



Potentiation of glycine-gated NR1/NR3A NMDA receptors relieves Ca²⁺-dependent outward rectification

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Glycine has diverse functions within the mammalian central nervous system. It inhibits postsynaptic neurons via strychnine-sensitive glycine receptors (GlyRs) and enhances neuronal excitation through co-activation of N-methyl-D-aspartate (NMDA) receptors. Classical Ca²⁺-permeable NMDA receptors are composed of glycine-binding NR1 and glutamate-binding NR2 subunits, and hence require both glutamate and glycine for efficient activation. In contrast, recombinant receptors composed of NR1 and the glycine binding NR3A and/or NR3B subunits lack glutamate binding sites and can be activated by glycine alone. Therefore these receptors are also named “excitatory glycine receptors.” Co-application of antagonists of the NR1 glycine-binding site or of the divalent cation Zn²⁺ markedly enhances the glycine responses of these receptors. To gain further insight into the properties of these glycine-gated NMDA receptors, we investigated their current-voltage (I–V) dependence. Whole-cell current-voltage relations of glycine currents recorded from NR1/NR3B and NR1/NR3A/NR3B expressing oocytes were found to be linear under our recording conditions. In contrast, NR1/NR3A receptors displayed a strong outwardly rectifying I–V relation. Interestingly, the voltage-dependent inward current block was abolished in the presence of NR1 antagonists, Zn²⁺ or a combination of both. Further analysis revealed that Ca²⁺ (1.8 mM) present in our recording solutions was responsible for the voltage-dependent inhibition of ion flux through NR1/NR3A receptors. Since physiological concentrations of the divalent cation Mg²⁺ did not affect the I–V dependence, our data suggest that relief of the voltage-dependent Ca²⁺ block of NR1/NR3A receptors by Zn²⁺ may be important for the regulation of excitatory glycinergic transmission, according to the Mg²⁺-block of conventional NR1/NR2 NMDA receptors.

Keywords: NMDA receptor, excitatory glycine receptor, voltage block, NR3 subunit, supralinear potentiation, Zn²⁺, NR1 antagonist, ligand-binding domain

INTRODUCTION

The simplest of all amino acids, glycine, has diverse functions within the mammalian central nervous system (CNS). Glycine mediates synaptic inhibition through hyperpolarizing glycine receptors (GlyRs) and contributes to neuronal excitation by acting as a co-agonist at glutamate-type N-methyl-D-aspartate (NMDA) receptors (see overview in Dingledine et al., 1999; Betz and Laube, 2006). The GlyR is a pentameric protein composed of two types (α and β) of membrane-spanning subunits (Grudzinska et al., 2005). In contrast, the NMDA receptor is a hetero-tetrameric membrane protein (Laube et al., 1998) composed of glycine-binding NR1 and glutamate-binding NR2 subunits (Laube et al., 1997; overview in Cull-Candy et al., 2001) and/or the recently discovered glycine-binding NR3A and NR3B subunits (Sucher et al., 1995; Nishi et al., 2001; Yao and Mayer, 2006). The ligand-binding domains (LBDs) of the NMDA receptor subunits are formed by two extracellular segments S1 and S2 and are thought to be arranged in a dimer-of-heterodimer orientation (Furukawa et al., 2005; Schüler et al., 2008). Binding of the agonist occurs between the two extracellular segments and results in a closure of the LBD and subsequent opening of the ion channel (Armstrong and Gouaux, 2000; overview in Mayer, 2006).

Conventional NMDA receptors composed of two NR1 and two NR2 subunits require the simultaneous binding of glutamate and glycine for efficient channel opening (Johnson and Ascher, 1987; Kuryatov et al., 1994). In contrast, NMDA receptors composed of NR1 and NR3 subunits result in nonselective cation-channels exclusively activated by glycine, termed “excitatory glycine receptors”, which are unaffected by glutamate (Chatterton et al., 2002). The physiological role of NR1/NR3 NMDA receptors is however still discussed controversially (Chatterton et al., 2002; Tong et al., 2008). Recombinantly expressed NR1/NR3A and NR1/NR3B receptors generate only small excitatory currents upon activation by glycine (Awobuluyi et al., 2007; Madry et al., 2007a; Smothers and Woodward, 2009). This low functionality cannot be attributed to impaired receptor assembly or decreased surface insertion but derives from opposite roles of glycine which acts agonistically at NR3 subunits and inhibitory through binding to the NR1 subunit (Awobuluyi et al., 2007; Madry et al., 2007a). NR1/NR3A receptor activation by glycine is strongly potentiated in the presence of either the divalent cation Zn²⁺ (ten-fold) or NR1 glycine-site antagonists such as MDL-29951 (MDL; >20-fold). Coapplication of both Zn²⁺ and MDL generates a >120-fold “supralinear” potentiation of

glycine-induced currents (Madry et al., 2008). In addition, Zn^{2+} alone elicits receptor currents with similar efficacy as glycine at concentrations $> 100 \mu M$ (Madry et al., 2008).

In the present study we investigated the current-voltage (I–V) dependence and permeation properties of glycine-gated NR1/NR3 NMDA receptors. We found that NR1/NR3A receptors display a strong outwardly rectifying I–V relationship, whereas NR3B subunit-containing receptors (NR1/NR3B and NR1/NR3A/NR3B) do not show a voltage-dependent inward current block at physiological ion concentrations. Further analyses revealed that the voltage-dependent inhibition of ion flux seen with NR1/NR3A receptors is (i) due to Ca^{2+} present in our extracellular recording solution, and is (ii) relieved by potentiating ligands like Zn^{2+} or/and MDL acting at the NR1 subunit. We conclude from these data that voltage-dependent inhibition of NR1/NR3A receptors by physiological concentrations of extracellular Ca^{2+} may be important for regulating excitatory glycinergic transmission, as described for the voltage-dependent Mg^{2+} block of conventional NR1/NR2 NMDA receptors important for glutamatergic signalling.

