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The Selectivity Filter of the *N*-Methyl-D-Aspartate Receptor: A Tryptophan Residue Controls Block and Permeation of Mg^{2+}

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ABSTRACT

A hallmark feature of *N*-methyl-D-aspartate (NMDA) receptors is their voltage-dependent block by extracellular Mg^{2+}. The structural basis for Mg^{2+} block is not fully understood. Although asparagine residues in the pore-forming M2 regions of NR1 and NR2 subunits influence Mg^{2+} block, it has been speculated that additional residues are likely to be involved. Here, we report the unexpected finding that a tryptophan residue in the M2 region of NR2 subunits controls Mg^{2+} block. An NR2B(W607L) mutation abolished block and greatly increased permeation of extracellular Mg^{2+}. A similar effect was seen with a mutation at the equivalent residue in NR2A but not with mutations at the equivalent residue or adjacent residues in NR1. In NR2B, mutations that changed NR2B(W607) to asparagine (W607N) or alanine (W607A) also greatly reduced Mg^{2+} block, whereas mutations that changed W607 to the aromatic residues tyrosine (W607Y) or phenylalanine (W607F) had little or no effect on Mg^{2+} block. Furthermore, the W607L, W607N, and W607A mutants, but not the W607Y and W607F mutants, decreased Ba^{2+} permeability of NMDA channels. Thus, residue NR2B(W607) may be involved in binding of divalent cations, in particular Mg^{2+}, through a cation-π interaction with the electron-rich aromatic ring of the tryptophan. We previously suggested that NR2B(W607) may contribute to the narrow constriction of the NMDA channel. A model is now proposed in which the M2 loop of NR2B is folded in such a way that NR2B(W607) is positioned at the narrow constriction, at a level similar to NR2B(N616) and NR1(N616), with these three residues forming a binding site for Mg^{2+}.

NMDA receptors are glutamate-gated ion channels that are involved in synaptic plasticity and ischemic neuronal cell death. A hallmark feature of NMDA receptors is their voltage-dependent block by extracellular Mg^{2+} (Mayer *et al.*, 1984; Nowak *et al.*, 1984). NMDA channels are blocked by Mg^{2+} at resting membrane potentials, and the block is relieved as neurons are depolarized, which allows the receptors to function as synaptic coincidence detectors.

NMDA receptors are hetero-oligomers containing combinations of NR1 and NR2 subunits in as-yet-undefined ratios and stoichiometries (Luo *et al.*, 1997; Monyer *et al.*, 1992; Moriyoshi *et al.*, 1991; Sheng *et al.*, 1994). The receptor subunits are thought to contain three membrane-spanning regions (M1, M3, and M4) and a reentrant loop (M2) that contributes to the permeation pathway of the ion channel (Bennett and Dingledine, 1995; Wo and Oswald, 1995; Wood *et al.*, 1995). Asparagine residues in the M2 segments of NR1 and NR2 subunits influence block by Mg^{2+} and permeation of Ca^{2+} or Ba^{2+} (Burnashev *et al.*, 1992; Mori *et al.*, 1992; Kawajiri and Dingledine, 1993; Sakurada *et al.*, 1993; Kupper *et al.*, 1996). These residues, which include N616 in NR1, N615 in NR2A, and N616 in NR2B, seem to contribute to the narrowest constriction of the channel pore (Kuner *et al.*, 1996; Wollmuth *et al.*, 1996) and may form part of a binding site for Mg^{2+}. It is conceivable that other residues in the channel pore may be involved in block by Mg^{2+}, but such residues have not yet been identified. Many studies of NMDA channel function have focused initially on amino acid residues in the NR1 subunit, followed by studies of the equivalent or adjacent residues in NR2 subunits. This strategy identified an important role of the asparagines at NR1(N616) and NR2B(N616) in Mg^{2+} block, but other residues that have been studied in the M2 region of NR1 have no effect or only modest effects on Mg^{2+} block (Burnashev *et al.*, 1992; Mori *et al.*, 1992; Kawajiri and Dingledine, 1993; Sakurada *et al.*,

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; N^1-DnsSpm, N^1-dansyl-spermine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GluR, glutamate receptor; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid; I-V, current-voltage.

1993; Kupper *et al.*, 1996). We recently found that trypto-phan residues in the M2 regions of NR2 subunits, including W607 in NR2B, influence permeation of the novel polyamine channel blocker N^1-DnsSpm (Kashiwagi *et al.*, 1997). Here, we show that mutations at NR2B(W607), but not at the equivalent position in the NR1 subunit, greatly influence block by extracellular Mg^{2+}. This tryptophan residue may form part of the selectivity filter and the Mg^{2+} binding site of the NMDA channel, and the results of this study have impli-cations for understanding the structure of the M2 loop and the roles of different NMDA receptor subunits in channel function.

Materials and Methods

cDNA clones and site-directed mutagenesis. The wild-type NR1 clone (Moriyoshi *et al.*, 1991) and the NR1(W608L) and NR1(F609L) mutants (Sakurada *et al.*, 1993) were gifts from Dr. S. Nakanishi (Institute for Immunology, Kyoto University Faculty of Medicine, Kyoto, Japan). The NR1(N616R) mutant (Kawajiri and Dingledine, 1993) was a gift from Dr. R. J. Dingledine (Department of Pharmacology, Emory University, Atlanta, GA). The wild-type NR2A and NR2B clones (Monyer *et al.*, 1992) were gifts from Dr. P. H. Seeburg (Center for Molecular Biology, University of Heidel-berg, Germany). NR1 and NR2 mutants were prepared by site-directed mutagenesis using the M13 phage system (Kunkel *et al.*, 1987; Sayers *et al.*, 1992), and mutations were confirmed by DNA sequencing. In some experiments, we used a rat brain NR2B clone containing the W607L mutation (Kashiwagi *et al.*, 1997). In other experiments, we used a mouse brain NR2B clone, ε2 (Kutsuwada *et al.*, 1992) (a gift from Dr. M. Mishina, University of Tokyo, Tokyo, Japan), containing a 1.7-kb *Hin*dIII/*Sph*I fragment of the rat NR2B clone with the W607L mutation. A similar construct was used to prepare the NR2B(W607N), NR2B(W607A), NR2B(W607Y), and NR2B(W607F) mutants. In control experiments, we found that an ε2 clone containing the wild-type *Hin*dIII/*Sph*I fragment of rat NR2B had properties indistinguishable from wild-type NR2B (data not shown). Oligonucleotides (sense strands) used for preparation of NR2B mutants were 5'-CTG GTG TTT AAC GGC TCC GTA CCT GT-3' for NR2B(N616G), 5'-GGC AAA GCA ATT AAT TTA CTC TGG GGT C-3' for NR2B(W607N), 5'-GGC AAA GCA ATT GCC TTA CTC TGG GGT-3' for NR2B(W607A), 5'-GCA AAG CAA TTT ACT TAC TCT GGG GT-3' for NR2B(W607Y), and 5'-GCA AAG CAA TTT TCT TAC TCT GGG GT-3' for NR2B(W607F). The oligonucleotide for NR1(N616W) (antisense) was 5'-CCC CAA TGC CGG ACC AGA GCA GGA CGC CC-3'. Underlined nucleotides indicate the position of the mutations. The double mutant, NR2B(W607L,N616G) was prepared by using the oligonucleotide for the NR2B(N616G) muta-tion with a single-strand DNA fragment of NR2B that contained the W607L mutation. Other NR1 and NR2 mutants were prepared as described previously (Chao *et al.*, 1997; Kashiwagi *et al.*, 1997). Most of the 5'-UTR was removed from the NR2B (Williams, 1993) and ε2 clones to improve expression in oocytes. The 5'-UTR of the ε2 clone was truncated at the *Sal*I site, leaving a 5'-UTR of 50 nucleotides.

