A Site in the Fourth Membrane-associated Domain of the N-Methyl-d-aspartate Receptor Regulates Desensitization and Ion Channel Gating*

Hong Ren, Yumiko Honse, Brian J. Karp, Robert H. Lipsky, and Robert W. Peoples†

From the Unit on Cellular Neuropharmacology, Laboratories of Molecular and Cellular Neurobiology and Neurogenetics, National Institute on Alcohol Abuse and Alcoholism, Bethesda, Maryland 20892-8115

The N-methyl-D-aspartate (NMDA) receptor has four membrane-associated domains, three of which are membrane-spanning (M1, M3, and M4) and one of which is a re-entrant pore loop (M2). The M1–M3 domains have been demonstrated to influence the function of the ion channel, but a similar role for the M4 domain has not been reported. We have identified a methionine residue (Met823) in the M4 domain of the NR2A subunit that regulates desensitization and ion channel gating. A tryptophan substitution at this site did not alter the EC₅₀ for glycine or the peak NMDA EC₅₀ but decreased the steady-state NMDA EC₅₀ and markedly increased apparent desensitization, mean open time, and peak current density. Results of rapid solution exchange experiments revealed that changes in microscopic desensitization rates and closing rates could account for the changes in macroscopic desensitization, steady-state NMDA EC₅₀, and current density. Other amino acid substitutions at this site could increase or decrease the rate of desensitization and mean open time of the ion channel. Both mean open time and desensitization were dependent primarily upon the hydrophobic character of the amino acid at the position. These results demonstrate an important role for hydrophobic interactions at Met823 in regulation of NMDA receptor function.

The N-methyl-d-aspartate (NMDA) receptor is a subtype of ionotropic glutamate receptor that plays essential roles in neuronal development, synaptic plasticity, and several types of neurological disorders (1). NMDA receptors are heteromeric containing NR1 subunits, which bind the co-agonist glycine (2, 3), and NR2 subunits, which bind the agonist glutamate (4, 5). The agonist-binding domains of all ionotropic glutamate receptor subunits consist of two lobes (S1 and S2) that are formed by the region of the extracellular N-terminal domain preceding the first membrane-associated (M) domain and the loop between the M3 and M4 domain, respectively (5–8), and that together form a clamshell structure that undergoes a conformational change to enclose the ligand upon binding (6, 9, 10).

Observations from x-ray crystallographic studies on non-NMDA glutamate receptor constructs suggest that the degree of binding domain closure induced by a particular agonist appears to determine both the degree of receptor activation and the extent of desensitization produced by that agonist (10) and that desensitization in these receptors results from the dissociation of dimers of the ligand-binding domains of adjacent subunits (11). NMDA receptors exhibit apparent desensitization sensitive to glycine or intracellular Ca²⁺ (1, 12) in addition to true glycine-insensitive desensitization (subsequently referred to simply as desensitization). NMDA receptor desensitization is physiologically relevant, as it can influence the amplitude, duration, and following frequency of NMDA receptor-mediated synaptic events (13–16). Determinants of NMDA receptor desensitization have been localized to two regions in or near the S1 ligand-binding site in the N-terminal domain, one that shows homology to leucine/isoleucine/valine-binding proteins and one located in the region immediately preceding the M1 domain (17, 18), as well as to a highly conserved motif (YTANLAAF) in the C-terminal portion of the M3 domain preceding the S2 ligand-binding lobe (19). The pre-M1 and M3 domains have been suggested to be involved in transducing the conformational changes induced by agonist binding into those responsible for ion channel gating (17, 19), but the nature of the conformational changes and molecular determinants underlying NMDA receptor ion channel gating remain unclear. The YTANLAAF motif in M3 has been shown to play an important role in the regulation of ion channel gating, as a point mutation in this region of the NR1 subunit increases mean open time in receptors formed from coexpression with NR2A subunits (19). Mutations at a tryptophan residue in the M2 domain of the NR1 or NR2A subunit have also been reported to subtly affect mean open time and opening frequency (20). We report here that a residue in the M4 domain of the NR2A subunit exerts a powerful regulatory influence on the desensitization and gating of the NMDA receptor ion channel.