MATERIALS AND METHODS

MDL-29951 was purchased from Tocris (Biotrend, Cologne, Germany). All other chemicals used were obtained from Sigma (Taufkirchen, Germany). All experimental procedures were done according to German law (Animal licence no. V 54–19c20/15-F126/13; Regierungspräsident Darmstadt).

cDNA CONSTRUCTS, OOCYTE EXPRESSION AND ELECTROPHYSIOLOGY

The NR1-1a, NR1-1a^{F466A}, NR3A and NR3B expression constructs have been described previously (Kuryatov et al., 1994; Madry et al., 2007a). *In vitro* synthesis of cRNA (mCAP mRNA Capping Kit, Ambion, Austin, TX, USA) was performed as described (Laube et al., 2004; Madry et al., 2007a) in the presence of 115 mM NaCl, 1 mM KCl, 1.8 mM $CaCl_2$ and 10 mM Hepes (pH 7.2). For heterologous expression of NR1/NR3 receptors, 25 ng of cRNA was injected at a NR1:NR3 ratio of 1:3 into *Xenopus laevis* oocytes. Oocytes were isolated and maintained as described previously (Laube et al., 1995). Two-electrode voltage-clamp recording of whole-cell currents was performed according to Madry et al. (2007b). N-methyl-D-Glutamine chloride (NMDG-Cl) was used in external solutions in which NaCl was replaced for analysis of divalent permeability. To monitor the voltage dependence of NR1, NR3A and NR3B receptor combinations, whole-cell current-voltage relationships of saturating glycine-induced currents were recorded in 20 mV-intervals ranging from -90 mV to $+30$ mV and normalized to the current value obtained at $+30$ mV. Data points were aligned by using a 3rd-order polynomial fit according to Geiger et al. (1995). To quantify the extent of rectification, the current ratios at 40 mV above and 80 mV below the individual reversal potentials (E_{rev} between -10 and 0 mV) were determined as rectification indices (R_i). The relative divalent to monovalent permeability (P_{div}/P_{mono}) was calculated by the Goldman-Hodgkin-Katz constant field (GHK) voltage equation assuming no anion permeability as described previously (Geiger et al., 1995). The internal concentrations of Na^+ and K^+ used in the calculations were 20 mM and 150 mM, respectively (Katz et al., 2000; Weisstaub et al., 2002). Permeability ratios were calculated for each oocyte and then averaged. In order to avoid the activation of the oocytes' native

Ca^{2+} -sensitive chloride currents, all experiments using Ca^{2+} containing extracellular solutions were carried out in oocytes incubated for 30 min at room temperature with the membrane-permeant Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-acetoxymethyl ester (BAPTA-AM, 100 μM) prior to electrophysiological recordings (Weisstaub et al., 2002).

STATISTICAL ANALYSES

Values given represent means \pm SEM. Statistical significance was determined at the $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) levels using a Student's two-tailed, unpaired t-test.

RESULTS

GLYCINE-GATED NR1/NR3A AND NR1/NR3B RECEPTORS DIFFER IN THEIR CURRENT-VOLTAGE (I–V) RELATIONSHIPS

In a previous study, we described a potentiating effect of the NR1 glycine-binding site antagonist MDL-29951 (MDL) on NR1/NR3A and NR1/NR3B receptors expressed in oocytes (Madry et al., 2007a). When further analyzing the effect of MDL on NR1/NR3A and NR1/NR3B receptor currents elicited by glycine (1 mM), we found that at a negative holding potential (-90 mV) NR1/NR3A receptors were significantly more potentiated by 200 nM MDL than NR1/NR3B receptors, with a potentiation of 8.8 ± 1.2 -fold and 2.5 ± 0.1 -fold, respectively ($p < 0.001$; **Figures 1A,B**). However, at a positive holding potential ($+30$ mV), potentiation of the glycine-induced currents by MDL was not different between the two receptor combinations (3.1 ± 0.5 -fold and 2.4 ± 0.4 -fold, respectively; $p > 0.05$; **Figures 1A,B**). We therefore analyzed whole-cell current-voltage relationships (I–V curves) of glycine-induced currents from NR1/NR3A and NR1/NR3B receptors over a voltage range of -90 mV to $+30$ mV (**Figures 1C,D**). I–V curves of NR1/NR3B receptors were found to be linear with a reversal potential of around -10 mV (**Figure 1D**), whereas those obtained from NR1/NR3A receptors showed an identical reversal potential but a strong outwardly rectifying behavior, with an inward current block emerging at a holding potential ≤ -30 mV (**Figure 1C**). Interestingly, co-application of 200 nM MDL caused a linearization of the I–V curve for glycine-activated NR1/NR3A receptors (**Figure 1C**), whereas the linear I–V relation of receptors containing the NR3B subunit was not altered in the presence of MDL (**Figure 1D**).

