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ACCELERATED COMMUNICATION

Molecular Determinants of Proton-Sensitive *N*-Methyl-D-aspartate Receptor Gating

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ABSTRACT

Extracellular protons inhibit *N*-methyl-D-aspartate (NMDA) receptors with an IC_{50} value in the physiological pH range. To identify the molecular determinants of proton sensitivity, we used scanning mutagenesis of the NR1 subunit to search for residues that control proton inhibition of NMDA receptors. Homology modeling of the extracellular domains suggested that residues at which mutations perturbed pH sensitivity were localized in discrete regions. The majority of mutations that strongly affected proton sensitivity were clustered in the extracellular end of the second transmembrane domain (M3) and adjacent linker leading to the S2 portion of the glycine-binding domain of NR1. Mutations in NR2A confirmed that the analogous region controls the pH sensitivity of this subunit and also identified the linker region between the third transmembrane domain (M4) and the S2 portion of the NR2 glutamate binding domain as an additional determinant of proton sensitivity. One mutant receptor, NR1(A649C)/NR2A(A651T), showed a 145-fold reduction in the IC_{50} for protons (IC_{50}, 17.3 μM corresponding to pH 4.9). The M3-S2 linker region has been suggested to control NMDA receptor gating, leading to the hypothesis that the proton sensor and receptor gate may be structurally and functionally integrated.

NMDA receptors play important roles in excitatory synaptic transmission, synaptic plasticity, memory formation, and neuronal development (Dingledine et al., 1999). However, overactivation of these receptors can lead to cytotoxic accumulation of Ca^{2+} in neurons. NMDA receptor-dependent neurotoxicity has been suggested to be a component of neuronal death associated with ischemic stroke (Dirnagl et al., 1999; Lee et al., 1999) and head trauma (Obrenovitch and Urenjak, 1997). NMDA receptor activation has also been proposed to be an important component of the initiation and maintenance of seizures (Meldrum et al., 1999).

NMDA receptor activation is tightly controlled by a number of endogenous modulators, including protons. An extracellular site sensitive to pH has emerged as an essential feature of gating. Protonation of one or a few residues completely inhibits NMDA receptor activation (Giffard et al., 1990; Tang et al., 1990; Traynelis and Cull-Candy, 1990), and mildly acidic extracellular pH is neuroprotective in a variety of models of glutamate-induced damage (Tombaugh and Sapolsky, 1993). NMDA receptors are under tonic inhibition of ~50% at physiological pH, suggesting that any shift in the pH sensitivity of the proton sensor can up- or down-regulate receptor function under normal conditions. At least three extracellular modulatory compounds or ions have been suggested to exert their actions by changing the pK_a of the proton sensor. Proton sensitivity of gating is a common downstream substrate for high-affinity Zn^{2+} inhibition of NR2A-containing receptors (Choi and Lipton, 1999; Low et al., 2000), for polyamine potentiation of NR2B-containing receptors (Traynelis et al., 1995), and for ifenprodil inhibition of

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

NR2B-containing receptors (Pahk and Williams, 1997; Mott et al., 1998). That is, these allosteric regulators all shift the proton sensitivity of channel function in a way that can quantitatively account for their inhibitory or potentiating effects at physiological pH.

The glutamate receptor family shares a number of structural features with pH-sensitive potassium channels (MacKinnon, 1995; Panchenko et al., 2001). The most primitive glutamate receptors still retain the K^+-selective filter but are gated by both protons and glutamate (Chen et al., 1999; Cui and Mayer, 2001). Recent work on inwardly rectifying potassium channels suggests that the molecular determinants of pH-sensitive gating are localized to one or a few residues near the intracellular side of the transmembrane domains (e.g., Schulte et al., 1999; Schulte and Fakler, 2000; Yang et al., 2000). Given that several structural features are shared between inward rectifier potassium channels and glutamate receptors, we hypothesized that the molecular determinants of proton inhibition of NMDA receptors are also localized to discrete portions of the receptor. To evaluate this hypothesis, we tested the pH sensitivity of mutations at 88 residues in NR1 coexpressed with NR2A or NR2B. These data were combined with previously reported effects on pH sensitivity of 53 NR1 mutations (Sullivan et al., 1994; Kashiwagi et al., 1996, 1997; Traynelis et al., 1998; Masuko et al., 1999) and homology modeling of the extracellular domains of NR1 to show that determinants of proton sensitivity are highly localized. Data in this report suggest that residues near the extracellular end of the second transmembrane domain (membrane spanning domain M3) and residues in the adjacent linker leading to the ligand binding domain S2 that were previously proposed to be critical gating elements (Kohda et al., 2000; Taverna et al., 2000; Jones et al., 2002) control the pH sensitivity of the NR1 and NR2A subunits of NMDA receptors (Low et al., 1999; Lyuboslavsky et al., 2001; French et al., 2002).

Materials and Methods

Stage V–VI oocytes from *Xenopus laevis* were isolated, injected with NMDA receptor cRNA synthesized in vitro, and recorded under voltage clamp as described previously (Traynelis et al., 1998, Kashiwagi et al., 2002). Oocytes were injected with 1 to 10 ng of cRNA encoding NR1-1a (hereafter referred to as NR1; GenBank accession numbers U11418, U08261), NR2A subunits (GenBank accession number D13211), NR2B (GenBank accession number U11419), and NR2C (GenBank accession numbers M91563; NR1:NR2 ratio 1:2 or 1:5); in a minority of experiments, NMDA receptor cDNAs within CMV-driven vectors were injected directly into the nucleus. Membrane currents in response to 10 to 100 μM glutamate and 10 to 60 μM glycine were recorded from oocytes 1 to 7 days after injection using a two-electrode voltage clamp (V_{hold} -20 to -50 mV). Lower concentrations of glutamate were used for receptors containing NR1(T648C), NR1(A649C), or NR1(A653T), which decreased the glutamate EC_{50} by more than an order of magnitude (data not shown). The recording solution contained 90 mM NaCl, 3 mM KCl, 10 mM HEPES, and 0.5 mM $BaCl_2$ (22°C); pH was adjusted with NaOH. In some experiments screening mutations for effects on pH sensitivity, the recording solution contained 2.5 mM $BaCl_2$ and oocytes were injected with BAPTA on the day of recording. Recording electrodes were filled with 0.3 M KCl. A trace amount of EDTA (10 μM) or 10 mM tricine was added to all extracellular solutions in experiments with NR1/NR2A to chelate contaminant extracellular Zn^{2+} (Paoletti et al., 1997). Temperature was 23°C. Responses to glutamate applied at different pH values were compared with control responses at pH 7.6 recorded before and after the pH change. Oocytes were prewashed with buffer at the new pH for 1 min before application of glutamate. Composite proton inhibition curves were constructed by normalizing the responses from individual oocytes at different pH values to the response amplitude at pH 7.6 (or pH 8.4 for some mutants). These measurements from different oocytes were then averaged. Proton inhibition curves were fitted with the equation Response = Maximal response/$[1 + ([H^+]/IC_{50})^{nH}]$, where n_H is the Hill slope. When responses from mutant NR1/NR2 receptors were of low amplitude (<100 nA), we compared the proton sensitivity of these receptors to that of homomeric NR1. For example, we found that oocytes injected only with cRNA for NR1(A653T) ($n = 10$ oocytes) show a significantly greater sensitivity to protons than responses from oocytes coinjected with NR1(A653T)/NR2A(A651T) ($p < 0.01$). Similarly, homomeric NR1(T648C) ($n = 5$ oocytes) also are more sensitive to protons than NR1(T648C)/NR2A(T646C) ($p < 0.01$). These observations suggest that we may have underestimated the shift in proton sensitivity caused by the mutation of these two residues if functional homomeric NR1 receptors are present, which would render the summed current more sensitive to protons. Proton concentrations were calculated using an activity coefficient of 0.8.

Site-directed mutagenesis was performed as described previously using the QuikChange kit (Stratagene, La Jolla, CA) or using the M13 phage system (Williams et al., 1995; Low et al., 2000; Kashiwagi et al., 2002). The region of interest was sequenced (500–600 bp) for all mutations.

