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Principal role of NR3 subunits in NR1/NR3 excitatory glycine receptor function

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Abstract

Calcium-permeable *N*-methyl-D-aspartate (NMDA) receptors are tetrameric cation channels composed of glycine-binding NR1 and glutamate-binding NR2 subunits, which require binding of both glutamate and glycine for efficient channel gating. In contrast, receptors assembled from NR1 and NR3 subunits function as calcium-impermeable excitatory glycine receptors that respond to agonist application only with low efficacy. Here, we show that antagonists of and substitutions within the glycine-binding site of NR1 potentiate NR1/NR3 receptor function up to 25-fold, but inhibition or mutation of the NR3 glycine binding site reduces or abolishes receptor activation. Thus, glycine bound to the NR1 subunit causes auto-inhibition of NR1/NR3 receptors whereas glycine binding to the NR3 subunits is required for opening of the ion channel. Our results establish differential roles of the high-affinity NR3 and low-affinity NR1 glycine-binding sites in excitatory glycine receptor function.

Keywords: NMDA receptor; Glutamate receptor; Agonist binding; Ligand-gated ion channel; Glycine binding site

The *N*-methyl-D-aspartate (NMDA) subtypes of ionotropic glutamate receptors (iGluRs) are calcium-permeable ligand-gated cation channels which mediate excitatory neurotransmission in the central nervous system [1]. "Conventional" NMDA receptors are composed of two homologous NR1 and NR2 subunits, each, and require both glutamate and glycine for efficient channel activation [2]. Their glycine- and glutamate-binding sites are formed by homologous segments of the NR1 and NR2 subunits, respectively [3,4]. Mutational analysis and crystal structures of the ligand-binding domains (LBDs) from both subunits have shown that agonist binding occurs in a cleft at the interface between two extracellular lobes (segments S1, S2) [4,5] and results in LBD closure and opening of the ion channel.

In addition to the NR1 and NR2A–D subunits (overview in [6]), two other NMDA receptor polypeptides have been identified, NR3A and NR3B [7,8]. Upon co-expression with NR1, the NR3 subunits form receptors that are activated by glycine alone, and hence can be classified as excitatory glycine receptors [9]. NR1/NR3 receptors are calcium-impermeable and unaffected by ligands acting at the NR2 glutamate-binding site. Consistent with these findings, the isolated LBD of the NR3A subunit has recently been shown to bind glycine with high-affinity [10]. Further functional characterization of NR1/NR3 receptors has, however, been hampered by the small amplitude of their glycine-elicited currents. Here, we show that glycine binding to the NR1 subunits causes rapid inactivation of NR1/NR3 receptors. In contrast, glycine binding to the NR3 subunit is essential for channel activation. Our results disclose differential roles of the NR1 and

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NR3 glycine-binding sites in excitatory glycine receptor function.

Materials and methods

DNA constructs, oocyte expression and electrophysiology. cDNAs of the NR1a, NR2A [3], and NR3A [7] subunits were subcloned in the pNKS2 vector. The NR3B clone [9] was kindly provided by D. Zhang, Burnham Institute, La Jolla (CA), USA. The NR2A*-His construct was generated by replacing the C-terminal region from amino acid 930 with a 6× His tag. Mutations were generated by site-directed mutagenesis (QuikChange XL Site-Directed Mutagenesis Kit, Stratagene) and confirmed by DNA sequencing. *In vitro* synthesis of cRNA (mCAP mRNA Capping Kit, Ambion) was performed as described [3]. For the expression of NMDARs, 25 ng cRNA/oocyte was injected at a NR1:NR3 (NR2A) ratio of 1:3. *Xenopus* oocytes were isolated and maintained as described [3]. Two-electrode voltage-clamp recording of whole-cell currents was performed according to [4].

Metabolic labelling and purification of NMDA receptor complexes. Injected oocytes were metabolically labelled by overnight incubation with [^{35}S]methionine as described [11]. After an additional 24 h chase interval, the resulting receptor complexes were purified by Ni^{2+}–NTA chromatography from 0.5% (w/v) dodecylmaltoside extracts of the labelled occytes as described [11].

Surface protein labelling using fluorescent dyes. After 4 days in culture, oocytes expressing NR1 and NR3A-His subunits were incubated for 20 min at 4 °C in 65 nmol/oocyte of the Cy-NHS-ester dye Cy5 (Amersham Biosciences) in phosphate buffer (20 mM Na_2HPO_4/NaH_2PO_4 pH 8.5; 110 mM NaCl; and 1 mM $MgCl_2$). After washing, NMDA receptor complexes were purified as outlined above.