In order to quantify the extent of rectification of NR1/NR3 receptor currents, we determined current ratios at $+30$ mV and -90 mV and calculated rectification indices (R_i). Based on a reversal potential of -10 mV, linear I–V relationships result in a R_i value of about 0.5 whereas outwardly rectifying I–V curves display R_i values > 1.5 . Consistent with the data shown above, MDL potentiation caused a significant change of the R_i for NR1/NR3A receptors ($-MDL$: 1.65 ± 0.15 , $+MDL$: 0.62 ± 0.08 ; $p < 0.001$), whereas no difference was seen for NR1/NR3B receptors in the absence and presence of the antagonist ($-MDL$: 0.43 ± 0.08 , $+MDL$: 0.34 ± 0.02 ; $p > 0.05$; **Figure 1F**). Finally, we analyzed the I–V curve of triheteromeric receptors composed of NR1, NR3A and NR3B subunits. Maximal inducible currents of these tri-heteromeric NR1/NR3A/NR3B receptors were more than 10-fold larger than those obtained from di-heteromeric NR1/NR3A and NR1/NR3B receptors (data not shown), which is consistent with an efficient tri-heteromeric NR1/NR3A/NR3B receptor assembly (Ulbrich and

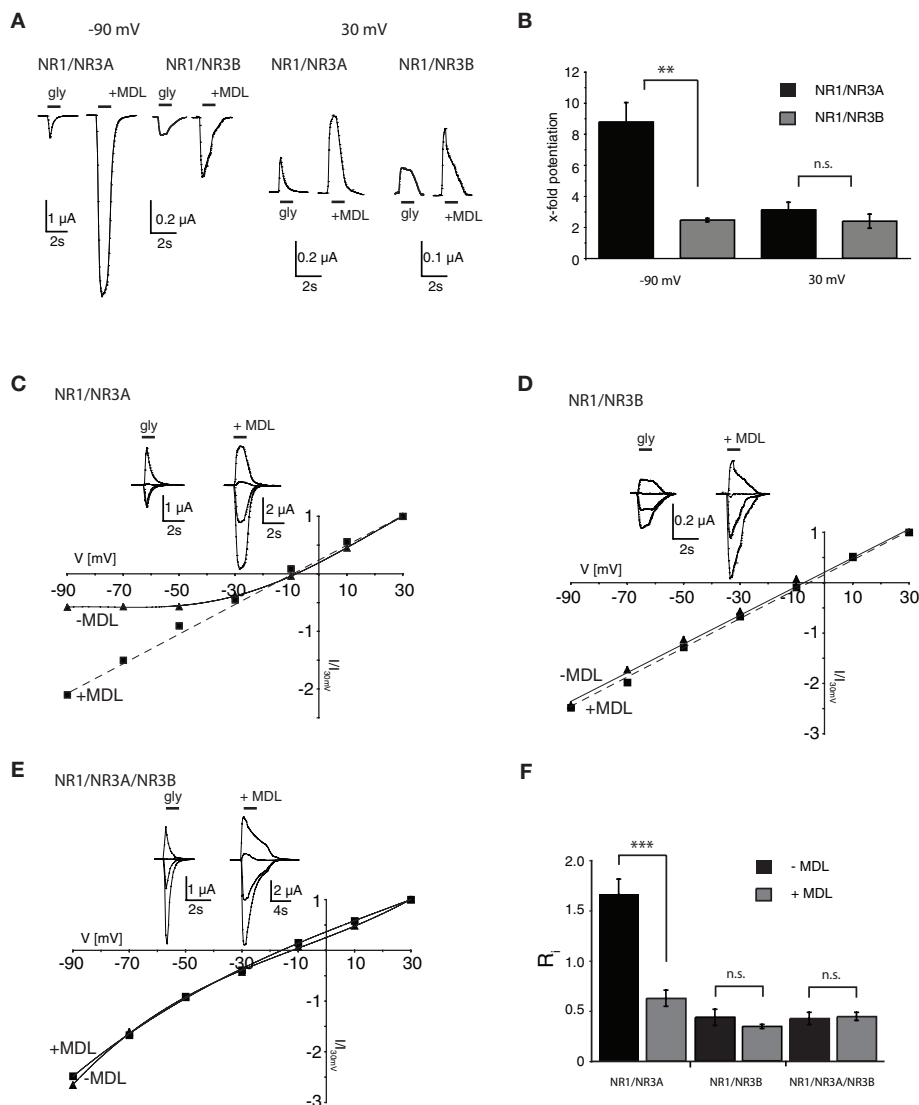


FIGURE 1 | I-V relationships of glycine-gated NR1/NR3 receptors.

(A,B) Effect of holding-potential on MDL-29951 (MDL) potentiated glycine currents of NR1/NR3A and NR1/NR3B receptors. (A) Sample traces at -90 mV (left) and $+30$ mV (right) activated with a saturating glycine concentration (1 mM) in the absence or presence of 200 nM MDL. (B) Relative potentiation by MDL of NR1/NR3A (black bars) and NR1/NR3B (gray bars) receptor currents at -90 and $+30$ mV. Note that MDL-potentiation was at -90 mV about 3-fold larger for NR1/NR3A receptors compared to NR1/NR3B ($p < 0.01$; $n = 5$).

(C-E) Normalized I-V plots of NR1/NR3A (C), NR1/NR3B (D) and

NR1/NR3A/NR3B (E) receptor currents recorded from -90 to $+30$ mV in 20 -mV intervals activated by a saturated glycine concentration in the absence (triangle) and presence (square) of 200 nM MDL. Respective sample traces are shown above. Note that NR1/NR3A receptors display an outwardly rectifying I-V curve in the presence of glycine alone, which becomes linear upon MDL-potentiation. (F) Quantification of I-V relationships of NR1/NR3 receptors in the absence (black bars) and presence (gray bars) of 200 nM MDL by determining the rectification index (R_1) of the currents measured at 40 mV above ($\Delta+40$ mV) and 80 mV below ($\Delta-80$ mV) the respective reversal potential.

