7. In the aforementioned mammalian experiments (*5, 6*), electrical currents or application of neuromodulators were used as stimuli. These stimuli could activate intrinsic modulating systems (that is, noradrenergic, cholinergic, serotoninergic, or dopaminergic) in addition to the tested neuronal pair. In fact, when the effect of such modulators on neuronal plasticity was tested, it was found that they play a crucial role [P. K. Stanton and J. M. Sarvey, *J. Neurosci.* **5**, 2169 (1985)].
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9. Nevertheless, modifications of cellular parameters had been studied during behavioral conditioning in mammals [N. W. Weinberger *et al.*, in *Learning and Computational Neuroscience*, M. Gabriel and J. W. Moore, Eds. (MIT Press, Cambridge, MA, 1990), pp. 91–138; S. Aou, C. D. Woody, D. Birt, *J. Neurosci.* **12**, 560 (1992)] or after training [G. R. Recanzone, M. M. Merzenich, W. M. Jenkins, K. A. Grajski, H. R. Dinse, *J. Neurophysiol.* **67**, 1031 (1992)].
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11. Two monkeys were used in this study: Monkey A (*Macaque fascicularis*, 5 kg) was trained to perform an auditory discrimination task (see text for details) and monkey B (*Macaque mulatta*, 4.5 kg) was trained to sit quietly during recordings. The recording techniques and surgery procedures were detailed elsewhere [M. Ahissar, E. Ahissar, H. Bergman, E. Vaadia, *J. Neurophysiol.* **67**, 203 (1992)]. We recorded simultaneously from five glass-coated tungsten microelectrodes that were confined within a cylindrical space (500-μm diameter). Neuronal activity from each electrode was classified as belonging to different neurons by template-matching algorithms realized in fast hardware machines [M. Abeles and M. H. Goldstein, *Proc. IEEE* **65**, 762 (1977)] and by window discriminators (Frederick-Haer, Bak).
12. This is a version of the "cellular classical conditioning" [E. R. Kandel and W. A. Spencer, *Physiol. Rev.* **48**, 65 (1968); E. R. Kandel, *Cellular Basis of Behavior* (Freeman, San Francisco, 1976)].
13. This pairing increased (or decreased) the covariance, namely the co-occurrence of spikes above (or below) the expected value. Thus, the contingency, and not merely the contiguity (which is represented by the overall co-occurrence), was modified.
14. D. H. Perkel, G. L. Gerstein, G. P. Moore, *Biophys. J.* **7**, 391 (1967). For each histogram, a band of 99% confidence limits for the equivalent, independent Poisson processes is shown by broken lines [M. Abeles, *J. Neurosci. Methods* **5**, 83 (1982)].
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16. To eliminate the effect of nonstationarity that may accompany extracellular recording, we took the following precautions: (i) only pairs for which each of the neurons exhibited a stable spike shape and firing pattern (determined by the ACH) all through the experiment were analyzed; and (ii) each pair was tested in blocks that were repeated several times. Each block contained the various conditioning paradigms.
17. Three types of "strength evaluators" were used in this study: gain, contribution, and synchronous gain (*15*). There was no significant difference between the results obtained with these three evaluators. The gain, a simple and straightforward evaluator, is used in this paper.
18. There were few examples in which connections were depressed when contingency was either not changed or slightly increased (Fig. 4), which suggests the existence of an additional, non-Hebbian, mechanism. For example, input activation associated with small postsynaptic depolarization (smaller than a certain threshold) yields a depression [A. Artola, S. Bröcher, W. Singer, *Nature* **347**, 69 (1990)].
19. We use the term "Hebbian-like" as representing the family of models that essentially require presynaptic and postsynaptic correlation for induction of synaptic modifications. [However, see (*20*), pp. 408–411.]
20. For a more recent theory that includes both principles, see S. Grossberg and D. S. Levine, in *Neural Networks and Natural Intelligence*, S. Grossberg, Ed. (MIT Press, Cambridge, MA, 1988), pp. 405–440.
21. We thank N. Donegan, Y. Dudai, A. Grinvald, and M. Segal for reading the manuscript and for helpful comments. This research was supported in part by the Fund for Basic Research, administered by the Israel Academy of Sciences and Humanities.

1 April 1992; accepted 9 July 1992

Control by Asparagine Residues of Calcium Permeability and Magnesium Blockade in the NMDA Receptor

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The *N*-methyl-D-aspartate (NMDA) receptor forms a cation-selective channel with a high calcium permeability and sensitivity to channel block by extracellular magnesium. These properties, which are believed to be important for the induction of long-term changes in synaptic strength, are imparted by asparagine residues in a putative channel-forming segment of the protein, transmembrane 2 (TM2). In the NR1 subunit, replacement of this asparagine by a glutamine residue decreases calcium permeability of the channel and slightly reduces magnesium block. The same substitution in NR2 subunits strongly reduces magnesium block and increases the magnesium permeability but barely affects calcium permeability. These asparagines are in a position homologous to the site in the TM2 region (Q/R site) of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors that is occupied by either glutamine (Q) or arginine (R) and that controls divalent cation permeability of the AMPA receptor channel. Hence AMPA and NMDA receptor channels contain common structural motifs in their TM2 segments that are responsible for some of their ion selectivity and conductance properties.