Expression in oocytes and voltage-clamp recording. Defol-liculated oocytes were prepared and maintained as described previ-ously (Williams *et al.*, 1993). Oocytes were injected with NR1 plus NR2 cRNAs in a ratio of 1:5 (0.2–4 ng of NR1 plus 1–20 ng of NR2). Macroscopic currents were recorded with a two-electrode voltage-clamp using a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA) or an OC-725 amplifier (Warner Instruments, Hamden, CT) as described previously (Williams, 1993, 1994; Williams *et al.*, 1993). Oocytes were continuously superfused with a saline solution (96 mM NaCl, 2 mM KCl, 1.8 mM BaCl$_2$, 10 mM HEPES, pH 7.5) (Na$^+$ saline). In most experiments, oocytes were injected with K$^+$-BAPTA (100 nl of 40 mM, pH 7.0–7.4) on the day of recording (Williams, 1993).

Reversal potentials were calculated by linear regression of data over a 10-mV range, 5 mV positive and 5 mV negative to an esti-mated reversal potential. The slope conductances at −50 mV and at +50 mV were measured by linear regression of data from −45 to −55 mV and from +45 to +55 mV. In experiments using Mg^{2+} as the extracellular charge carrier, the Na^+ saline was replaced with a solution that contained 64 mM MgCl$_2$, 2 mM KCl, and 10 mM HEPES, pH 7.5 (Mg^{2+} saline). In most experiments, the pH of this solution was adjusted to pH 7.5 using NaOH, and the Mg^{2+} saline therefore contained ~6 mM Na^+. In some experiments, the pH of the solution was adjusted using $Mg(OH)_2$, and the solution did not contain KCl; thus Mg^{2+} was the only extracellular cation in those experiments. No differences were observed between the reversal potentials measured in Mg^{2+} saline in which pH was adjusted with NaOH or with $Mg(OH)_2$. In experiments using Ba^{2+} as the extracellular charge carrier, the Na^+ saline was replaced with a solution that contained 64 mM BaCl$_2$, 2 mM KCl, and 10 mM HEPES, pH 7.5 (Ba^{2+} saline). There were small liquid junction potentials (+3 to +8 mV) when changing from Na^+ saline to Mg^{2+} or Ba^{2+} saline. The reported values of V_{rev} have been corrected for these junction potentials.

Results and Discussion

NR2B(W607) controls Mg^{2+} block. We initially studied W-to-L mutations at several positions in the M2 loop of the NR1 and NR2B subunits (Fig. 1A). Wild-type NR1/NR2B receptors were inhibited by Mg^{2+} with an IC$_{50}$ value of 19 μM at −70 mV (Fig. 1, B and C; Table 1). Mutations in the NR1 subunit at W608L, W611L, or an adjacent aromatic residue, F609L, had no effect on Mg^{2+} block. In contrast, mutation NR2B(W607L) almost abolished block by extracellular Mg^{2+}, which had little or no effect at concentrations up to 300 μM (Fig. 1C; Table 1). At higher concentrations (1–3 mM), Mg^{2+} potentiated glutamate-induced currents at NR1/NR2B(W607L) receptors, presumably due to an effect of Mg^{2+} at the stimulatory polyamine site (Paoletti *et al.*, 1995). Stimulation by 1–3 mM Mg^{2+} was also seen at wild-type NR1/NR2B receptors at depolarized potentials (+40 to +60 mV) (data not shown). This form of Mg^{2+} stimulation, like that of spermine, seems to be voltage-independent and is presumably unmasked at negative membrane potentials at NR1/NR2B(W607L) receptors because of the lack of Mg^{2+} block at these receptors. A W-to-L mutation at residue NR2B(W610L), three residues downstream of W607 (Fig. 1A), also had a small effect on Mg^{2+} block, increasing the IC$_{50}$ by ~3-fold (Table 1). Block by Mg^{2+} was also reduced in receptors containing a W-to-L mutation in the NR2A subunit at position NR2A(W606), although the effect of this mutation on the IC$_{50}$ for Mg^{2+} was smaller than that of the equivalent mutation (W607L) in NR2B (Table 1).

We studied four other mutations, W-to-N, W-to-A, W-to-Y, and W-to-F, at NR2B(W607). The W-to-N and W-to-A mu-tants drastically reduced Mg^{2+} block, whereas the W-to-Y and W-to-F mutants had little or no effect on the potency of Mg^{2+} measured at −70 mV (Table 1). Thus, the presence of an amino acid with an aromatic side chain (Y or F) at position NR2B(W607) can restore block by extracellular Mg^{2+}.

Block by Mg^{2+} is voltage dependent, and we therefore studied I-V relationships of glutamate-induced currents in the absence and presence of Mg^{2+}. At wild-type NR1/NR2B receptors, block by Mg^{2+} (1–1000 μM) was strongly concen-tration- and voltage-dependent (Fig. 1D). In contrast, at con-centrations below 100 μM, Mg^{2+} had no effect on the I-V relationship at NR1/NR2B(W607L) receptors (data not

shown), and at concentrations of 100-1000 μM Mg^{2+} produced a very small block at hyperpolarized membrane potentials (Fig. 1E). The NR2B(W607Y) mutation does not affect the potency of Mg^{2+} measured at -70 mV (Table 1). However, voltage-dependent block by Mg^{2+} at NR1/NR2B(W607Y) receptors was more shallow than at wild-type receptors. At

