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Title Page

The Pore Region of *N*-Methyl-D-Aspartate Receptors Differentially Influences Stimulation and Block by Spermine

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Running title: Characteristics of pore region of NMDA receptors

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Abbreviations:

NMDA, *N*-methyl-D-aspartate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*,-tetraacetic acid; I-V, current-voltage.

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ABSTRACT

The transmembrane and pore-forming regions of *N*-methyl-D-aspartate (NMDA) receptors containing the NR1 and NR2B subunits were studied by measuring the effects of various NR1 and NR2B mutants on stimulation and block by spermine. Block by spermine was predominantly affected by mutations in the M3 segment of NR1 and especially in the M1 and M3 segments of NR2B. These regions are in the outer vestibule of the channel pore and may contribute to a spermine binding site. Mutations in different regions — predominantly the M3 segment and M2 loop of NR1 and the M3 segment of NR2B — influenced spermine stimulation, a surprising finding since spermine stimulation is thought to involve a spermine binding site in the distal, exracellular regulatory (R) domain. However, some of these mutations also influence sensitivity to ifenprodil and protons, and changes in spermine sensitivity may be secondary to changes in proton sensitivity. The results are consistent with the proposal that the relative positions of the M1 and M3 transmembrane segments and M2 loops are staggered or asymmetric in NR1 and NR2 subunits, and with the idea that stimulation and block by spermine involve separate binding sites and distinct mechanisms, although some residues in the receptor subunits can affect both stimulation and block.

Introduction

N-Methyl-D-aspartate (NMDA) receptors are subtypes of ionotropic glutamate receptors that have distinct characteristics including the need for two different agonists (glutamate and glycine) for activation, high permeability to Ca^{2+}, and voltage-dependent block by Mg^{2+} (Dingledine et al., 1999). NMDA receptors are involved in synaptic plasticity and may also play a role in seizure activity (Rogawski, 1992; Hollmann and Heinemann, 1994; Dingledine et al., 1999). The receptors are probably tetramers composed of combinations of three types of subunits — NR1, NR2, and NR3. Most NMDA receptors in the adult central nervous system contain combinations of NR1 and NR2, with NR2A and NR2B predominating in forebrain areas such as the cerebral cortex (Hollmann and Heinemann, 1994; Dingledine et al., 1999). Each subunit is proposed to have a large extracellular N-terminal region which comprises a "regulatory domain" (R domain), also termed the "amino terminal domain" (ATD or NTD), and part of the agonist binding domain; a core region comprising three membrane-spanning domains (M1, M3, and M4) and a re-entrant loop (M2) that forms part of the channel pore and selectivity filter; a large extracellular loop between M3 and M4 that also contributes to the agonist binding domain; and an intracellular C-terminal domain (Dingledine et al., 1999; Madden, 2002). The M2 loop region in NR1 and NR2 subunits is a critical determinant of divalent cation permeability and Mg^{2+} block. In particular, asparagine residues in this region form part of a Mg^{2+} binding site and contribute to the selectivity filter of the channel (Sakurada et al., 1993; Dingledine et al., 1999). Results of several studies have led to the proposal that residues at the tip of the M2 loops — NR1 (Asn616) and NR2B (Asn616), but not NR2B (Asn615) — make the narrowest constriction of channel pore, and that the M3 segments from the two subunits,

which form the outer vestibule and entrance to the channel, are staggered relative to each other in the vertical axis of the channel (Kuner et al., 1996; Beck et al., 1999; Kashiwagi et al., 2002; Sobolevsky et al., 2002, 2007).

Polyamines (putrescine, spermidine and spermine) are present at millimolar concentrations in cells and play important roles in cell proliferation and differentiation (Igarashi and Kashiwagi, 2000). In the nervous system, spermine may be released from neurons and/or glial cells (Masuko et al., 2003). Polyamines, in particular spermine, have multiple effects on NMDA receptors, including stimulation and a weak voltage-dependent inhibition that likely represents an open-channel block (Benveniste and Mayer, 1993; Williams, 1997). Spermine stimulation — an enhancement of the current gated by glutamate and glycine — appears to be due to binding of spermine to the extracellular R domain (Masuko et al., 1999). The R domain is also involved in inhibition by ifenprodil, an atypical NMDA antagonist, and can affect inhibition by protons (Traynelis et al., 1995; Masuko et al., 1999; Perin-Dureau et al., 2002). Proton inhibition, which predominantly involves residues at the top of the M3 segment and in the M3-M4 linker, may represent a common “end point” for effects of spermine and ifenprodil, with spermine causing relief of tonic proton inhibition, and ifenprodil increasing proton inhibition (Traynelis et al., 1995; Low et al, 2003; Mott et al., 1998).

In this study, we have examined effects of mutations in the M1 through M4 regions on block and stimulation by spermine. This work extends earlier studies in which we made the surprising discovery that several mutations in and around the channel pore can influence

not only block but also stimulation by spermine (Kashiwagi et al., 1997). Here, we show that this is true for many residues in the M3 region and M2 loop of the NR1 subunit, but that, to a large extent, different residues and regions in the NR1 and NR2B subunits have differential effects on stimulation and block by spermine.

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Materials and Methods

NMDA Clones and Site-Directed Mutagenesis. The rat NR1 clone used in these studies is the NR1A variant (Moriyoshi et al., 1991), which lacks the 21-amino acid insert encoded by exon 5. This clone, and some of the NR1 mutants in the M2 and M1-M2 linker region (Moriyoshi et al., 1991; Sakurada et al., 1993), were gifts from Dr. S. Nakanishi (Osaka Bioscience Institute, Osaka, Japan). The wild-type mouse and rat NR2B clones (Kutsuwada et al., 1992; Monyer et al., 1992) were gifts from Drs. M. Mishina (Graduate School of Medicine, University of Tokyo, Tokyo, Japan) and P. H. Seeburg (Center for Molecular Biology, University of Heidelberg, Heidelberg, Germany). The preparation of most other NR1 and NR2B mutants has been described previously (Kashiwagi et al., 1996, 1997, 2002, 2004; Williams et al., 1998; Masuko et al., 1999; Jin et al., 2007). Site-directed mutagenesis to construct additional NR1 and NR2B mutants was carried out by the method of Ho et al. (1989) using the polymerase chain reaction. For mutations in NR2B we used the rat NR2B clone for residues N806-S832 and the mouse (ε2) NR2B clone including a 1.7 kb HindIII-SphI fragment of the rat NR2B clone (Williams et al., 1998) for other residues. Amino acids are numbered from the initiator methionine in each subunit. This differs from the numbering system used in some laboratories, in which residues are numbered from the start of the mature peptide. In the case of NR1, there is an 18-amino acid signal peptide; for example, residue NR1(Asn616) described in this study corresponds to residue NR1(Asn598) using the alternative numbering system (Kuner et al., 1996).