Excitatory synaptic transmission in the vertebrate central nervous system is mediated by glutamate, which activates glutamate receptor ion channels. These receptors are divided into two functional subtypes: the AMPA receptors mediate fast synaptic currents and NMDA receptors mediate currents that are slower and longer lasting. NMDA receptors mediate an inflow of Ca^{2+} into the postsynaptic cell, which is controlled by extracellular Mg^{2+} in a voltage-dependent manner (*1*). The NMDA receptor subunits NR1 (*2*) and NR2 (*3*) are distantly related to each other and contain, in their transmembrane 2 (TM2) segment, an asparagine at a position homologous to the Q/R site (*4*) of the AMPA receptor subunits (Fig. 1A). The amino acid occupying the Q/R site of AMPA receptor subunits is controlled by RNA editing (*5*) and determines conductance rectification and the divalent cation permeability of the ion channel (*6*). Arginine in this site imparts a much lower Ca^{2+} and Mg^{2+} permeability to the AMPA receptor channel than the glutamine residue (*6*). Engineering an asparagine into this critical position of AMPA receptor subunits imparts to the altered channel a high permeability for Ca^{2+} compared to Mg^{2+} (*4*). We therefore investigated the effect of replacing the asparagine (*7*) in the position homologous to the Q/R site of AMPA receptor subunits on the Ca^{2+} and Mg^{2+} permeability of recombinantly expressed NMDA receptors.

Whole-cell currents were activated by fast application of L-glutamate to transfected 293 cells expressing NMDA receptor channels (*7*). Voltage ramps were applied before and during agonist application to compare changes in reversal potentials when the extracellular solution was changed from high Na^+ (140 mM) to high Ca^{2+} (110 mM) or high Mg^{2+} (110 mM). The difference in the reversal potential between the wild-type NR1-NR2A and the mutant NR1(N598Q)-NR2A channel after a switch from high Na^+ to high Ca^{2+}

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extracellular solution is illustrated in Fig. 1, B and C. The reversal potential in cells expressing the wild-type subunits shifted from approximately 0 mV in high Na^+ solution to a positive membrane potential close to 20 mV in high Ca^{2+} solution (Fig. 1B, arrow) (*3*). In cells expressing the NR1(N598Q)-NR2A subunits, the reversal potential shifted on average to about −6 mV. This difference indicates that the mutant channel has a much lower Ca^{2+} permeability and, hence, that Ca^{2+} permeability is critically dependent on the asparagine residue NR1(N598) in the TM2 segment. This conclusion is supported by the observation that NR1(N598Q)-NR2C channels also have a reduced Ca^{2+} permeability. The wild-type NR1-NR2C channel is characterized by a reversal potential of about 19 mV in high Ca^{2+} solution (*3*), whereas for the NR1(N598Q)-NR2C channel the reversal potential is close to 7 mV (Table 1). We investigated whether the homologous asparagine in the NR2 subunits has a similar function in determining Ca^{2+} permeability of heteromeric channels. In NR1-NR2A(N595Q) and NR1-NR2C(N593Q) channels, the reversal potential in high Ca^{2+} extracellular solution did not, however, differ significantly from that of the wild-type channel (Table 1).

In contrast to the mutation in the NR1 subunit, which did not measurably increase the channels' low Mg^{2+} permeability, the NR2 subunit mutations generated channels with increased Mg^{2+} permeability. Reversal potentials in high Mg^{2+} extracellular solution were close to −12 mV; those of the corresponding wild-type channels were more negative than −50 mV in these conditions (legend to Fig. 1, B and D, and Table 1).

In native NMDA receptor channels, extracellular Mg^{2+} ions are strong blockers of Na^+ inward current through the channel (*8*). To assess the effects of mutations in the TM2 segment on channel block by Mg^{2+}, we compared whole-cell current-voltage (*I-V*) relations in nominally divalent ion–free rat Ringer's solution with those measured after adding small amounts of Mg^{2+} to the extracellular solution. The strength of the block by 0.1 mM Mg^{2+} in the wild-type channel was reduced in the NR1(N598Q)-NR2A mutant channel. The response amplitude in the presence of 0.1 mM Mg^{2+} was, in cells expressing the wild-type channel, 9 ± 3% (mean ± SEM, n = 4) of the control value in divalent ion–free Ringer's solution and 30 ± 6% (n = 4) of control in the mutant channel when measured at −100 mV (Fig. 2, A and B, traces a and b). The replacement of the homologous asparagine by glutamine in the TM2 segment of the NR2A subunit reduced the channels' sensitivity to block by 0.1 mM extracellular