SDS–PAGE. Proteins were supplemented with SDS sample buffer containing 20 mM dithiotreitol (DTT) and electrophoresed in parallel with molecular mass markers (Precision Plus Protein All Blue Standard, Bio-Rad) on 10% tricine-SDS–polyacrylamide gels. Gels were fixed, dried, and exposed to BioMax MR films (Kodak, Stuttgart, Germany) at −80 °C. Gels containing Cy5-labelled protein samples were scanned with a gel imager (*Typhoon 9400*, Amersham).

Glycosylation analysis. For analysis of the glycosylation status, 5–10 μl aliquots of affinity-purified receptor protein were supplemented with reducing (20 mM DTT) SDS sample buffer and 1% (w/v) octylglucoside (Calbiochem, La Jolla, CA, USA), and incubated for 2 h at 37 °C with 5 IUB milliunits Endo H or PNGase F (NEB, Frankfurt, Germany).

Results

Assembly and plasma membrane insertion of recombinant NR1/NR3A receptors

NR3 subunits form excitatory glycine receptors upon co-expression with the NR1 subunit [9]. However, these receptors generate only small currents upon agonist application. In order to disclose whether this might reflect inefficient assembly or surface expression, we compared the subunit composition and agonist responses of recombinant NR1/NR3A receptors with those of "conventional" NR1/NR2A NMDA receptors. To this end, hexahistidyl-tagged NR3A (NR3-His) and NR2A* (NR2A*-His; see Materials and methods) subunits were co-expressed with the wild-type (wt) NR1 subunit in *Xenopus* oocytes. After metabolic labelling with [^{35}S]methionine, the recombinant receptors were affinity-purified and analyzed by SDS–PAGE and autoradiography. This revealed comparable amounts of ^{35}S-labelled NR1/NR3A-His and NR1/NR2A*-His receptor subunits (Fig. 1A). Hence, NR1/NR3A receptors are efficiently synthesized and assembled in the oocyte expression system. However, the maximal inducible currents generated upon superfusion of NR1/NR3A-His and NR1/NR2A*-His expressing oocytes with saturating agonist concentrations differed by a factor of about 25 (0.15 μA vs. >5 μA, Fig. 1B).

To exclude that the small glycine evoked currents of NR1/NR3A receptors are due to an impaired plasma membrane insertion, we performed affinity purification of NR1/NR3A-His receptors from oocytes surface-labelled with a Cy5-NHS ester. SDS–PAGE revealed that the assembled receptor was present at the cell surface (Fig. 1C) at similar quantities than obtained upon co-expression of the NR1/NR2A*-His subunits (data not shown). In contrast, singly expressed NR1-His subunits were not surface-labelled under identical conditions. Furthermore, the apparent molecular masses of the Cy5-labelled subunits were much less reduced upon Endo H as compared to PNGaseF treatment (Fig. 1C), indicating complex *N*-glycosylated proteins located at the cell surface. Thus, the small glycine currents of NR1/NR3A receptors cannot be attributed to intracellular retention.

Comparison of NR1/NR3A and NR1/NR3B receptor currents

Similar to our findings obtained with the NR1/NR3A receptors, oocytes co-expressing the NR1 and NR3B subunits displayed small and rapidly desensitizing maximal glycine-inducible currents (Fig. 1D and E and Table 1) of similar apparent agonist affinity (Fig. 1F and Table 1). However, in contrast to NR1/NR3A receptor responses NR1/NR3B-mediated currents showed a marked decline of the peak current at elevated glycine concentrations (>20 μM) and a pronounced rebound effect upon glycine wash-out (Fig. 1E and F).

Differential effects of NR1 and NR3 glycine site antagonists

To disclose the contributions of the NR1 and NR3 LBDs to receptor activation, we examined the effects of antagonists on glycine-induced currents. Application of 5 μM CNQX, an AMPA receptor antagonist that also binds to the glycine sites of the NR3 and, with lower affinity, NR1 subunits [10], caused an about 80–90% inhibition of the currents evoked by 20 μM glycine at both NR1/NR3A and NR1/NR3B receptors (Fig. 2A and E). This is in agreement with the K_d value of CNQX determined with a soluble NR3A-S1S2 LBD construct (2.51 ± 0.35 μM; [10]). In contrast, the NR1-specific glycine site antagonists MDL-29951 (MDL) and L-689560 (L689) did not inhibit but strongly potentiated glycine-evoked currents of NR1/NR3A receptors (Fig. 2B and F). At saturating glycine concentrations (1 mM), currents were potentiated about 25-fold in the presence of 200 nM MDL (Fig. 2B and Table 1). At 500 μM glycine, half-max-