Expression in Oocytes and Voltage-Clamp Recording. The preparation of

capped cRNAs and the preparation, injection, and maintenance of oocytes were carried out mostly as described previously (Williams, 1993). Oocytes were injected with NR1 plus NR2 cRNAs in a ratio of 1:5 (0.1-0.4 ng of NR1 plus 0.5-2 ng of NR2). Macroscopic currents were recorded with a two-electrode voltage clamp using a GeneClamp 500 amplifier (Molecular Devices, Union City, CA). Electrodes were filled with 3 M KCl and had resistances of 0.4 to 4 MΩ. Oocytes were continuously superfused with a saline solution (100 mM NaCl, 2 mM KCl, 1.8 mM $BaCl_2$, and 10 mM HEPES, pH 7.5), and in most experiments, oocytes were injected with 40 mM K^+-BAPTA (100 nl; pH 7.0-7.4) on the day of recording. Receptors were activated by superfusion of glutamate and glycine (10 μM).

To study the voltage dependence of block, voltage ramps were constructed by ramping the command signal from –150 to +50 mV over 6 s. Leak currents, measured in the absence of agonist and blockers, were digitally subtracted.

Chemicals. Ifenprodil tartrate, (1*RS*, 2*SR*)-4-[2-(Benzylpiperidin-1-yl)-1-hydroxypropyl]phenol hemi-(2*R*, 3*R*)tartrate, and spermine tetrahydrochloride were obtained from Sigma and Nacalai Tesque, respectively.

Results

Effects of Mutations on Stimulation and Inhibition by Spermine. We made a series of individual point mutations in and around the membrane-spanning and pore-forming regions of the NR1 and NR2B subunits. The mutants were constructed in most cases to alter the functional side chains, for example by neutralizing the charge (E-to-Q, D-to-N), removing an aromatic ring (W-to-L, Y-to-L), removing a hydroxyl group (S-to-A, T-to-A) or substituting an amino acid with A, C or S. We screened each mutant by measuring the effects of 100 μM spermine on currents induced by 10 μM glutamate and glycine at two holding potentials, –20 mV and –100 mV. At –20 mV, voltage-dependent block by spermine is minimized and the predominant effect in wild-type receptors is spermine stimulation. At –100 mV, inhibition is seen at wild-type receptors and presumably masks spermine stimulation. Although spermine stimulation can be influenced by agonist concentration (Benveniste and Mayer, 1993; Williams, 1994), we used saturating concentrations of glutamate and glycine in these studies.

Some mutations reduced or increased stimulation by spermine without having large effects on block by spermine. Conversely, other mutations reduced or increased block by spermine without effects on stimulation. However, other mutants had effects on both stimulation and block by spermine. The interpretation of the effects of these mutants is complex – for example, some mutants increased stimulation by spermine and not only abolished block but actually produced a large stimulation by spermine in the experimental paradigm designed to study spermine block (or the loss of block). To aid in the interpretation

of the results from these mutants, we adopted the following strategy: If a given mutant showed reduced spermine stimulation at –20 mV and increased spermine block at –100 mV, it was judged to predominantly affect spermine stimulation (i.e., reducing stimulation and unmasking block). Conversely, if a given mutant produced a decrease in spermine block at –100 mV and an increase in spermine stimulation at –20 mV, it was judged to predominantly affect spermine block (i.e., reducing block and unmasking stimulation). However, it is worth noting that for mutations that generate a complex phenotype, these interpretations may be an over-simplification and the actual mechanisms underlying the measured effects may be more complex than simply a reduced stimulation that unmasks the block or a reduced block that unmasks the stimulation.

At wild-type NR1/NR2B receptors at –20 mV, spermine increased the current by 40 ± 3% (Fig. 1A and C). If spermine stimulation decreased by ≥20% (an arbitrary threshold that we selected), the effect of the mutation was judged to be significant and the residue is highlighted by white letters in black boxes in Figs. 1A (NR1 mutants) and 2A (NR2B mutants). Similarly, if spermine stimulation at –20 mV increased by more than twice that seen at the wild type without a significant effect on block at –100 mV, the effect of the mutation on stimulation was judged to be significant, and the residue is highlighted by an open box in Figs. 1A and 2A. With regard to spermine inhibition, wild-type NR1/NR2B receptors were inhibited by 32 ± 4% at –100 mV (Fig. 1B and C). If spermine block was abolished (in some cases unmasking a stimulation), the effect of the mutation was judged to be significant, and the residue is shown by white letters in black boxes in Figs. 1B and 2B. If spermine block at –100 mV was increased by more than twice that seen at the wild type but

without a significant effect on stimulation at –20 mV, the effect of the mutation on block was judged to be significant, and the residue is highlighted by an open box in Figs. 1B and 2B.

Results of studies with NR1 mutants are shown in Fig. 1 and are summarized schematically in Fig. 3B. The effects of some of these mutations, primarily in the M2 region, on spermine stimulation but not spermine block at NR1/NR2B receptors have been previously reported (Kashiwagi et al., 1997). We repeated the studies of those mutants in this paper and included studies of spermine block at the same receptor subtype (NR1/NR2B) together with many additional mutants in the M1 through M4 regions whose effects on spermine stimulation and block have not been previously studied. Mutations that reduced spermine stimulation were at residues P557 in the pre-M1 region, W608, W611 and N616 in the M2 loop, F639, V644, S646, Y647, T648, A649, A653, L655, V656 and L657 in the M3 segment, and D669 and L808 in the post-M3 and pre-M4 region. With mutations at P557, W608, T648, A649, A653, L657 and L808 shown with asterisks in Figs. 1A and 1B, a more than 2-fold increase in spermine block was observed at –100 mV. These residues may be involved in both spermine stimulation and block.

The pattern was different with regard to spermine block — block was reduced by mutations at F554 and F558 in the pre-M1 region, W563 in the M1 segment, N650 and A652 in the M3 segment, E662 and T807 in the post-M3 and pre-M4 segments, and G815 and F817 in M4. With mutations at N650, A652 and T807 shown with asterisks in Figs. 1A and 1B, more than 200% of control current was observed in the presence of spermine at –100 mV. These residues may have a more direct effect on spermine stimulation. Furthermore,

significant spermine stimulation without an effect on block was observed in D581 in the M1 segment, E621 in the M2 loop, and I828 in M4, and significant spermine block without effect on stimulation was observed in H571 in the M1 segment, A606 in the M2 loop, and V820 and I824 in M4. Those residues were shown by open boxes in Fig. 1. Overall, although there was some overlap between residues that affect stimulation and block by spermine, the effects were largely separable.