EXPERIMENTAL PROCEDURES

Mutagenesis, Transfection, and Cell Culture—Site-directed mutagenesis in plasmids containing NR1-1a and NR2A subunit cDNA (Drs. D. R. Lynch, University of Pennsylvania; and D. M. Lovinger, Vanderbilt University) was performed using the QuickChange kit (Stratagene, La Jolla, CA), and all mutations were verified by DNA sequencing. Human embryonic kidney (HEK) 293 cells were seeded in 35-mm dishes, allowed to grow to 70–85% confluence, and transfected with cDNA for the wild-type or mutant NR1 and NR2A subunits and green fluorescent protein at a 2:2:1 ratio, respectively, using LipofectAMINE 2000 or calcium phosphate (both from Invitrogen). The culture medium during and after the transfection step contained 100 µM ketamine and 200 µM mGlu-5-agonist (2-amino-5-phosphonovaleric acid) to minimize cell death due to excitotoxicity. Cells were used in experiments 18–72 h after transfection.

Western Blot Analysis—Biotinylation and membrane solubilization

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†To whom correspondence should be addressed: Unit on Cellular Neuropharmacology, LMCN, NIAAA, Park 5 Bldg., Rm. 150, 12420 Parklawn Dr., MSC 8115, Bethesda, MD 20892-8115. Tel.: 301-443-1236; Fax: 301-480-6882; E-mail: bpeoples@helix.nih.gov.

‡The abbreviations used are: NMDA, N-methyl-D-aspartate; HEK, human embryonic kidney; BAPTA, 1,2-bis(2-aminophosphorylethane-N,N,N’,N’-tetraacetic acid; ANOVA, analysis of variance.

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were performed as described by Chen et al. (21) and Hu and Wenthold (22). Transfected HEK-293 cells were washed with phosphate-buffered saline and incubated with the membrane-impermeable reagent sulfoxyvocinimidyl 2-biotinamidoethyl-1,3,1'-dithiopropionate (1.5 mg/ml; Pierce) in Buffer A (0.5 mM MgCl2 and 1 mM CaCl2 in phosphate-buffered saline) for 30 min at 4 °C. Cells were washed and incubated with Buffer A containing 100 mM glycine for 30 min at 37 °C and then harvested in Buffer B (50 mM Tris (pH 7.5), 150 mM NaCl, 0.02% NaN3, 1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and 2 

μg/ml aprotinin). After centrifugation (2000 × g for 15 min), pellets were solubilized with 2% SDS in Buffer B for 3 min at 90 °C. The solubilized membrane fraction was centrifuged at 49,000 × g for 30 min at 15 °C, and then the supernatant was diluted six times with 2% Triton X-100 in Buffer B. Aliquots were subjected to 7% SDS-PAGE (lysat) and protein assay (bicinchoninic acid protein assay kit, Pierce), and the remainder was incubated with NeutrAvidin-linked beads (Pierce) overnight at 4 °C. Following washing and centrifugation, the samples were subjected to 7% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blocked for 1 h in TBST (Tris-buffered saline with 0.1% Tween 20) containing 5% nonfat milk. Nitrocellulose membranes were incubated with rabbit polyclonal anti-NR2A antibody (1:1000; Chemicon International, Inc., Temecula, CA) in TBST with 1% nonfat milk overnight at 4 °C. After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG as the secondary antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Proteins were visualized by enhanced chemiluminescence (PerkinElmer Life Sciences) and quantitated using Typhoon and ImageQuant Version 5.1 (Amersham Biosciences).