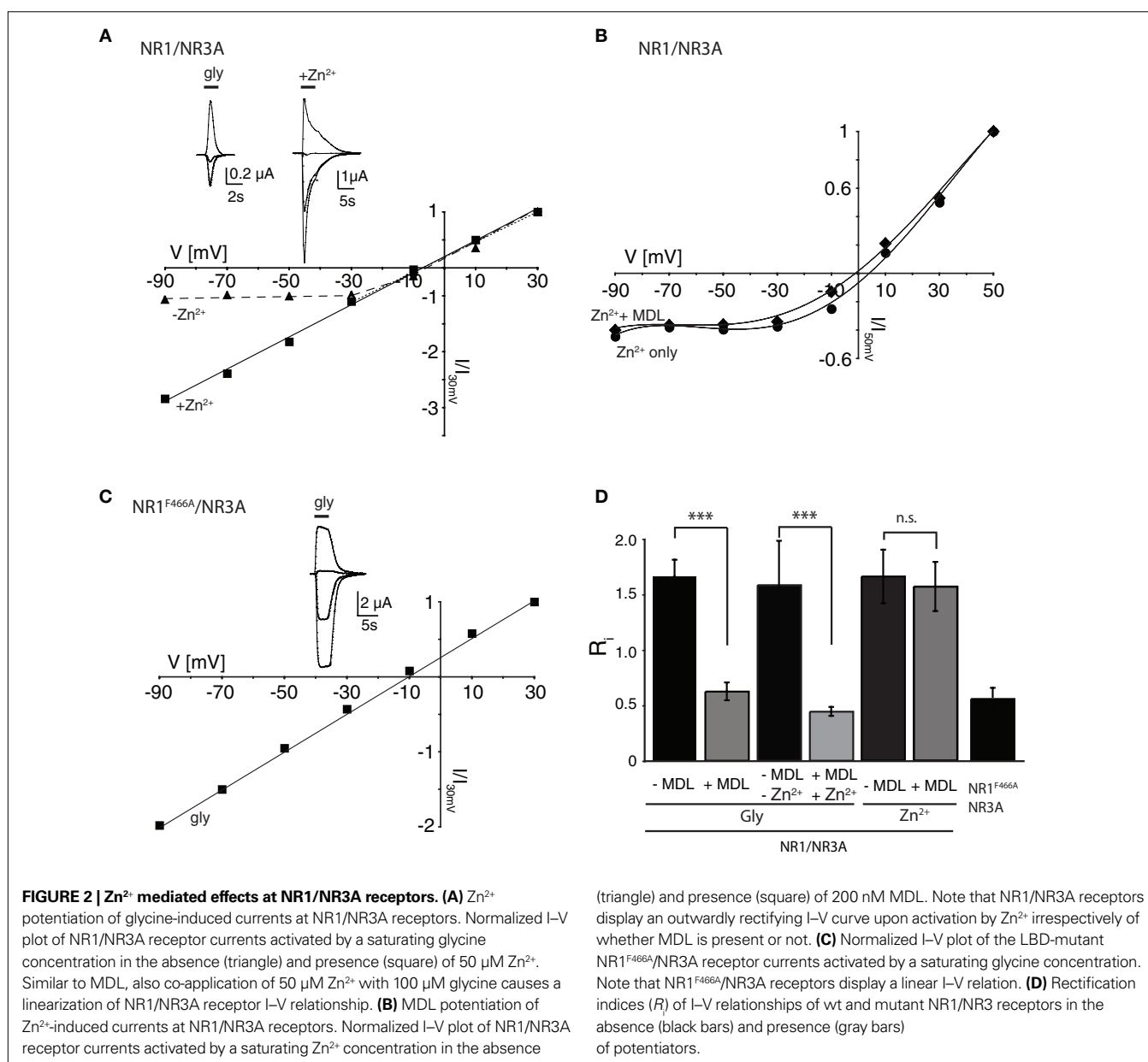
Isacoff, 2008; Smothers and Woodward, 2009). I-V curves from NR1/NR3A/NR3B expressing oocytes were found to be linear in both, the absence and presence of MDL (Figure 1E). Analyses of the R_1 revealed values of 0.42 ± 0.06 vs. 0.44 ± 0.04 in the absence and presence of 200 nM MDL for NR1/NR3A/NR3B-receptors, respectively ($p > 0.05$; Figure 1F). Thus, MDL caused a linearization of the outwardly rectifying I-V curve of NR1/NR3A receptors by a relief of the voltage-dependent inward current block, whereas NR3B containing combinations showed a linear I-V-relationship irrespective whether MDL was present or not.

DIFFERENTIAL EFFECTS OF Zn^{2+} ON NR1/NR3A RECEPTOR I-V RELATIONS

The divalent cation Zn^{2+} exerts complex and opposing effects at NR1/NR3 receptors. At NR1/NR3A receptors it acts in the lower micromolar concentration range as a positive modulator of glycine-currents and as a full principal agonist at Zn^{2+} concentrations > 100 μ M (Madry et al., 2008). In contrast, NR1/NR3B receptors neither become potentiated nor activated by Zn^{2+} . Therefore we wondered whether Zn^{2+} -potentiation of NR1/NR3A receptor currents would display a linear I-V relationship similar to that found for MDL-potentiated receptor

currents. Indeed, co-application of glycine and 50 μM Zn^{2+} fully linearized the outwardly rectifying I–V curve seen in the absence of Zn^{2+} to R_i values resembling those found in the presence of MDL (**Figures 2A,D**). Since maximal potentiation of NR1/NR3A receptors is observed upon co-application of the modulators Zn^{2+} and MDL (Madry et al., 2008), we also analyzed the I–V curves of these supralinearly potentiated NR1/NR3A receptors. In line with a relief of the glycine-mediated inward current block by MDL and Zn^{2+} alone, we found a linear I–V relationship of the glycine-induced currents when both 200 nM MDL and 100 μM Zn^{2+} were co-applied (**Figure 2D**, and data not shown). In conclusion, potentiation of glycine-induced NR1/NR3A receptor currents by Zn^{2+} , NR1 antagonists or a combination of both abrogated the inward current block seen upon application of glycine alone.