Results of studies with NR2B mutants are shown in Fig. 2 and summarized in Fig. 3B. Mutations at residues N616 in the M2 loop, S645, A648, L650 and A652 in the M3 segment, and D814 in M4 reduced spermine stimulation, whereas spermine block was affected by mutations at F550, P553 in the pre M1-region, A556, D557, W559, M561, M562, F563, V564, M565, L566 and L567 in the M1 segment, W607 and W610 in the M2 loop, F638, A644, Y646, T647, N649, F653 and M654 in the M3 segment, Q656, D661, D668, V808, Q812 and L813 in the post-M3 and pre-M4 regions, and I815 and N817 in M4. Similar to the results with NR1, in the NR2B mutants that reduced spermine stimulation at –20 mV, the degree of block was enhanced at –100 mV. Similarly, many mutants that reduced spermine block at –100 mV enhanced spermine stimulation at –20 mV. In the case of mutations at W559, W607, F638, Y646, N649, F653, M654 and L813 shown with asterisks in Figs. 2A and 2B, more than 200% of control current was observed in the presence of spermine at –100 mV, suggesting that these amino acid residues may also have a more direct effect on spermine stimulation. Furthermore, significant spermine stimulation without effect on block was observed in F642 in the M3 segment and L825 in M4.

Assuming mutations that reduce spermine block reflect a reduced interaction of spermine with a binding site within the vestibule or pore of the channel, the results with mutations in NR1 and NR2B, summarized in Fig. 3B, suggest that the binding site responsible for block by spermine mainly involves the M3 segment of NR1 together with the M1 and M3 segments of NR2B (red circles and split circles with red over pink on Fig. 3B). Residues at the top of the M4 segment may be located around the mouth of the channel and affect entry of spermine into the channel as it moves toward a binding site located in the vestibule.

With regard to the relative positions of the M2 loops in NR1 and NR2B, W608 and W611 in NR1 predominantly affected spermine stimulation, and the equivalent residues W607 and W610 in NR2B predominantly affected spermine block (Fig. 3B). These results are consistent with the proposal that the relative positions of the M2 loops are different in NR1 and NR2B. Also, it is particularly notable that N-to-Q mutations at the Asn residues at the "tip" of the M2 loop – N616 in NR1 and N616 and N615 in NR2B – have little or no effect on block by spermine. N-to-Q mutations at these positions greatly reduce the effects of "typical" NMDA channel blockers such as Mg^{2+} and MK-801 (Sakurada et al., 1993; Kashiwagi et al, 2002). Thus, the results of the current study would be consistent with the proposal that the spermine binding site is more peripheral, located predominantly in the vestibule formed by the M1 and M3 regions rather than at the selectivity filter formed by the M2 loop. A more drastic mutation at N616 in NR1, N-to-R, has, however, been shown to abolish spermine block in NR1/NR2A receptors (Kashiwagi et al, 1997). The presence of a positively charged Arg residue at NR1-616 may greatly reduce spermine block by simple electrostatic repulsion (Kashiwagi et al, 1997), which would not be the case with the N-to-Q

mutation used in the present study.

In some mutants such as N650A and A652T in NR1 and F653A and M654S in NR2B, spermine greatly enhanced the currents at both –20 mV and –100 mV. We studied the concentration-dependence of the effects of spermine using the key mutants identified in the M1 and M3 segments to determine whether inhibition is seen in mutants where stimulation is reduced or abolished, and vice-versa. As shown in Figs. 4A and 4B, concentration-dependent inhibition by spermine was still observed with mutants such as NR1 T648A and NR2B A648G in which spermine stimulation was reduced or abolished. Conversely, in mutants such as NR1 N650A and NR2B M654S, where spermine block was reduced or abolished, concentration-dependent stimulation by spermine was still observed (Figs. 4C and 4D). This suggests that the stimulatory and inhibitory effects of spermine are indeed separable since one effect can be abolished while the other persists. However, this does not rule out the possibility that some mutations may abolish the inhibitory effect of spermine and simultaneously increase its stimulatory effect.

I-V curves were constructed with a number of mutants that reduced spermine stimulation or spermine inhibition. At wild-type NR1/NR2B receptors, spermine produces a net inhibition at very negative membrane potentials, and this inhibition is relieved and gives rise to a net stimulation at positive membrane potentials (Fig. 5). At receptors containing the NR1(T648A) and NR2B(S645A) mutants (at which spermine stimulation was reduced when studied at –20 mV; see Figs 1 and 2), spermine blocked currents in a voltage-dependent manner and no stimulation was seen at voltages up to +50 mV. At receptors containing the

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NR1(W563L), NR1(A652T), NR2B(L566A) or NR2B(T647A) mutants (at which spermine inhibition was reduced when studied at –100 mV; see Figs 1 and 2), stimulation but not inhibition by spermine was seen, even at very negative membrane potentials (Fig. 5). This is again consistent with separate stimulatory and inhibitory effects of spermine, and suggests that the mutations do not have general, non-specific effects on channel properties since one effect of spermine is preserved even as the other is abolished.

Amino Acid Residues That Affect Inhibition by Ifenprodil and Protons. As described above, some residues in the transmembrane and pore-forming regions affect spermine stimulation at NR1/NR2B receptors. There is evidence that spermine enhances, and ifenprodil inhibits NMDA receptors through binding to the R domains (Masuko et al., 1999; Perin-Dureau et al., 2002). In addition, some residues in the R domain have small effects on sensitivity to protons, but residues near the top of the M3 segment and in the proximal part of the M3-M4 linker have the largest effects on proton sensitivity and may contribute to the proton sensor (Low et al., 2003). Spermine stimulation and ifenprodil inhibition appear to involve changes in tonic proton inhibition (Traynelis et al., 1995; Mott et al., 1998; Low et al., 2003). Thus, we studied the effects of protons and ifenprodil using the various mutants that affect sensitivity to spermine (Fig. 6). Effects of 1 μM ifenprodil on responses to 10 μM glutamate and glycine were measured at –20 mV. As an index of proton sensitivity, responses to 10 μM glutamate and glycine were measured at pH 8.0 and 6.0, and current at pH 6.0 is expressed as a percentage of current at pH 8.0. As shown in Fig. 6, inhibition by ifenprodil and protons was reduced by mutations in NR1 and NR2B that affect spermine stimulation, but not by mutations that affect spermine block. The results suggest

that amino acid residues on the M3 segment of NR1 and NR2B that are involved in spermine stimulation can influence the functions of the R domains or their downstream effects on agonist-induced gating, directly or indirectly, possibly through changes in proton sensitivity.