Electrophysiological Recording—Patch-clamp recording was performed at room temperature using an Axopatch 1D or 200B amplifier (Axon Instruments, Inc., Union City, CA). For whole-cell recording, gigahm seals were formed using patch pipettes with tip resistances of 1–7 megohms, and series resistances of 4–10 megohms were compensated by 80%. In some experiments, cells were lifted off the surface of the dish after obtaining a gigaohm seal to increase the speed of the solution exchange; in these experiments, patch pipettes were pulled of the dish after obtaining a gigaohm seal to increase the speed of the solution exchange. For single-channel recordings, patch pipettes were coated with R6101 elastomer (Dow-Corning), and had tip resistances of 7–15 megohms following fire polishing. Cells were voltage-clamped at −50 mV, unless noted otherwise. Data were filtered (2-kHz low-pass 8-pole Bessel for whole-cell recording and 1-kHz low-pass 8-pole Butterworth for fluctuation analysis) and acquired at 5–20 kHz on a computer using a DigiData interface and pClamp software (Axon Instruments, Inc.). Cells were superfused at 1–2 ml/min in extracellular medium containing 150 mM NaCl, 5 mM KCl, 0.2 mM CaCl2, 10 mM HEPES, and 10 mM glucose; the pH was adjusted to 7.4 using NaOH, and the osmolality was adjusted to 340 mmol/kg using sucrose. The patch pipette solution in the cell was composed of 140 mM NaCl, 2 mM MgCl2, 4 mM CaCl2, 10 mM BAPTA, and 10 mM HEPES; the pH was adjusted to 7.4 using CH3OH, and the osmolality was adjusted to 310 mmol/kg using sucrose. In recordings from cell-attached patches, the patch pipette solution contained 1 μM NMDA, 50 μM glycine, and 10 μM EDTA in extracellular medium. Solutions of agonists and drugs were prepared fresh daily in extracellular medium and applied to cells using a stepper motor-driven rapid solution exchange apparatus used without the metallic manifolds supplied by the manufacturer (Fast-Step, Warner Instrument Corp.) and 600-μm inner diameter square three-barrel glass tubing. The 10–90% rise time for solution exchange in lifted cells was ≈1.5 ms, which was determined using steps from 0 to 150 mM Na+ in the continuous presence of agonist.

Concentration-response data were analyzed using the nonlinear curve-fitting program ALLFIT (26), which allows statistical comparison of parameters from multiple curves by measuring the degradation in goodness of fit (using an F test for the sums of squares of the residuals) that results from constraining these parameters to be equal. Values reported for half-maximal concentration (EC50s) and slope factor (n) are those obtained by fitting the data to Equation 1:

\[ y = \frac{EC_{\text{max}}}{1 + (EC_{50}/y^*)^{n}} \]

where \( y \) and \( y^* \) are concentration and response, respectively, and \( EC_{\text{max}} \) is the maximal response. In fluctuation analysis experiments, fast Fourier transformations of 25–60 data sweeps of 600–800 ms were averaged; background spectra were subtracted; and the data were fitted with a lorentzian function of the form shown in Equation 2,

\[ S_f = \frac{S_0}{1 + (s^2)^2} \]

where \( S_f \) is the spectral density at frequency \( f \) (in hertz), \( S_0 \) is the zero frequency asymptote, and \( s \) is the corner frequency. Time constants (\( \tau \)) were obtained from the relationship \( \gamma = 1/2\pi\tau \). Mean current amplitude and variance of the data were obtained using Clampfit Version 8.0 (Axon Instruments, Inc.); single-channel conductances (\( \gamma \), in picosiemens) were determined from the relationship \( \gamma = e\psi_{\text{m}} \), where \( e\psi_{\text{m}} \) is the membrane holding potential (in volts) and \( \psi \) is the slope (in picoamperes) of the least-squares linear fit to the initial region of a current variance versus amplitude plot. Data from single-channel recordings were idealized using the program SKM (QUB suite) (27), which utilizes a hidden Markov event detection algorithm. These data were filtered at 10 kHz (8-pole Bessel) to ensure optimal detection of brief (submillisecond) openings and closings using SKM; similar results were obtained using 2-kHz filtering and a 50% threshold event detection criterion. Maximal likelihood multiple exponentials were fitted to dwell time histograms using Clampfit, and gaussian functions were fitted to all-points histograms using TAC (Bruxton Corp., Seattle, WA). No attempt was made to correct for missed events, as no appropriate correction procedure is currently available for NMDA receptors (28).