The glycine binding core of the NR1 subunit (residues Thr396-Thr550/Asp658-Cys798) was modeled using the GluR2 S1S2 crystal structure as a template (1GR2; Armstrong et al., 1998). An alignment was made between secondary structure elements predicted for NR1 using PredictProtein (*http://www.embl-heidelberg.de/predictprotein/predictprotein.html*; Levin et al., 1993; Rost and Sander, 1993a,b, 1994) and those known for 1GR2. Modeler (v3; Sali and Blundell, 1993; Marti-Renom et al., 2000) was subsequently used to create a homology model of the NR1 ligand-binding core. The model was visually compared with 1GR2 using SYBYL (ver. 6.0; Tripos, St. Louis, MO), and analyzed using SYBYL's ProTable to find inappropriate backbone conformations and high-energy regions. The alignment was modified at these problem regions by introducing gaps to increase the degrees of freedom and rotate side chains of high-energy residues. The modified alignment was resubmitted to Modeler and a new model was obtained. Seventeen iterations of viewing the model, changing alignment, and resubmitting to Modeler-3 were done to minimize the total energy of the modeled protein. A homology model of the amino terminal domain of NR1 (residues Pro24-Ile356) was created using the crystal structure coordinates for mGluR1 (1EWK, 1EWV; Kunishima et al., 2000). Seven iterations of the same process of modeling, evaluation, and realignment described above were performed for the NR1 amino terminal domain.

A homology model of the pore-forming elements of the NMDA receptor was constructed from an alignment between alternating NR1 (residues Ser553-Arg659) and NR2 (residues Phe549-Glu657) subunits and the subunits of the KcsA potassium channel (1BL8; Doyle et al., 1998). Sequences were aligned on the first transmembrane forming domain and this was submitted to Modeler, assuming an arbitrary subunit arrangement of NR1-NR2-NR1-NR2

Results

To evaluate regions of the NMDA receptor that are important for proton-sensitive gating, we used scanning mutagenesis of the NR1 subunit. We made 78 single and 5 double

mutations throughout all regions of the NR1 subunit, coexpressed these mutated subunits with NR2B in *X. laevis* oocytes, and assessed the proton sensitivity from the ratio of currents evoked by saturating concentrations of glutamate and glycine and recorded at pH 6.8 and 7.6. The proton IC_{50} was determined for all mutations that showed a significant change in the ratio of the response at pH 6.8 and 7.6 ($p < 0.05$, ANOVA, Tukey-Kramer post hoc test; $n = 3$–20 oocytes per mutant). The results of this analysis are summarized in Table 1. None of the mutations we tested in the amino terminal domain or S1 region had marked (>2-fold) effects on the IC_{50} values for protons. By contrast, a variety of mutations that altered the proton sensitivity of NMDA receptors by more than 2-fold were found in the M3 region and adjacent linker to S2 (T648C, A649C, A653T, V656A, L657A, R659L, R671L/R673L; see Table 1, Fig. 1, A and B). A comprehensive plot combining the sensitivity of proton inhibition to mutational analysis across the NR1 subunit in this and other studies (Fig. 1C) suggests that residues within M3 and the adjacent linker leading to the S2 domain may control proton sensitive gating.

Molecular Determinants of Proton Sensitivity within Extracellular Domains of NR1. To evaluate tertiary locations of the residues identified above as well as previously described residues at which mutations alter proton sensitivity of NR1/NR2B receptors, we constructed homology models of extracellular domains of the NR1 subunit. mGluR1 (1EWK, 1EWV) and GluR2 (1GR2) crystal structures were selected as templates for the amino terminal domain and the S1-S2 glycine binding domain of NR1, respectively. These models approximate main chain configurations used to aid development of general structural hypotheses concerning proton sensitive gating and are not intended to represent refined structures with atomic resolution. Thus, the results of homology modeling should be interpreted with appropriate caution. Figure 2 shows the positions within these models of the polypeptide chain for key residues at which mutations alter proton sensitivity. Mutations with no effect on proton inhibition or spermine potentiation are shown as black and blue, respectively. In the amino-terminal domain (Fig. 2A), two previously reported mutations (E181Q, E185Q; Masuko et al., 1999) that reduce proton sensitivity lie near the exon5 splice site, a region known to influence pH sensitivity (Traynelis et al., 1995). The other mutations described previously (Y109A, E342Q, E297Q; Williams et al., 1995; Masuko et al., 1999) that increased the proton IC_{50} by more than 3-fold all lie along the back plane that links the two lobes (Fig. 2A). Interestingly, the corresponding positions of these residues vary in the open and closed conformations of mGluR1 ligand-binding domain, with E297 showing movement away from the hinge region adjoining the two domains. The amino-terminal domain of NR2A harbors a high-affinity Zn^{2+} binding site (Choi and Lipton, 1999; Fayyazuddin et al., 2000; Low et al., 2000; Paoletti et al., 2000). Zn^{2+} binding to this site enhances proton sensitivity, which can qualitatively account for the degree of Zn^{2+} inhibition over a wide range of pH values (Choi and Lipton, 1999; Low et al., 2000). To test whether the proton sensor might reside in the amino-terminal domain, we evaluated a deletion construct for NR2A that lacked the amino terminal domain. Replacement of the first 385 residues of the amino terminal domain of NR2A with MKTIIALSYIFCLVFADYKDDDDATRYM modestly enhanced proton sensitivity, shifting IC_{50} value from 119 nM ($n = 66$) for NR1/NR2A to 63 nM ($n = 25$) for NR1/NR2A(Δ385). These data are consistent with the idea that the proton sensor is not contained in the NR2A amino terminal domain but can be modestly influenced by this region (Low et al., 2000).

We have identified two new residues in the NR1 S2 region (R659L, R671L/R673L; Table 1) at which mutations decrease proton sensitivity. We assumed that R671L exerts the greatest effect within the double NR1 Arg mutant because R673L is without effect. Figure 2B shows how these residues and three previously described NR1 mutations (D669Q, D789Q, C798S; Sullivan et al., 1994; Kashiwagi et al., 1996) are clustered in or near the linkers that connect the S2 region of the ligand binding core to the M3 and M4 transmembrane domains. Another NR1 residue (Cys744) that influenced pH sensitivity probably forms a disulfide bond with Cys798 (Sullivan et al., 1994) and thus is also clustered within this group of residues. This colocalization of otherwise nonadjacent residues within the S2 region supports the idea that the molecular determinants of proton sensitive gating are highly localized rather than diffusely scattered throughout the protein.

Molecular Determinants of Proton Sensitivity within the M3 and the M3-S2 Linker of the NR1 Subunit. Previous studies have suggested that all glutamate receptors contain a conserved extracellular motif (SYTANLAAF) that is critical for gating just downstream of the M3 transmembrane domain. A mutation of one residue in this motif in the δ2 glutamate receptor subunit generates constitutively open channels and is responsible for the "lurcher" mouse phenotype, which has led many to refer to this region

TABLE 1

NR1 mutations that alter NMDA receptor proton sensitivity

The pH sensitivity of the following NR1 mutations were evaluated: H38A, H53A, H67A, H94A, H101A, T122A/T123A, H134A, H146A, H162A, H171I, H280A, H293A, H371A, H449A, H477A, K543A/K544A, R548A, D552N, F554L, M555L, P557G, F558L, S560A, L562S, L564S, L565S, V566S, G567S, L568S, H571A, Y578L, T602G, S604A, S605A, A606S, F609L, G620C, V644S, A645S, S646A, Y647C, T648C, A649C, N650A, N650C, L651C, A652T, A652C, A653T, A653C, F654A, F654C, L655A, L655C, V656A, L657A, R659A, R659L, E661Q, R663L, R671L/R673L, R673L, D677N, K678L, S688A, R694L/R695L, H705A/H709A, R722L, H727A, R755L, R763L, H780A, W792L, R794L, R801L, T807C, T807V, L808A, T809A, F810A, E811Q, N812A, A814L, F817A. The NR1 mutations listed below had statistically significant effects on the ratio of NR1/NR2B and NR1/NR2A current recorded at pH 6.8 and pH 7.6, and thus IC_{50} values were determined. The position of mutations within the lurcher region were indexed to the lurcher site (assigned '0').