wild-type receptors, there was a clear region of negative slope conductance with 10–1000 μM Mg^{2+} and a complete block at extreme negative potentials with 1000 μM Mg^{2+}. In contrast, at NR1/NR2B(W607Y) receptors, the slope conductance with 10–1000 μM Mg^{2+} was shallow, and there was an incomplete block with 1000 μM Mg^{2+} (Fig. 1F) The difference between
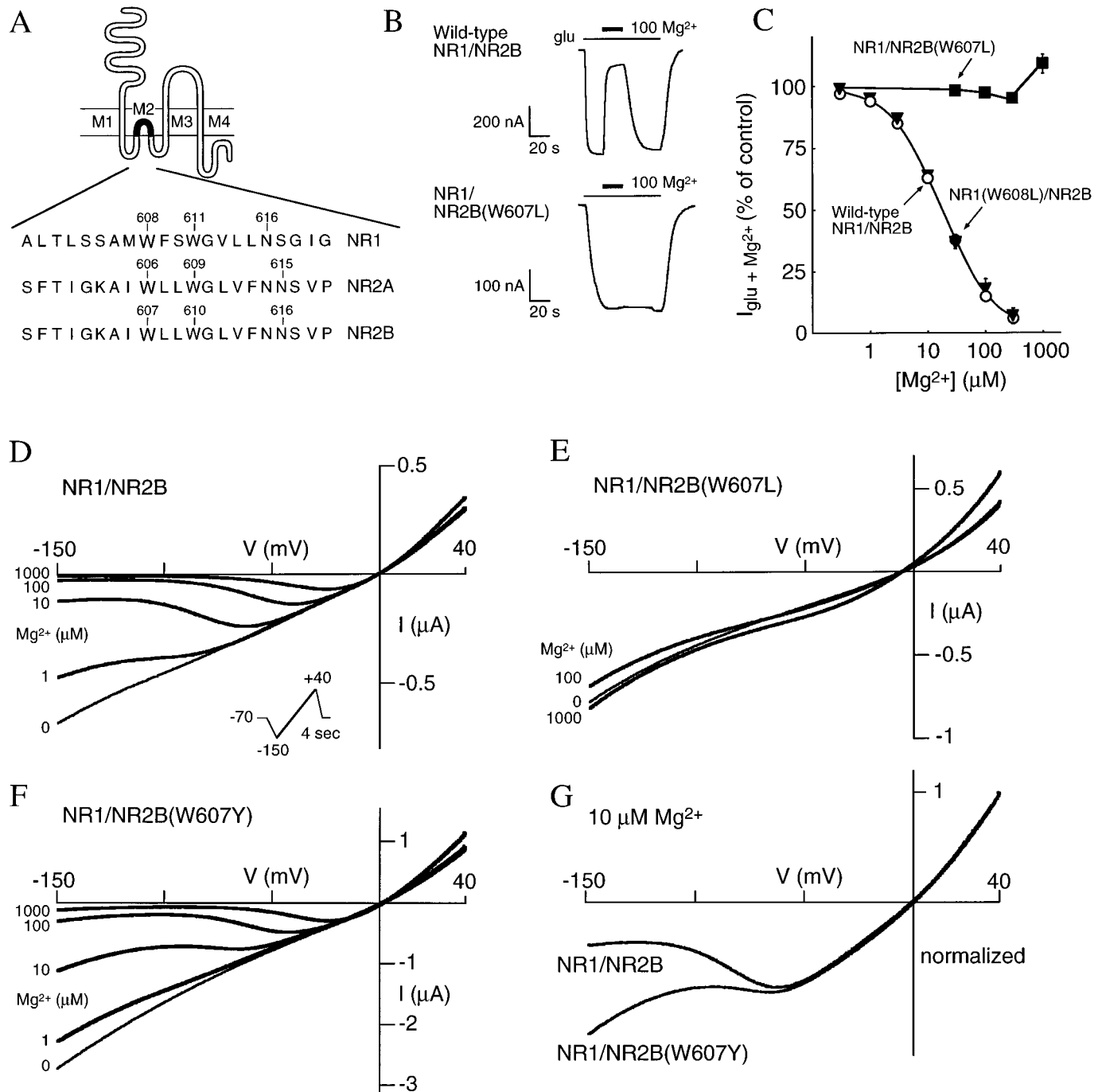
Fig. 1. Mutations at NR2B(W607L) alter block of NMDA receptors by Mg^{2+}. A, Schematic showing the putative topology of NMDA receptor subunits, with three membrane-spanning domains (M1, M3, M4) and a reentrant loop (M2). The amino acid sequences (*single letter code*) in the M2 regions of NR1, NR2A, and NR2B are shown; amino acids are numbered from the initiator methionine in each subunit. B, Effects of 100 μM Mg^{2+} on inward currents induced by glutamate (*glu*, 10 μM; with 10 μM glycine) were measured in oocytes expressing wild-type and mutant receptors and voltage-clamped at -70 mV. C, Concentration-inhibition curves were measured at NR1/NR2B (○), NR1(W608L)/NR2B (▲), and NR1/NR2B(W607L) (■) at -70 mV (mean ± standard error from three or four oocytes for each subunit combination). D–F, I-V curves were constructed by using voltage ramps (*inset* in D) in oocytes activated by glutamate and glycine (10 μM each) in the absence and presence of Mg^{2+} (1–1000 μM) in cells expressing NR1/NR2B (D), NR1/NR2B(W607L) (E), and NR1/NR2B(W607Y) (F) receptors. Leak currents, measured by ramps applied in the absence of glutamate, glycine, and Mg^{2+}, have been subtracted. G, Currents measured in the presence of 10 μM Mg^{2+} at NR1/NR2B and NR1/NR2B(W607Y) receptors were normalized to the current measured at $+40$ mV in each oocyte and the traces superimposed.

wild-type NR1/NR2B and NR1/NR2B(W607Y) receptors is illustrated in Fig. 1G. These data suggest that Mg^{2+} is able to permeate NR1/NR2B(W607Y) channels more easily than wild-type channels, manifest as a partial relief from block at very negative potentials, although Mg^{2+} block at −70 mV is unaffected by W607Y (Table 1). In experiments using Mg^{2+} as the main extracellular charge carrier, we subsequently found that this is indeed the case (see below). The profile seen with NR2B(W607F) was similar to that of the wild-type channels, with a steep region of negative slope conductance in the presence of extracellular Mg^{2+} (data not shown).

Some mutations in the M2 region of NR1, including NR1(N616Q), reduce the sensitivity of NMDA receptors to proton inhibition and increase the potencies of glutamate and glycine (Kashiwagi *et al.*, 1997). These effects presumably reflect long range allosteric changes or disruptions to channel gating because NR1(N616) lies in the ion permeation pathway and is not directly involved in binding of protons or agonists. We determined the effects of NR2B(W607) mutations on sensitivity to glutamate, glycine, and pH. These parameters were unaffected by mutations at NR2B(W607) (Table 2).