Data obtained in rapid solution exchange experiments were fitted to a simple five-state kinetic model (Scheme 1) in which A represents agonist; R represents receptor; and the subscripts C, O, and D represent closed, open, and desensitized states of the channel, respectively. In this model, \( k_1 \) and \( k_2 \) are the respective agonist binding and unbinding rates; \( \beta \) and \( \alpha \) are the respective channel opening and closing rates; and \( d_1 \) and \( d_2 \) are the rates for entry into and recovery from the desensitized state, respectively. Data from rapid solution exchange experiments were fitted to the model using the program SCoPFit (Simulation Resources, Redlands, CA), with \( k_1 \), constrained to the value determined in cultured hippocampal neurons (under conditions in which NMDA receptor subunit expression should have been predominantly NR1/ NMDA (25), with 0.005 μm−1 ms−1 and 0.0010 μm−1 ms−1, respectively); and with all other rates allowed to vary. Simulated current values were generated using the program Scop, with all parameters in the model set to their respective average values determined from fits to the data. Analysis of variance (ANOVA), correlation analysis, and linear regression analysis were performed using the program StatView (SAS Institute, Inc., Cary, NC). Fisher’s protected least significant difference test was used to determine differences among means following ANOVA. All values are reported as the means ± S.E.

Results

Effect of the NR2A(M823W) Mutation on the NMDA Concentration Response—The results of a previous study have demonstrated that Val823Ala (29) in the M4 domain of the NMDA receptor NR1 subunit are not exposed to the lumen of the ion channel (31). Mutation of these residues to tryptophan, which, as the largest and most hydrophobic amino acid, should be the substitution most likely to disrupt protein-protein interactions (29), produced little or no change in receptor function (as measured by apparent desensitization) (data not shown), with the exception of the NR1(M818W) mutant, which was nonfunctional. This methionine residue is conserved in NMDA receptor NR1 and NR2 subunits (Fig. 1). However, tryptophan substitution of the corresponding residue in the NR2A subunit had a pronounced effect on receptor function. Fig. 2A illustrates cur-
Met823 (NR2A(M823W) mutation did not alter the EC50 of glycine for not altered by the NR2A(M823W) mutant (Fig. 2). In contrast, the concentration-response curve for glycine was versus 0.424 NMDA concentration in receptors containing both wild-type the slower component was significantly dependent upon binding domains S1 and S2 (thick lines), and the presumed position of M4, ligand-NR2A subunit showing membrane-associated domains M1–M4, ligand-binding domains S1 and S2 (thick lines), and the presumed position of MetM (shaded band).

As is evident, the NR2A(M823W) mutant exhibited a greater degree of apparent desensitization at all NMDA concentrations tested. The NR2A(M823W) mutation did not alter the EC50 of NMDA for activation of peak current (35.1 ± 4.39 versus 40.2 ± 6.39 μM; ALLFIT analysis; p > 0.05) (Fig. 2B), but significantly lowered the EC50 of NMDA for activation of steady-state current (4.98 ± 1.08 versus 23.4 ± 3.76 μM; ALLFIT analysis; p < 0.05). In contrast, the concentration-response curve for glycine was not altered by the NR2A(M823W) mutant (Fig. 2C). The NR2A(M823W) mutation did not alter the EC50 of glycine for activation of peak current (4.03 ± 0.322 versus 3.76 ± 0.442 μM; ALLFIT analysis; p > 0.05) or steady-state current (2.97 ± 0.424 versus 3.41 ± 0.274 μM; ALLFIT analysis; p > 0.05).

**Effect of the NR2A(M823W) Mutation on Desensitization—**As noted above, the NR2A(M823W) mutation dramatically altered the apparent desensitization of the NMDA-activated current. We accordingly tested the effect of the mutation on the desensitization kinetics of current activated by different concentrations of NMDA in lifted cells using a fast solution exchange apparatus. In the majority of cells expressing wild-type NR2A or mutant NR2A(M823W) subunits, desensitization of NMDA-activated current following prolonged application of NMDA exhibited two exponential components (Fig. 3A). The time constant for the faster component was not altered by NMDA concentration in wild-type or mutant receptors (ANOVA; p > 0.05) (Fig. 3B) and did not differ between wild-type and mutant receptors (ANOVA; p > 0.05). This fast component of desensitization was most probably attributable to the presence of ambient Zn2+ (33, 34), as it was abolished in the presence of 10 μM EDTA (data not shown). In contrast, the time constant for the slower component was significantly dependent upon NMDA concentration in receptors containing both wild-type and mutant NR2A(M823W) subunits (ANOVA; p < 0.001) and was ~3–7-fold lower in NR1/NR2A(M823W) receptors compared with wild-type receptors (ANOVA; p < 0.0001).