Higher concentrations of Zn^{2+} (>100 μM) activate NR1/NR3A receptors with a comparable efficacy as glycine (Madry et al., 2008). Hence, we wanted to know whether both agonists display similar I–V relationships. We found that Zn^{2+} -gated NR1/NR3A receptors displayed a similar inward current block than seen with glycine (**Figure 2B**). Strikingly, MDL potentiation of Zn^{2+} -induced currents was not accompanied by a linearization of the I–V curve, and thus did not cause a decrease of the rectification index (**Figures 2B,D**). Apparently, the voltage-dependent blocks seen with both glycine and Zn^{2+} are differentially affected by MDL. Since both Zn^{2+} and MDL are thought to act via the NR1-LBD, we analyzed the effect of a mutation within the glycine-binding site of the NR1 subunit (phenylalanine 466 to alanine) on glycine-gated I–V relations of NR1/NR3A receptors (**Figure 2C**). Previous mutational analyses have shown that the affinity of both glycine and MDL to the NR1



subunit is reduced by the NR1^{F466A} substitution (Kuryatov et al., 1994; Madry et al., 2007a), and that the potentiating and activating effects of Zn²⁺ at NR1/NR3A receptors are abolished (Madry et al., 2008) by this mutation. Here we found a linear I–V relationship of glycine-induced currents recorded from NR1^{F466A}/NR3A receptors (R_i : 0.49 ± 0.02 ; **Figures 2C,D**). This indicates that the NR1 LBD is crucial for the rectification behavior of NR1/NR3A receptors.

PHYSIOLOGICAL CONCENTRATIONS OF CA²⁺ CAUSE VOLTAGE-DEPENDENT INHIBITION OF NR1/NR3A RECEPTOR-MEDIATED ION FLUX

Mg²⁺ ions are known to block NR1/NR2 NMDA receptor channels at negative membrane potentials (overview in Cull-Candy et al., 2001). We therefore analyzed whether divalent cations are responsible for the voltage-dependent inward current block seen with

NR1/NR3A receptors. First we measured glycine-mediated currents of NR1/NR3A receptors in the absence of divalent cations. I–V relationships obtained under divalent-free conditions were found to be linear, with a rectification index in the range of those seen with glycine-gated NR1/NR3B and NR1/NR3A/NR3B, or potentiated NR1/NR3A receptors (R_i : 0.43 ± 0.04 ; **Figures 3A,B**). To further test whether the voltage-dependent inhibition of NR1/NR3A receptor-mediated ion flux is due to a specific divalent cation, we analyzed I–V relations of glycine-induced currents in the presence of Ca²⁺, Ba²⁺ and Mg²⁺ (1.8 mM each). The presence of either 1.8 mM Ba²⁺ or Mg²⁺ resulted only in a minor inhibition of inward current flow with R_i -values of 0.84 ± 0.05 and 0.84 ± 0.03 , respectively (**Figure 3B**). In contrast, I–V relations in the presence of 1.8 mM Ca²⁺ revealed a strong inward rectification with a highly significant larger R_{iCa}