Table 1. Reversal potentials of glutamate-activated whole-cell currents in transfected 293 cells expressing combinations of wild-type and mutant NMDA receptor subunits. 100 μM L-glutamate was applied. The recording pipette contained high Cs^+ (140 mM). Reversal potentials V_{rev} (mV) were measured first for high Na^+ (140 mM) solution and then for high Ca^{2+} (110 mM) or high Mg^{2+} (110 mM) solutions. The values represent the mean ± SEM determined from the number of cells indicated in parentheses; nd, not determined. The reversal potentials in high Na^+ solution are close to 0 mV (within 3 mV) for wild-type and mutant channels. In the double mutant, Ca^{2+} and Mg^{2+} permeability were similar.

Subunit combination	V_{rev} (mV) Ca^{2+}/Cs^+	V_{rev} (mV) Mg^{2+}/Cs^+
NR1-NR2A	23.4 ± 1.6 (5)*	< −50 (4)
NR1-NR2C	19.5 ± 0.7 (6)*	< −100 (3)
NR1(N598Q)-NR2A	−5.9 ± 1.6 (10)	< −100 (3)
NR1(N598Q)-NR2C	6.6 ± 1.0 (3)	< −100 (1)
NR1-NR2A(N595Q)	18.1 ± 1.3 (6)*	−12.7 ± 0.9 (4)
NR1-NR2C(N593Q)	17.6 ± 1.0 (3)*	−11.0 ± 3.9 (3)
NR1(N598Q)-NR2A(N595Q)	3.4 ± 1.3 (3)	−6.4 (1)
NR1(N598R)-NR2A	−82.5 ± 2.2 (4)	nd

*Values are not significantly different from each other (P = 0.01). A t test was used for significance tests.