Receptor	ΔLurcher	IC_{50}	IC_{50}(MUT)/IC_{50}(WT)
		nM	
NR1/NR2B		49	1.0 ($n = 16$)
NR1(H477A)/NR2B		80	1.6 ($n = 20$)
NR1(K543A/K544A)/NR2B		31	0.6 ($n = 11$)
NR1(R548A)/NR2B		71	1.5 ($n = 9$)
NR1(T648C)/NR2B	−5	371	7.6 ($n = 10$)
NR1(A649C)/NR2B	−4	400	8.2 ($n = 26$)
NR1(A652C)/NR2B	−1	31	0.6 ($n = 17$)
NR1(A653T)/NR2B	0	369	7.6 ($n = 4$)
NR1(A653C)/NR2B	0	101	2.1 ($n = 16$)
NR1(V656A)/NR2B	+3	261	5.4 ($n = 3$)
NR1(L657A)/NR2B	+4	198	4.1 ($n = 3$)
NR1(R659L)/NR2B	+6	165	3.4 ($n = 8$)
NR1(R671L/R673L)/NR2B		173	3.5 ($n = 8$)
NR1/NR2A		119	1.0 ($n = 56$)
NR1(T648C)/NR2A	−5	1833	15.4 ($n = 13$)
NR1(A649C)/NR2A	−4	3402	28.7 ($n = 11$)
NR1(A653T)/NR2A	0	650	5.5 ($n = 12$)

MUT, mutant; WT, wild-type.

as the "lurcher region" (Kohda et al., 2000). The analogous mutation in GluR1 seems to greatly increase the affinity of agonists (Taverna et al., 2000). The equivalent mutation in NMDA receptor subunits does not generate constitutively open channels, but mutations at some nearby residues within this region do generate constitutively open NMDA channels (Kashiwagi et al., 2002). Studies using scanning cysteine mutagenesis to probe the structural changes associated with NMDA receptor gating have shown agonist-dependent movements of this region (Jones et al., 2002). Potassium channel gating has also been suggested to involve movement of M2 region in bacterial potassium channels, which corresponds to M3 in NMDA receptors (Jiang et al., 2002a,b). We postulate that the equivalent region in NMDA receptors (M3) may undergo a pH-sensitive movement in gating. To test this hypothesis, we mutated the 16 consecutive residues surrounding the lurcher motif of NR1 and determined the effects of these mutations on the pH sensitivity. The results were evaluated with the assumption that the M3-lurcher region of NR1 has structural similarity with the analogous region of KcsA and MthK.

Data shown in Fig. 3A and Table 1 suggest that a number of residues in and adjacent to the lurcher motif of NR1 influence the pH sensitivity of NR1/NR2B receptors. Indeed, this region showed the highest sensitivity within NR1 to mutation-induced perturbations of proton-sensitive gating (Fig. 1C). Large shifts in the proton sensitivity occurred at three residues. NR1(T648C) increased the IC_{50} for protons 7.6-fold when it was coexpressed with NR2B. Mutation of an adjacent residue, NR1(A649C), also reduced the proton sensitivity of NR1/NR2B to similar degree (8.2-fold). The NR1 lurcher mutation (A653T) causes a 7.6-fold decrease in the proton sensitivity of NR1/NR2B receptors. Figure 3, B and C, shows the superposition of NR1 residues at which mutations reduce proton sensitivity on the KcsA and MthK K^+ channel structures (see *Materials and Methods*). Interestingly, several NR1 residues that influence the pH sensitivity line up along one side of the M3 helix near its extracellular end (Fig. 3D). Furthermore, although NR1(Y647C)/NR2B shows normal pH sensitivity, NR1(Y647L) has been previously reported to reduce pH sensitivity by ~3-fold (Kashiwagi et al., 1997). Thus, Tyr647-Thr648-Ala649 occupy nearly a full turn of the helix and seem to influence pH sensitivity. These results suggest that a side-chain hydrogen bond acceptor such as threonine, cysteine, or tyrosine can critically influ-

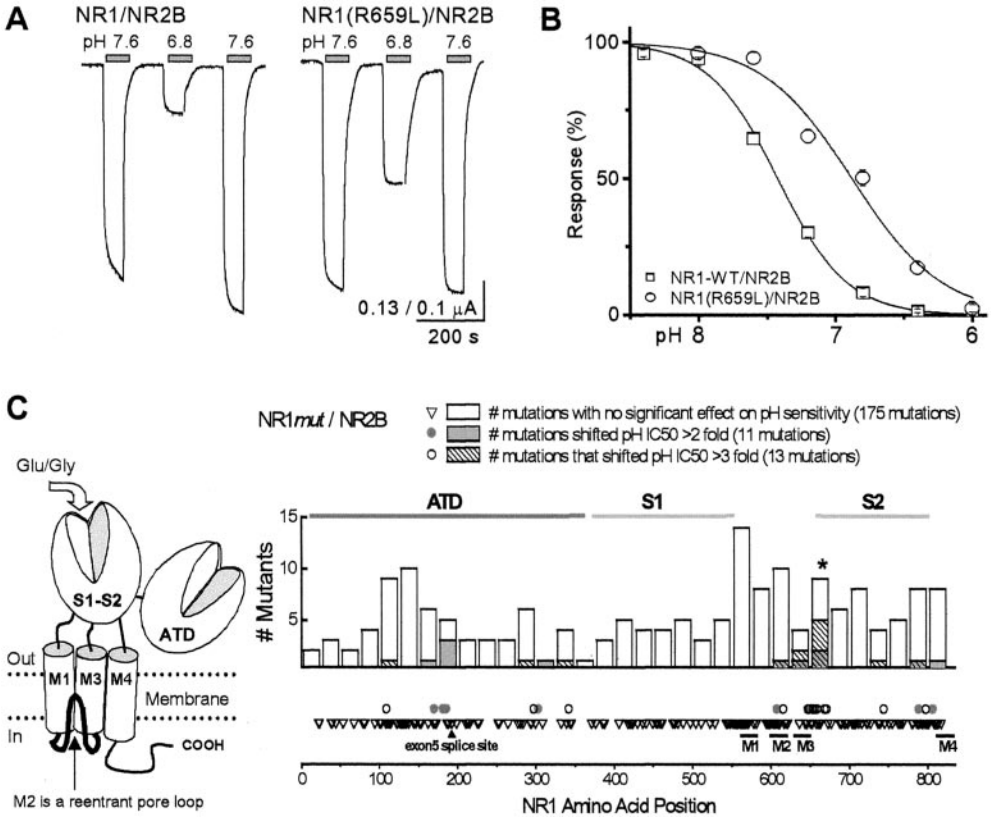

Fig. 1. Structural determinants of proton sensitivity in the NR1 subunit. A, current recordings from *X. laevis* oocytes expressing NR1/NR2B and NR1(R659L)/NR2B receptors recorded under two-electrode voltage clamp (V_m −40 mV) are shown in response to maximally effective concentrations of glutamate and glycine. B, composite proton inhibition curve for the receptors shown in A. Error bars are S.E.M. and are shown when larger than symbol. C, NR1 mutations (83) are plotted on a linear representation of the NR1 gene product according to their effect on proton inhibition; the IC_{50} values of mutations with significant effects on proton sensitivity are given in Table 1. The effects of 53 previously described NR1 mutations on proton sensitivity are also plotted, and were drawn from Kashiwagi et al. (1996, 2 mutations; 1997, 8 mutations), Masuko et al. (1999, 25 mutations), Sullivan et al. (1994, 10 mutations), and Traynelis et al. (1998, eight mutations). Because spermine potentiation reflects relief of proton inhibition (Traynelis et al., 1995), we also plotted 63 previously described NR1 mutations that did not disrupt spermine potentiation; these data were drawn from Kashiwagi et al. (1996, eight mutations; 1997, seven mutations), Masuko et al. (1999, 44 mutations), Williams et al. (1995, 4 mutations). Plot of mutations in NR1 that caused a 2-fold shift of the proton IC_{50} (11 of 199) are shown as gray symbols and bars on the histogram. Mutations with more than 3-fold shift of the proton IC_{50} (13 of 199) are shown as hollow symbols and hatched bars. Histogram shows number of effective or ineffective mutations per 25 residues. The position of exon5, the presence of which reduces proton sensitivity, is shown as a solid triangle.

ence gating at certain positions within the M3-S2 linker region.

Molecular Determinants of Proton Sensitivity within the M3 and M3-S2 Linker of NR2 Subunits. NMDA receptors are heteromeric receptors composed of NR1 and NR2 subunits, which bind the coagonists glycine and glutamate, respectively. NMDA receptor activation requires the binding of both agonists, suggesting that NR1 and NR2 independently contribute to gating (Jones et al., 2002; Banke and Traynelis, 2003). To determine whether NR1/NR2B and NR1/NR2A receptors share structural similarities in their pH sensitivity of gating, we coexpressed the three NR1 mutations having the largest effect on pH sensitivity with NR2A as well as with NR2B. NR1(T648C) and NR1(A649C) showed even greater reductions in proton sensitivity when coexpressed with NR2A than with NR2B (Table 1). In particular, NR1(A649C)/NR2A showed the largest reduction (~30-fold) in proton sensitivity of any single mutation reported. We subsequently tested whether the conserved lurcher motif in NR2 subunits also influences pH sensitive gating. Eight consecutive residues in the M3-lurcher motif of NR2A were mutated and the proton sensitivity of these mutant receptors examined (Table 2). The NR2A mutations of T646C, A647C, and A651T all altered the proton sensitivity in a fashion similar to that of the corresponding mutations in NR1 subunits. These data suggest that the M3 region of NR1 and NR2A may adopt a similar structure and play a similar role in the gating of NMDA receptors.

