Expression of Glycine-Activated Diheteromeric NR1/NR3 Receptors in Human Embryonic Kidney 293 Cells Is NR1 Splice Variant-Dependent

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ABSTRACT

In oocytes, glycine activates receptors formed by diheteromeric combinations of *N*-methyl-D-aspartate (NMDA) NR1 and NR3 subunits. In contrast, functional receptors in mammalian cells require the simultaneous expression of NR1 and both NR3A and NR3B subunits. In vivo, NR3A and NR3B subunits show differential expression patterns and thus may not naturally form triheteromeric receptors. In this study, we examined whether NR1 splice variants play a role in allowing assembly of functional diheteromeric receptors in mammalian cells. Little current was found in human embryonic kidney 293 cells coexpressing either NR3A or NR3B and the NR1-1a splice variant. However, robust glycine-activated currents were generated in cells transfected with NR3(A or B) and either NR1-2a, NR1-3a, or NR1-4a, and current density was correlated with NR1 C-terminal length. Truncation of the NR1-1a C terminus modestly enhanced NR1-

1a/NR3A currents, whereas only small increases were observed with mutations of C-terminal residues that control trafficking or phosphorylation. In contrast, large currents were observed when an extracellular phenylalanine in NR1-1a that influences glycine access was mutated to alanine. A separate mutation in NR1-1a that disrupts glycine binding did not generate responses in NR1-1a/NR3A receptors alone, but it produced a greater than 30-fold potentiation of currents during coapplication of glycine and the glycine antagonist 7-chlorokynurenic acid. Finally, transfection of cells with the NR1-4a subunit along with NR2 and NR3 subunits resulted in the expression of both NR1/NR3 receptors and conventional NMDA receptor currents. These results indicate a prominent role for NR1 splice variants in the functional expression of NR1/NR3 receptors in mammalian cells.

N-Methyl-D-aspartate (NMDA) receptors are a subtype of the ionotropic glutamate receptor family. These receptors play important roles in excitatory synaptic transmission, learning, and memory and have been implicated as important mediators of various neurological disorders (Cull-Candy et al., 2001; Nakazawa et al., 2004). The subunits that comprise NMDA receptors are assembled from three separate gene families: NR1, NR2, and NR3 (McBain and Mayer, 1994; Dingledine et al., 1999). The NR1 gene contains exons subject to alternative RNA splicing leading to the generation of eight splice variants (Zukin and Bennett, 1995). The NR2 gene family has four members, NR2A, NR2B, NR2C, and NR2D, whereas the NR3 gene family contains the NR3A and NR3B subunits. Conventional NMDA receptors are thought

to be tetramers composed of two NR1 subunits, and two NR2 (A-D) subunits (Dingledine et al., 1999). Recent studies have shown that incorporation of NR3 subunits into conventional NMDA receptors results in smaller unitary conductance states and decreases in current amplitude, calcium permeability, and magnesium sensitivity (Cia-barra et al., 1995; Sucher et al., 1995; Das et al., 1998; Perez-Otano et al., 2001; Matsuda et al., 2002; Sasaki et al., 2002). Although the NR3 subunit associates with conventional NMDA receptors, recent studies have also shown that NR1 and NR3 subunits assemble to form novel excitatory glycine-activated receptors (Chatterton et al., 2002; Awobuluyi et al., 2007; Madry et al., 2007; Smothers and Woodward, 2007). NR1/NR3 receptors are cationic channels with low calcium permeability and, unlike most conventional NR1/NR2 receptors, are insensitive to block by magnesium (Chatterton et al., 2002). NR1/NR3 receptors are activated by glycine or D-serine, but not glutamate, and are unaffected by conventional NMDA receptor antago-

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; ANOVA, analysis of variance; HEK, human embryonic kidney; APV, D-2-amino-5-phosphonovaleric acid; 7-CK, 7-chlorokynurenic acid; 5,7-DCK, 5,7-dichlorokynurenic acid; BS³, bis[sulfosuccinimidyl] suberate; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate.

nists such as APV, ketamine, memantine, and MK-801 (Chatterton et al., 2002; Smothers and Woodward, 2007).