Changes in Agonist Sensitivity Caused by Mutations in the Channel Pore. Several mutations in the M3 segments, including NR1 T648A and L657A, have been previously found to generate constitutively open channels (Kashiwagi et al., 2002). Because some of these mutants also affect spermine stimulation, we determined whether sensitivity to agonists, that is, glycine and glutamate, is also changed. As shown in Fig. 7, the effective concentrations of both glycine and glutamate decreased greatly by these mutants. The results are consistent with the idea that NR1 and NR2B function as a heterodimer within the tetrameric receptor (Schorge and Colquhoun, 2003; Furukawa et al., 2005), because mutations in NR1 influence the affinity for glutamate, which binds to NR2B, and mutations in NR2B influence the affinity for glycine, which binds to NR1.

Discussion

We studied the effects of mutations in the channel pore and vestibule regions of NR1/NR2B receptors with a focus on how mutations affect stimulation and block by spermine. Our data are consistent with previous proposals that corresponding or equivalent portions of the NR1 and NR2 subunits are arranged asymmetrically and that the M3 segment is strongly involved in binding of channel blockers (Sobolevsky et al., 2002, 2007; Kashiwagi et al., 2002). Thus, residues in the M3 segments of NR1 and NR2B have effects on spermine block, but these effects are different in NR1 and NR2B. In this study, we also found that the M1 segment of NR2B contains many residues at which mutations affect block by spermine – a pattern that is not reflected in the M1 segment of the NR1 subunit. Thus, the channel pore and outer vestibule may be comprised of the M3 segments of NR1 and NR2B and the M1 segment of NR2B (see Fig. 3A). This would be at least partly consistent with published reports concerning the accessibility of residues in the upper (extracellular) portions of the M1 and M3 domains (Beck et al., 1999). The differential effects of mutations in the M2 loop are also consistent with an asymmetric arrangement of the NR1 and NR2B subunits.

Although it is assumed that mutations in and around the M1 and M3 segments have direct effects on block by spermine, this may not always be the case. For example, if the relative packing of the M1, M3, and M4 segments in the tertiary structure of the subunit is different in NR1 and NR2B, mutations in one segment, say M1, may have a greater effect on the structure of another segment, say M3, in the NR2B subunit than they do in NR1, and thus a greater effect on residues in M3 that interact directly with spermine.

An interesting finding that extends our earlier observations (Kashiwagi et al., 1997) was that residues in the M3 segments of NR1 and NR2B and in the M2 loop can influence spermine stimulation; this is surprising because spermine stimulation is proposed to involve binding of spermine to the extracellular R domain (amino terminal domain) rather than binding within the channel vestibule or pore. Some of these amino acid residues also affect inhibition by protons and ifenprodil, and this region is close to or overlaps the major proton-sensitive region and the regions involved in channel gating (Jones et al., 2002; Low et. al., 2003; Chang and Kuo, 2008). Thus, the effects on spermine stimulation of mutations in this region may be secondary to changes in proton sensitivity and/or gating.

The tetrameric NMDA receptor is likely assembled as a dimer of dimers, with a non-alternating subunit arrangement (Fig. 3A; Schorge and Colquhoun, 2003). The structure of the NMDA channel pore and outer vestibule has been studied extensively using the substituted cysteine accessibility method (e.g., Beck et al., 1999; Sobolevsky et al., 2002, 2007). Our model of this region in NR1/NR2B receptors, shown in Fig. 3B, was constructed based in part on the putative transmembrane segments described by Ishii et al. (1993) and the structure of the KcsA potassium channel (Doyle et al., 1998), and is similar to the model proposed for NR1 and NR2C (Sobolevsky et al., 2007). The top few amino acids of the M3 domain may lie outside the cell membrane or outside the region of the vestibule (Sobolevsky et al., 2007). In particular, this region is involved in channel gating (Jones et al., 2002; Chang and Kuo, 2008) which may account for some of the effects seen in the present study with mutations near the top of the M3 region.

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The relative positions of the R domain and the S1/S2 agonist-binding domain *in situ*, and their proximity to the channel vestibule and pore are unknown. The R domains appear to contain binding sites for modulators such as spermine (NR1), ifenprodil (NR2B), and Zn^{2+} (NR2A) (Masuko et al., 1999; Paoletti et. al., 2000; Perin-Dureau et al., 2002). It may be that modulators that bind to the R domains affect gating (and, ultimately, macroscopic currents) via changes in the conformation of the S1/S2 agonist binding domains or via changes in coupling of the S1/S2 domains to the gating and pore-forming regions. Alternatively, it is conceivable that the R domain may lie close to the membrane and perhaps interact directly with some portion of the membrane-spanning region, perhaps the top part of the M3 segment just outside the membrane or another exterior portion of the outer vestibule. This may explain the effects of mutations in and around the membrane-spanning domains on stimulation by spermine, if spermine binds to the R domain of NR1 and that domain, in turn, is close to or can interact with the top end of the M3 region of the same subunit. A recent paper has proposed a model in which the dimer interfaces of the S1/S2 domains are critical for coupling between the R domains and channel gating (Gielen et al., 2008), although that model does not directly address the location of the R domains relative to the channel pore.

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Footnotes

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Legends for figures

Fig. 1. Effects of spermine at NR1/NR2B receptors containing NR1 mutants. Effects of spermine (100 μM) were determined in oocytes expressing NR1/NR2B receptors with wild-type (WT) or mutant NR1 subunits, voltage-clamped at –20 mV (A) and –100 mV (B) and activated by 10 μM glutamate and 10 μM glycine. Values are mean ± SEM from 20 oocytes for wild type and four oocytes for each mutant. A, mutations that reduced stimulation by ≥ 20% at –20 mV compared with wild type are highlighted by white letters in black boxes. Mutations at which spermine stimulation was increased rather than decreased without significant effect on block are shown by open boxes. B, mutations that abolished block at –100 mV are indicated by white letters in black boxes. Mutations at which inhibition by spermine was increased rather than decreased without significant effect on stimulation are shown by open boxes. Asterisks indicate the residues which may be involved in both stimulation and block. In A and B, the solid vertical lines indicate the effect of spermine in WT receptors, and the broken vertical lines indicate the threshold for considering the effect of a mutation to be significant with regard to a reduction in spermine stimulation or spermine block. C, representative traces to illustrate the effects of spermine on currents activated by 10 μM glutamate and 10 μM glycine (Glu-Gly) at –20 mV (stimulation, upper panels) and –100 mV (block, lower panels) in wild-type and mutant receptors.

Fig. 2. Effects of spermine at NR1/NR2B receptors containing NR2B mutants. For details, see the legend to Figure 1A and B.