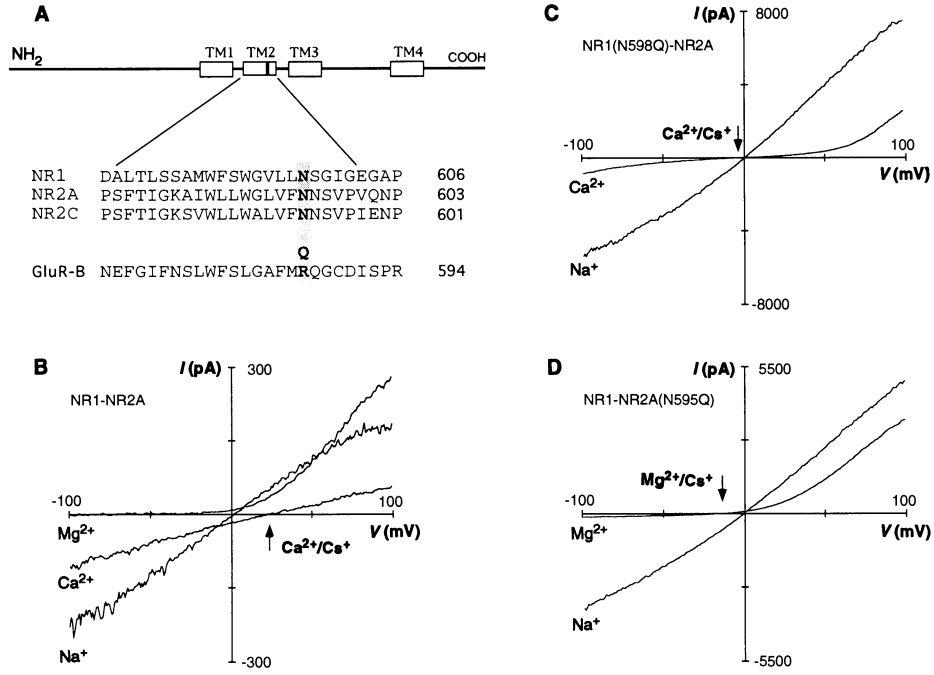

Fig. 1. Difference in Ca^{2+} and Mg^{2+} permeability between wild-type and mutant NMDA receptor channels. (**A**) Ionotropic glutamate receptor subunit with predicted transmembrane regions and TM2 sequence alignment of NMDA receptor subunits NR1 (*2*), NR2A (*3*), NR2C (*3*), and of AMPA receptor subunit GluR-B or GluR-2 (*13*) in its edited and unedited version (*5*). The residues determining functional channel properties are indicated in bold. Numbers refer to amino acid positions within the mature subunits. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (**B**) Wild-type channel (NR1-NR2A) expressed in 293 cells. Whole-cell *I-V* relation of glutamate-activated currents in high Na^+ (140 mM), in high Ca^{2+} (110 mM), and in high Mg^{2+} (110 mM) extracellular solutions measured during ramped changes in membrane potential. The reversal potential in high Na^+ solution is close to 0 mV. The reversal potential in high Ca^{2+} extracellular solution is indicated by arrow (22.3 mV). Because of the small size of the current (<0 mV), a Mg^{2+}/Cs^+ reversal potential could not be determined accurately for this wild-type channel. The reversal potential in high Mg^{2+} solution is more negative than −50 mV, since outward current was detected up to −50 mV, and up to −100 mV no inward current was measurable. This suggests that Mg^{2+} ions are impermeant. For the other subunit combinations with low Mg^{2+} permeability listed in Table 1, outward current was measurable up to −100 mV under these conditions. (**C**) Mutant channel comprising NR1(N598Q) and NR2A subunits. Whole-cell *I-V* relation of glutamate-activated current. Reversal potential in high Ca^{2+} extracellular solution is indicated by arrow (−3.0 mV). Reversal potential in high Na^+ extracellular solution is close to 0 mV. (**D**) Mutant channel comprising NR2A(N595Q) and NR1 subunits. *I-V* relations in high Na^+ and in high Mg^{2+} extracellular solutions. The reversal potential in high Mg^{2+} extracellular solution is −11 mV (arrow); in high Na^+ solution it is close to 0 mV.

Mg^{2+} even more strongly. Reduction of inward current by extracellular Mg^{2+} in the mutant NR2A(N595Q) channel was significantly smaller [71 ± 4% (n = 4) of control] than in the wild type (Fig. 2, A and C, traces a and b), as expected from the increased Mg^{2+} permeability. Qualitatively comparable changes in Mg^{2+} block were observed in cells expressing mutant NR1(N598Q)-NR2C or NR1-NR2C(N593Q) subunit combinations.

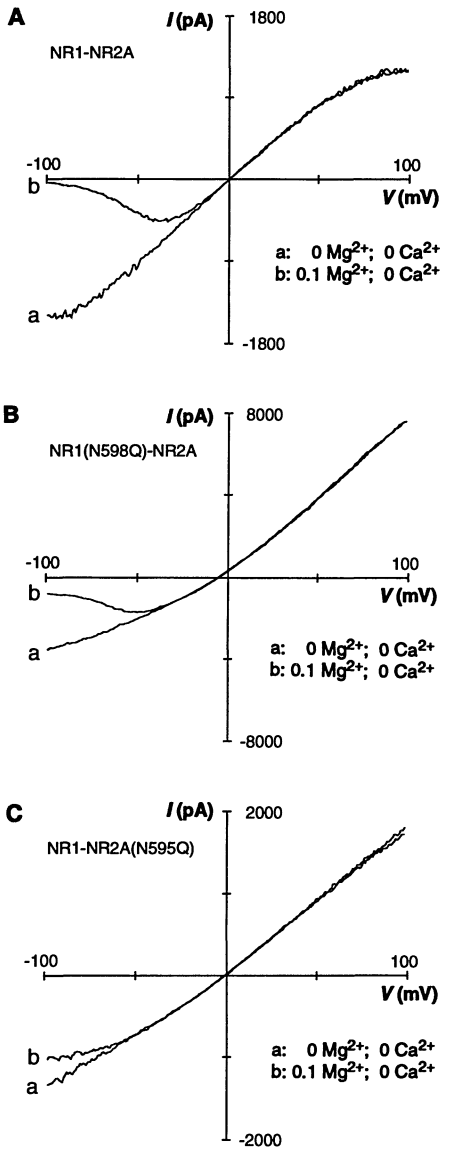

Fig. 2. Difference in channel block by extracellular Mg^{2+} between wild-type and mutant NMDA receptor channels in 293 cells. (**A**) Wild-type channel comprising NR1 and NR2A subunits. Whole-cell I-V relation of glutamate-activated currents measured in (a) divalent ion–free rat Ringer's solution and (b) after addition of 0.1 mM Mg^{2+}. (**B**) Mutant channel comprising NR1(N598Q) and NR2A subunits. Same experimental conditions as in (A). Whole-cell I-V relation in (a) divalent ion–free Ringer's solution and (b) after addition of 0.1 mM Mg^{2+}. (**C**) Mutant channel comprising NR1 and NR2A(N595Q) subunits. Same experimental conditions as in (A) and (B). Whole-cell I-V relation in (trace a) divalent ion–free Ringer's solution and (trace b) after addition of 0.1 mM Mg^{2+}.