Despite evidence suggesting that recombinant NR1/NR3 subunits form functional glycine-activated receptors in oocytes, currents generated by these subunits have not been unequivocally observed in neurons (Chatterton et al., 2002; Tong et al., 2008). In fact, expression of NR1/NR3A or NR1/NR3B subunits in mammalian cells fails to generate measurable currents (Nishi et al., 2001; Matsuda et al., 2002), whereas robust responses are produced in cells transfected with NR1 and both NR3(A,B) subunits (Smothers and Woodward, 2007). The reason for this discrepancy is not known, but could be due to differences in receptor expression or trafficking between oocytes and mammalian cells or to factors that regulate receptor function. In this study, we report that the expression of functional NR1/NR3 dimeric receptors in mammalian cells is NR1 splice variant-dependent with NR1-4 variants generating currents 10 to 20 times larger than those obtained with NR1-1. This effect seems to be due to both increases in surface expression and reduced desensitization of glycine evoked currents.

Materials and Methods

Molecular Biology and Site-Directed Mutagenesis. The cDNA clones of NR1 and NR3A used in these experiments were kindly provided by S. Heinemann (NR1 splice variants, with the exception of NR1-1a that was provided by Nakanishi), and S. Lipton (NR3A, NR3B). Specific mutations of residues in the NR1 subunit were done by use of the QuikChange mutagenesis protocol (Stratagene, La Jolla, CA). All mutations were verified by DNA sequencing. The coding region of the NR3A subunit was subcloned into the green fluorescent protein expression vector, pGFP-N3, as described previously (Smothers and Woodward, 2003).

Maintenance of HEK293 Cells and Recombinant Receptor Expression. In brief, HEK293 cells (American Type Culture Collection, Manassas, VA) were cultured as described previously (Smothers and Woodward, 2003). Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and grown at 37°C in a 5% CO₂ environment. Low-density cultures (approximately 5×10^4 cells per dish) plated onto 35-mm dishes were transfected with equal amounts of cDNA (1 µg) coding for NR1 (wild-type or mutant) NR3A or NR3B with use of the Lipofectamine 2000 reagent (Invitrogen). Cells were used for electrophysiological recordings 24 to 48 h after transfection.

Western Blotting and Cross-Linking. In brief, SDS-polyacrylamide gel electrophoresis was used to separate proteins that were then transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk and then probed with primary NR1 antibody (BD Bioscience, San Jose, CA; 1:1000) overnight at 4°C. Membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Bands were identified by use of enhanced chemiluminescence (GE Healthcare, Piscataway, NJ) and images were scanned, inverted and quantified using Image J (version 1.38; National Institutes of Health, Bethesda, MD).

Cross-linking experiments were performed as described by Grosshans et al. (2002). Transfected HEK293 cells were washed with ice-cold phosphate-buffered saline and treated in the absence or presence of 2 mg/ml of BS³ cross-linking reagent for 1 h at 4°C. After treatment the cells were washed twice with ice-cold Tris buffer and sonicated in homogenization buffer containing protease and phosphatase inhibitors. Protein concentration was determined by bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA).

Electrophysiological Recording Conditions. All recordings were performed as described previously (Smothers and Woodward,

2007). In brief, cells were perfused with an external solution containing 135 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 5 mM HEPES, and 10 mM glucose (pH adjusted to 7.2 with NaOH, and osmolarity adjusted to 325 mOsm/kg with sucrose). The pipette-filling internal solution was composed of 100 mM *N*-methyl-D-glucamine, 40 mM CsCl, 2 mM NaATP, 2 mM MgCl₂, 10 mM HEPES, 10 mM EGTA (pH was adjusted to 7.2 with KOH, and osmolarity was adjusted to 320 mOsm/kg with sucrose). All internal solutions used for each experiment were from frozen stocks. All drug solutions were prepared fresh for each experiment from frozen stocks. Stock solutions of glycine were prepared in water and diluted into external solution. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Whole-cell voltage-clamp recordings (Hamill et al., 1981) were performed at room temperature by use of the Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA). Cells were voltage-clamped at -60 mV, and current records were filtered at 1 kHz (eight-pole Bessel filter) and digitized at 2 kHz by use of an ITC-16 interface (Instrutech Corp., Port Washington, NY). Software control of data acquisition was provided by Pulse Control running within the Igor Pro program (version 4.03; WaveMetrics, Portland, OR) on an Apple Macintosh G3 computer (Apple Computer, Cupertino, CA). Patch electrodes were fabricated from thick-walled borosilicate glass (B150; WPI, Sarasota, FL) and filled with internal solution (tip resistance, 4–7 MΩ). A three-barrel perfusion apparatus (barrel internal diameter, 0.6 mm; SF-77B; Warner Instruments, Hamden, CT) was used to switch between control and drug-containing solutions.