Permeation of Mg^{2+} and Ba^{2+} through NMDA channels. Although the NMDA channel is a relatively nonselective cation channel, the pore must contain a selectivity filter that allows passage of Ca^{2+} and Ba^{2+}, which are highly permeable, but not of Mg^{2+}, which permeates native NMDA channels very poorly (Mayer and Westbrook, 1987; Stout *et*

al., 1996). To determine whether NR2B(W607) mutations alter Mg^{2+} permeability and to study the relationship between block and permeation of Mg^{2+}, we measured currents through wild-type and mutant channels with Na^+ or Mg^{2+} as the major extracellular cation. In the absence of Mg^{2+}, and with Na^+ as the main extracellular cation (Na^+ saline), currents through NR1/NR2B and NR1/NR2B(W607L) receptors had similar reversal potentials, close to 0 mV (Fig. 2, B and C). Under conditions where extracellular Na^+ was replaced by Mg^{2+}, outward currents were observed at wild-type NR1/NR2B receptors and these currents asymptotically approached zero as the oocytes were hyperpolarized (Fig. 2B). This suggests that Mg^{2+} does not permeate these channels or that the reversal potential (V_{rev}) for Mg^{2+} is more negative than −100 mV. In contrast, large inward currents were seen in Mg^{2+} saline at NR1/NR2B(W607L) receptors, and V_{rev} in Mg^{2+} saline was −25 ± 1 mV (16 oocytes) (Fig. 2). A similar increase in Mg^{2+} permeability was seen with a mutation at NR2A(W606L), a position equivalent to NR2B(W607L), and V_{rev} in Mg^{2+} saline at NR1/NR2A(W606L) receptors was −28 ± 2 mV (nine oocytes).

We could measure reversal potentials in Mg^{2+} saline for each of the five NR2B(W607) mutants (Fig. 2C). The W607L, W607N, and W607A mutants all produced large inward currents in Mg^{2+} saline, whereas currents through receptors with the W607Y and, in particular, W607F mutants were very small (shown for W607Y in Fig. 2B). Differences in the size of macroscopic currents carried by Mg^{2+} are shown quantitatively in Fig. 2D, in which the ratio of the slope conductance at −50 mV and +50 mV is plotted for each mutant. Thus, although the W607Y and W607F mutants are more permeable to Mg^{2+} than wild-type channels, the Mg^{2+} block at these mutants is much more profound than at the W607L, W607N, or W607A mutants. The values of V_{rev} for Mg^{2+} observed with the different NR2B(W607) mutants were very similar (Fig. 2C) even though the W-to-Y and W-to-F mutants produced very small currents (Fig. 2D). This suggests that residue NR2B(W607) predominantly affects block by Mg^{2+} *per se* rather than affecting the rate of permeation of the ion after the block is relieved. Thus, the strength of the block at wild-type channels that contain a tryptophan at NR2B(W607) presumably accounts for the lack of permeation of Mg^{2+} through those channels.

To determine whether the NR2B(W607L) mutations alter Ba^{2+} permeability, we compared V_{rev} in Na^+ saline and in a solution containing 64 mM Ba^{2+} (Ba^{2+} saline). Because wild-type NMDA channels are highly permeable to Ba^{2+}, there is a large positive shift in V_{rev} when switching from Na^+ saline

TABLE 1

Block of mutant NMDA receptors by Mg^{2+}

IC_{50} values for Mg^{2+} were determined from concentration-response curves, similar to those shown in Fig. 1C, in oocytes voltage-clamped at −70 mV and activated by 10 μM glutamate plus 10 μM glycine. Values are the geometric mean (− standard error, + standard error).

| Subunit combination	Mg^{2+} IC_{50}	No. of oocytes
	μM	
Wild-type NR1/NR2B	19 (18, 20)	6
NR1(W608L)/NR2B	19 (17, 21)	3
NR1(F609L)/NR2B	15 (14, 16)	4
NR1(W611L)/NR2B	21 (20, 23)	6
NR1/NR2B(W607L)[a]	>300	5
NR1/NR2B(W607N)[a]	>300	7
NR1/NR2B(W607A)	217 (160, 293)	6
NR1/NR2B(W607Y)	22 (20, 24)	6
NR1/NR2B(W607F)	29 (26, 32)	7
NR1/NR2B(W610L)	58 (50, 67)	3
Wild-type NR1/NR2A	17 (15, 18)	3
NR1/NR2A(W606L)	61 (54, 68)	6

[a] 300 μM Mg^{2+} inhibited glutamate responses by 5 ± 2% at NR1/NR2B(W607L) and by 3 ± 2% at NR1/NR2B(W607N) receptors.

TABLE 2

Sensitivity of NR2B(W607) mutants to pH, glutamate, and glycine

IC_{50} values (mean ± standard error) for pH inhibition were determined by measuring responses to glutamate over a pH range of 6.5 to 8.5 as described previously (Kashiwagi *et al.*, 1997). EC_{50} values for glutamate and glycine were determined from concentration-response curves for each agonist in the presence of 10 μM glycine (glutamate curves) or 10 μM glutamate (glycine curves). Values are the geometric mean (− standard error, + standard error).

Subunit combination	pH IC_{50}	No. of oocytes	Glutamate EC_{50}	No. of oocytes	Glycine EC_{50}	No. of oocytes
			μM		μM	
Wild-type NR1/NR2B	7.3 ± 0.02	5	1.4 (1.3, 1.5)	6	0.14 (0.12, 0.16)	6
NR1/NR2B(W607L)[a]	7.5 ± 0.01	4	2.5 (2.4, 2.6)	4	0.22 (0.21, 0.23)	4
NR1/NR2B(W607N)	7.4 ± 0.01	3	2.7 (2.5, 3.0)	4	0.25 (0.24, 0.26)	4
NR1/NR2B(W607A)	7.3 ± 0.01	3	2.0 (1.8, 2.3)	4	0.42 (0.35, 0.51)	4
NR1/NR2B(W607Y)	7.3 ± 0.01	3	1.8 (1.7, 2.0)	4	0.28 (0.25, 0.31)	4
NR1/NR2B(W607F)	7.2 ± 0.01	3	1.6 (1.4, 1.8)	4	0.12 (0.12, 0.13)	4