In the native NMDA receptor channel, extracellular Ca^{2+} acts as a weak channel blocker, reducing the amplitude of single channel inward currents carried by Na^+ (9). Adding 1.8 mM Ca^{2+} to divalent ion–free Ringer's solution caused, in the wild-type NR1-NR2A channel, a slight reduction of inward current [77 ± 5% (n = 4) of control response measured in divalent ion–free Ringer's solution at −100 mV]. In contrast, in the NR1(N598Q)-NR2A and in the NR1-NR2(N595Q) channels the reduction in inward current by adding Ca^{2+} is stronger [45 ± 7% (n = 5) and 44 ± 5% (n = 5) of control]. The increase in blocking effects of extracellular Ca^{2+} suggests that the asparagines in TM2 of NR1 and NR2 subunits contribute to channel sites that interact with both Ca^{2+} and Mg^{2+}. Consistent with this view is the observation that blockade of inward currents by extracellular Mg^{2+} depends, in one of the mutants, on extracellular Ca^{2+} concentration. In the wild-type channel the Mg^{2+} block is weakly independent on Ca^{2+} [13 ± 6% (n = 4) of control response measured in Mg^{2+}-free Ringer's solution containing 1.8 mM Ca^{2+}; membrane potential of −100 mV)], whereas in the NR1(N598Q)-NR2A channel the Mg^{2+} block of the response is strongly reduced in the presence of 1.8 mM Ca^{2+} [78 ± 4% (n = 3) of control]. In the NR1-NR2A(N598Q) channel the Mg^{2+} block of inward current is, as expected from the relatively high Ca^{2+} and Mg^{2+} permeability of this channel, only weakly affected by extracellular Ca^{2+} [91 ± 0.4% (n = 3) of control].

To quantify the differences in Mg^{2+} blockade caused by mutations in different subunits, glutamate-activated whole-cell currents were measured in *Xenopus* oocytes (7) expressing wild-type and mutant channels in conditions of low (0.18 mM) extracellular Ca^{2+}. The results show that wild-type and mutant channels have different sensitivities to Mg^{2+} blockade (Fig. 3), as suggested by the experiments with transfected 293 cells. The apparent inhibitory constant (IC_{50}) values for Mg^{2+} channel block at −100 mV were 3.6 μM for wild type and 20.6 μM and 78.5 μM for the NR1 and NR2A mutants, respectively. This confirms, under slightly different experimental conditions, the view that the Mg^{2+} blockade is more strongly reduced by the mutation in the NR2 subunit.

Replacing the asparagine by arginine in NR1(N598R)-NR2A subunit receptors generated channels that did not exhibit a measurable Ca^{2+} permeability, suggesting that the size and charge of the critical amino acid present in TM2 is important for the Ca^{2+} permeability of NMDA receptor channels. In cells expressing this mutant channel the reversal potential of the glutamate response shifts from close to 0 mV in high Na^+ solution to below −70 mV in high Ca^{2+} extracellular solution (Fig. 4, A and B, and Table 1). In cells expressing the NR1(N598R)-NR2A receptor, the shape of the I-V relation of glutamate-activated currents in high Na^+ solution was also insensitive to addition of extracellular Mg^{2+}.

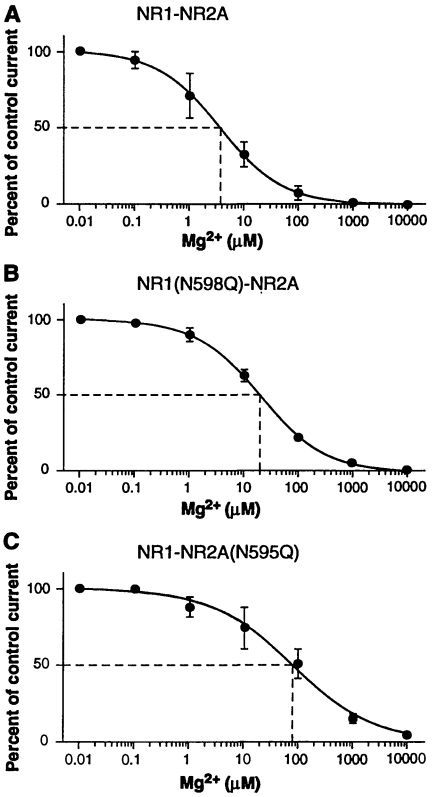

Fig. 3. Dependence of Mg^{2+} blockade of glutamate-activated currents on Mg^{2+} concentration in wild-type and mutant channels. Whole-cell currents elicited by bath applied glutamate (100 μM) at −100 mV membrane potential in *Xenopus* oocytes injected previously with subunit-specific mRNAs. Mean and SD of reduction of currents by Mg^{2+} are plotted as a function of extracellular Mg^{2+}. The extracellular solution contained 0.18 mM Ca^{2+}. (**A**) Coexpression of wild-type NR1 and NR2A subunits. (**B**) Co-expression of NR1(N598Q) and NR2A subunits. (**C**) Coexpression of NR1 and NR2A(N595Q) subunits. Percentage of current amplitude (100% is the control current amplitude measured in the absence of Mg^{2+}) is plotted as a function of extracellular Mg^{2+} concentration in semilogarithmic coordinates. Data points were fitted by the function $y = 100\%/(1 + [Mg^{2+}]/IC_{50})^{n_H}$, with y = percentage of control current; $[Mg^{2+}]$ = concentration of Mg^{2+}; and n_H = Hill coefficient. IC_{50} values (mean ± SEM) for Mg^{2+} block estimated by this fit are 3.6 ± 0.5 μM (n = 6), 20.6 ± 0.8 μM (n = 6), and 78.5 ± 19.6 μM (n = 8); Hill coefficients are 0.75, 0.79, and 0.58, respectively.