Molecular Determinants of Proton Sensitivity within the M4-S2 Linker of NR2. NMDA receptors composed of NR1/NR2C show a reduced sensitivity to protons compared with receptors containing other NR2 subunits (Traynelis et al., 1995). The IC_{50} values for proton inhibition of NR2A and NR2C receptors were 119 nM (pH 7.0; $n = 30$) and 825 nM (pH 6.2; $n = 15$). Although the S2 region is highly conserved, NR2C and NR2A share only 68% amino acid identity immediately downstream of the lurcher region. To deter-

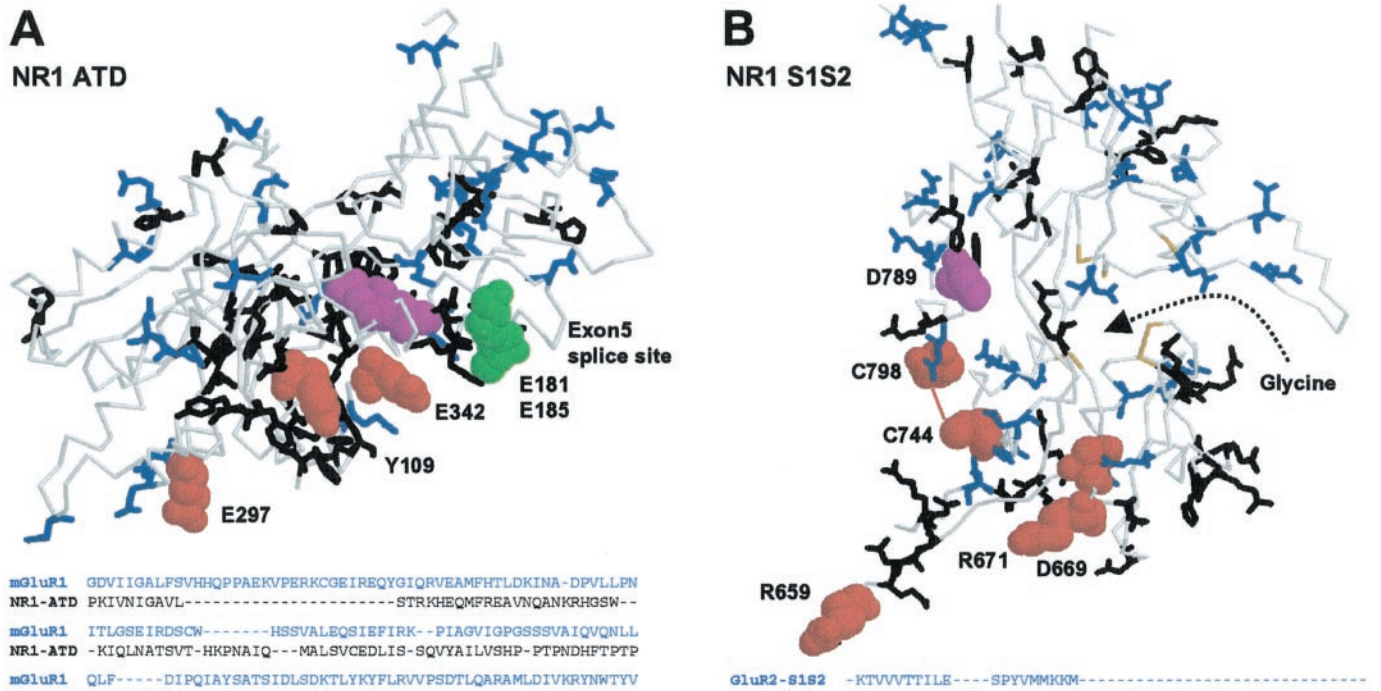

Fig. 2. Homology models of the extracellular portions of the NR1 subunit. A, a homology model of the amino terminal domain was constructed as described under *Materials and Methods* using the mGluR1 crystal structure (1EWV) as a template (Kunishima et al., 2000). Mutations that reduced pH sensitivity by 2-fold are space-filled magenta (E181Q, E185Q), and those that reduced pH sensitivity by more than 3-fold are space-filled red (Y109A, E297Q, E342Q). Mutations causing less than a 2-fold shift of the proton IC_{50} are colored black, and mutations with no effect on spermine potentiation are blue (see Fig. 1 legend for reference citations). The exon5 splice site is shown in green. B, a homology model of the S1-S2 glycine binding domain for NR1 was constructed as described under *Materials and Methods* using the GluR2 ligand binding core 1GR2 as a template (Armstrong et al., 1998). D789Q increased the IC_{50} value for proton inhibition by more than 2-fold, and is shown in magenta. Mutations at residues that alter pH sensitivity by more than 3-fold are space filled red (R659L, R671L/R673L, D669Q, C744S, C798S). Mutations with no effect on pH sensitivity or spermine potentiation are colored black and blue, respectively (see Fig. 1 legend for reference citations). Residues (Phe424-Pro447, italics) within an insertion were modeled as a loop and are not shown. Residues corresponding to those in GluR2 that form hydrogen bonds with the ligand glutamate are orange.

mine whether the differential proton sensitivity between these subunits might involve residues near the lurcher region, we evaluated the current response at pH 6.4 and 7.3 of four chimeric subunits: NR2C/A(564), NR2C/A(697), and NR2C/A(826), and NR2A/C(553) (see Fig. 4A for chimeric junctions). In these experiments, the differential pH sensitivity of the chimeric receptors was controlled by residues 697 to 826 in the S2 extracellular region. Chimeric NR2C-containing receptors with residues 697 to 826 of NR2A were significantly more sensitive to protons than wild-type NR1/NR2C receptors (Fig. 4A). We examined the pH sensitivity of mutations that exchanged, individually or pair-wise, seven uniquely divergent or nonconserved residues in NR2C for those in NR2A within this region (R710P/D711Y, H715S, H781P, Q800E/K801E, Q812H). These experiments identified a His residue conserved in NR2A, -B, and -D but replaced by Gln in NR2C (n = 3–12 oocytes per mutant receptor) as an essential determinant of the reduced pH sensitivity of NR2C. NR1/NR2C(Q812H) receptors showed an increased proton sensitivity that was shifted from that of NR1/NR2C (IC_{50} = 825 nM) to a value similar (IC_{50} = 240 nM; n = 9) to wild-type NR2A (119 nM; Fig. 4B). Surprisingly, introduction of Gln into the analogous position of NR2A did not fully convert the pH sensitivity to that of wild-type NR2C receptors. Receptors composed of NR1/NR2A(H801Q) had an IC_{50} value of 171 nM (n = 10), compared with wild-type NR1/NR2A (119 nM). These data suggest that other determinants of the NR2C pH insensitivity reside between residues 697 and 826.

Whereas these data lead us to reject the hypothesis that residues downstream of lurcher are responsible for reduced pH sensitivity of NR2C-containing receptors, these data nevertheless identify a second linker region between the S2 region and the M4 transmembrane domain as a molecular determinant of proton sensitivity of both NR2A and NR2C receptors; NR1 residues in this region also influence pH sensitivity (Fig. 2B; Sullivan et al., 1994). Table 2 summarizes the effects of a number of mutations at ionizable residues in the M4-S2 linker region of the NR2A subunit. Of these mutations, NR1/NR2(D813A,D815A) had the largest