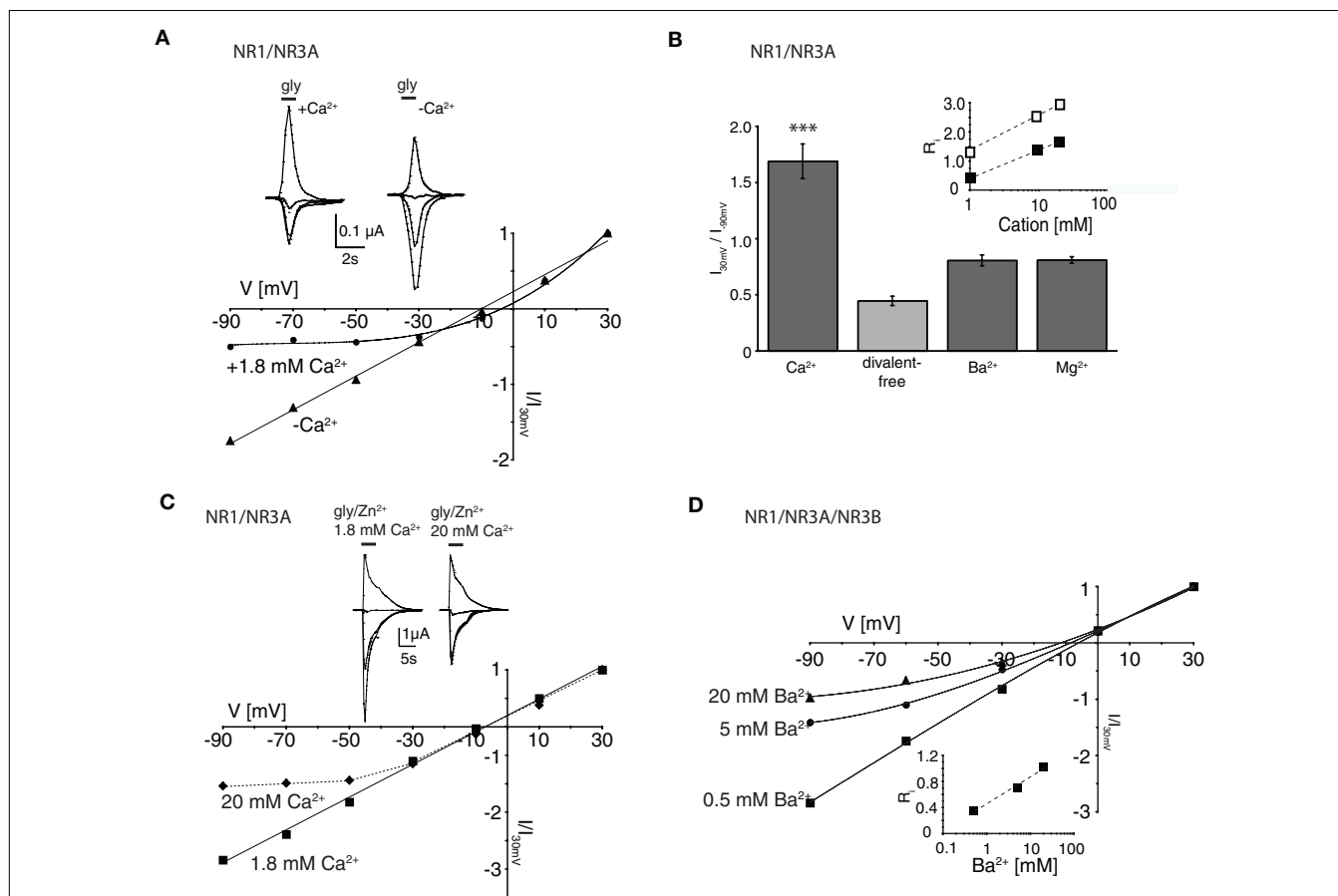


FIGURE 3 | Dependency of I–V relationship on divalent cations for glycine-activated NR1/NR3A receptors. (A) Normalized I–V plot of NR1/NR3A receptor currents activated by a saturating glycine concentration in the absence (triangle) and presence (square) of 1.8 mM Ca²⁺. Note, that application of a saturating glycine concentration (100 μM) in the absence of any divalent cations results in a linear I–V relationship, whereas 1.8 mM Ca²⁺ causes an inward current block (see also **Figure 1C**). Sample traces are shown above the I–V plot. **(B)** Quantification of divalent-dependent inward current block of NR1/NR3A receptors. Rectification indices (R_i) of I–V relationships of NR1/NR3A receptors in the absence of divalent cations and in the presence of 1.8 mM Ca²⁺, Ba²⁺ and Mg²⁺ are shown. Inset shows a plot of the three different rectification indices (R_i) fitted against the respective log of (Ca²⁺) (open symbol) and (Mg²⁺) (closed symbol). **(C)** Effect of different Ca²⁺ concentrations on Zn²⁺

potentiated glycine-activated currents of NR1/NR3A receptors. Normalized I–V plot of potentiated NR1/NR3A receptor currents activated by a saturating glycine concentration and 50 μM Zn²⁺ in the presence of 1.8 mM (square) and 20 mM (triangle) Ca²⁺. Note that an increase of the extracellular Ca²⁺ concentration from 1.8 to 20 mM led to an outwardly rectifying I–V-relationship similar to those found under non-potentiated conditions in the presence of low Ca²⁺. Sample traces are shown above the I–V plot. **(D)** Increasing divalent cation concentrations lead to outwardly rectifying I–V relationships in NR3B containing NR1/NR3 receptors. Normalized I–V plot of NR1/NR3A/NR3B receptor currents activated by a saturating glycine concentration in the presence of 0.5 mM (square), 5 mM (circle) and 20 mM Ba²⁺ (triangle). Inset shows a plot of the three different rectification indices (R_i) fitted against the respective log [Ba²⁺].

of 1.68 ± 0.09 ($p < 0.001$; **Figure 3B**). To estimate the efficacy of Ca^{2+} and Mg^{2+} to block inward currents, I–V relationships with increasing concentrations (1, 10 and 20 mM) of the two divalent cations were measured. Only higher Mg^{2+} concentrations (>10 mM) resulted in a pronounced inward rectification with R_i -values > 1 similar to those found with low Ca^{2+} concentrations, whereas I–V curves in the presence of 1 mM Mg^{2+} were linear (**Figure 3B**, inset). This is consistent with different affinities of the two cations tested for ion channel block and shows that under physiological divalent cation concentrations Ca^{2+} and not Mg^{2+} determines the I–V relationship of NR1/NR3A receptors.

To test whether potentiated NR1/NR3A glycine currents might be affected at non-physiological elevated Ca^{2+} concentrations, we analyzed the I–V relationship of Zn^{2+} -potentiated (50 μM) glycine-induced currents in the presence of 20 mM Ca^{2+} . This increased Ca^{2+} concentration produced an inward current block at holding potentials < -30 mV (**Figure 3C**) as seen in the absence of Zn^{2+} at low Ca^{2+} (1.8 mM). Based on this result, we re-investigated the divalent cation dependency of the I–V curves of NR1/NR3B and NR1/NR3A/NR3B receptors, which both exhibit linear I–V relationships under physiological salt concentrations. Similarly, increasing the extracellular Ca^{2+} or Ba^{2+} concentration to 20 mM led to the emergence of an outwardly rectifying I–V curve at both NR1/NR3B and NR1/NR3A/NR3B receptors (**Figure 3D**). This implies that in the presence of elevated divalent cation concentrations NR3B subunit containing receptor combinations display an outwardly rectifying I–V relationship as found for NR1/NR3A receptors in the presence of physiological Ca^{2+} concentrations.

In summary, physiological Ca^{2+} conditions are responsible for the outward rectification of glycine-gated NR1/NR3A receptors, whereas potentiated NR1/NR3A and NR3B containing receptors are blocked only at higher Ca^{2+} concentrations. Thus, differences in the affinity of the Ca^{2+} block seem to underlie the differential rectification behavior of NR3A and NR3B subunit containing receptors.