Fig. 3. Models to illustrate possible subunit arrangements (A) and residues that affect

spermine stimulation and block in the channel pore and vestibule of NR1/NR2B receptors (B). A, the contribution of each helix of NR1 and NR2B to the pore and outer vestibule of the channel is represented. The arrangement is asymmetric, with both the M1 and M3 segments of NR2B and, predominantly, the M3 segment of NR1 contributing to the outer vestibule/entrance to the pore. The non-alternating subunit arrangement is based on the model proposed by Schorge and Colquhoun (2003). B, the M1-M2-M3-M4 region is depicted with a helix-loop-helix (M1-M2-M3) core region similar to the structure reported for the KcsA potassium channel from *Streptomyces lividans* (Doyle et al., 1998). The M2 region contains a helix followed by a random coil structure. Results of mutations shown in Figs. 1 and 2 are summarized here. Blue and red circles indicate residues at which mutations reduced spermine stimulation and block, respectively. Dark-and-light blue split circles indicate residues at which mutations reduced spermine stimulation and increased block. Red-and-pink split circles indicate residues at which mutations abolished spermine block and increased stimulation. Light blue circles indicate residues where mutants increased spermine block without significant effect on stimulation. Pink circles indicate residues where mutants increased spermine stimulation without significant effect on block. White circles indicate residues at which mutations have little or no effect. Numbers next to each residue indicate the amino acid number in the mature peptide. Numbers in red indicate positions at which mutations generate constitutively active channels (Kashiwagi et al., 2002).

Fig. 4. Concentration-response curves for spermine at mutants that affect spermine stimulation and spermine block. Concentration–response curves for spermine were determined at receptors containing wild-type (WT) or mutant NR1 (A, C) or NR2B (B, D)

subunits in oocytes voltage-clamped at –20 mV and activated by 10 μM glutamate and glycine. A and B, effects of spermine were determined at NR1 mutants (A) and NR2B mutants (B) that were found to reduce spermine stimulation (see Figs. 1 and 2). C and D, effects of spermine were determined at NR1 mutants (C) and NR2B mutants (D) that were found to abolish spermine block (see Figs. 1 and 2). All data are expressed as a percentage of the Glu + Gly current measured in the absence of spermine. Values are mean ± SEM from 4-5 oocytes. Where error bars are not shown, they are within the size of the symbol.

Fig. 5. Current-voltage relationships at NMDA receptors in the presence and absence of spermine. I-V curves were constructed by voltage ramps from –150 to +50 mV in oocytes expressing wild-type and mutant receptors. Responses to 10 μM glutamate (with 10 μM glycine) were measured in the absence (black lines) and presence (blue or red lines) of spermine. Leak currents have been subtracted. The experiments were repeated four times with similar results.

Fig. 6. Inhibition of NR1/NR2B receptors by ifenprodil and protons. Effects of 1 μM ifenprodil on responses to 10 μM glutamate and glycine were measured in oocytes expressing wild-type and mutant NR1/NR2B receptors voltage-clamped at –20 mV. Currents measured in the presence of ifenprodil are expressed as % of the control (Glu + Gly) current. To study proton inhibition, currents induced by 10 μM glutamate and glycine were measured at pH 8.0 and 6.0, and the current at pH 6.0 is expressed as a percentage of the current measured at pH 8.0. Values are mean ± SEM from 4-5 oocytes. A, mutations in NR1; B, mutations in NR2B.

Fig. 7. Concentration-response curves for glycine and glutamate. A, Responses to various concentrations of glycine in the presence of 10 μM glutamate were measured in oocytes expressing wild-type or mutant NR1/NR2B receptors voltage clamped at –70 mV. Data are expressed as a % of the maximum current in the presence of 10 μM glycine. B, Responses to various concentrations of glycine in the presence of 30 μM glutamate were measured in oocytes expressing wild-type or mutant NR1/NR2B receptors voltage clamped at –70 mV. Data are expressed as a % of the maximum current in the presence of 30 μM glutamate. Values are mean ± SEM from 4-5 oocytes. Where error bars are not shown, they are within the size of the symbol.