Inward currents measured in divalent ion–free Ringer's solutions were not measurably blocked when 0.5 mM Mg^{2+} was added (Fig. 4C). Thus, when the positively charged arginine occupies the critical position in TM2 of the NR1 subunit, divalent cations appear to be prevented from entering the channel.

The strong effect on Ca^{2+} permeability and conductance of an asparagine to glutamine or arginine substitution in the putative transmembrane segment TM2 of the NR1 subunit suggests that this segment forms part of the channel lining of the NMDA receptor. The homologous asparagines in the NR2 subunits are also likely to form part of the channel wall because of the strong effect of their replacement by glutamine on Mg^{2+} permeability and channel block by Mg^{2+}. The effects of mutations on permeability and channel block could suggest that the asparagines from the two subunit partners form, at least in part, the selectivity filter of the NMDA receptor for both Mg^{2+} and Ca^{2+}. Homologous positions revealed by sequence alignment, however, need not reflect equivalent positions with respect to the lining of the assembled channel. The different NMDA receptor subunits may contribute distinct properties to the channel because of their asymmetric arrangement, as seen for other channels (*10*). Alternatively, the coordination sites for Ca^{2+} and Mg^{2+} in the channel's lumen may form as a function of a particular subunit stoichiometry, which may change as a result of introducing point mutations. Our experiments do not address the question of additional determinants of Ca^{2+} permeability and Mg^{2+} block. These are likely to exist because the strength of Mg^{2+} blockade differs between heteromeric NR1-NR2A and NR1-NR2C channels (*3*).

High Ca^{2+} permeability and voltage-dependent blockade by extracellular Mg^{2+} are thought to be important for the function of NMDA receptors in mediating long-term changes in synaptic efficacy (*11*) and the cytotoxic effects of glutamate in hypoxic conditions (*12*). Both Ca^{2+} permeability and Mg^{2+} blockade are critically dependent on the presence of single asparagine residues in the TM2 of NR1 and NR2 subunits. Hence, mutations affecting this residue in either subunit would cause dysfunctions in excitatory synaptic transmission by disrupting the voltage dependence of Ca^{2+} entry into the postsynaptic cell.