[a] Data for glutamate and glycine at NR2B(W607L) are from Kashiwagi *et al.* (1997).
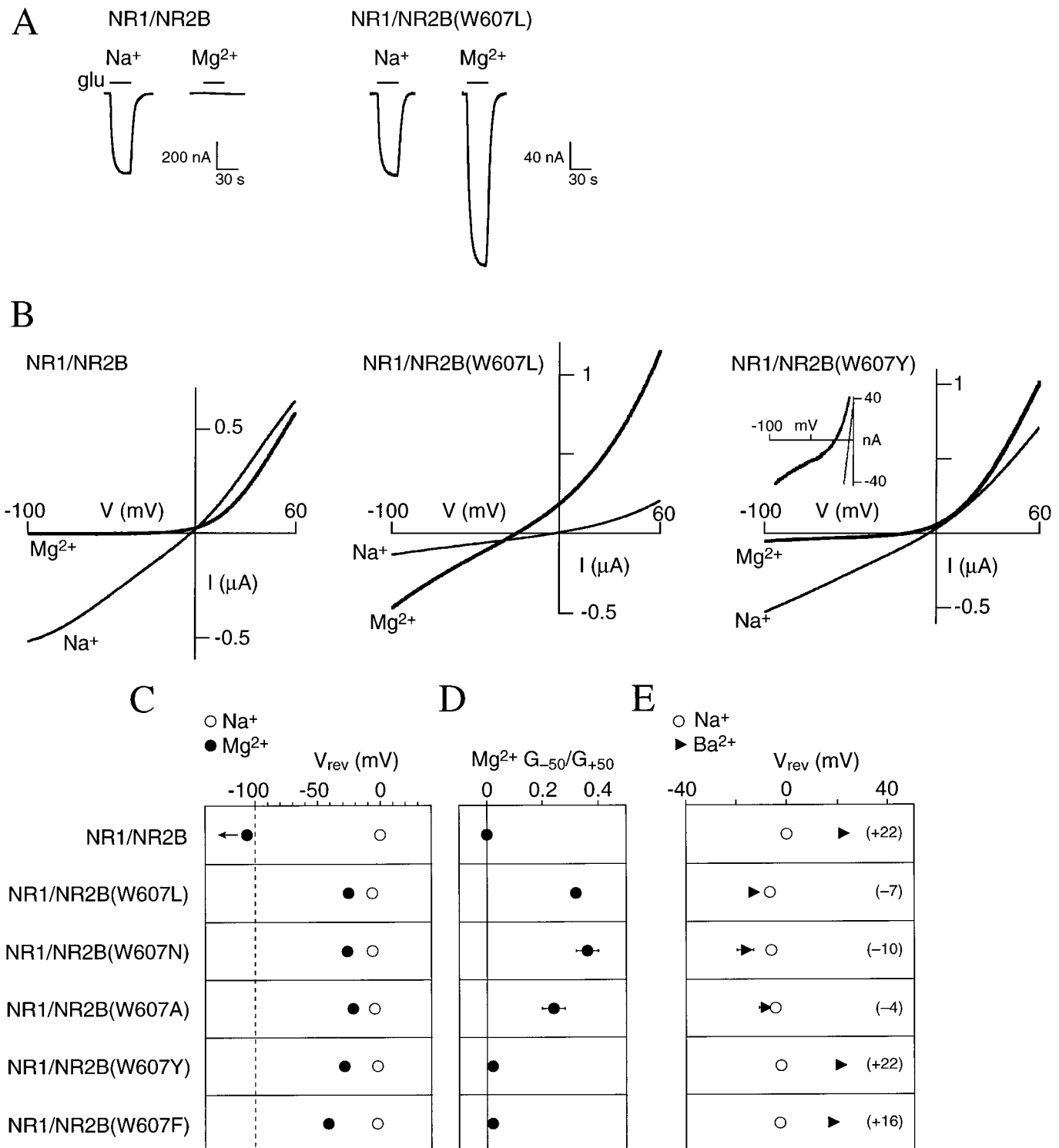
Fig. 2. Mg^{2+} permeates NR1/NR2B(W607L) channels. A, Responses to glutamate and glycine (10 μM each; *glu*) were measured in oocytes voltage-clamped at -70 mV in an extracellular solution containing 96 mM Na^+, 2 mM K^+, and 1.8 mM Ba^{2+} (Na^+ saline) or a solution containing 64 mM Mg^{2+} as the only cation (Mg^{2+} saline). B, I-V curves were constructed by using voltage ramps (-100 mV to $+60$ mV over 4 sec) in oocytes activated by glutamate and glycine (10 μM each) in Na^+ saline or Mg^{2+} saline. Leak currents have been subtracted. *Inset* for NR1/NR2B(W607Y) shows an expanded view (-40 to $+40$ nA) of the I-V curves over the range of -100 to 0 mV to illustrate the very small inward current carried by Mg^{2+} in this cell. C, Reversal potentials (V_{rev}) at wild-type and mutant receptors were determined in Na^+ saline (○) and Mg^{2+} saline (●) by using voltage ramps. At NR1/NR2B receptors, inward currents could not be detected in Mg^{2+} saline and a *symbol with an arrow* is drawn below -100 mV to indicate that Mg^{2+} is presumed to be impermeable or to have a V_{rev} more negative than -100 mV. D, The ratio of the slope conductance measured at -50 mV and $+50$ mV (G_{-50}/G_{+50}) in Mg^{2+} saline is shown for each mutant. E, Values for V_{rev} were determined in Na^+ saline (○) and Ba^{2+} saline (▲) by using voltage ramps. *Numbers in parentheses at the extreme right of each panel,* the mean shift in V_{rev} in Ba^{2+} compared with Na^+ for each receptor type. Values are mean $\pm$ standard error from 5–16 oocytes for each subunit combination. Values of V_{rev} have been corrected for small liquid junction potentials observed in Mg^{2+} saline and Ba^{2+} saline. Where errors are not shown, they are within the size of the symbol.

to Ba^{2+} saline (Hume *et al*., 1991). Even with Ba^{2+} in the extracellular solution it is possible to activate secondary Cl^- conductances that would influence the measured reversal potential. We attempted to eliminate these Cl^- currents by injecting oocytes with BAPTA, by limiting the duration of agonist application, and, in some experiments with Ba^{2+} saline, by reducing the concentration of glutamate from 10 to 1 μM. At wild-type NR1/NR2B receptors, the shift in V_{rev} was $+22 \pm 1$ mV (Fig. 2E). At receptors containing the NR2B mutants W607L, W607N, and W607A, but not W607Y or W607F, Ba^{2+} permeability was markedly reduced (Fig. 2E). Thus, residue NR2B(W607) influences Ba^{2+} permeability as well as Mg^{2+} block. It may be that transient binding of Ba^{2+} to NR2B(W607), and subsequent unbinding and passage of Ba^{2+} through the channel, is involved in Ba^{2+} flux through wild-type channels. These data are consistent with the reported interactions between Ba^{2+} (or Ca^{2+}) and Mg^{2+} within NMDA channels (Mayer and Westbrook, 1987).

Reversal potentials in extracellular Na^+, Mg^{2+}, and Ba^{2+} were measured for receptors containing various combinations of NR1 and NR2 mutants (Figs. 3 and 4). A W-to-L mutation at NR1(W608L) had no effect on Mg^{2+} permeation and produced a small decrease in Ba^{2+} permeability (Figs. 3A and 4A). Mutations at the second tryptophan residue in the M2 loop of NR2 subunits, NR2B(W610L) and NR2A(W609L), but not at the equivalent position in NR1, increased permeation of Mg^{2+} (Fig. 4, A and D), although inward currents carried by Mg^{2+} through these channels were very small (Fig. 3C) and reversal potentials were more negative than that seen with NR2B(W607L). When the

NR2B(W610L) mutant was studied in combination with the NR1(W608L) or NR1(W611L) mutants, there was no further increase in Mg^{2+} permeability and only a small change in Ba^{2+} permeability. Similarly, the combination of NR2B(W607L) with NR1(W608L) or NR1(W611L) did not produce any further change in Mg^{2+} permeability compared with NR1/NR2B(W607L) receptors (Fig. 4A). Thus, of the tryptophan residues present in this region of the pore, only the first tryptophan in NR2 subunits is a major determinant of Mg^{2+} block. GluR subunits of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and kainate receptors contain a leucine residue at the position equivalent to NR1(W611) and NR2B(W610). Mutation of this leucine to tryptophan in the GluR1 subunit has been reported to increase divalent cation permeability of homomeric GluR1 channels (Ferrer-Montiel *et al*., 1996). Our results show that mutations NR1(W611L) and NR2B(W610L), either alone or in combination, have little or no effect on Ba^{2+} permeability (Fig. 4A), suggesting that the tryptophan at this position in NMDA channels plays only in minor role in controlling Ba^{2+} permeability.