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Fig. 1

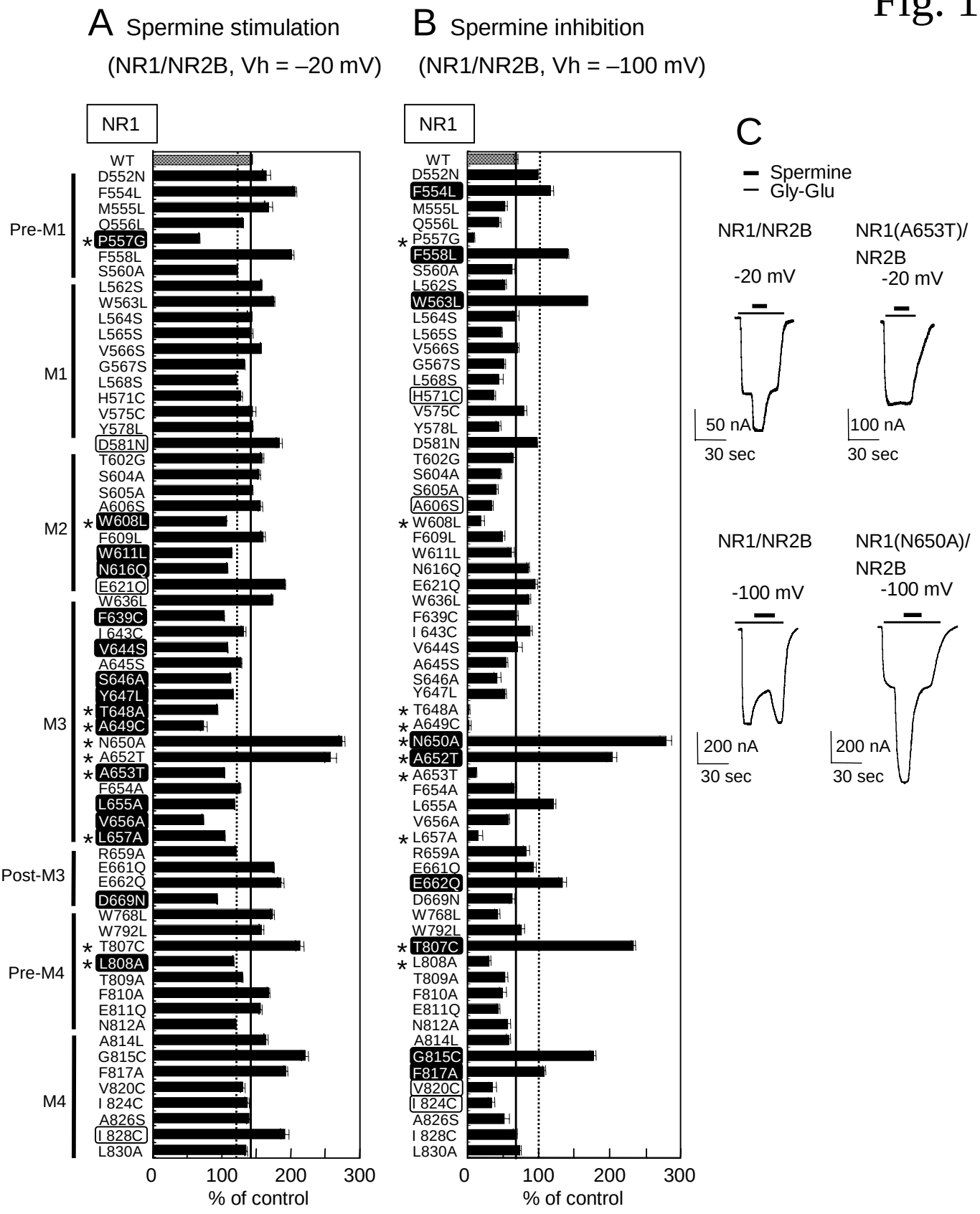

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Fig. 2

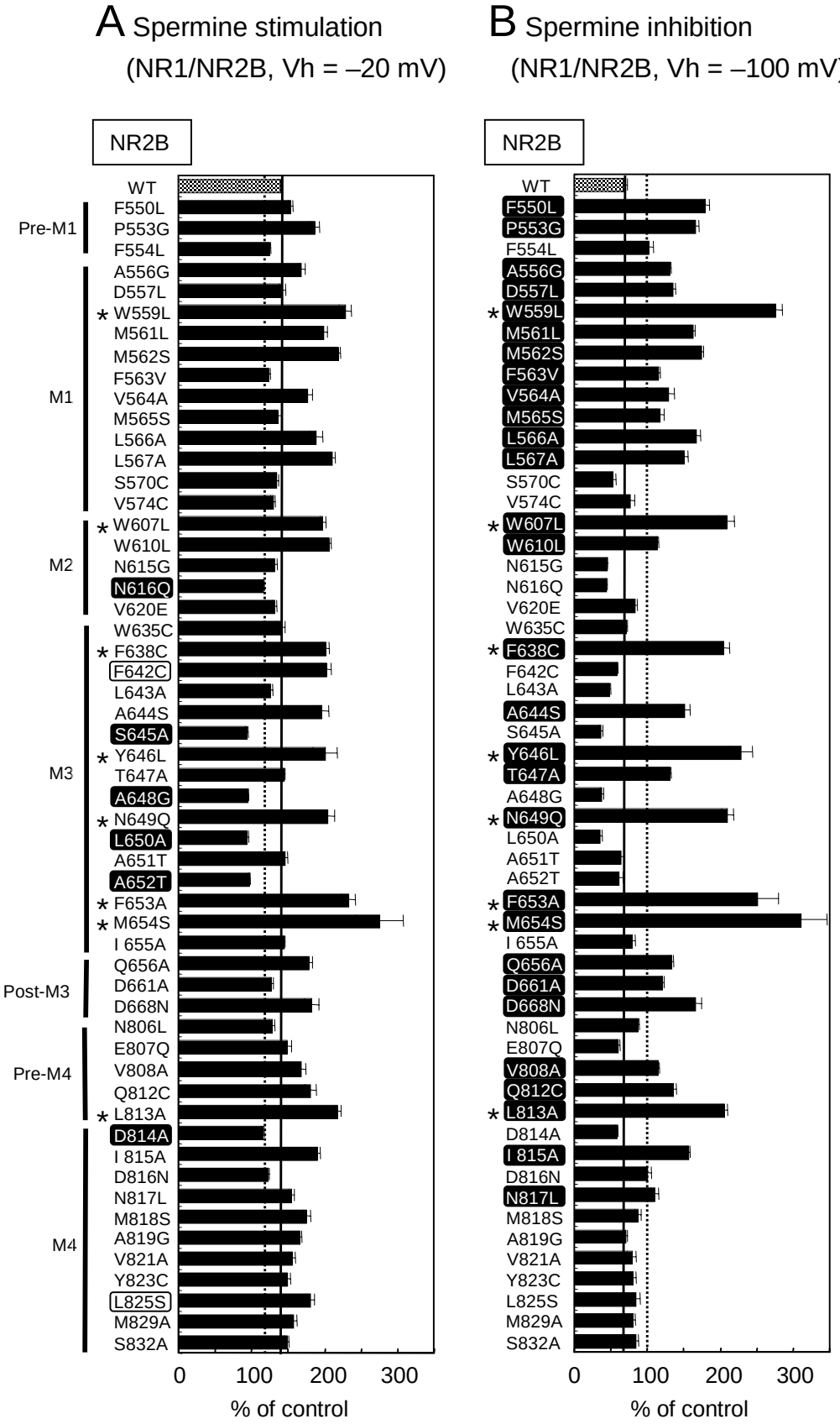
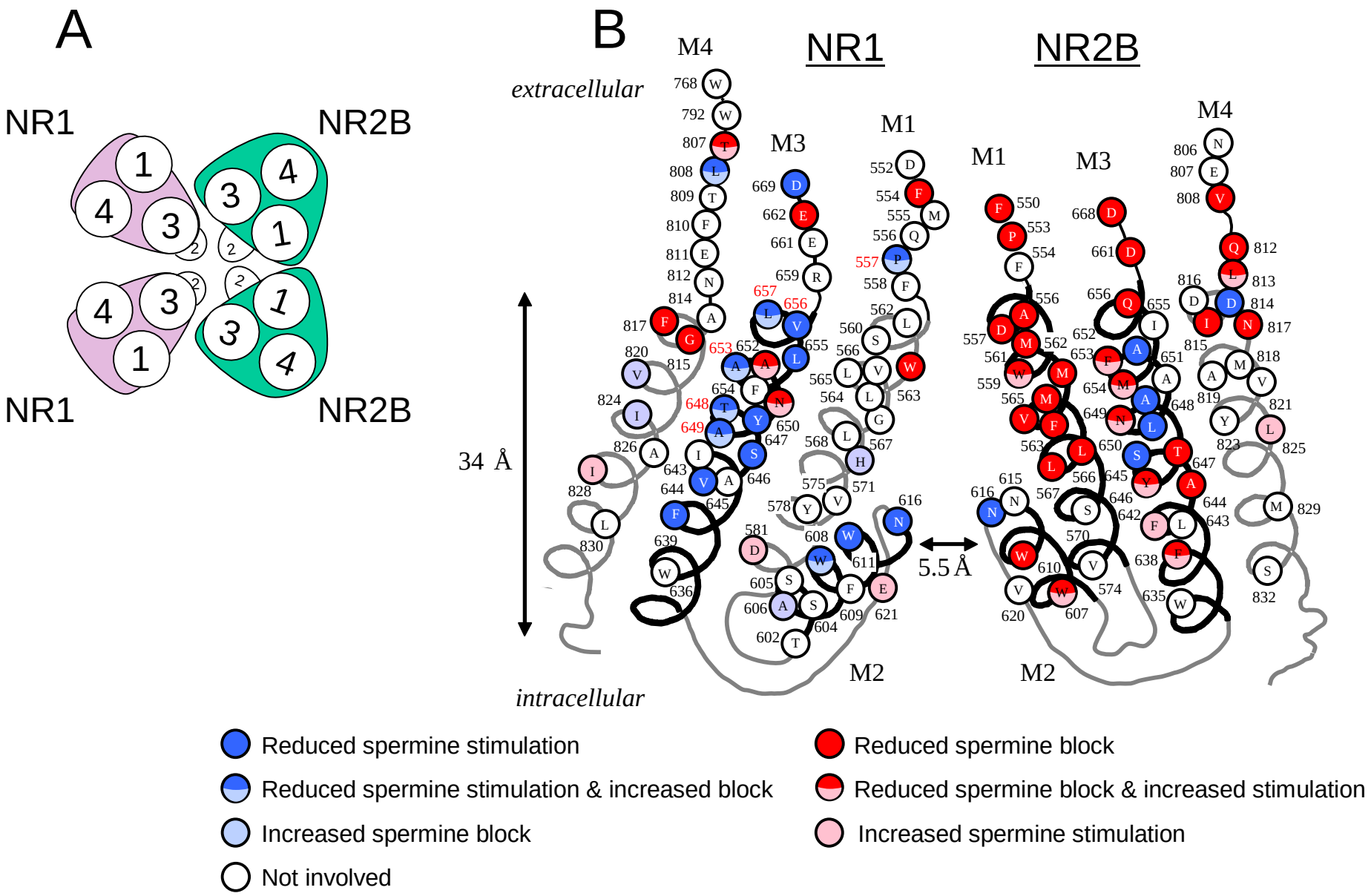