PERMEABILITY FOR DIVALENT CATIONS IS NOT ALTERED IN SUPRALINEARLY POTENTIATED NR1/NR3A RECEPTORS

Removal of a cation-dependent open channel block has been shown in both transient receptor potential (TRP) channels and conventional NMDA receptors by an increase in the passage-rate of the blocking ion through the channel pore (Parnas et al., 2009). Therefore the relief of the Ca^{2+} -mediated block seen with MDL and Zn^{2+} -potentiated NR1/NR3A receptors may derive from an increased Ca^{2+} permeability. To test this hypothesis, we substituted Na^+ with the ion channel pore impermeable compound N-methyl-D-glucamine chloride (NMDG-Cl) and determined I–V curves in the presence of different Ca^{2+} concentrations in order to obtain an approximate estimate of the relative divalent to monovalent cation permeability $P_{\text{Ca}}/P_{\text{Na}}$. **Figure 4A** shows that the reversal potentials pooled from three different oocytes become more depolarized as a function of the concentration of Ca^{2+} in the extracellular medium. However, the ratio of $P_{\text{Ca}}/P_{\text{Na}}$ was < 0.25 in all measurements as determined by the Goldman-Hodgkin-Katz voltage equation (see Geiger et al., 1995), confirming a low Ca^{2+} permeability of the NR1/NR3A receptor under non-potentiated conditions (Chatterton et al., 2002). In a next step we compared the

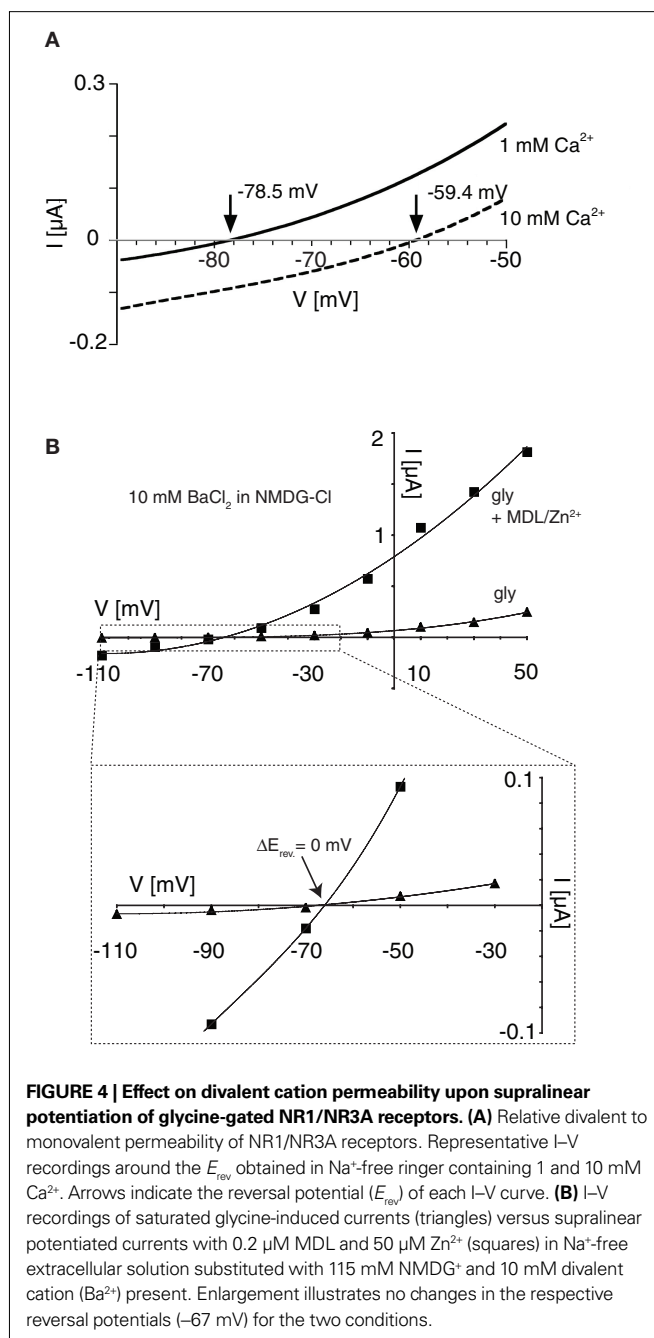


FIGURE 4 | Effect on divalent cation permeability upon supralinear potentiation of glycine-gated NR1/NR3A receptors. (A) Relative divalent to monovalent permeability of NR1/NR3A receptors. Representative I–V recordings around the E_{rev} obtained in Na^+ -free ringers containing 1 and 10 mM Ca^{2+} . Arrows indicate the reversal potential (E_{rev}) of each I–V curve. (B) I–V recordings of saturated glycine-induced currents (triangles) versus supralinearly potentiated currents with 0.2 μM MDL and 50 μM Zn^{2+} (squares) in Na^+ -free extracellular solution substituted with 115 mM NMDG $^+$ and 10 mM divalent cation (Ba^{2+}) present. Enlargement illustrates no changes in the respective reversal potentials (-67 mV) for the two conditions.

I–V relationships of glycine-induced currents with that of supralinearly potentiated currents in the presence of divalent cations. Neither the analysis of I–V curves in the presence of 10 mM Ba^{2+} nor using 10 mM Ca^{2+} in the extracellular solution with BAPTA-AM pre-incubated cells revealed any shifts in the reversal potentials (E_{rev} -67 mV; **Figure 4B**). We also tested the accessibility to MK-801, a classical ion channel pore blocker of NR1/NR2 NMDA receptors, which has no effect on glycine-gated NR1/NR3A receptors (Wong et al., 1986; Chatterton et al., 2002). However, even when activated with glycine in the presence of Zn^{2+} and MDL, MK-801 (100 μM) remained ineffective at NR1/NR3A receptors (data not shown). Together these data indicate that the loss of the

voltage-dependent Ca^{2+} -block seen with NR1/NR3A receptors in the presence of MDL and Zn^{2+} is not accompanied by an increased Ca^{2+} permeability of the ion channel.