Currents were evoked by a 6-s agonist application. The peak current amplitude was determined as the difference between the current immediately before agonist application and at the point where inward current was maximal. Leak currents were continually monitored as an indicator of seal and cell integrity. Cells that showed unstable leak currents were discarded from the data analysis.

Data Analysis. Data were analyzed by analysis of variance (ANOVA) and post hoc testing with use of Prism 4.0 (GraphPad Software, Inc., San Diego, CA). Curve fitting for dose-response curves used the following equation: $Y = Y_{\min} + (Y_{\max} - Y_{\min}) / (1 + 10^{(\log_{10}(EC_{50}) - X)})$, where X is $\log_{10}[\text{drug}]$, and Y is the percentage of maximal current. Drug inhibition of receptor currents (I_{Control}) was calculated by use of the formula $[1 - (I_{\text{Glycine+drug}}/I_{\text{Control}})] \times 100$, where $I_{\text{Glycine+drug}}$ represents the response to coapplication of glycine + drug, and I_{Control} represents the mean of two responses to glycine, one before and one after the coapplication of the drug. Western blot data were obtained from at least three separate experiments. Data are expressed as the mean $\pm$ S.E.M with the percentage of surface calculated as the ratio of BS³-treated per non-BS³-treated.

Results

Effect of NR1 Splice Variants. Alternative splicing of the NR1 subunit generates eight splice variants (for review, see Zukin and Bennett, 1995). Four splice variants are characterized by the presence or absence of cassettes (C1, C2, or C2') in the C-terminal domain and are termed NR1-1, NR1-2, NR1-3, and NR1-4 (Fig. 1A). Alternative splicing in the N-terminal domain generates additional variants where the absence or presence of cassette (N1) is denoted by the letter a or b, respectively. To determine whether NR1 splice variants influenced expression of dimeric NR1/NR3 receptors in a mammalian expression system, NR1-1a, NR1-2a, NR1-3a, and NR1-4a subunits were individually coexpressed with the NR3A subunit in HEK293 cells. In HEK293 cells transfected with NR1-1a and NR3A subunits, application of 100 µM glycine evoked only small currents in the range of 20 to 30 pA (Fig. 1B). However, in HEK293 cells transfected with NR3A and NR1-2a, NR1-3a, or NR1-4a subunits, glycine applica-

Fig. 1. Glycine activation of currents from HEK293 cells transfected with NR3A and NR1 splice variants. **A**, diagram of NR1 splice variants showing alternatively spliced cassettes in the N-terminal domain (N1 cassette, $\square$) and C-terminal domain (C1 cassette, $\blacksquare$; C2 cassette, $\blacksquare$; and C2' cassette, $\boxtimes$). Gray bars represent transmembrane domains. **B**, representative traces from NR1-1a/NR3A, NR1-2a/NR3A, NR1-3a/NR3A, and NR1-4a/NR3A recombinant receptors during exposure to 100 μ M glycine. The horizontal bar above the traces indicates a 6-s agonist application. Scale bar, 5 s. **C**, current density relationship for glycine activation of NR1/NR3 receptors containing NR1-1a, NR1-2a, NR1-3a, and NR1-4a splice variants. NR1-1a/NR3A relationship is shown as a dash line for emphasis. The data represent peak current measurements normalized to whole-cell capacitance and expressed as mean $\pm$ S.E.M. ($n = 9$ –14 cells per receptor combination). **D**, concentration-response relationship for glycine activation of NR1/NR3A receptors containing NR1-1a, NR1-2a, NR1-3a, and NR1-4a splice variants. The NR1-1a/NR3A relationship is shown as a dotted line for clarity. Data represent the peak current values expressed as a percentage (mean $\pm$ S.E.M.) of that obtained at the maximal glycine concentration (500 μ M) tested ($n = 10$ –15 determinations per glycine concentration).