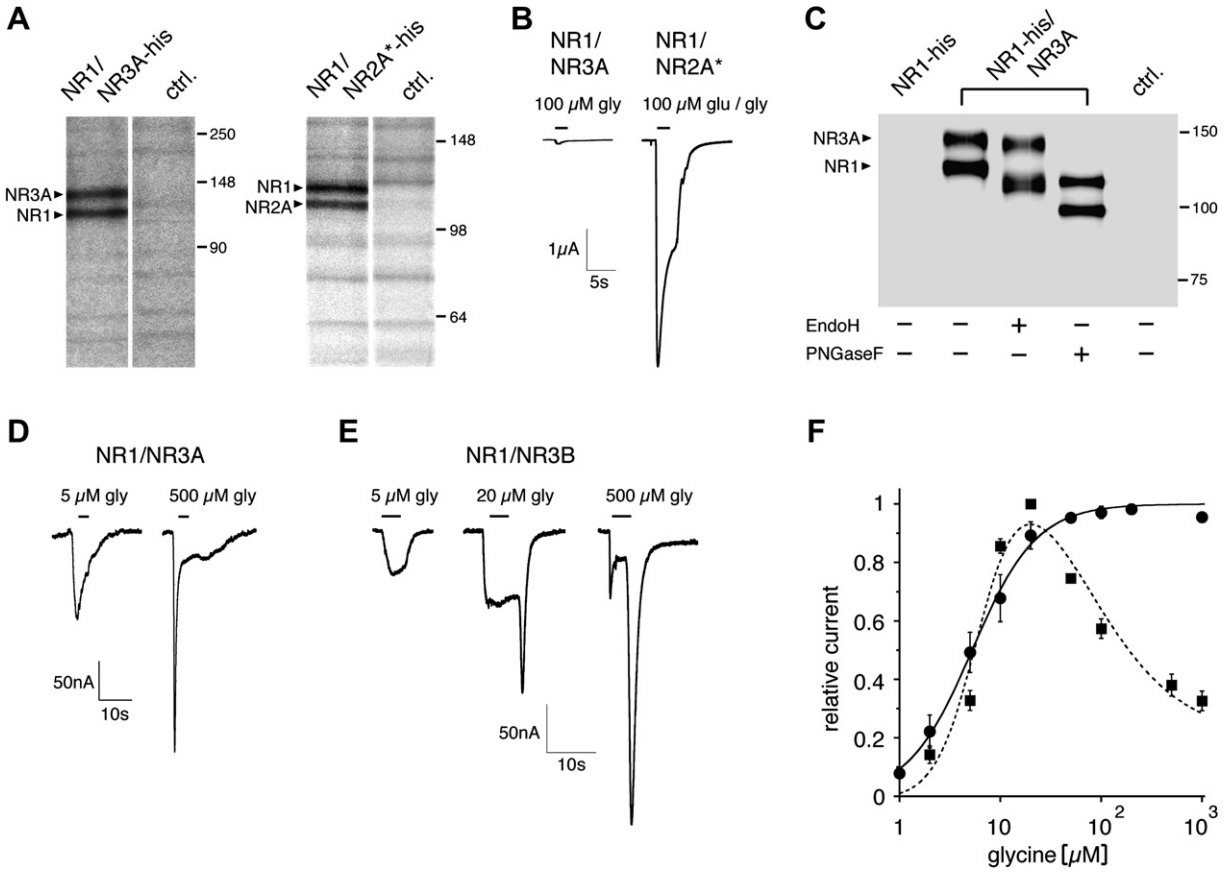

Fig. 1. Heterologous expression and functional characterization of recombinant NR1/NR3 receptors. (A) SDS–PAGE of metabolically labelled and affinity-purified receptors from oocytes co-expressing wt NR1 with His-tagged NR3A (left) and NR2A* (right) subunits, respectively. Note comparable protein levels of all subunits. (B) NR1/NR3A (left) and NR1/NR2A*-His (right) expressing oocytes displayed significant differences in maximal inducible glycine currents. (C) SDS–PAGE of NR1-His/NR3A receptors affinity purified after Cy5 surface labelling as compared to NR1-His expressed alone. Endo H and PNGase F treatment is indicated below respective lanes (ctrl., non-injected oocyte). (D,E) Comparison of glycine-evoked peak currents of NR1/NR3A (D) and NR1/NR3B (E) receptors. NR1/NR3A receptors desensitized more readily at high glycine concentrations (500 μM) than NR1/NR3B receptors; the latter exhibited a pronounced rebound effect upon agonist washout and a decrease of glycine currents at elevated agonist concentrations (>20 μM). (F) Dose–response curves of NR1/NR3A (filled circles) and NR1/NR3B (filled squares) receptors. Error bars in F indicate SE.

Table 1
Pharmacology of wt and mutant NR1/NR3 receptors

Subunit composition	EC_{50} (μM)	I_{max} (μA)	*n*
NR1/NR3A	6.5 ± 1.1	0.2 ± 0.06	7
NR1/NR3A (MDL)	1444 ± 200	5.1 ± 1.4	6
$NR1^{F466A}$/NR3A	82 ± 11	2.14 ± 1.0	4
NR1/NR3B	6 ± 0.3	0.064 ± 0.016	10
NR1/NR3B (MDL)	413 ± 18	0.34 ± 0.05	6
$NR1^{F466A}$/NR3B	148 ± 24	2.97 ± 0.7	7

