulse	High, slow	A_1 = 2.7 mM; τ_1 = 0.1 ms A_2 = 0.41 mM; τ_2 = 2.1 ms	37	100%
Double-exp-decay pulse	High, fast[c]	A_1 = 2.7 mM; τ_1 = 0.025 ms A_2 = 0.41 mM; τ_2 = 0.52 ms	22	46%
Double-exp-decay pulse	High, fast	A_1 = 2.7 mM; τ_1 = 0.025 ms A_1 = 0.41 mM; τ_2 = 0.52 ms	37	90%
Double-exp-decay pulse	Low,[b] slow	A_1 = 1.1 mM; τ_1 = 0.1 ms A_2 = 0.17 mM; τ_2 = 2.1 ms	22	66%
Double-exp-decay pulse	Low, slow	A_1 = 1.1 mM; τ_1 = 0.1 ms A_2 = 0.17 mM; τ_2 = 0.025 ms	37	97%
Double-exp-decay pulse	Low, fast	A_1 = 1.1 mM; τ_1 = 0.025 ms A_2 = 0.17 mM; τ_2 = 0.52 ms	22	16%
Double-exp-decay pulse	Low, fast	A_1 = 1.1 mM; τ_1 = 0.025 ms A_2 = 0.17 mM; τ_2 = 0.52 ms	37	63%

[a] Glutamate concentration stays constant for 0.8 ms after 0.2-ms ramp-up.
[b] High indicates that the initial peak glutamate concentration is high (2.7 mM + 0.41 mM), whereas low indicates that the initial peak glutamate concentration is low (1.1 mM + 0.17 mM); the 2.7 mM peak and 0.41 mM tail are from the revised estimates of synaptic cleft glutamate concentration made by Clements (1996); the 1.1 mM peak glutamate concentration is from the original estimate of synaptic cleft glutamate concentration made by Clements et al. (1992).
[c] Slow indicates that glutamate concentration decays with slower time constants (0.1 ms and 2.1 ms), whereas fast indicates that glutamate concentration decays with faster time constants (0.025 ms and 0.52 ms);
[d] Temperature at which parameters were determined.

The occupancy of NMDARs afterng synaptic release is likely to not be fixed and may increase in scenarios that favor multiquantal release, such as evoked release from terminals with multiple release sites (Tong and Jahr, 1994; Silver et al., 1996; Vincent and Marty, 1996). In contrast, spontaneous release or uniquantal-evoked release may lead to a relatively lower transmitter concentration in the synaptic cleft. Preparations with reduced extracellular diffusion barriers such as cell cultures may exhibit lower cleft glutamate concentration and receptor occupancy (Umemiya et al., 1999; McAllister and Stevens, 2000). However, it is notable that in slice preparations during minimal synaptic stimulation, NMDAR occupancy is not complete (Mainen et al., 1999), and studies suggest that at some CNS synaptic clefts, glutamate concentration may be relatively low and on the order of 100 μM (Choi et al., 2000). Other factors that would increase receptor occupancy and lower the EC_{50} value for glutamate include physiological temperature (as opposed to room temperature). Our simulation results using NMDAR rate constants corrected for physiological temperature suggest that nonsaturation of NMDARs could occur whether transmitter clearance rates were elevated and peak glutamate levels were of ~1 mM (Table 2). In contrast, using peak estimates of 2.7 mM and a double exponential clearance rate previously proposed by (Clements, 1996), we find that NMDARs (rates constants adjusted to that expected for 37°C) would be saturated with agonist during synaptic activity (Table 2). Perhaps transmitter clearance rates might also be elevated at physiological temperature because glutamate transporters are highly temperature dependent (Bergles and Jahr, 1998). Because most fast perfusion studies of NMDARs are done at room temperature, it is not clear that current estimates of transmitter clearance rates (based on these experiments) are also corrected for physiological temperature.

We observe a profound acceleration of both activation and deactivation of NMDARs at 37°C. Despite this, our data using 1-ms application of glutamate indicates that agonist potency is still dependent on application duration at physiological temperature. Given that temperature can have such a large affect on NMDAR kinetic parameters, we suggest that all experimental and/or modeling studies of these receptors be performed at 37°C (or use the appropriate kinetic parameters corrected for temperature). In support of our conclusions, it is interesting to note that despite the expected acceleration of NMDAR kinetics, the hippocampal slice study of (Mainen et al., 1999) indicated that NMDARs are not saturated by synaptic release of single quanta at 37°C.

Our results demonstrating that the peak current dose-response curve of high-affinity NMDARs shifts to the right as the exposure time to glutamate shortens can be predicted from various kinetic schemes that have been published previously (Lester et al., 1990; Clements et al., 1992) (see Table 1). For example, a previous modeling study (Perkel and Nicoll, 1993) suggested that the NMDA receptor dose-response curve would be shifted to the right during brief pulses of agonist. Our study extends this theoretical work by performing actual glutamate dose-response curves on native and recombinant NMDARs, using different agonist application durations. In support of our work, modeling (Holmes, 1995) and experimental data (Perkel and Nicoll, 1993; Mainen et al., 1999; Umemiya et al., 1999) also suggest that NMDARs are not saturated during brief synaptic-like agonist pulses. Furthermore, brief iontophoretic pulses of glutamate applied to individual synaptic boutons of cultured hippocampal neurons showed decreased agonist potency under these nonequilibrium conditions (Dube and Liu 1999). Although the latter study suggested that glutamate potency might vary with agonist duration, agonist concentration was not precisely