Fig. 3



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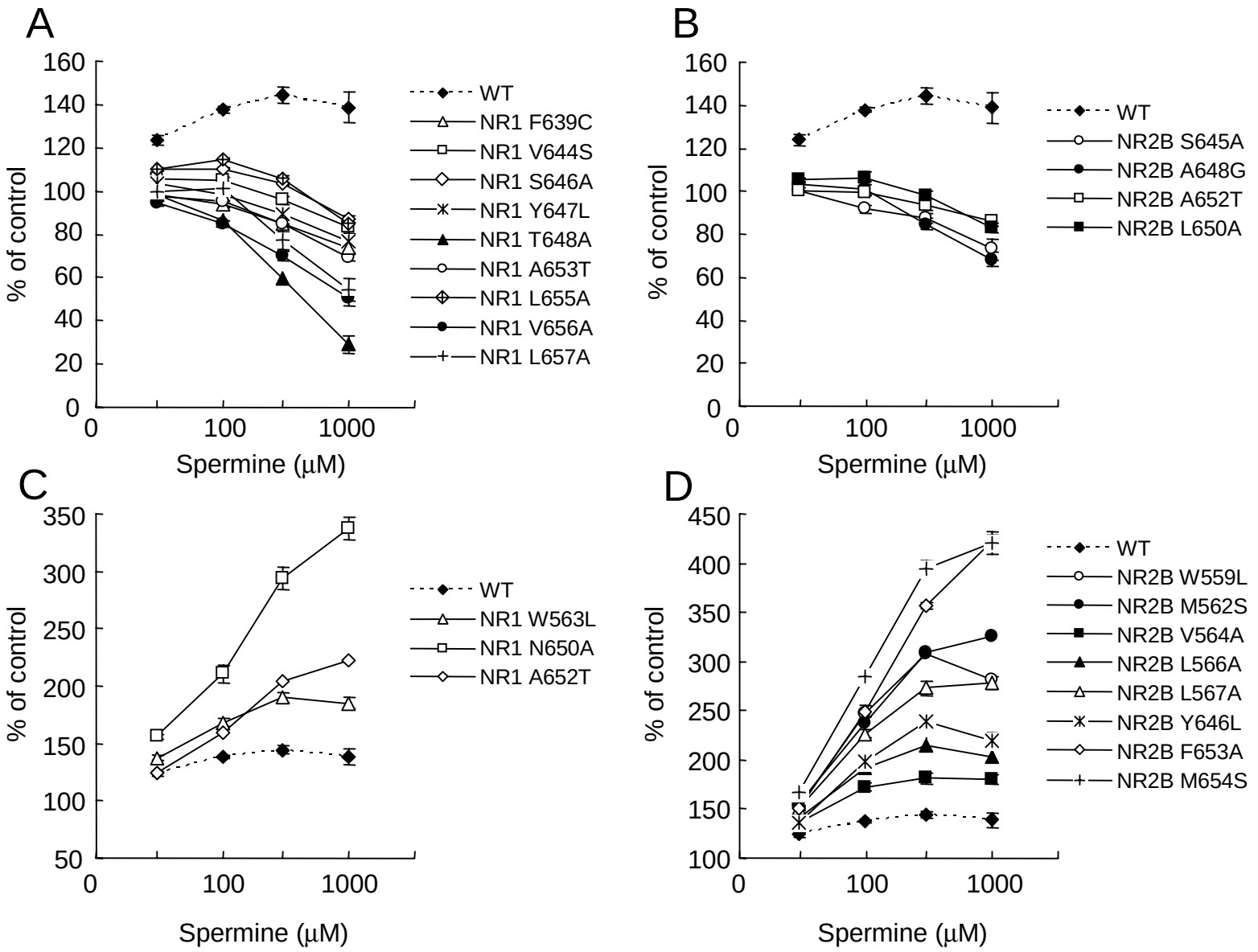