tion induced much larger currents that were dependent on the splice variant expressed. These currents were characterized by a fast peak response to glycine followed by rapid decay to a steady state. When normalized to whole-cell capacitance, current densities were highest for NR1-4a/NR3A receptors followed by NR1-3a, NR1-2a, and NR1-1a (Fig. 1C). Whole-cell capacitance was not different between groups (ANOVA, $p = 0.8765$). The increase in current produced by the NR1 C-terminal splice variants was not accompanied by significant changes in apparent glycine affinity. As shown in Fig. 1D, when currents were normalized to the maximum value obtained for each splice variant, the concentration-response curves were virtually identical. Analysis of the glycine dose-response data generated EC_{50} values of 56.5 μ M for NR1-1a, 38.8 μ M for NR1-2a, 33.9 μ M for NR1-3a, and 22.6 μ M for NR1-4a. The EC_{50} values were not significantly different (ANOVA, F test, $p > 0.05$).

The effect of NR1 splice variants on glycine-evoked currents in NR3A-transfected cells was also observed with the NR3B subunit (Fig. 2A). As with NR3A, little to no glycine-activated current was observed when NR3B was expressed with the NR1-1a splice variant. However, robust glycine-

activated currents were produced in cells expressing NR3B and NR1-2a, NR1-3a, and NR1-4a splice variants. As previously shown in oocytes (Awobuluyi et al., 2007; Madry et al., 2007), these currents displayed a rapid decay upon initial exposure to glycine (>10 μ M) followed by a rebound as agonist application was terminated. The complex nature of these currents prevents an accurate quantification of their maximum amplitude across the agonist concentration curve. Normalizing current density at the nondesensitizing concentration of 10 μ M glycine indicated a significant effect of splice variant on NR1/NR3B receptor current density (ANOVA, $p < 0.05$) (Fig. 2B). To quantify current density at glycine concentrations that evoked desensitization (>10 μ M), steady-state current was normalized to the steady-state response at 10 μ M glycine ($I/I_{10 \mu M}$) and then to whole-cell capacitance (Fig. 2C). Current density was observed to be significantly greater for the NR1-2a versus other splice variant at high glycine concentrations (>100 μ M) (ANOVA, $p < 0.0001$).

To determine whether the N-terminal N1 cassette also regulates NR3 receptor currents, we expressed NR1-1b or NR1-4b with either NR3A or NR3B in HEK293 cells. Glycine (100 μ M) evoked robust currents in NR3A and NR3B recep-

Fig. 2. Glycine activation of current expression from HEK293 cells transfected with NR3B and NR1 splice variants. A, representative currents from NR1-1a/NR3B, NR1-2a/NR3B, NR1-3a/NR3B, and NR1-4a/NR3B recombinant receptors during exposure (horizontal bar above traces) to indicated micromolar concentration of glycine. Scale bar, 5 s. B, effects of splice variants on NR1/NR3B receptor current density at 10 μ M glycine. Data represent the mean $\pm$ S.E.M. of 10 μ M glycine steady-state current normalized to whole-cell capacitance ($n = 3$ –11 cells). C, normalized glycine concentration-response relationship for NR1-1a/NR3B, NR1-2a/NR3B, NR1-3a/NR3B, and NR1-4a/NR3B recombinant receptors. Data represent the mean $\pm$ S.E.M. of steady-state current normalized to whole-cell capacitance and expressed as the ratio to current at 10 μ M glycine (I/I_{10}) ($n = 6$ –11 cells per receptor combination). D, current traces from HEK293 cells transfected with NR1-1b/NR3A, NR1-4b/NR3A, NR1-1b/NR3B, and NR1-4b/NR3B. Currents were activated with 100 μ M glycine for 6 s as indicated by bars above traces.

tors containing the NR1-4b splice variant, but little current was observed in cells expressing NR3A or NR3B and NR1-1b (Fig. 2D).