Glycine EC_{50} values and maximal inducible currents (I_{max}) were determined in the presence and absence of 200 nM MDL-29951 3–5 days after injection of 25 ng cRNA of the indicated NR1 and NR3 subunits. Values represent means ± SE; *n*, number of experiments.

imal potentiation by MDL was seen at 21 ± 1.7 nM (Fig. 2C), a value close to the K_d value of MDL determined at the soluble NR1-S1S2 LBD [12]. NR1/NR3B receptors were also potentiated by MDL, although to a lesser extent (5-fold) (Fig. 2F and Table 1). Notably, in the presence of 200 nM MDL the apparent EC_{50} values of glycine at NR1/NR3A and NR1/NR3B receptors were significantly increased (Fig. 2D and G, solid lines, and Table 1). MDL also reduced the rebound effect seen upon agonist washout, indicating that the latter is mediated by NR1. Similar potentiations were also observed with L689 at both NR1/NR3A and NR1/NR3B receptors (data not shown). In summary, antagonists acting at the glycine binding sites of the NR1 and NR3 subunits had opposite effects on channel activity.

Mutations within NR1 and NR3 LBDs mimick the effects of glycine site antagonists

To further corroborate different roles of the NR1 and NR3 glycine binding sites in NR1/NR3 receptor function, mutations were introduced into the respective LBDs. We have previously shown that the mutant $NR1^{F466A}$ causes a ca. 10,000-fold reduction in the apparent glycine affinity of NR1/NR2B receptors [3]. Co-expression of $NR1^{F466A}$ with the wt NR3A or NR3B subunits resulted in large gly-