We also carried out experiments to compare the effects of the NR2B(W607L) mutant with those of mutations at NR1(N616) (Figs. 3B and 4B) and NR2B(N616) (Figs. 3D and 4C), in some cases combining the NR1 and NR2 mutants to determine whether the effect of one mutant predominates. N-to-G, N-to-R, and N-to-W mutations at NR1(N616) all increased Mg^{2+} permeability and decreased Ba^{2+} permeability (Fig. 4B), although inward currents carried by Mg^{2+} through these mutants were very small (Fig. 3B). With the exception of NR1(N616R), the NR2B(W607L) mutant seemed to predominantly influence Mg^{2+} permeability (Fig. 4B). In contrast, the NR1(N616) mutants predominantly influenced Ba^{2+} permeability in NR1(N616X)/NR2B(W607L) channels (Fig. 4B).

The NR2B(N616G) mutant greatly increased Mg^{2+} permeability and reduced Ba^{2+} permeability, similar to the NR2B(W607L) mutant (Figs. 3D and 4C). A double mutant, NR2B(W607L,N616G), did not have a greater effect than either mutant alone, nor did the combination of NR2B(N616G) with NR1(N616G) (Fig. 4C). Similar results were seen with an N615G mutant in NR2A, which increased Mg^{2+} permeability comparable with the NR2A(W606L) mutant (Fig. 4D). The lack of additivity of the mutations at NR2B(W607) and NR2B(N616) suggests that these residues may make a similar or functionally overlapping contribution to the Mg^{2+} binding site and they may be closely positioned in the pore (Fig. 5).

Implications for understanding Mg^{2+} block and the structure of NMDA channels. A tryptophan residue is present in all glutamate receptor subunits, with the exception of NR-L, and in some K^+ channel subunits at a position equivalent to NR2B(W607). In NMDA channels (Kuner *et al*., 1996) and in *Shaker* K^+ channels (Lü and Miller, 1995), the tryptophan at this position has been reported to face the lumen of the channel pore. This would be consistent with a direct interaction of Mg^{2+} with NR2B(W607) in the NMDA channel lumen, possibly involving a cation-π interaction of Mg^{2+} with the aromatic ring of W607 (Kumpf and Dougherty, 1993). In support of this idea, block by Mg^{2+} was drastically reduced in mutants containing leucine, asparagine, or alanine but was largely unaffected in mutants containing tyrosine or phenylalanine. Based on the kinetics of block by
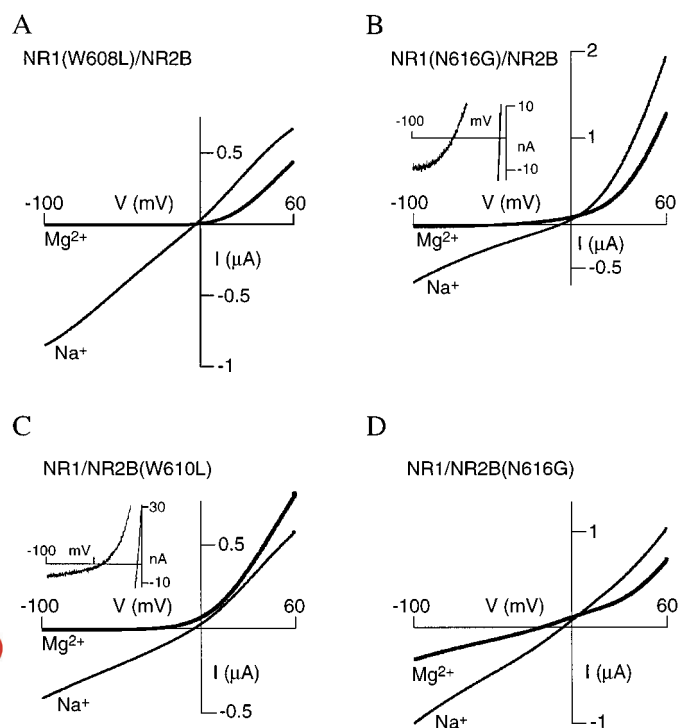

Fig. 3. Mg^{2+} permeation at mutant NMDA receptors. I-V curves were constructed by using voltage ramps (-100 mV to $+60$ mV over 4 sec) in oocytes activated by glutamate and glycine (10 μM each) in Na^+ saline or Mg^{2+} saline. Leak currents have been subtracted. B and C, *insets*, expanded view (-10 to $+30$ nA or -10 to $+10$ nA) of the I-V curves over the range of -100 mV to 0 mV to illustrate the very small inward currents carried by Mg^{2+} in these cells.

external and internal Mg^{2+}, a barrier-well energy profile has been proposed for Mg^{2+} block of the NMDA channel (Li-Smerin and Johnson, 1996). That profile contains a very high energy barrier that prevents crossover and permeation of internal Mg^{2+} (Li-Smerin and Johnson, 1996). It is tempting to speculate that the tryptophan residue at position NR2B(W607) is the actual physical determinant of that energy barrier, and it will be of interest to study the influence of NR2B(W607) on block by intracellular Mg^{2+}.