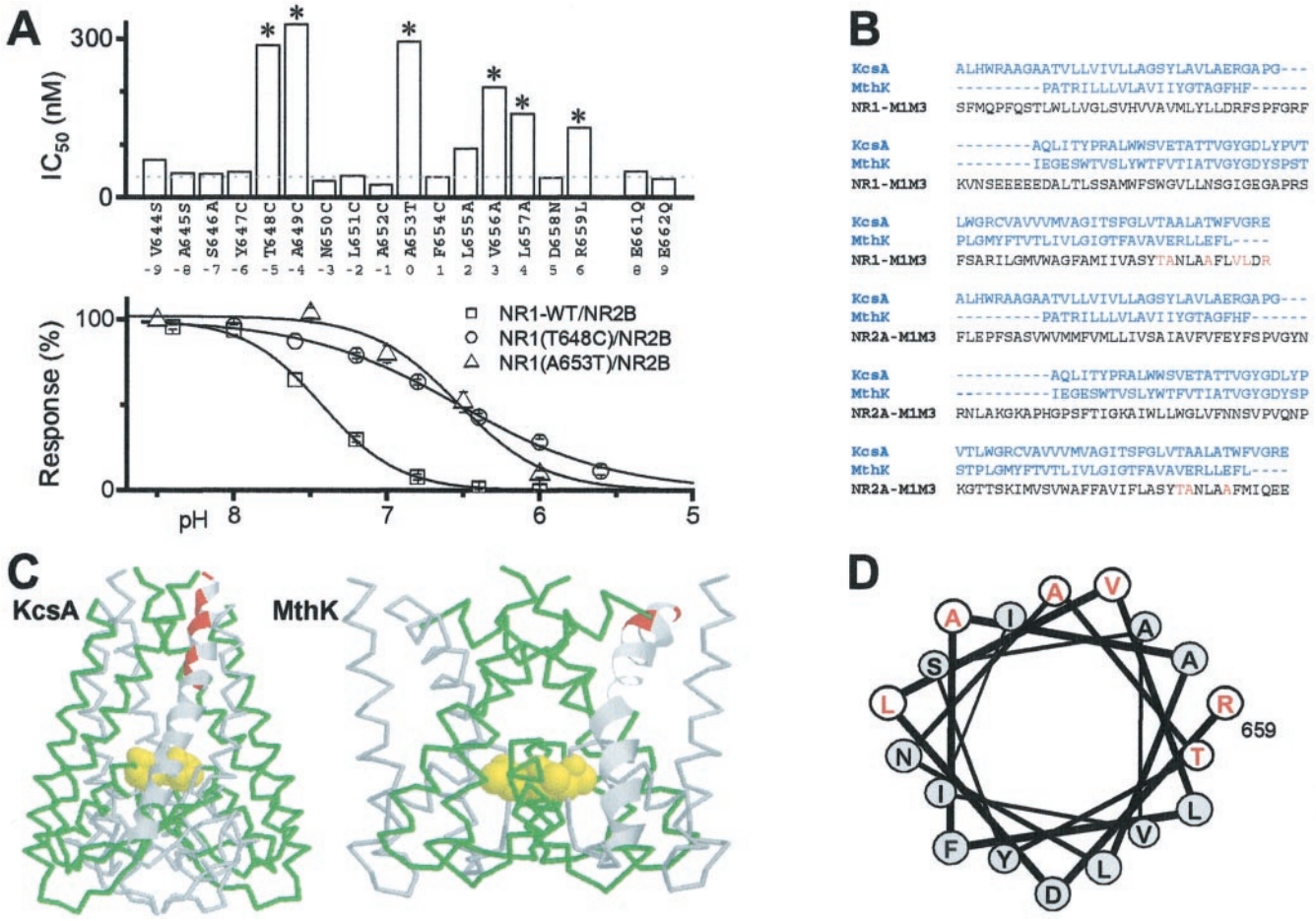

Fig. 3. Mutations in the M3-lurcher region of NR1 control proton sensitivity. A, top, bar graph showing IC_{50} values of mutations within the lurcher region of the NR1 subunit when coexpressed with NR2B. Data for NR1 (D658N) and NR1(E662Q) are from Kashiwagi et al. (1996). Bottom, composite proton inhibition curves for two mutant receptors that alter proton sensitivity. Alignment of NR1 and NR2 against KcsA (Doyle et al., 1998) and MthK (Jiang et al., 2002b). The horizontal line represents the IC_{50} of wild-type NR1/NR2B receptors. C, left, a homology model of the pore-forming elements of a hypothetical NMDA receptor with the arbitrary stoichiometry NR1-NR2A-NR1-NR2A using the closed conformation of KcsA. The M3-lurcher region of NR1 is shown as a gray ribbon structure with mutations causing >3-fold shift in proton IC_{50} shown as red. NR2A backbone is shown in green; Asn616 in NR1 and Asn614 in NR2A within the pore, which are known to influence ion permeation and block as well as proton sensitivity (Kashiwagi et al., 1997), are space-filled yellow. All M3-lurcher residues that shift proton IC_{50} value by more than three-fold are shown in red. Right, residues corresponding to the hypothetical position of NR1 Ala653 and Thr648 are also shown highlighted within the MthK open channel structure using the alignment described by Jiang et al. (2002a,b) between KcsA and MthK. Coloring is as described for KcsA. D, helical wheel plot for 18 residues within NR1 (642–659). Residues at which mutations caused more than a 2-fold shift in the proton IC_{50} value are shown as white symbols with red labels.

effect on proton IC_{50} of any NR2 mutations studied here (Fig. 4C). Consistent with this finding, mutations at a nearby position in NR2C(E814Q/E817Q) decreased proton inhibition at pH 6.4 by 5.3-fold ($p < 0.05$; $n = 4$). These data suggest that both the M3-S2 and M4-S2 linker domains, which are closely positioned between the channel pore and the agonist binding domain, can control the effects of protons on NMDA receptor function.

Independent Contribution of NR1 and NR2 Subunits to Proton Sensitivity. Do the various pH-sensitive regions in NR1 and NR2 operate independently or in a cooperative fashion? To evaluate this idea, we determined the proton IC_{50} for coexpressed pairs of NR1 and NR2 mutant subunits. We studied the three most effective NR1 mutations in combination with each of the three most effective NR2 mutations. We used thermodynamic cycles (Carter et al., 1984) to calcu-

TABLE 2

NR2 mutations that alter NMDA receptor proton sensitivity

Mutations were made in the lurcher region of NR2A to test whether this region also controls proton-sensitive gating. The position of mutations within the lurcher region were indexed to the lurcher site (assigned '0').

Receptor	Δ Lurcher	IC_{50}	IC_{50}(MUT)/IC_{50}(WT)
		nM	
NR1/NR2A		119	1.0 ($n = 56$)
M3-S2 Linker			
NR1/NR2A(T646C)	−5	356	3.0 ($n = 17$)
NR1/NR2A(A647C)	−4	150	1.3 ($n = 3$)
NR1/NR2A(N648S)	−3	68	0.6 ($n = 8$)
NR1/NR2A(L649C)	−2	194	1.6 ($n = 4$)
NR1/NR2A(A650C)	−1	96	0.8 ($n = 11$)
NR1/NR2A(A651C)	0	88	0.7 ($n = 6$)
NR1/NR2A(A651T)	0	571	4.8 ($n = 50$)
NR 1/ NR2A(F652C)	+1	61	0.5 ($n = 11$)
M4-S2 Linker			
NR1/NR2A(H801A)		338	2.8 ($n = 6$)
NR1/NR2A(K804L)		133	1.1 ($n = 8$)
NR1/NR2A(E803A, K804L,E806A)		108	0.9 ($n = 21$)
NR1/NR2A(D813A)		84	0.7 ($n = 14$)
NR1/NR2A(D813A,D815A)		810	6.8 ($n = 27$)
Coexpressed NR1-NR2 Linker Mutants			
NR1(T648C)/NR2A(T646C)	−5,−5	2098	18 ($n = 22$)
NR1(T648C)/NR2A(A651T)	−5, 0	2461	21 ($n = 24$)
NR1(T648C)/NR2A(D813A,D815A)	−5	9566	81 ($n = 31$)
NR1(A649C)/NR2A(T646C)	−4 −5	2793	24 ($n = 24$)
NR1(A649C)/NR2A(A651T)	−4,0	17288	146 ($n = 19$)
NR1(A649C)/NR2A(D813A,D815A)	−4	3166	27 ($n = 20$)
NR1(A653T)/NR2A(T646C)	0,−5	2033	17 ($n = 11$)
NR1(A653T)/NR2A(A651T)	0,0	3300	28 ($n = 66$)
NR1(A653T)/NR2A(D813A,D815A)	0	5000	42 ($n = 31$)

MUT, mutant; WT, wild-type.

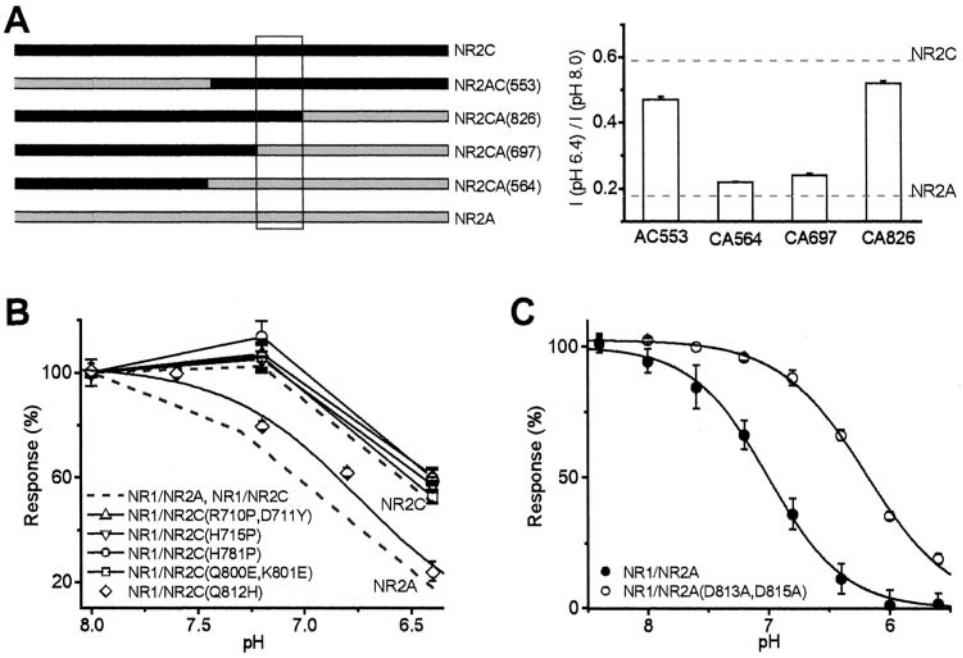

Fig. 4. Structural determinants of proton insensitivity of NR2C subunits. A, left, the cartoon shows the design and nomenclature of chimeric NR2A-C receptors. The region controlling reduced proton sensitivity of NR2C receptor is indicated by a box. Right, the ratio of current response of chimeric receptors to maximally effective concentrations of glutamate and glycine applied at pH 6.4 and 8.0 are shown ± S.E.M. ($n = 3$–6 oocytes). B, composite proton inhibition curves for mutant NR1/NR2C receptors. Proton inhibition curves for wild-type NR1/NR2C and NR1/NR2A receptors are shown as broken lines. C, composite proton inhibition curve for NR1/NR2A and NR1/NR2A(D813A,D815A).