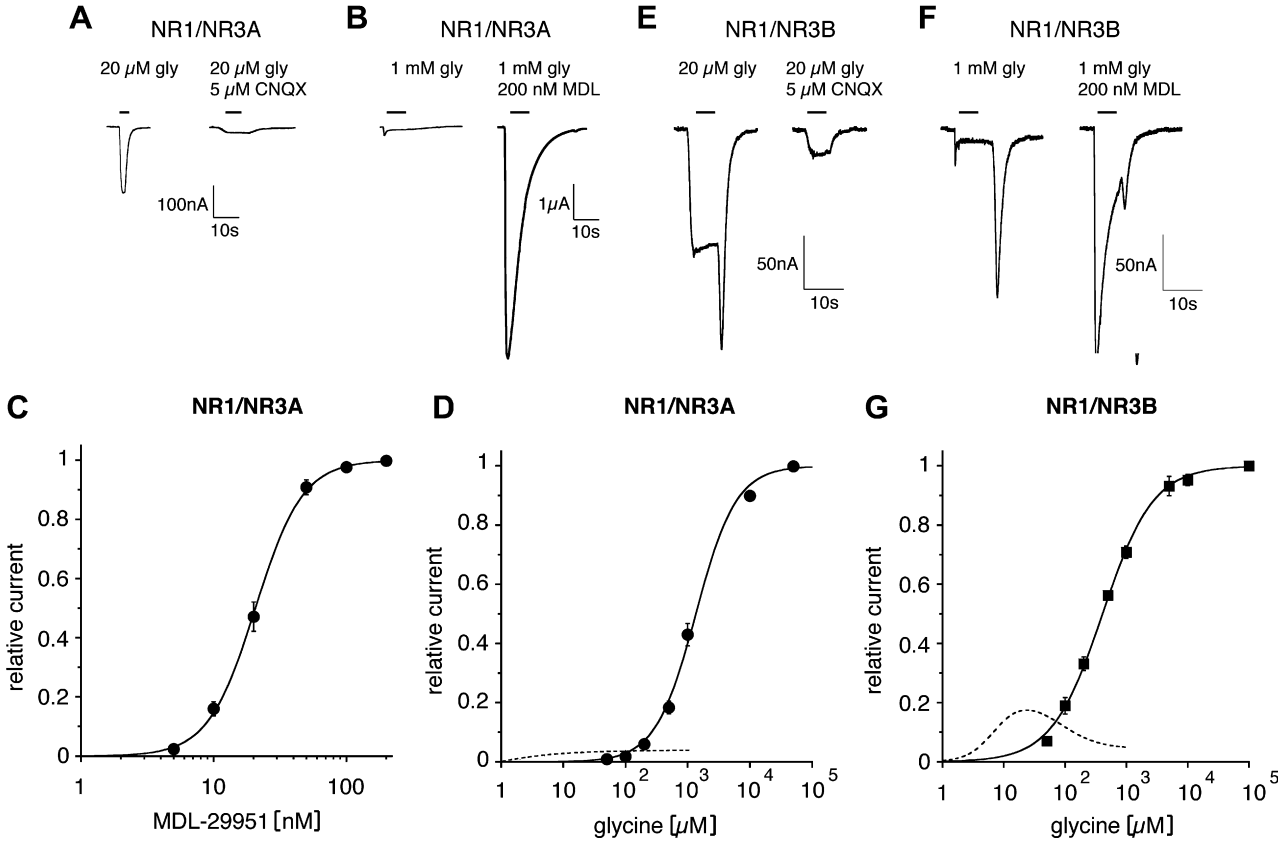

Fig. 2. Effects of glycine site antagonists on NR1/NR3 receptor currents. Glycine-induced currents of NR1/NR3A receptors were (A) inhibited by about 90% in the presence of CNQX, and (B) potentiated about 25-fold by MDL. (C) Dose-dependence of MDL potentiation of NR1/NR3A receptors in the presence of 500 μM glycine (EC_{50}: 21 ± 1.7 nM, $n = 8$). (D) Glycine dose–response curve in the presence of 200 nM MDL (solid line; see Table 1). Data obtained without MDL (dotted line) are plotted as a relative ratio compared to those with MDL. (E) Glycine currents of NR1/NR3B receptors were inhibited by about 80% by 5 μM CNQX, and (F) increased about 6-fold upon adding MDL. Note large rebound currents after glycine washout in the absence of antagonists (E, F). (G) Glycine dose–response curve of NR1/NR3B receptors in the presence (solid line) and absence (dotted line) of 0.2 μM MDL. Error bars in C, D, G indicate SE.

cine currents resembling those seen with NR1/NR3 receptors in the presence of MDL (Fig. 3A and Table 1). However, the shift in apparent glycine affinities was less pronounced than compared to wt receptors in the presence of MDL (Fig. 3C and Table 1). Similar results were also obtained with the mutants $NR1^{Q387A}$ and $NR1^{K465E}$ (data not shown). Thus, both antagonizing and mutating the NR1 glycine-binding site results in similar potentiation of receptor currents.

Further analysis revealed that, in contrast to wt NR1/NR3 channels, the $NR1^{F466A}$/NR3A and $NR1^{F466A}$/NR3B receptors were not potentiated by 200 nM MDL. Increasing the MDL concentration to 5 μM led to an inhibition of relative glycine-induced currents at both mutant receptors (Fig. 3B). Analogous effects were also obtained with L689 under identical conditions (data not shown). Thus, elevated concentrations of MDL and L689 also antagonize glycine binding to the NR3 subunit, which might explain the pronounced decrease in apparent glycine affinity seen with wt NR1/NR3 receptors upon MDL addition (Fig. 2D and G). Furthermore, negative cooperative interactions of the antagonist-occupied NR1 subunit with NR3 might contribute to the variability of glycine EC_{50} values observed here.