Effects of NR1 C-Terminal Domain Properties. The data presented above demonstrate that current amplitudes of NR3 receptors depend on NR1 splice variants that differ in their C terminus. These C-terminal domains have been labeled C0, C1, and C2 with all NR1 splice variants expressing the C0 domain, whereas C1 and C2 domains are subject to alternative splicing (Zukin and Bennett, 1995). To further explore the modulatory role of these C-terminal domains, a series of NR1 mutants was tested. No glycine-activated currents were observed in cells that coexpressed NR3A and a

NR1 mutant truncated at the end of the fourth transmembrane domain (TM4) (data not shown). Glycine did activate currents in cells expressing NR3A and an NR1 subunit truncated at the end of the C0 domain (NR1 863) that were approximately half the size of those observed for NR1-4a/NR3A receptors (Fig. 3, A and B).

The C1 cassette contains a triple arginine repeat that acts as an endoplasmic reticulum retention motif and limits export of the NR1 subunit (Standley et al., 2000; Scott et al., 2001). To examine the effect of this domain on NR3 currents, these arginine residues (RRR893–895) were mutated to glycine. Coexpression of the mutant NR1(RRR) with NR3A resulted in currents (60–100 pA) that were marginally greater

Fig. 3. Effect of NR1 C-terminal domain on NR1/NR3A current expression. A, representative currents from HEK293 cells expressing NR1-4a/NR3A and mutant NR1-1a/NR3A receptors containing a stop codon at residue 863 of the NR1 subunits. Traces from NR1-1a/NR3A receptors are omitted because glycine application does not result in currents from these receptors. Horizontal bars above traces indicate a 6-s application of 100 μ M glycine. B, concentration-response relationship of wild-type NR1-1a/NR3A, mutant NR1-1a 863/NR3A, and NR1-4a/NR3A receptors. The data represent peak current measurements normalized to whole-cell capacitance and expressed as mean $\pm$ S.E.M. ($n = 6-10$ cells per receptor combination). C, traces from NR1-4a/NR3A and mutant NR1-1a RRR/NR3A receptors. D, summary graph of concentration-response relationship between NR1-4a/NR3A and mutant NR1-1a RRR/NR3A receptors. Data represent peak current measurements normalized to whole-cell capacitance and expressed as mean $\pm$ S.E.M. ($n = 5-7$ cells per receptor combination). E, traces from NR1-4a/NR3A and mutant NR1-1a S897A-, NR1-1a S897D-, and NR1-1a SS896DD-containing NR1/NR3A receptors. F, summary graph of concentration-response relationship between NR1-4a/NR3A and mutant NR1-1a S897A, NR1-1a S897D and NR1-1a SS896DD receptors. Data represent peak current measurements normalized to whole-cell capacitance and expressed as mean $\pm$ S.E.M. ($n = 4-5$ cells per receptor combination).

than those of NR1-1a/NR3A receptors, but were significantly less than those in cells expressing NR1-4a/NR3A subunits (Fig. 3, C and D).

In addition to the RRR motif, the C1 cassette also contains major phosphorylation sites for protein kinase C and protein kinase A, and serine residues at 896 and 897 have been shown to play a role in subunit trafficking (Ehlers et al., 1995; Scott et al., 2001). To determine whether these residues affected NR1/NR3 receptor function, serines 896 and 897 in the NR1 subunit were mutated to amino acids that mimic (S897D, SS896-7DD) or negate phosphorylation (S897A). When combined with the NR3A subunit, glycine activated small currents in these receptors that were only

slightly greater than that of wild-type NR1-1a/NR3A receptors (Fig. 3, E and F).

Receptor Subunit Trafficking. The reduced current density of NR1-1a-containing receptors compared with those expressing NR1-4a could be due to differences in surface expression as observed for conventional NMDA receptors (Okabe et al., 1999). To address this issue, we used BS³ cross-linking analysis to determine the relative surface expression of NR1-1a and NR1-4a. BS³ is a membrane-impermeable cross-linking compound that aggregates surface receptors while leaving intracellular subunits unaffected (Grosshans et al., 2002). Because BS³ cross-linked receptors cannot enter SDS-polyacrylamide gel electrophoresis gels,

the quantity of intracellular and surface pools of receptors can be estimated. HEK293 cells were transfected with NR3A and NR1-1a or NR1-4a splice variants. Receptors expressed at the plasma membrane were cross-linked with BS³, and immunoblots were probed with a pan antibody to NR1. As shown in Fig. 4, untransfected cells showed no signal, whereas prominent bands were present at approximately 120 kDa in lanes for NR1-1a/NR3A and NR1-4a/NR3A in all samples (Fig. 4A). As we expected, the band in NR1-4a/NR3A transfected cells was slightly lower than that for NR1-1a/NR3A because of the shorter C terminus of this splice variant. After cross-linking, signal intensity decreased and this effect was greater for the NR1-4a splice variant compared with the NR1-1a (Fig. 4B). The percentage of the total signal present at the surface (total cross-linked) indicates that significantly more NR1-4a-containing receptors are present at the surface than NR1-1a receptors (Fig. 4C).