DISCUSSION

In this article, we show that glycine-gated NR1/NR3A and NR1/NR3B NMDA receptors display a differential sensitivity for Ca^{2+} upon heterologous expression in *Xenopus* oocytes. At negative holding potentials, physiological concentrations of Ca^{2+} (1.8 mM) caused a pronounced inward current block of NR1/NR3A receptors, whereas receptors containing the NR3B subunit were only inhibited at elevated Ca^{2+} concentrations (>10 mM). Interestingly, the voltage-dependent inhibition of NR1/NR3A receptor currents by external Ca^{2+} was abrogated upon co-application of Zn^{2+} , glycine-site antagonist or mutations within the glycine-binding site of the NR1 subunit; all these conditions resulted in a linear I–V relationship. Ion substitution experiments revealed that neither MDL or Zn^{2+} potentiation nor the relief of the Ca^{2+} -block was accompanied by changes in Ca^{2+} permeability. Notably, when NR1/NR3A receptor mediated currents were elicited by Zn^{2+} , MDL co-application did not result in a linear I–V, consistent with different mechanisms underlying glycine and Zn^{2+} agonism.

An interesting finding of this study is that the difference in maximal MDL potentiation of glycine currents seen between NR1/NR3A and NR1/NR3B receptors (Madry et al., 2007a) is due to a pronounced Ca^{2+} -dependent outward rectification of NR1/NR3A receptors. Thus, at physiological Ca^{2+} concentrations NR1/NR3A receptor channels are blocked at negative holding potentials whereas NR1/NR3 receptors containing the NR3B subunit are not affected. Notably, a similar outward rectification of the here described voltage-dependent Ca^{2+} block of the NR1/NR3A receptor exists in conventional NMDA receptors composed of NR1/NR2 subunits. Their voltage-dependent block at resting membrane potentials is mediated by extracellular Mg^{2+} (overview in Cull-Candy et al., 2001). Molecular structures responsible for the Mg^{2+} block have been partially identified and comprise sites in the middle and at the entrance of the channel forming segments of NMDA receptor subunits (overview in Dingledine et al., 1999). For example, asparagine residues of the QRN site in the M2 segment of NR1 and NR2 subunits have been shown to determine the block by Mg^{2+} (Kuner et al., 1996). In addition, a DRPEER motif in NR1 (Watanabe et al., 2002), a tryptophan residue in the M2 regions of NR2 subunits (Williams et al., 1998) and the common SYTANLAAF motif in TM3 (Yuan et al., 2005; Wada et al., 2006) affect the Mg^{2+} block. Comparing the sequences of NR1, NR2 and NR3 subunits reveals a remarkable conservation of these regions, although especially within the QRN site and the SYTANLAAF motif several exchanges between NR1, NR2 and NR3 subunits are found. For example, the corresponding NR3 residue of the QRN site is a glycine. Although all residues mentioned above are highly conserved in NR2 subunits, channels containing NR2A or NR2B subunits are more sensitive to Mg^{2+} block compared with NR2C or NR2D-containing channels, suggesting that additional elements exist that determine subunit specificity to divalent cations. However, the well known physiological function of conventional NMDA receptors in the

mammalian brain is to serve as coincidence detectors of presynaptic and postsynaptic activity. This function is achieved through removal of the Mg^{2+} block upon postsynaptic membrane depolarization (Cull-Candy et al., 2001). Likewise, a similar mechanism can be envisaged for NR1/NR3A receptors where release of both, the principal agonist glycine and a second so far unknown ligand may result in a pronounced potentiation of glycine-currents and relief of the voltage-dependent Ca^{2+} block (this study). A previous report has disclosed that the neuromodulator Zn^{2+} (overview in Frederickson et al., 2005) is essential for proper functioning of glycinergic inhibitory neurotransmission (Hirzel et al., 2006). Thus, Zn^{2+} may be similarly essential for efficient activation of NR1/NR3A receptors (Madry et al., 2008).

A second important result of this study is that at least two ligands have to bind simultaneously for abrogating Ca^{2+} -dependent outward rectification of NR1/NR3A receptors. Accordingly, efficient channel gating of NR1/NR3 receptors requires simultaneous occupancy of the NR1 and NR3 LBDs (Awobuluyi et al., 2007; Madry et al., 2007a). Here we show that only ligand-binding to both, the NR3A and NR1 LBD resulted in a linearization of the I–V curve, whereas co-application of the full agonist Zn^{2+} and the NR1 antagonist MDL, both binding within the NR1 LBD, did not abrogate the inward-rectifying Ca^{2+} block. This suggests a remarkable mechanistic similarity in ion channel activation between NR1/NR3A and conventional NR1/NR2 NMDA receptors. Both conventional and glycine-gated NMDA receptors require binding of two ligands within the LBDs of both subunits for efficient channel opening. Thus, only highly cooperative interactions between fully liganded subunits enable the conformational transition of NMDA receptors to the fully open state. Notably, although a remarkable potentiation is found upon binding of both the full agonist Zn^{2+} and MDL, the voltage-dependent Ca^{2+} block is not diminished due to the lack of NR3 LBD occupation. A similar contribution of multiply liganded subunits to efficient channel activation has been proposed for the AMPA receptor, a related member of the iGluR family (Rosenmund et al., 1998). According to this view, occupancy of the NR1 or NR3 binding sites is in principle sufficient to drive channel opening, but additional occupancy of the respective other subunits increases receptor current which might be mediated due to changes in the open probability and/or sub-conductance states of the ion channel. Whatever the precise mechanism(s) of glycine vs. Zn^{2+} action may be, our results show that ion permeability is not changed upon supralinear potentiation of the glycine current. Thus, removal of the Ca^{2+} -dependent channel block is not accompanied by an increase in the passage-rate of Ca^{2+} through the channel pore.

Taken together our data demonstrate fundamental differences in the I–V dependence of di-heteromeric NR1/NR3 receptors that might provide a versatile electrophysiological tool to discriminate NR1/NR3A and NR1/NR3B receptors *in vivo*.

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