It is clear that the presence of a tryptophan by itself is not sufficient to confer channel block by extracellular Mg^{2+} because other subtypes of glutamate receptors are not blocked by external Mg^{2+} and, as shown here, W608 in the NR1 subunit does not influence Mg^{2+} block. Additional features of the NMDA channel must also control binding and permeation of Mg^{2+}, possibly including an interaction of Mg^{2+} with the carbonyl oxygens of the M2 asparagine residues (Burna-

shev *et al.*, 1992; Kawajiri and Dingledine, 1993; Kupper *et al.*, 1996). Another possibility is that the structure, folding, or positioning of the M2 loop in NR2 subunits is different from that in subunits of non-NMDA receptors and, perhaps, different from that in NR1, and that this accounts for the role of NR2B(W607) in Mg^{2+} block

We previously found that the NR2B(W607L) but not the NR1(W608L) mutation reduced block and increased the apparent permeation of the polyamine-derivative N^1-DnsSpm (Kashiwagi *et al.*, 1997). Those results led us to propose that residue NR2B(W607) may contribute to the narrowest constriction of the channel pore (Kashiwagi *et al.*, 1997) together with the asparagines at positions NR1(N616) and NR2B(N616) (Kuner *et al.*, 1996; Wollmuth *et al.*, 1996), although this is not consistent with current models of the proposed structure of the M2 loop region in which NR2B(W607) lies some distance below NR2B(N616) (Kuner
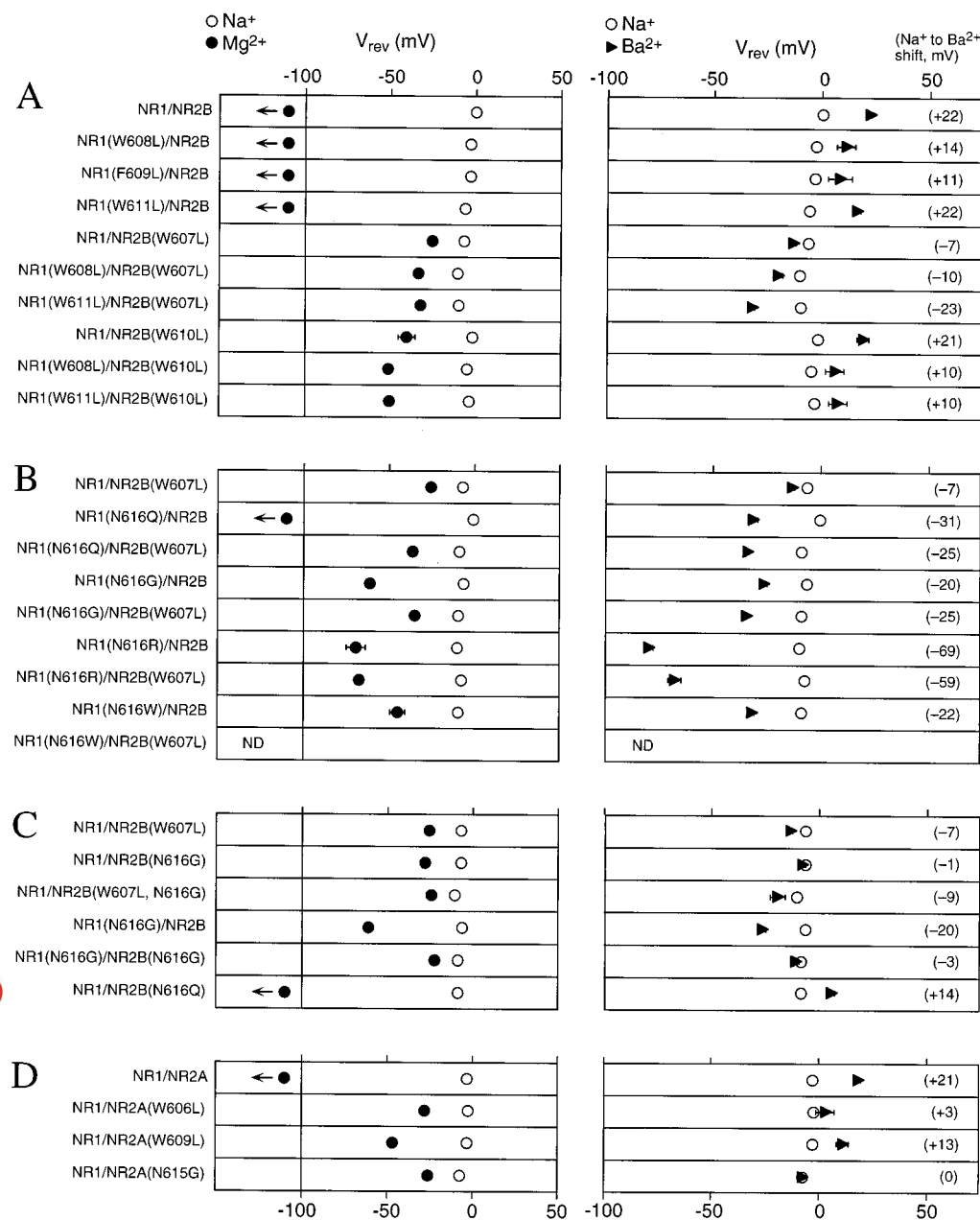
Fig. 4. Properties of mutant NMDA receptors. *Left*, reversal potentials (V_{rev}) at wild-type and mutant receptors were determined in Na^+ saline (○) and Mg^{2+} saline (●) by using voltage ramps. In oocytes in which inward currents could not be detected in Mg^{2+} saline (e.g., NR1/NR2B), V_{rev} for Mg^{2+} is not known, and the *symbol with an arrow*, below -100 mV, is drawn to indicate that Mg^{2+} is presumed to be impermeable or to have a V_{rev} more negative than -100 mV. *Right*, values for V_{rev} were determined in Na^+ saline (○) and Ba^{2+} saline (▲) by using voltage ramps. *Numbers in parentheses*, mean shift in V_{rev} in Ba^{2+} compared with Na^+ for each receptor type. Data are presented in four groups (A–D) to facilitate comparison between various mutants or combinations of mutants, and some data, for example, NR1/NR2B(W607L), are presented in two or more groups to facilitate comparison with related mutants or groups of mutants. A–C, Data from receptors containing NR2B. D, Data from receptors containing NR2A. Values, which are mean ± standard error from 4–16 oocytes for each subunit combination, have been corrected for small liquid junction potentials observed in Mg^{2+} saline and Ba^{2+} saline. Where errors are not shown, they are within the size of the symbol. *ND*, not determined; macroscopic currents through NR1(N616W)/NR2B(W607L) channels were only 1–7 nA in Na^+ saline.
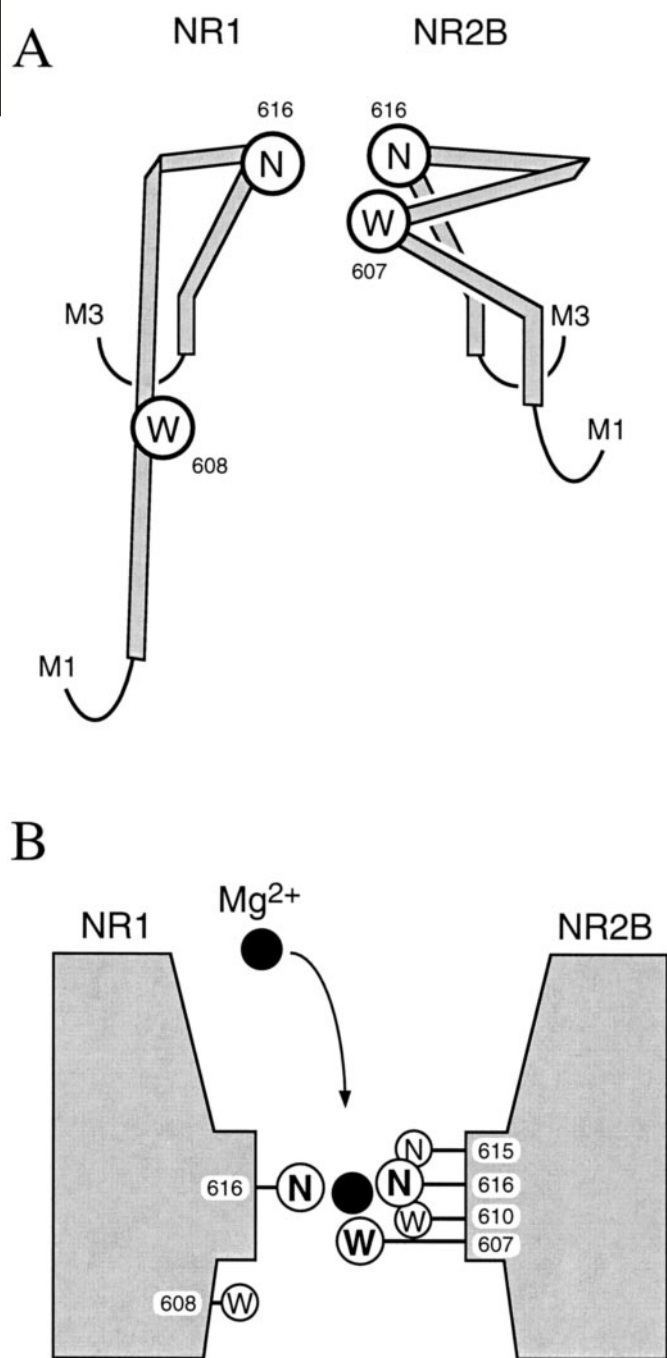
Fig. 5. Models of M2 loop structures and Mg^{2+} block. A, The M2 loop regions of NR1 and NR2B are shown (*shaded ribbons*) with the positions of some N and W residues (*circles*). The structure of the M2 loop in NR1 is based on that proposed by Kuner *et al.* (1996), with N616 being at the tip of the loop and forming part of the narrow constriction of the channel, and W608 lying some distance below N616 but still being in the lumen of the channel. The structure of the M2 loop in NR2 subunits was previously suggested to be similar to that of NR1, with the second asparagine (N616 in NR2B) forming the narrowest part of the channel (Kuner *et al., 1996*). However, we proposed that residue W607 in NR2B also contributes to the narrow constriction of the channel (Kashiwagi *et al.*, 1997) and forms part of the selectivity filter and Mg^{2+} binding site. To account for the effects seen with mutations at NR2B(W607), the M2 loop of NR2B is proposed to be folded in such a way that NR2B(W607) lies at a level in the channel similar to NR2B(N616) and NR1(N616). It is also possible that a similar structure exists for the M2 loop of NR1 (not illustrated), although mutations at NR1(W608) do not produce phenotypes like those seen with mutations at NR2B(W607). B, In NR1/NR2B receptors, the Mg^{2+} binding