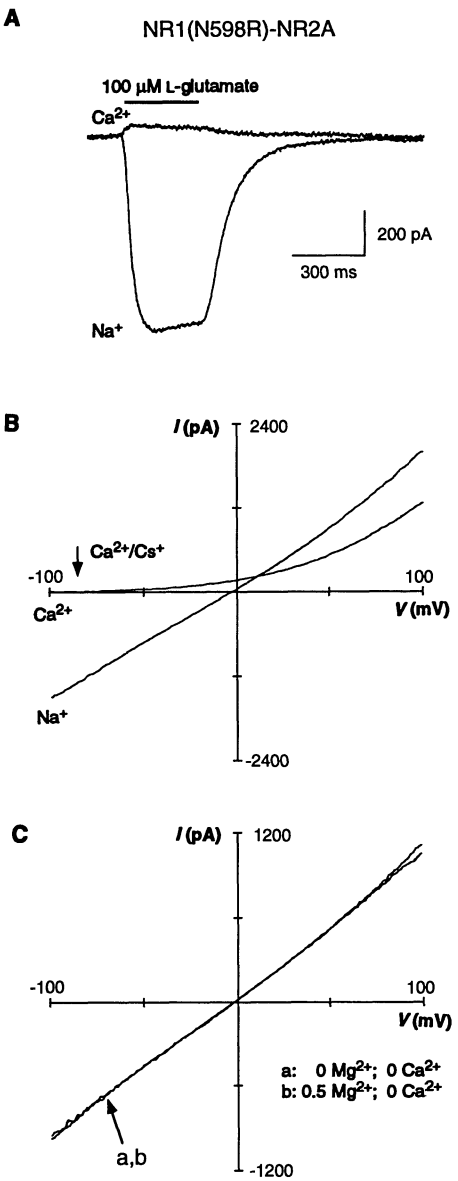

Fig. 4. Reduction of Ca^{2+} permeability and channel block by extracellular Mg^{2+} in mutant channel where asparagine in TM2 of the NR1 subunit is replaced by arginine. NR1(N598R) subunit co-expressed with NR2A subunit in 293 cells. (**A**) Whole-cell currents elicited by 100 µM glutamate (horizontal bar) at −60 mV in high Na^+ (inward current, downward deflection) or high Ca^{2+} (outward current, upward deflection) extracellular solution. Outward current at −60 mV in high Ca^{2+} solution indicates low Ca^{2+} permeability of this mutant channel. (**B**) Whole-cell *I-V* relations of glutamate-activated currents in high Na^+ solution and high Ca^{2+} solution as indicated. Reversal potentials are close to 0 mV and −87 mV (arrow). (**C**) Whole-cell *I-V* relations in (trace a) divalent ion–free rat Ringer's solution and (trace b) after adding 0.5 mM Mg^{2+} to the Ringer's solution. The two traces superimpose almost completely.

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7. Codon substitutions within TM2 were introduced into the coding sequences of cloned NR1 (*2*), NR2A (*3*), and NR2C (*3*) cDNAs by oligonucleotide-mediated mutagenesis [P. H. Seeburg, W. W. Colby, D. J. Capon, D. V. Goeddel, A. D. Levinson, *Nature* **312**, 71 (1984)] by using single-stranded phagemid DNAs as templates. All oligonucleotides (antisense, 24 nucleotides) were designed to engineer single codon substitutions into cloned NMDA receptor DNAs (*2, 3*) to generate subunits carrying amino acid replacements in TM2. Codon substitutions were confirmed by DNA sequencing [F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)]. Expression vectors [C. M. Gorman, D. R. Gies, G. McGray, *DNA Protein Eng. Technol.* **2**, 3 (1990)] constructed with these mutated cDNAs were used along with the wild-type constructs (*2*) for co-expression in 293 (ATCC CRL 1573) cells [C. Chen and H. Okayama, *Mol. Cell. Biol.* **7**, 2745 (1987); D. B. Pritchett, H. Lüddens, P. H. Seeburg, *Science* **245**, 1389 (1989)]. Typically, 5 × 10^4 cells were transfected with 300 ng of recombinant vector per subunit. The rat NR2C cDNA [H. Monyer *et al.*, *Science* **256**, 1217 (1992)] used by us has a single nucleotide deletion (nucleotide 2866 of coding sequence) relative to the mouse homolog [T. Katsuwada *et al.*, *Nature* **358**, 38 (1992)], as revealed by sequencing another rat NR2C encoding clone. The latter cDNA directs the expression of a polypeptide with an additional 275 COOH-terminal amino acid residues. Its sequence deposited in EMBL-GenBank (M91568) now replaces the earlier version. Its co-expression with NR1 in 293 cells generates glutamate-activated channels with permeability properties and current kinetics identical to the shorter version. Cells on cover slips were transferred 48 hours after transfection to the stage of an inverted microscope and kept in a chamber continuously perfused with rat Ringer's solution containing 135 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM Hepes (pH 7.2; NaOH). Whole-cell current recordings (List EPC-7 amplifier, Darmstadt, Germany) were made at room temperature [O. P. Hamill, A. Marty, E. Neher, B. Sakmann, F. J. Sigworth, *Pfluegers Arch.* **391**, 85 (1981)] from single cells lifted from the bottom of the dish. Pipette solution contained 140 mM CsCl, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM Hepes (pH 7.2; CsOH). Divalent ion–free Ringer's solution was used as an extracellular solution in most of the experiments when Mg^{2+} and Ca^{2+} blockade were studied. $MgCl_2$ (0.1 mM or 0.5 mM) or $CaCl_2$ (1.8 mM) (or both) was added to this solution. High Na^+ solution contained 140 mM NaCl, 5 mM Hepes (pH 7.2; NaOH). High Mg^{2+} solution contained 110 mM $MgCl_2$, 5 mM Hepes [pH 7.2; $Mg(OH)_2$], and high Ca^{2+} solution contained 110 mM $CaCl_2$, 5 mM Hepes [pH 7.2; $Ca(OH)_2$]. L-glutamate (100 µM) and 10 µM glycine dissolved in one of the tested extracellular solutions were applied together rapidly to the cells by means of a Piezo-driven double-barreled system (*14*). Before application of agonists, cells were equilibrated for at least 30 s with control solution. Data collection and analysis were as in (*4*). For experiments with *Xenopus* oocytes, mRNA synthesized in vitro was prepared and injected (10 ng per subunit and oocyte) as described [C. Methfessel *et al.*, *Pfluegers Arch.* **407**, 577 (1986)]. Whole-cell currents were measured with a conventional two-microelectrode voltage-clamp amplifier (n.p.i., Tamm, Germany) 3 to 5 days after RNA injection in response to 100 µM glutamate added to the extracellular solution and

applied by bath perfusion. Extracellular solution contained 115 mM NaCl, 2.5 mM KCl, 0.18 mM $CaCl_2$, 10 mM Hepes, pH 7.2 (NaOH). $MgCl_2$ was added to the extracellular solution as indicated. Voltage ramps were applied before and 30 s after the beginning of the response to glutamate. Currents recorded in response to the first voltage ramp were then digitally subtracted from the second ramp to obtain the *I-V* relation of glutamate-activated current. Extracellular solutions contained 10 μM glycine. All experiments were carried out at room temperature.