To analyze the role of the NR3 subunit in channel activation, we mutated the NR3A-binding site residues R638, V797 and D844, which are homologous to positions of the NR1 subunit known to be crucial for glycine binding [3,13]. Co-expression of $NR3A^{V797L}$ with NR1 generated small responses to 1 mM glycine that were modestly potentiated by MDL, whereas the $NR3A^{R638K}$ and $NR3A^{D844E}$ mutants failed to produce detectable glycine currents even in the presence of MDL or L689 (Fig. 3D). To investigate whether the non-functional NR3 subunits co-assemble with NR1, we affinity-purified wt and mutant NR1-His/$NR3A^{R638K}$ and NR1-His/$NR3A^{D844E}$ receptors from [^{35}S]methionine labelled oocytes. SDS–PAGE revealed that both mutant subunits were present in the purified receptor complexes at intensities comparable to those found in wt NR1/NR3A receptors (Fig. 3E). Hence, the loss of channel activity seen upon NR3A LBD substitutions is due to impaired glycine binding rather than altered subunit expression or assembly.

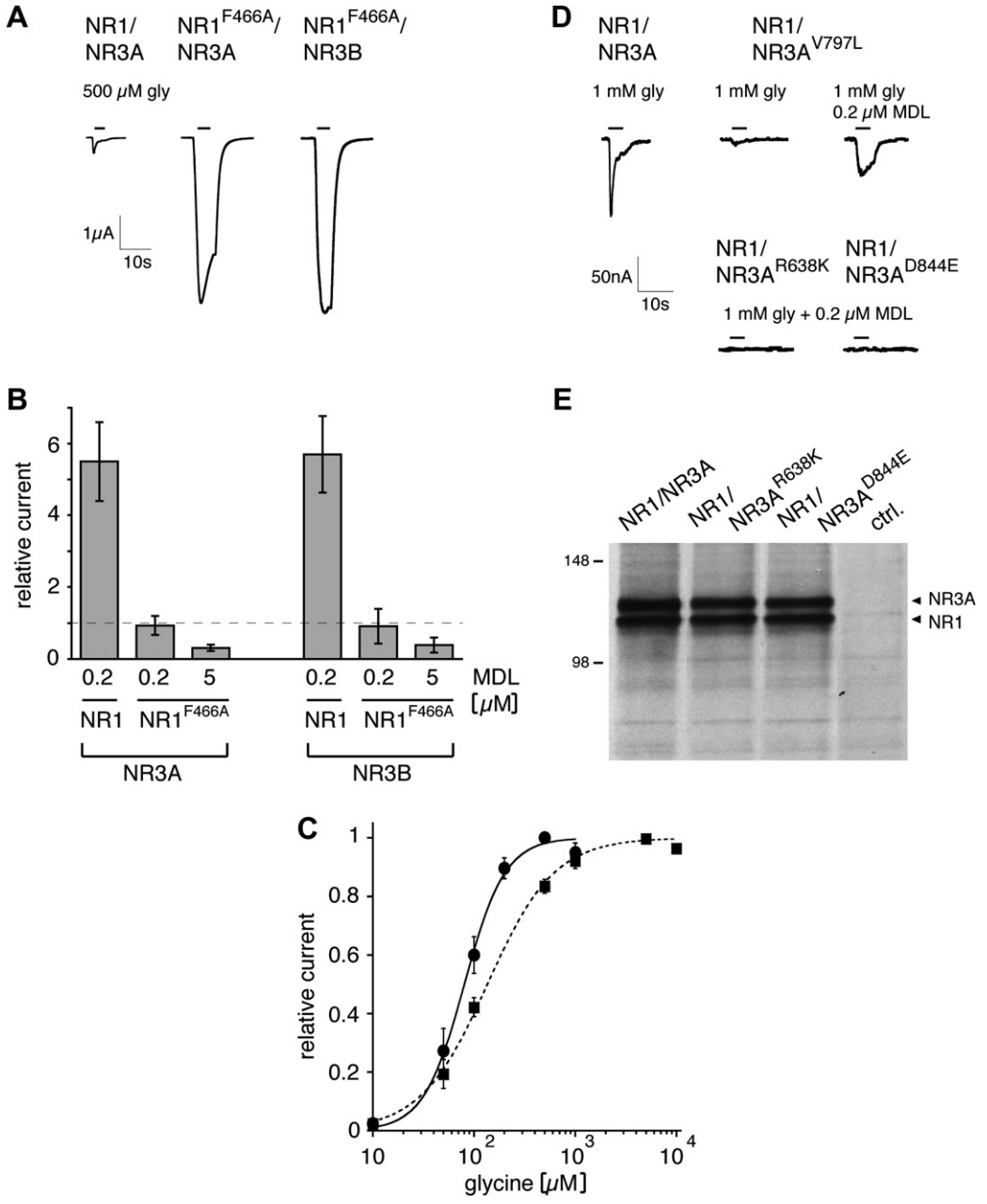

Fig. 3. Effects of NR1 and NR3 binding site mutations. (A) Mutant NR1^{F466A} increases maximal inducible glycine currents of NR1^{F466A}/NR3A and NR1^{F466A}/NR3B receptors. (B) Effects of MDL on currents elicited by 500 μM glycine. In contrast to NR1/NR3A and NR1/NR3B receptors, NR1^{F466A}/NR3A and NR1^{F466A}/NR3B receptors were not potentiated by 0.2 μM MDL ($I_{rel.}$ 5.5 ± 1.1 and 5.7 ± 1.1 vs. 0.93 ± 0.26 and 0.91 ± 0.48; $n = 4$); 5 μM MDL even inhibited both mutant channels ($I_{rel.}$ 0.31 ± 0.09 and 0.39 ± 0.21; $n = 4$). Dashed line represents normalized current values in the absence of MDL. (C) Glycine dose–response curves of NR1^{F466A}/NR3A (filled circles) and NR1^{F466A}/NR3B receptors (filled squares). (D) Mutations in the NR3A ligand binding site impair NR1/NR3A receptor function. In contrast to wt NR1/NR3A receptors, NR1/NR3A$^{V797\ L}$ receptors were only marginally potentiated by MDL. NR1/NR3A^{R638K} and NR1/NR3A^{D844E} receptors were non-functional, although (E) similar amounts of receptor polypeptides were purified from ^{35}S-labelled oocytes, as revealed by SDS–PAGE. Error bars in B and C indicate SE.

Discussion