**Effect of the NR2A(M823W) Mutation on Ion Channel Gating—**To determine whether the NR2A(M823W) mutation could alter other ion channel gating parameters in addition to desensitization, we used single-channel recording in patches from cells expressing wild-type or mutant receptors (Fig. 4). Single-
channel conductance measured at −50 mV was not altered by the NR2A(M823W) mutation (55.0 ± 1.26 vs 52.7 ± 1.16 pSi for wild-type versus NR2A(M823W) receptors, respectively; t test; p > 0.05). Single-channel currents in cell-attached patches from cells expressing wild-type NR1/NR2A receptors yielded open time distributions that could be fitted with three exponential components with average time constants of 51.7 ± 6.39 μs (59.6%), 0.831 ± 0.0616 ms (20.6%), and 3.66 ± 0.547 ms (28.4%) (n = five patches) (Fig. 4B). Open time distributions of single-channel currents in cells expressing NR1/NR2A(M823W) receptors exhibited four exponential components with average time constants of 51.7 and 10.9 ms.

Similar observations were obtained in experiments using fluctuation analysis. Variance analysis of current activated by NMDA at concentrations of 1–10 μM revealed that the unitary conductance of the ion channel was not changed by the NR2A(M823W) mutation (48.0 ± 1.87 vs 48.4 ± 2.11 pSi).

FIG. 4. The NR2A(M823W) mutation increases the mean open time of the NMDA receptor. A, currents activated by 1 μM NMDA in the presence of 50 μM glycine and 10 μM EDTA in cell-attached patches from cells expressing wild-type NR1/NR2A (left panel) or mutant NR1/NR2A(M823W) (right panel) subunits. The membrane holding potential was +50 mV; channel openings are upward. Lower traces are segments of the upper traces shown with an expanded time scale. B, open time histograms from cell-attached patches containing wild-type NR1/NR2A (left panel) or mutant NR1/NR2A(M823W) (right panel) subunits. Dashed curves indicate maximal likelihood multiple exponential fits to the data, and dotted curves indicate the individual exponential components. The traces in A and the corresponding distributions in B are from the same individual patches; similar results were obtained in four other patches. C, power density spectra of NMDA-activated current in cells expressing wild-type NR1/NR2A (left panel) or mutant NR1/NR2A(M823W) (right panel) subunits. Spectra from individual data sweeps were averaged following fast Fourier transformation. The curves are the best fits of the data to the lorentzian function (Equation 2) under “Experimental Procedures.” The corner frequencies of 47.8 and 14.6 Hz obtained in cells expressing wild-type NR1/NR2A and mutant NR1/NR2A(M823W) subunits, respectively, correspond to time constants of 3.33 and 10.9 ms.

FIG. 3. Effect of the NR2A(M823W) mutation on desensitization kinetics. A, traces illustrating desensitization of current activated by various micromolar concentrations of NMDA in lifted cells expressing wild-type NR1/NR2A or mutant NR1/NR2A(M823W) subunits. The curves are double-exponential fits to the data. B, average time constants for desensitization of NMDA-activated current. At 30 μM NMDA, three of seven cells expressing wild-type (WT) receptors exhibited the fast component of desensitization; for all other treatment conditions, n = 5–13 cells. Asterisks denote significant differences from the same component of desensitization in cells expressing wild-type receptors (ANOVA and Fisher’s protected least significant difference test; p < 0.0001). The fast and slow components at each NMDA concentration in each subunit combination differed significantly (ANOVA and Fisher’s protected least significant difference test; p < 0.001).
contribution of the second open time constant, under these conditions, the values obtained in noise analysis appear to represent primarily the contribution of the two longest time constants. These values agreed well with the proportionally weighted means of the third and fourth open time constants obtained in single-channel analysis (11.5 ± 1.24 and 3.66 ± 0.550 ms for mutant and wild-type receptors, respectively).