late a coupling coefficient (Ω) for each pair of mutations. Coupling coefficients of 1.0 indicate independent and additive effects of the mutations, whereas those less than 1.0 suggest nonadditive effects. Our calculations assumed that both the proton IC_{50} value approximated the K_d for proton binding to a single site and that protonation did not induce major conformational changes in the protein. The data summarized in Table 1 for mutant NR1 subunits expressed with NR2A and in Table 2 for coexpressed mutant NR1 and mutant NR2A subunits indicate that some but not all pairs of NR1/NR2 mutations exert independent effects on proton sensitivity. For example, the lurcher point mutation in NR1(A653T)/NR2A or NR1/NR2A(A651T) alone produced shifts of comparable magnitude (Table 1); coexpression of both mutant subunits NR1(A653T)/NR2A(A651T) produced an additive shift (Table 2). Of the pairs of mutations tested, NR1(A649C) and NR1(A653T) had additive effects with NR2(A651T). Only the NR1(A653T) lurcher mutation showed additive effects with NR2A(T646C). These data suggest that the proton sensitivity of NMDA receptor gating can be incrementally influenced by certain mutations in the M3-S2 linker regions of different subunits. This additivity for coexpressed mutant receptors suggests that the proton sensor may reside within regions that are either in contact with both subunits or jointly influenced by NR1 and NR2 M3-S2 linker regions.

Coexpression of NR1(A649C)/NR2A(A651T) resulted in the largest shift in pH sensitivity described to date for NMDA receptors (145-fold; Fig. 5A). It seems likely that the proton sensitivity of this mutant receptor (IC_{50} 17 μM or pH 4.9) is near a detectable limit that is set by the inevitable protonation of many side chains at acid pH that could inhibit receptor function in ways unrelated to the mechanism studied here. For example, pH values below 5.5 will probably cause substantial changes in ionization of accessible histidine residues, acidic residues, or other residues whose pK_a values have been shifted by the local microenvironment within the protein. Thus, the double mutant NR1(A649C)/NR2A(A651T) may have fully eliminated the pH sensitivity that we are interested in at the gate, only to have other cryptic protonation sites inhibit receptor function by different (nonphysiological) mechanisms at acidic pH values less than ~5.5.

Discussion

The most important finding of this study is the close integration of the molecular determinants of proton sensitivity with the gating machinery of NMDA receptor subunits. Our data suggest that residues at which mutations perturb proton sensitivity are localized to the regions lying between the ligand binding core and transmembrane helices involved in gating (Fig. 6), rather than broadly scattered throughout extracellular domains. We further propose that the residues to which protons bind are near the linker regions in each subunit. It seems reasonable to propose that these linker regions, which probably communicate the effects of domain closure to movement of transmembrane domains, are regions in which allosteric regulators such as protons could inhibit gating.

The only available structural information addressing the potential location of the pH sensor before this study was the finding that protons inhibit receptor function from the extracellular side of the channel (Traynelis and Cull-Candy, 1991). Mutations at a number of extracellular and pore forming residues in NR1 and NR2 had been known to modestly shift the proton IC_{50} (see references in Fig. 1 legend), but the structural relationship between these residues was unclear. The present work implicates the regions that link transmembrane domains M3 and M4 to the ligand binding core as key molecular determinants of proton sensitivity in both NR1 and NR2 subunits (Fig. 6). Furthermore, the finding that mutations in both NR1 and NR2 can alter pH sensitivity suggests that the contribution of each subunit to gating may be pH sensitive, consistent with the idea that all subunits contribute to gating. The additive effect of some mutations in NR1 and NR2 subunits (Fig. 5) also supports this idea. The contribution of both NR1 and NR2 subunits to proton sensitivity of gating also sets a ceiling on the magnitude of the pH shift caused by a mutation in any one subunit, because pH sensitivity of the other subunits may remain intact.

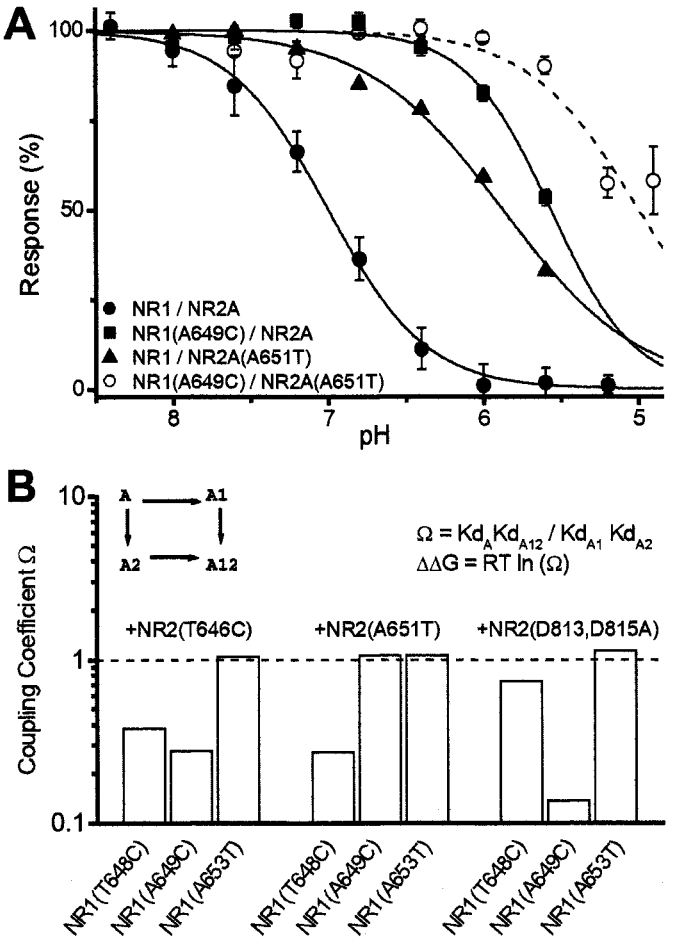

Fig. 5. Effects of coexpressed mutations in the lurcher region of NR1 and NR2A subunits are additive. A, composite proton inhibition curves for wild-type NR1/NR2A receptors, NR1(A649C)/NR2A, NR1/NR2A(A651T), and NR1(A649C)/NR2A(A651T) receptors. The proton IC_{50} values are given in Table 2. B, the coupling coefficients for pairs of NR1 and NR2 mutations were calculated using mutant thermodynamic cycles. Mutations with independent effects have a coupling coefficient of 1.0. The coupling energy ΔΔG in kilocalories per mole for mutations with negative cooperativity were −0.6 for NR1(T648C)/NR2A(T646C), −0.8 for NR1(T648C)/NR2A(A651T) and NR1(A649C)/NR2A(T646C), and −1.2 for NR1(A649C)/NR2A(D813A,D815A).

Biochemical Basis of Proton Sensitivity. There are multiple ways by which extracellular protons can influence ion channel function. The simplest working hypothesis of how extracellular protons could inhibit NMDA receptor function is that protonation of a few specific side chains creates the opportunity for the formation or dissolution of hydrogen bonds or ionic interactions. This change in structure might increase the activation energy for conformational changes or domain movements that lead to opening of the pore. If the protonation-induced increase in the activation energy is beyond that provided by binding of glutamate to NR2 or glycine to NR1, then the protonated receptor will be nonfunctional. In this scenario, the term proton sensor would describe a few amino acid residues in the extracellular domain of NMDA receptor subunits that have an ionizable side chain and inhibit gating once protonated. Obviously, a large number of ionizable residues are scattered throughout the extracellular portion of the NMDA receptor. Thus, one might expect diffuse localization of multiple proton sensors that act in concert to cause an overall inhibition of gating. In this way, gating could be sensitive to extracellular proton concentration with an IC_{50} value of pH 7.0 (100 nM), even though no single ionizable residue would necessarily have a pK_a of 7.0. To date, no ionizable residue within either NR1 or NR2 has been identified that can fully control the proton sensitivity, as does in fact occur at Kir channels (Schulte et al., 1999; Schulte and Fakler, 2000), but we cannot rule out this possibility for NMDA receptors. However, mutations at more than 100 ionizable residues scattered throughout the extracellular domains of the NR1 subunit do not change proton sensitivity. Rather, we find that the residues that most strongly influence the proton sensitivity of NMDA receptors reside in two localized clusters.