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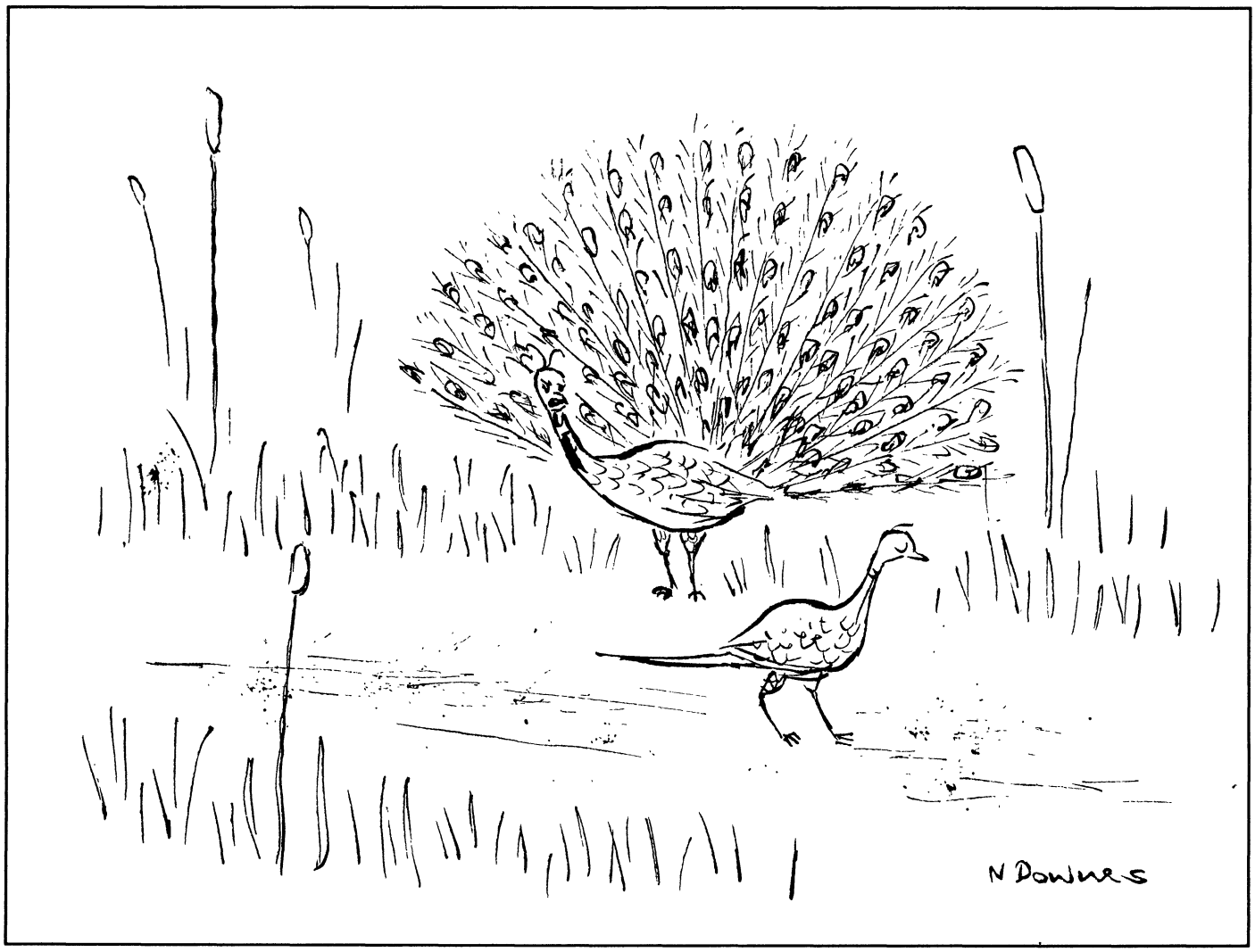
15. We thank S. Grünewald for cell culture, T. Kuner for help with mutagenesis, and A. Herold for DNA sequencing. This work was funded, in part, by the German Ministry for Research and Technology (BMFT) grant BCT 364 AZ 321/7291 (P.H.S.).

9 July 1992; accepted 13 August 1992



"May I remind you that ninety-nine percent of all species that ever existed are today extinct."