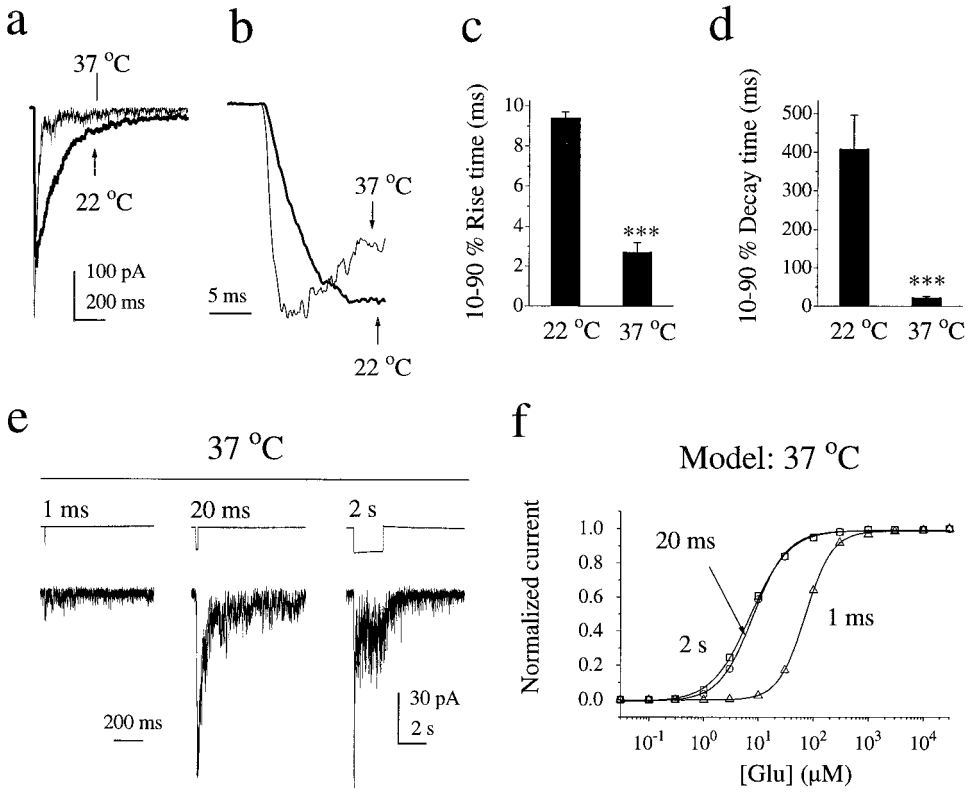


Fig. 4. Temperature-dependent kinetics of cortical neuron NMDA receptor-mediated currents. a, representative glutamate-evoked currents recorded from a single outside-out patch excised from cultured cortical neuron at 22 and 37°C (the thick trace is at 22°C). [Glu] = 1 mM, [Gly] = 50 μM, [CNQX] = 20 μM, [TTX] = 0.5 μM, [Mg^{2+}] = nominal 0, V_h = −60 mV. The agonist application duration was 20 ms. b, expanded records from a, showing the activation time course at 22 and 37°C (records were normalized to peak amplitude; thick trace is at 22°C). c, rise time (10–90%) for glutamate currents recorded at 22 (n = 5) and 37°C (n = 8), respectively. d, decay time (10–90%) for current recorded at 22 (n = 5) and 37°C (n = 8), respectively (1 mM glutamate application for 20 ms was used in the c and d group data). e, application duration-dependent increase in NMDAR peak current amplitude for an outside-out patch at 37°C (10 μM glutamate applied for indicated times). Above each record is the open tip response showing the solution exchange time course. f, model depicting the NMDAR dose-response curve for different agonist application durations using NR1A/NR2A parameters adjusted for 37°C (ramped rectangular agonist pulses as in Table 2 were used). Unpaired t tests were used to assess differences in activation and deactivation time courses (***$P < 0.001$).

controlled, making it impossible to construct accurate dose-response curves. Nor did the previous studies examine the sensitivity of recombinant NMDAR subtypes to this phenomenon. Therefore, the results we present here represent the first experimental evidence (to our knowledge) that the peak response EC_{50} value for NMDARs varies with duration of agonist application.

Because the NMDAR exhibits agonist duration-dependent changes in EC_{50} value, it is possible that this mechanism may allow the receptors to be tuned to different temporal properties of transmitter release. For example, if NMDARs were exposed to a low concentration of glutamate for a relatively long period of time, they could become effectively activated. Perhaps extended exposure of NMDARs to glutamate occurs with extrasynaptic spillover of transmitter during intense periods of activity (Barbour and Hausser, 1997; Min et al., 1998; Rusakov et al., 1999). The high potency of extended application of glutamate at NMDARs would also be expected to contribute to glutamate excitotoxicity triggered by ischemia, where exposure of neurons to micromolar levels of glutamate for minutes at a time is well documented (Benveniste et al., 1984). In contrast, exposure of receptors to the same glutamate concentration for a short time would limit activation and increase response fidelity. Furthermore, our data suggest that brief glutamate exposures result in similar peak response EC_{50} values for NR1A/NR2A and NR1A/NR2B, whereas more sustained exposures to low glutamate concentrations would activate a higher percentage of NR1A/NR2B- than NR1A/NR2A-type receptors at the peak of the current response. Thus, brief synaptic exposure to glutamate combined with the relatively slow activation rate reduces NMDAR saturation and may allow changes in transmitter release to be manifested at the postsynaptic level.

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