**Kinetic Modeling of the Effect of the NR2A(M823W) Mutation**—The data obtained for either wild-type NR1/NR2A or mutant NR1/NR2A(M823W) receptors in fast exchange experiments could be adequately fitted to a simple five-state kinetic model (Fig. 5A). Average values for rate constants obtained from the fits of the data to the model indicated that the NR2A(M823W) mutation increased the rate of entry into the slow desensitized state (9.09 versus 0.696 s⁻¹ for mutant and wild-type receptors, respectively; ANOVA; p < 0.0001) and decreased the rate of exit from this state (0.0582 versus 0.798 s⁻¹ for mutant and wild-type receptors, respectively; ANOVA; p < 0.001), but did not significantly alter the rates of ion channel opening (0.137 versus 0.102 ms⁻¹ for mutant and wild-type receptors, respectively; ANOVA; p > 0.5) or agonist unbinding (0.217 versus 0.183 ms⁻¹ for mutant and wild-type receptors, respectively; ANOVA; p > 0.5). Comparison of simulated traces generated from the model (Fig. 5B) using the average values obtained from fits to the data showed that the model can account not only for the marked increase in macroscopic desensitization produced by the NR2A(M823W) mutation, but also for the >-2-fold increase in peak current density and decrease in steady-state current density observed in cells expressing the mutant subunit (Fig. 5C). This increase in peak current density was not attributable to an increase in receptor expression, as the NR2A(M823W) mutation did not alter cell-surface receptor expression as determined in Western blot experiments (Fig. 5, D and E).

**Effect of Other NR2A Met823 Mutations on Ion Channel Gating**—In an attempt to identify the physicochemical parameters of the substituent at the NR2A Met823 site responsible for regulating the gating of the ion channel, we constructed and tested a panel of substitution mutants at this site. Substitution of highly polar residues (Asp, His, and Lys) or glycine at this site did not yield functional receptors. As was observed for the NR2A(M823W) mutant, a number of the remaining mutant subunits showed marked differences in the EC₅₀ for NMDA activation of steady-state current, the maximal steady-state current/peak current ratio, and the mean open time (Table 1), whereas the EC₅₀ for NMDA activation of peak current and the unitary conductance were unchanged. The single exception to this was the NR2A(M823I) mutant, which exhibited a unitary conductance of 40 picoSiemens and an increased EC₅₀ for NMDA activation of peak current compared with wild-type receptors. The mean open time of NR1/NR2A(M823I) receptors was, however, dramatically attenuated compared with that of wild-type receptors (0.77 ms), which could account for an apparent reduction in unitary conductance due to the short duration of many channel opening events relative to the rise time of the recording system. Linear regression analysis of the results obtained with the series of NR2A Met823 mutants revealed a significant linear relation between the steady-state current/peak current ratio and the substituent amino acid hydrophathy (R² = 0.516; linear regression ANOVA; p < 0.05) (Fig. 6A), but not molecular volume, hydrophilicity, or polarity (R² = 0.001, 1.01 × 10⁻⁶, and 0.288, respectively; linear regression ANOVA; p > 0.05). Similarly, the mean open time of the series of mutants was linearly related to hydrophathy (R² = 0.424; linear regression ANOVA; p < 0.01) (Fig. 6B) as well as to polarity (R² = 0.427; linear regression ANOVA; p < 0.05), but not to molecular volume or hydrophilicity (R² = 0.262 and 0.217, respectively; linear regression ANOVA; p > 0.05). Although there was an apparent trend toward an inverse relationship between steady-state current/peak current ratio and mean open time in the various NR2A Met823 mutants, these parameters were not significantly correlated (R² = 0.3036; Fisher’s z test; p > 0.05) (Fig. 7A). The values for the steady-state NMDA EC₅₀ were, however, highly correlated with steady-state current/peak current ratios (R² = 0.9332; Fisher’s z test; p < 0.0001), whereas there was no correlation between the values for the peak NMDA EC₅₀ and steady-state current/
peak current ratios ($R^2 = 0.0144$; Fisher’s $z$ test; $p > 0.05$) (Fig. 7B).