et al., 1996; Sutcliffe *et al.*, 1996). The finding that W607 in NR2B but not W608 in NR1 affects Mg^{2+} block reinforces the idea that the M2 regions of NR1 and NR2 make nonsymmetrical contributions to channel structure. It may be that the secondary structure of at least part of the M2 loop in NR1 and NR2 subunits is different, leading to placement of NR2B(W607) at a position in the channel pore such that it can interact with Mg^{2+} whereas NR1(W608) cannot (Fig. 5). If residue NR2B(W607) is located at a level in the channel similar to NR1(N616) and NR2B(N616) (Fig. 5A), it is possible that some or all of the M2 region extends in a plane parallel, rather than perpendicular, to the plane of the membrane, as has been proposed for the pore-forming loop of a cyclic nucleotide-gated channel (Sun *et al.*, 1996). A limitation of models such as those shown in Fig. 5 is that they are "static," and it is possible that the M2 loops are very flexible, as has been proposed for pore-forming loops of voltage-gated Na^+ and K^+ channels (Bénitah *et al.*, 1997; Liu *et al.*, 1996; Tsushima *et al.*, 1997) and that under some conditions they can arrange themselves such that NR2B(W607) is very close to or forms part of the narrow constriction and the selectivity filter in the channel.

We found that a W-to-L mutation at the second tryptophan in NR2B, NR2B(W610), also had a small effect on Mg^{2+} block. In the study by Kuner *et al.* (1996), the equivalent tryptophan residues in NR1 and NR2C were not accessible to thiol reagents after mutagenesis to cysteine, suggesting that this tryptophan is not solvent accessible in the channel. If NR2B(W610) does not face the channel lumen, it is possible that the W-to-L mutation causes a nonspecific disruption of channel structure, perhaps influencing the position of the NR2B(W607) or NR2B(N616) residues. In this context, it is notable that mutations at NR1(S617) have a small effect on Mg^{2+} block (Kawajiri and Dingledine, 1993), although this position is also not accessible to thiol reagents after cysteine mutagenesis (Kuner *et al.*, 1996). Another possibility is that residues such as NR2B(W610) and NR1(S617) do face the channel lumen but their accessibility is limited because of shielding by other residues or by ions passing through the channel.

In conclusion, we propose that there are at least three residues that make critical contributions to the narrow constriction and selectivity filter of the NMDA channel. These include N616 in NR1 and N616 and W607 in NR2B (Fig. 5). The N and W residues in NR2 subunits predominantly influence block and permeation of extracellular Mg^{2+} and probably form part of a Mg^{2+} binding site. Although the schematics shown in Fig. 5 are undoubtedly a gross oversimplification, future models of the M2 region will need to take into account a

site of the NMDA channel is proposed to be formed by the asparagines at NR1(N616) and NR2B(N616) and the tryptophan at NR2B(W607) (*bold letters in large circles*). Block by Mg^{2+} could involve interactions with the carbonyl oxygens of the asparagine residues and a cation-π interaction with the electron-dense aromatic ring of the tryptophan. An aromatic ring at position NR2B(607) also influences permeation of Ba^{2+}, and it is conceivable that tryptophan 607 is important for transient binding and subsequent passage of Ba^{2+} through the channel (not illustrated). Mutations at NR2B(N615) and at NR2B(W610) also have small effects on Mg^{2+} block, and these residues may make weak contributions to the Mg^{2+} binding site (*small circles*). Residue NR1(W608), which is solvent accessible in the channel (Kuner *et al.*, 1996), does not influence Mg^{2+} block and may lie some distance below NR1(N616), as shown here (also see Kuner *et al.*, 1996) if the M2 loop structure is as shown in A.

critical role for the conserved tryptophan in NR2 subunits in block by Mg^{2+}, as well as the potential positioning of this residue at the narrow constriction of the channel (Kashiwagi *et al.*, 1997).

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