The most prominent cluster of residues that affect pH sensitivity is located within or near the lurcher region, an area suggested by a variety of studies to control gating (Kohda et al., 2000; Jones et al., 2002). Our present data show that mutations in about half of the residues within the lurcher motif can alter the proton sensitivity. Although the lurcher region does not contain residues that are conventionally considered ionizable (with the exception of NR1 Tyr647 and NR2 Tyr645), peptide linkage and some side chains in this region could be hydrogen bond acceptors with a critically placed residue at another location that is protonated with a pK_a near pH 7.0. In Ca^{2+}-activated potassium channels, gating has been proposed to occur as the inner helices corresponding to the lurcher domain either rotate or bend away from the pore (Liu et al., 2001, Jiang et al., 2002a). Recent data suggest that the lurcher region moves during gating of NMDA receptors (Jones et al., 2002). The homologous region within KcsA and MthK is adjacent to the hinge that is hypothesized to control movement of the transmembrane helix and opening of the pore. Specifically, the position within MthK corresponding to NR1 Ala640, which is two helix turns upstream from Thr648 and Ala649, has been suggested as the pivot point for the movement of the M2 helix in gating (Jiang et al., 2002a). A pivoting point near T648 and A649 is consistent with our data showing that this position can critically influence pH-sensitive gating of NMDA receptors. If the M3 transmembrane domain in NR1 is a helix that extends through the lurcher motif, then the residues at which mutations strongly influence pH sensitivity would reside on one side of this helix (Fig. 3D). It is possible that a portion of the helix acts in concert with adjacent motifs as a proton sensor and such interactions modulate the movement of M3 during gating. Additional sets of residues at which mutations alter the proton sensitivity are located within the S2 domain just beyond lurcher motif, as well as in the adjacent linkers leading from M4 to the S2 domain. These regions are well positioned to communicate binding effects to the regions that control gating, because they link the back of the agonist-binding clamshell to the transmembrane domains M3 and M4.

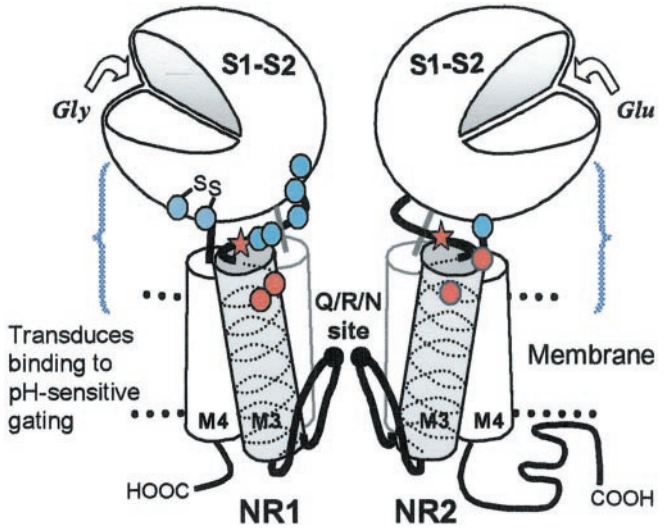

Fig. 6. Co-localization of residues that influence proton sensitivity. The region between the ligand binding core and the transmembrane domains is ideally positioned to control the interaction between the ligand binding and pH-sensitive gating (blue bracket); the amino terminal domain is not shown. Hypothetical positions of residues at which mutations alter the proton IC_{50} by more than 3-fold are blue; residues showing larger pH effects are red (NR1 Thr648, Ala649, Ala653; NR2 Thr646, Ala651, Asp815). The residues corresponding to the lurcher position are indicated as stars.

An alternative hypothesis is that multiple residues that are distant to the M3-S2 and M4-S2 linkers together coordinate the movement of gating machinery near the lurcher region in a pH-sensitive fashion. In this model, the effects of mutations in M3 that perturb proton sensitivity would reflect allosteric interaction between the gate and the proton sensor. That is, because protonation can influence gating, perturbations of gating machinery should influence the proton sensor. Consistent with this idea is the observation that mutations at N616 at the Q/R/N site of the M2 loop, which lies deep in the ion channel pore, can affect pH sensitivity (Kashiwagi et al., 1997). Although this alternative hypothesis cannot be eliminated, several features of our current data cannot easily be explained by this model. For example, mutations that shift proton sensitivity do not markedly alter the Hill slope for proton inhibition, suggesting that if multiple sites of protonation exist, the pK_a for each site must be shifted equally by a variety of different mutations in the gating region. Furthermore, all extracellular NR1 mutations that perturb proton sensitivity described to date reside near the gate control elements, except for three mutations in the amino terminal domain. Our data suggests that the amino terminal domain of

NR2A does not contain the proton sensor. Thus, there are no candidate regions outside of M3-S2 and M4-S2 linkers that might contain a distributed and diffuse proton sensor.

Conclusion

The nature of NMDA receptor gating is complex and poorly understood. However, our work has helped define the regions of the receptor that control pH sensitivity of gating. Regardless of whether the proton sensor is composed of a single ionizable residue or multiple residues, our data suggest that the molecular determinants of proton sensitivity are integrally associated with the regions of the channel that control opening of the gate. Numerous parallel properties between potassium channels and glutamate receptors have led to the generalization that these two channels share a similar molecular architecture (MacKinnon, 1995; Chen et al., 1999; Panchenko et al., 2001). Our data expand this idea by suggesting that the pH sensitivity of glutamate receptors may share structural features with proton control of potassium channel gating.

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References

Armstrong N, SunY, Chen GQ, Gouaux E (1998) Structure of a glutamate receptor ligand binding core in complex with kainate. *Nature (Lond)* **395:**913–917.

Banke TG and Traynelis SF (2003) Activation of NMDA receptors. *Nat Neurosci* **6:**144–152.

Carter PJ, Winter G, Wilkinson AJ, and Fersht AR (1984) The use of double mutants to detect structural changes in the active site of the tyrosyl-tRNA synthetase (*Bacillus stearothermophilus*). *Cell* **38:**835–840.

Chen GQ, Cui C, Mayer ML, and Gouaux E (1999) Functional characterization of a potassium-selective prokaryotic glutamate receptor. *Nature (Lond)* **16:**817–821.

Choi Y-B and Lipton SA (1999) Identification and mechanism of action of two histidine residues underlying high affinity Zn^{2+} inhibition of NMDA receptor. *Neuron* **23:**171–180.

Cui C and Mayer ML (2001) Gating mechanisms of GluR0 (Abstract). *Soc Neurosci Abstr* **27:**1269.

Dingledine R, Borges K, Bowie D, and Traynelis SF (1999) The glutamate receptor ion channels. *Pharmacol Rev* **51:**7–61.

Dirnagl U, Iadecola C, and Moskowitz MA (1999) Pathobiology of ischemic stroke: an integrated view. *Trends Neurosci* **22:**391–397.

Doyle DA, Cabral JM, Pfuetzner RA, Kuo A, Gulbis JM, Cohen SL, Chait BT, and MacKinnon R (1998) The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science (Wash DC)* **280:**69–77.

Fayyazuddin A, Villarroel A, Le Goff A, Lerma J, and Neyton J (2000) Four residues of the extracellular N-terminal domain of the NR2A subunit control high-affinity Zn^{2+} binding to NMDA receptors. *Neuron* **25:**683–694.

French A, Le P, Lyuboslavsky P, Wyatte K, Gernert KM, Neyton J, Paoletti P, Low C-M, Williams K, Traynelis SF, and Zheng F (2002) Control of NMDA receptor proton sensitivity by residues in the lurcher motif (Abstract). *Soc Neurosci Abstr* **28:**639.3.

Giffard RG, Monyer H, Christine CW, and Choi DW (1990) Acidosis reduces NMDA receptor activation, glutamate neurotoxicity and oxygen-glucose deprivation neuronal injury in cortical cultures. *Brain Res* **506:**339–342.

Jiang Y, Lee A, Chen J, Cadene M, Chait BT, and MacKinnon R (2002a) The open pore conformation of potassium channels. *Nature (Lond)* **417:**523–526.