**DISCUSSION**

In this study, mutation of a highly conserved methionine residue (Met<sup>823</sup>) in the NR2A subunit of the NMDA receptor to tryptophan dramatically increased the rate of macroscopic desensitization as well as the mean open time of the ion channel. In wild-type NR1/NR2A receptors, the extent of desensitization ($I_{ss}/I_{p} = 0.58$ at 1 mM NMDA) and the slow time constant of desensitization ($\tau_{ss} = 2.0$ s at 300 nM NMDA) were similar to those previously observed under similar conditions ($I_{ss}/I_{p} = 0.47–0.65$ and $\tau_{ss} = 1.1–1.9$ s) (17, 34, 35). The faster of the two components of apparent desensitization was presumably attributable primarily to the presence of Zn<sup>2+</sup> in the extracellular solution, as its value was in the range (200–300 ms) reported for apparent desensitization due to high-affinity Zn<sup>2+</sup> inhibition (34), and as it was not observed in the presence of the chelator EDTA. This fast component of desensitization was also not significantly altered by the NR2A(M823W) mutation. In contrast, the slower component of desensitization was markedly altered by the NR2A(M823W) mutation. A marked alteration in the rates of entry into and exit from the desensitized state in receptors containing the NR2A(M823W) mutant was also observed when the data were fitted to a simple kinetic model. Although it is possible that the mutation may have introduced a new desensitized state, this should have been evident as an additional component of desensitization rather than as a change in the rate of an existing component. This
residue in the M4 domain thus appears to be critical in regulating the transition of the ion channel into and out of the slow desensitized state. In addition, NR2A Met\textsuperscript{823} regulates the rate of ion channel closing, as was observed in noise analysis experiments in which the NR2A(M823W) mutation increased the mean open time of the ion channel. Results from single-channel recordings indicated that this increase in mean open time in receptors containing the NR2A(M823W) mutant was attributable to the introduction of an additional long open time not present in wild-type channels.

Results of previous studies support a physiological role for mean open time and desensitization of NMDA receptors in the regulation of synaptic transmission. Alterations in the phosphorylation state of NMDA receptors can produce changes in mean open time that significantly affect the amplitude of NMDA receptor-mediated synaptic events (36, 37), and drugs such as ethanol that decrease NMDA receptor mean open time (38, 39) depress the amplitude of the NMDA receptor component of postsynaptic potentials (40). NMDA receptor desensitization can influence the duration and following frequency as well as the amplitude of synaptic events (13–16). Although the time constant of desensitization affected by the NR2A(M823W) mutation in this study is slow relative to that of the glutamate concentration transient in the synaptic cleft (~1 ms) (52), this component of desensitization may nevertheless participate in the regulation of NMDA receptor function. NMDA receptor channel gating kinetics slow the dissociation of glutamate (15, 41), with the result that the time constant for decay of NMDA receptor-activated synaptic currents can be in the range 150–300 ms (41, 42). Furthermore, time constants for recovery from desensitization may exceed 500 ms (43). The contribution of the slow component of NMDA receptor desensitization to regulation of synaptic activity would thus be predicted to increase with prolonged receptor stimulation, such as would occur during repetitive firing, which could cause a significant proportion of the receptors to accumulate in the desensitized state.

The trend toward a relation between desensitization and open time in the various NR2A Met\textsuperscript{823} mutants observed in this study may indicate that, in general, the longer the channel is open, the more likely it is to enter the desensitized state. This would seem to agree with the molecular mechanism of desensitization recently proposed for non-NMDA glutamate receptors (11). The lack of a significant correlation between steady-state current/peak current ratio and mean open time in the various mutants may indicate that other factors such as structural differences differentially regulate desensitization and open time at this site. Mutations at NR2A Met\textsuperscript{823} did not influence the NMDA EC\textsubscript{50} for peak current, with the exception of the NR2A(M823I) mutant, in which case it is likely that the very brief duration of ion channel opening seen in this mutant was responsible for the increase in the NMDA EC\textsubscript{50} for activation of peak current due to a reciprocal influence of ion channel gating on agonist binding (44). In contrast to the results obtained for the NMDA EC\textsubscript{50} for peak current, six of nine NR2A Met\textsuperscript{823} mutations significantly decreased the NMDA EC\textsubscript{50} for steady-state current activation. The decreased steady-state EC\textsubscript{50} values were most probably attributable to the increased desensitization observed in these mutant receptors, as they were highly correlated with steady-state current/peak current ratios and were also predicted by the kinetic model (data not shown). If this interpretation is correct, the increase in apparent affinity for mutants exhibiting a high degree of desensitization would result from an increase in the number of agonist molecules trapped on desensitized receptors (45), as has been observed in \gamma-aminobutyric acid type A receptors (46), rather than from an increase in the affinity of the binding site for agonist. This interpretation is also consistent with the observed lack of correspondence between peak and steady-state NMDA EC\textsubscript{50} values in the various mutants, the lack of correlation between NMDA EC\textsubscript{50} values for peak current and steady-state current/peak current ratios, and the absence of a change in the agonist unbinding rate determined using kinetic modeling.