Effects of NR1 Glycine Site Mutations and Antagonists. The BS³ analysis suggests that at least some NR1-1a/NR3A receptors are trafficked to the plasma membrane; however, virtually no currents can be elicited from this subunit combination. Previous studies using oocytes show that NR1-1a/NR3A currents are potentiated in the presence of certain NR1 glycine site antagonists, suggesting that these receptors desensitize quickly during glycine activation thus limiting the observed current (Awobuluyi et al., 2007; Madry et al., 2007). To further examine this relationship, NR1 glycine site mutants and glycine site antagonists were tested. Phenylalanine 484 in the S1 domain affects glycine sensitivity in conventional NMDA receptors and substitution of an alanine residue at this site (F484A) results in a ~6000-fold reduction in apparent glycine affinity (Kuryatov et al., 1994). F484A mutations were generated in both the NR1-1a and NR1-4a subunits and expressed along with NR3A in HEK293 cells. Glycine-activated robust and nondesensitizing currents in cells expressing NR3A and either NR1-1a F484A or NR1-4a

F484A (Fig. 5A). However, although the current densities for these two mutant receptors were 3- to 4-fold higher than the wild-type combinations (see Fig. 1C), receptors containing the NR1-4 splice variant still displayed higher currents (Fig. 5B).

In oocytes, NR1 glycine-site antagonists have been reported to inhibit the desensitization of NR1/NR3 receptors; and thus could promote the appearance of these receptor currents (Awobuluyi et al., 2007; Madry et al., 2007). When this was examined in HEK293 cells expressing wild-type NR1/NR3A receptors, the NR1 glycine-site antagonist 7-CK potentiated currents to a similar degree in NR1-1a/NR3 and NR1-4a/NR3A receptors when coapplied with glycine (Fig. 6, A and B). The effect of 7-CK was manifested primarily as an increase in the steady-state component of NR1-1a/NR3A and NR1-4a/NR3A receptor currents. However, the peak current of NR1-4a/NR3A receptors was blocked by 7-CK.

The NR1(F484A) and 7-CK antagonist findings suggest that limiting glycine binding may promote NR1/NR3 receptor activation. In addition to residue Phe484 in the S1 domain of the NR1 subunit, an aspartate residue at position 732 in the S2 domain (D732) directly contributes to glycine binding (Furukawa and Gouaux, 2003). Mutating this residue to asparagine (D732N) reduces glycine affinity by over 14,000 times in conventional NMDA receptors (Williams et al., 1996). NR1-1a and NR1-4a splice variants containing the D732N mutation were generated and expressed along with NR3A in HEK293 cells. Cells expressing NR1-1a(D732N)/NR3A subunits showed little to no glycine-activated current, whereas larger currents were obtained in NR1-4a(D732N)/NR3A receptors (Fig. 6C). It is striking that, when glycine was coapplied with the NR1 antagonist 7-CK in these mutants, extremely large (>1 nA) nondesensitizing currents were generated (Fig. 6C). In both NR1-1a(D732N) and NR1-4a(D732N) containing receptors, currents were potentiated by 4000 to 5000% over wild-type values (Fig. 6D).

Fig. 4. Effects of NR1 splice variants on membrane surface expression. HEK293 cells were sham transfected (con) or transfected with NR1-1a/NR3A or NR1-4a/NR3A and processed as described in *Materials and Methods*. The cross-linker (BS³) was used to label the recombinant receptor pool at the surface, and samples were analyzed by Western blot with anti-NR1. A, representative immunoblots of sham-transfected (con) and NR1-1a/NR3A- and NR1-4a/NR3A-transfected HEK293 cells along with corresponding BS³-treated samples. B, densitometry analysis of band intensity from NR1-1a/NR3A- and NR1-4a/NR3A-transfected tissue. Data represent the mean ± S.E.M. of three separate experiments. C, band intensity expressed as the ratio of BS³-treated to corresponding non-BS³-treated sample. Data represent the mean ± S.E.M. of three separate experiments.