Jiang Y, Lee A, Chen J, Cadene M, Chait BT, and MacKinnon R (2002b) Crystal structure and mechanism of a calcium-gated potassium channel. *Nature (Lond)* **417:**515–522.

Jones KS, VanDongen HMA, and VanDongen AMJ (2002) The NMDA receptor M3 segment is a conserved transduction element coupling ligand binding to channel opening *J Neurosci* **22:**2044–2053.

Kashiwagi K, Fukuchi J-I, Chao J, Igarashi K, and Williams K (1996) As aspartate residue in the extracellular loop of the *N*-methyl-D-aspartate receptor controls sensitivity to spermine and protons. *Mol Pharmacol* **49:**1131–1141.

Kashiwagi K, Masuko T, Nguyen CD, Kuno T, Tanaka I, Igarashi K, and Williams K (2002) Channel blockers acting at N-methyl-D-aspartate receptors: differential effects of mutations in the vestibule and ion channel pore. *Mol Pharmacol* **61:**533–545.

Kashiwagi K, Pahk AJ, Masuko T, Igarashi K, and Williams K (1997) Block and modulation of *N*-methyl-D-aspartate receptors by polyamines and protons: role of amino acid residues in the transmembrane and pore forming regions of NR1 and NR2 subunits. *Mol Pharmacol* **52:**701–713.

Kohda K, Wang Y, and Yuzaki M (2000) Mutation of a glutamate receptor motif reveals its role in gating and delta2 receptor channel properties. *Nat Neurosci* **3:**315–322.

Kunishima N, Shimada Y, Tsuji Y, Sato T, Yamamoto M, Kumasaka T, Nakanishi S, Jingami H, and Morikawa K (2000) Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature (Lond)* **407:**971–977.

Lee J-M, Zipfel G-J, and Choi DW (1999) The changing landscape of ischaemic brain injury mechanisms. *Nature (Lond)* **399:**A7–A14.

Levin JM, Pascarella S, Argos P, and Garnier J (1993) Quantification of secondary structure prediction improvement using multiple alignments. *Protein Eng* **6:**849–854.

Liu YS, Sompornpisut P, and Perozo E (2001) Structure of the KcsA channel intracellular gate in the open state. *Nat Struct Biol* **8:**883–887.

Low C-M, Lyuboslavsky P, Marchan E, and Traynelis SF (1999) Structural basis of zinc and proton inhibition of NMDA receptors. *Soc Neurosci Abstr* **25:**1714.

Low C-M, Zheng F, Lyuboslavsky P, and Traynelis SF (2000) Molecular determinants of coordinated proton and zinc inhibition of NMDA NR1/NR2A receptors. *Proc Natl Acad Sci USA* **97:**11062–11067.

Lyuboslavsky P, Thiel B, Paoletti P, Neyton J, Zheng F, and Traynelis SF (2001) Control of NMDA receptor proton sensitivity by extracellular residues in the linker region of S2 domain of the NR2 subunit. *Soc Neurosci Abstr* **27:**1849.

MacKinnon R (1995) Pore loops: an emerging theme in ion channel structure. *Neuron* **14:**889–892.

Marti-Renom MA, Stuart AC, Fiser A, Sánchez R, Melo F, and Sali A (2000) Comparative protein structure modeling of genes and genomes. *Annu Rev Biophys Biomol Struct* **29:**291–325.

Masuko T, Kashiwagi K, Kuno T, Nguyen ND, Pahk AJ, Fukuchi J-I, Igarashi K, Williams K (1999) A regulatory domain (R1–R2) in the amino terminus of the *N*-methyl-D-aspartate receptor: effects of spermine, protons, and ifenprodil and structural similarity to bacterial leucine/isoleucine/valine binding protein. *Mol Pharmacol* **55:**957–969.

Meldrum BS, Akbar MT, and Chapman AG (1999) Glutamate receptors and transporters in genetic and acquired models of epilepsy. *Epilepsy Res* **36:**189–204.

Mott DD, Doherty JJ, Zhang S, Washburn MS, Fendley MJ, Lyuboslavsky P, Traynelis SF, and Dingledine R (1998) Phenylethanolamines inhibit NMDA receptors by enhancement of proton inhibition. *Nat Neurosci* **1:**659–667.

Obrenovitch TP and Urenjak J (1997) Is high extracellular glutamate the key to excitotoxicity in traumatic brain injury. *J Neurotrauma* **14:**677–698.

Pahk AJ and Williams K (1997) Influence of extracellular pH on inhibition by ifenprodil at *N*-methyl-D-aspartate receptors in *Xenopus* oocytes. *Neurosci Lett* **225:**29–32.

Panchenko VA, Glasser CR, and Mayer ML (2001) Structural similarities between glutamate receptor channels and K^+ channels examined by scanning mutagenesis. *J Gen Physiol* **117:**345–360.

Paoletti P, Ascher P, and Neyton J (1997) High affinity zinc inhibition of NMDA NR1-NR2A receptors [published erratum appears in *J Neurosci* **17**(20) 1997]. *J Neurosci* **17:**5711–5725.

Paoletti P, Perin-Dureau F, Fayyazuddin A, Le Goff A, Callebaut I, and Neyton J (2000) Molecular organization of a zinc binding N-terminal modulatory domain in a NMDA receptor subunit. *Neuron* **28:**911–925.

Rost B and Sander C (1993a) Improved prediction of protein secondary structure by use of sequence profiles and neural networks. *Proc Natl Acad Sci USA* **90:**7558–7562.

Rost B and Sander C (1993b) Prediction of protein secondary structure at better than 70% accuracy. *J Mol Biol* **232:**584–599.

Rost B and Sander C (1994) Combining evolutionary information and neural networks to predict protein secondary structure. *Proteins* **19:**55–72.

Sali A and Blundell TL (1993) Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol* **234:**779–815.

Schulte U and Fakler B (2000) Gating of inward-rectifier K^+ channels by intracellular pH. *Eur J Biochem* **267:**5837–5841.

Schulte U, Hahn H, Konrad M, Jeck N, Derst C, Wild K, Weidemann S, Ruppersberg JP, Fakler B, and Ludwig J (1999) pH gating of ROMK (Kir1.1) channels: control by an Arg-Lys-Arg triad disrupted in antenatal Bartter syndrome. *Proc Natl Acad Sci USA* **96:**15298–15303.

Sullivan JM, Traynelis SF, Chen H-SV, Escobar W, Heinemann SF, and Lipton SA (1994) Identification of two cysteine residues on the NR1 subunit that are required for redox modulation. *Neuron* **13:**929–936.

Tang CM, Dichter M, and Morad M (1990) Modulation of N-methyl-D-aspartate channel by extracellular H^+. *Proc Natl Acad Sci USA* **87:**6445–6449.

Taverna F, Xiong Z-G, Brandes L, Roder JC, Salter MW, and MacDonald JF (2000) The lurcher mutation of an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subunit enhances potency of glutamate and converts an antagonist to an agonist. *J Biol Chem* **275:**8475–8479.

Tombaugh GC and Sapolsky RM (1993) Evolving concepts about the role of acidosis in ischemic neuropathology. *J Neurochem* **61:**793–803.

Traynelis SF, Burgess MF, Zheng F, Lyuboslavsky P, and Powers JL (1998) Control of voltage-independent zinc inhibition of NMDA receptors by the NR1 subunit. *J Neurosci* **18:**6163–6175.

Traynelis SF and Cull-Candy SG (1990) Proton inhibition of *N*-methyl-D-aspartate receptors in cerebellar neurons. *Nature (Lond)* **345:**347–350.

Traynelis SF and Cull-Candy SG (1991) Pharmacological properties and H^+ sensitivity of excitatory amino acid receptor channels in rat cerebellar granule neurones. *J Physiol* **433:**727–763.

Traynelis SF, Hartley M, and Heinemann SF (1995) Control of proton sensitivity of the NMDA receptor by RNA splicing and polyamines. *Science (Wash DC)* **268:**873–876.

Williams K, Kashiwagi K, Fukuchi J-I, and Igarashi K (1995) An acidic amino acid in the *N*-methyl-D-aspartate receptor that is important for spermine stimulation. *Mol Pharmacol* **48:**1087–1098.

Yang Z, Xu H, Cui N, Qu Z, Chanchevalap S, Shen W, and Jiang C (2000) Biophysical and molecular mechanisms underlying the modulation of heteromeric Kir4.1-Kir5.1 channels by CO_2 and pH. *J Gen Physiol* **116:**33–45.

Zheng F, Erreger K, Low C-M, Banke T, Lee CJ, Conn PJ, and Traynelis SF (2001) Allosteric interaction between the amino terminal domain and the ligand binding domain of NR2A. *Nat Neurosci* **4:**894–901.

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