In this study, we present strong evidence for differential roles of the NR1 and NR3 subunits in excitatory glycine receptor function. Antagonists of or mutations within the glycine-binding site of the NR1 subunit produced large NR1/NR3 receptor currents, presumably by suppressing receptor desensitization. In contrast, antagonizing or mutating the NR3-binding site resulted in a severe reduction or even complete loss of glycine responses. This is consistent with a pivotal role of the NR3 subunit in NR1/NR3 receptor activation.

Binding studies with soluble LBDs have shown that NR3 and NR1 subunits bind glycine with high and low affinity, respectively [10,12]. Based on the different glycine affinities, we propose that sequential occupation of the

NR3 and NR1 sites results in two separate effects on receptor function: first, activation of the ion channel by glycine binding to the high-affinity NR3 sites, and second, inhibition through glycine binding to the low-affinity NR1 sites. Hence, exposure to saturating glycine concentrations leads to transient, rapidly desensitizing receptor responses. Differential kinetics of glycine binding/unbinding to the NR3 and NR1 subunit would also explain the pronounced rebound effect seen after washout of glycine ([9]; this study), which likely reflects a slower rate of glycine dissociation from the NR3 LBD. Our concept of differential contributions of the two glycine-binding sites to distinct receptor conformational states (Fig. 4) is compatible with accepted models of ligand binding and channel activation in the iGluR family [14]. Accordingly, glycine binding to the NR3 subunits is thought to generate sufficient conformational strain within their LBDs to open the ion channel (Fig. 4A). Subsequent binding of glycine to the low-affinity NR1 sites would weaken interdomain interactions between the NR1 and NR3 LBDs (Fig. 4A), thereby allowing for rapid receptor desensitization [15]. This assumption is supported by our observation that the small and rapidly desensitizing glycine currents of NR1/NR3 receptors are potentiated and converted into barely desensitizing steady-state currents by antagonists of or mutations within the NR1 glycine-binding site (Fig. 4B and C). In contrast, NR3 antagonists and mutations counteract receptor activation by interfering with NR3 LBD closure, which is essential for inducing channel opening (Fig. 4D).