The results of linear regression analyses using a number of physicochemical measures of amino acids were consistent with an important influence of the hydrophobicity of the substituent at NR2A Met\textsuperscript{823} upon both mean open time and desensitization. Thus, hydrophobic interactions between NR2A Met\textsuperscript{823} and a closely apposed residue or region of the protein may stabilize the ion channel in the closed and desensitized states. The observation that the molecular volume of the substituent did not influence either parameter in a systematic manner suggests that the regulation exerted by the residue at this site on ion channel gating behavior does not involve a simple volume occupation, as appears to be the case for similar residues in \gamma-aminobutyric acid type A and glycine receptors (47–50). The inverted V shape of the I\textsubscript{ss}/I\textsubscript{ps} versus molecular volume plot may indicate, however, the existence of an optimal value for molecular volume at this position at which desensitization is minimal. The lack of an absolute relationship between ion channel open time and desensitization in the various mutants tested is consistent with a differential influence of this site on the transition rates for the closed and desensitized states of the ion channel. It is likely that these differences may be attributable to subtle structural characteristics of the amino acid at the site, which are not well represented by relatively crude measures such as overall molecular volume and hydrophobicity of the side chain.

A previous study investigating the aqueous accessibility of residues in the proximal region of M4 of the NR1 subunit (31) did not report any alteration in ion channel function following cysteine substitution at NR1 Met\textsuperscript{518}, the cognate site to NR2A Met\textsuperscript{823}. In the present study, substitution of a tryptophan at this position resulted in nonfunctional receptors. Because tryptophan is the largest and most hydrophobic amino acid, the lack of function of this mutant NR1 subunit is most likely due to an intolerance of this position to the increase in molecular volume or hydrophobicity. This observation, coupled with the observation that tryptophan substitutions at the adjacent residues in the NR1 subunit did not alter receptor function, suggests that this residue may influence ion channel function, albeit not in a manner identical to that of its cognate site in the NR2A subunit.

Results of studies on the NR1 subunit (31, 51) suggest that part of the N-terminal end of the region originally identified as

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**Fig. 7.** Relation between NMDA receptor desensitization and mean open time or NMDA EC\textsubscript{50}. The graphs plot steady-state/peak ratios (I\textsubscript{ss}/I\textsubscript{ps}) of current activated by 1000 \mu M NMDA and 10 \mu M glycine against the mean open time of channels activated by 5 \mu M NMDA and 10 \mu M glycine (A) or the EC\textsubscript{50} for NMDA activation of peak or steady-state current (B).
M4 is located outside of the membrane. Thus, NR2A Met823 may be located much closer to the extracellular face of the membrane than would be originally inferred based upon its position in the sequence of the M4 domain. Assuming that the segment of the M4 domain that actually spans the membrane is α-helical in structure, NR2A Met823 is most likely oriented toward one or more of the other membrane-associated domains, rather than toward the lipid membrane, because small changes in the residue at this position (e.g., leucine versus isoleucine) could dramatically influence ion channel function in a manner that is consistent with a protein-protein interaction, but difficult to envision for a protein-lipid interaction. In addition, the results of this study clearly demonstrate that this site influences the behavior of the ion channel gating region. Sites in the pre-M1 and M3 domains also influence ion channel gating and desensitization and may be involved in transducing agonist binding into ion channel gating (17, 19). In non-NMDA glutamate receptors, agonist binding appears to induce a movement of the S2 ligand-binding domain relative to S1 (11), which may indicate that NR2A Met823 influences ion channel gating via hydrophobic interactions with an adjacent site in M3 or another membrane-associated domain.

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A Site in the Fourth Membrane-associated Domain of the N-Methyl-d-aspartate Receptor Regulates Desensitization and Ion Channel Gating
Hong Ren, Yumiko Honse, Brian J. Karp, Robert H. Lipsky and Robert W. Peoples

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