Fig. 5

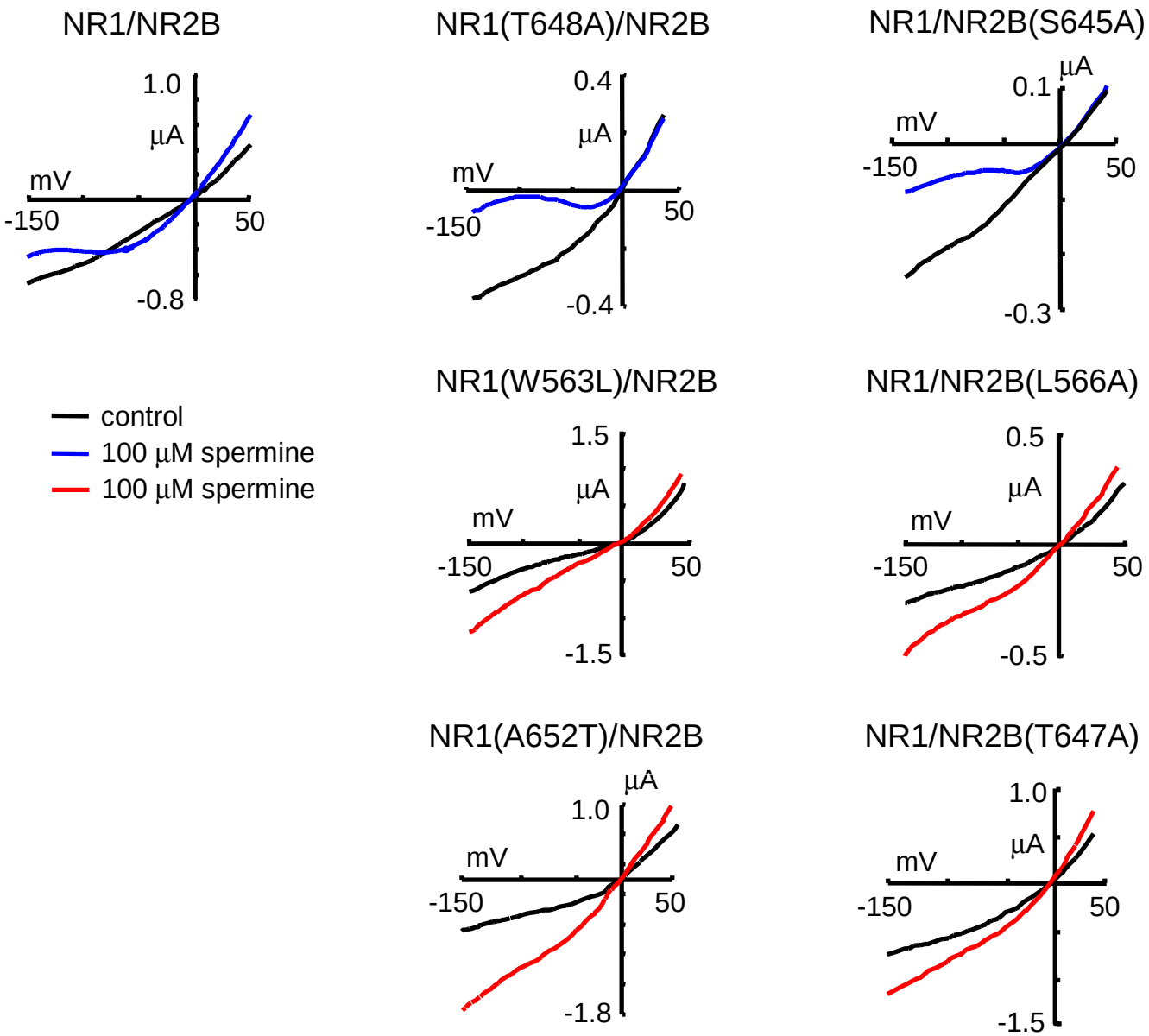

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Fig. 6

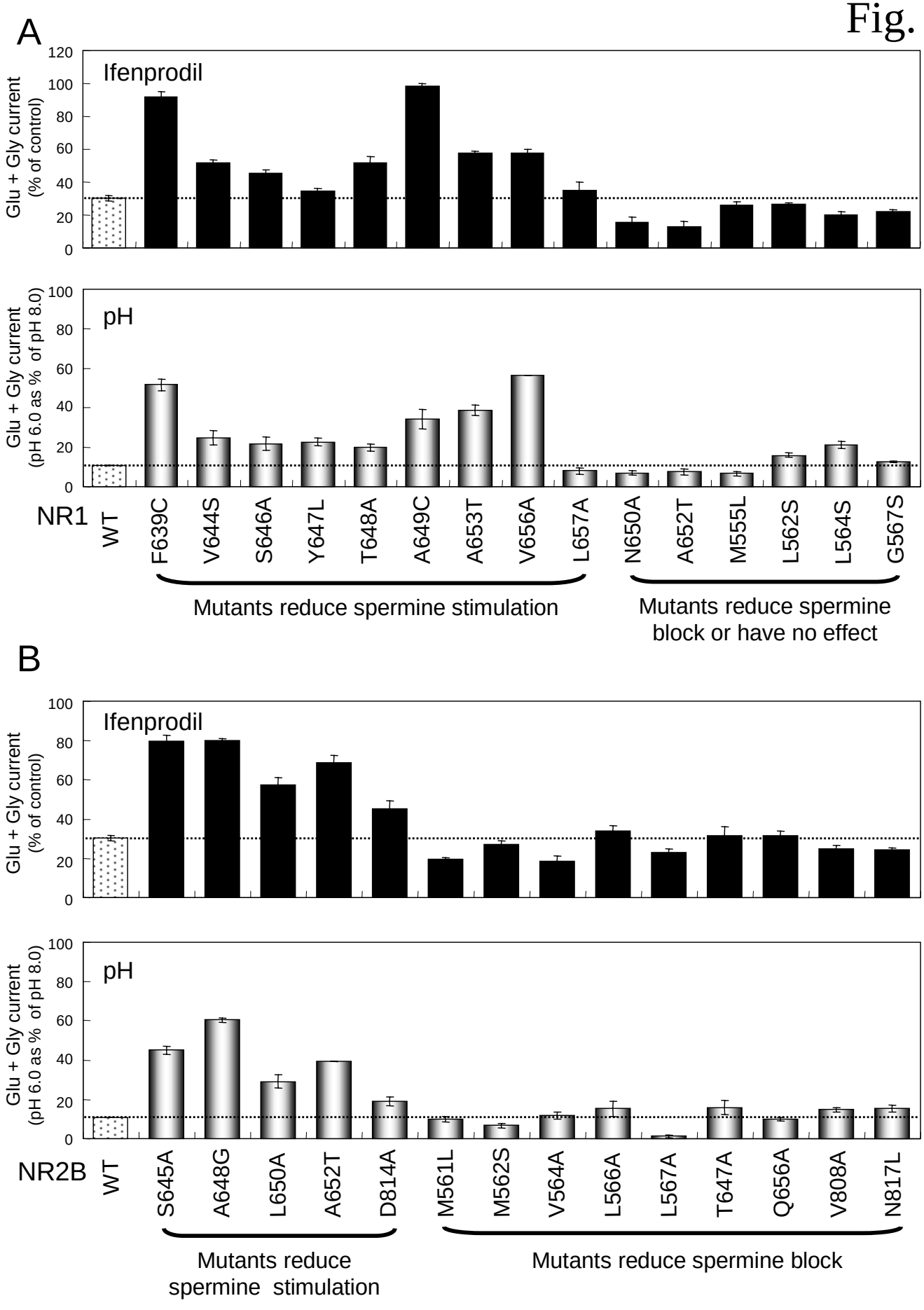

Fig. 7