Together with the model of NR1/NR3 receptor activation proposed above, our data suggest a unique role of glycine at NR3 subunit containing receptors. Excitatory glycine receptors matching the NR1/NR3 receptor pharmacology described here have so far not been found *in vivo*. This could be due to a pronounced desensitization of NR1/NR3 receptors by free glycine. In addition, the widespread use of the non-NMDA receptor antagonist CNQX might mask excitatory glycine currents. Although the existence of native NR1/NR3 receptors needs to be confirmed, incorporation of NR3 subunits into NR1/NR2 di-heteromeric receptors has been shown to result in a decrease in single channel conductance [16]. Based on the recently established NR1/NR2 dimer-of-dimer arrangement in NMDA receptors [17], we attribute the reduced agonist response of tri-heteromeric NR1/NR2/NR3 NMDA receptors to glycine-dependent silencing mediated by the NR1/NR3 dimer. We speculate that the opposite effects of glycine on the current responses of NR1/NR2 and NR1/NR3

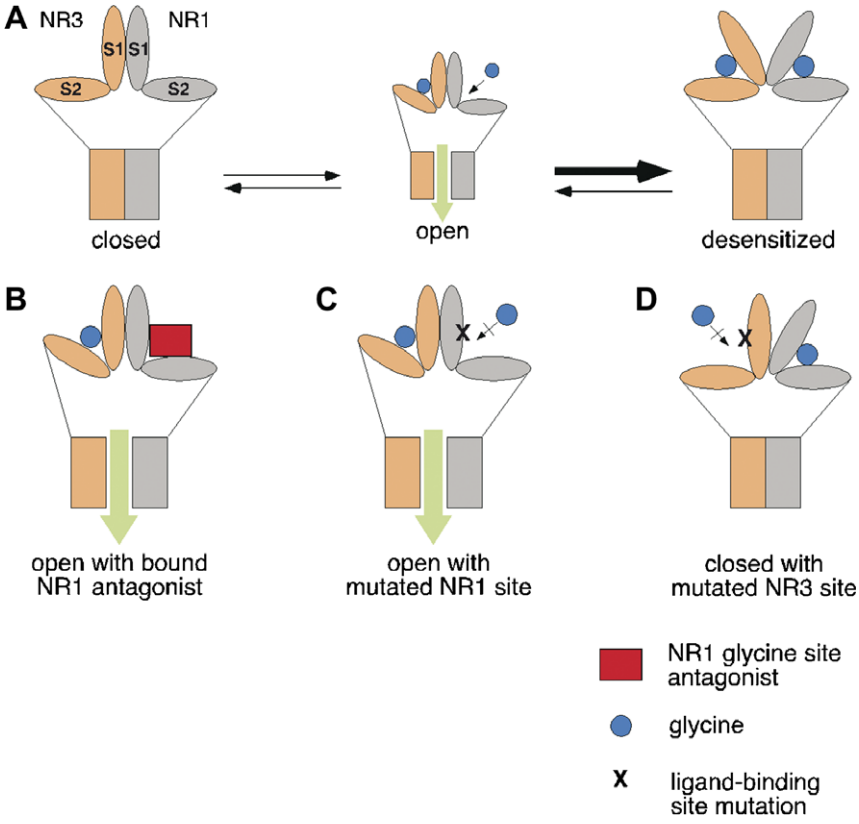

Fig. 4. Schematic model of NR1/NR3 receptor states. (A) Reaction scheme of NR1/NR3 receptor gating depicting the hetero-dimeric arrangement of the LBDs of the NR1 (grey) and NR3 (orange) subunits. Sequential occupation of the high- and low-affinity glycine sites of the NR3 and NR1 subunits results in channel opening (middle) followed by desensitization (right). (B–D) Differential effects of antagonists of and mutations within the NR1 and NR3 LBDs on channel function. (B) Binding of glycine to the NR3 subunit in the presence of an NR1 antagonist results in non-desensitizing channel opening. (C) Mutations affecting glycine binding to the NR1 LBD similarly lead to channel activation without desensitization. (D) In contrast, mutation of (or antagonist binding to, not shown) the LBD of NR3 results in an inactive or desensitized receptor. For clarity, only two subunits of the receptor are shown. (For color coding described in this figure legend, the reader is referred to the web version of this article.)

receptors reflect differences in the strength of LBD interactions between NR1/NR2 and NR1/NR3 dimers. Depending on the side-chains located at the interface formed by these LBD dimers, glycine binding would result in potentiation at conventional NR1/NR2 receptors, whereas at NR1/NR3 receptors it would cause rapid receptor deactivation. The different consequences of glycine binding to NR1/NR2 and NR1/NR3 dimers might reflect evolutionary adaptation of channel gating to particular physiological conditions.

Acknowledgments

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