Fig. 5. Influence of glycine sensitivity on NR1/NR3 receptor expression. **A**, representative traces from NR1-1a F484A/NR3A and NR1-4a F484A/NR3A receptors. **B**, summary graph of glycine activation of NR1-1a F484A/NR3A and NR1-4a F484A/NR3A receptors. The data represent steady-state current measurements normalized to whole-cell capacitance and expressed as mean $\pm$ S.E.M. ($n = 3$ –9 determinations per glycine concentration).

Glycine Activation in Cells Expressing NR1, NR2, and NR3 Receptors. A recent study indicates that coexpression of NR1, NR2, and NR3 subunits in oocytes yields separate populations of NR1/NR2 and NR1/NR3 receptors (Ulbrich and Isacoff, 2008). To examine whether NR1 splice variants modulate expression of NR1/NR3 and/or NR1/NR2 receptors in a mammalian expression system, HEK293 cells were transfected with subunit combinations of NR1-4a, NR2A, and NR3A, or NR3B. Coapplication of glutamate (10 μ M) and glycine (100 μ M) induced large currents in cells expressing NR1-4a/NR2A/NR3A (Fig. 7A) and NR1-4a/NR2A/NR3B (Fig. 7B) subunits. When applied alone, glutamate (10 μ M) or glycine (100 μ M) induced smaller currents in both subunit combinations. When APV (100 μ M) was added to block the NR2 glutamate binding site, currents induced by glutamate and glycine were similar to those produced by glycine alone. To determine whether glycine-activated cur-

rents shared properties with that of NR1/NR3 receptors, NR1 glycine-site antagonists 7-CK and 5,7-DCK were coapplied with glycine. Coapplication of glycine and 7-CK (100 μ M) enhanced the steady-state current from HEK293 cells expressing NR1-4a/NR2A/NR3A and NR1-4a/NR2A/NR3B subunits (Fig. 7C). Conversely, 5,7-DCK (10 μ M) inhibited the currents in HEK293 cells expressing NR1-4a/NR2A/NR3A and NR1-4a/NR2A/NR3B subunits (Fig. 7C). Neither the enhancement nor inhibition of steady-state current by these compounds differed by subunit combination.

Discussion

Glycine-activated NR3-containing receptors represent a novel class of receptors. Despite being discovered almost 15 years ago, the physiological role of NR3-containing receptors is still largely unknown. In initial studies, expression of the NR3A subunit in oocytes did not yield functional receptors, suggesting that this subunit may, like classic NMDA receptors, require additional subunits for function (Ciabarra et al., 1995; Sucher et al., 1995). This was indeed found because NR3A and NR3B formed novel glycine-activated channels when combined with the NR1 subunit, and these receptors were unaffected by ligands acting on the glutamate binding site of traditional NMDA receptors (Chatterton et al., 2002). Despite these intriguing findings, robust channel activity of NR3-containing receptors seemed to be restricted to the oocyte expression system, because dimeric combinations of NR1/NR3 subunits expressed in HEK293 cells fail to generate noticeable currents (Smothers and Woodward, 2007). In this study, we show that NR1 splice variants play a major role in the expression of functional NR1/NR3 receptors in mammalian cells. In HEK293 cells, virtually no currents were observed with NR1-1/NR3A and NR1-1/NR3B receptors. In contrast, NR3 subunits coexpressed with NR1-2a, NR1-3a, or NR1-4a splice variants resulted in robust currents similar to those observed in oocytes (Chatterton et al., 2002; Awobuluyi et al., 2007; Madry et al., 2007; Cavara et al., 2009). In HEK293 cells NR1/NR3A and NR1/NR3B receptors displayed rapidly desensitizing currents that were observed with all splice variants. This desensitization was especially complex for NR1/NR3B receptors that showed a prominent rebound in current after termination of agonist application. The magnitude of glycine-activated currents in NR1/NR3 receptors was splice variant-dependent in the order of NR1-1 < NR1-2 < NR1-3 < NR1-4. The effect of NR1 C-terminal domains on NR1/NR3 receptor current contrasts with findings reported by Cavara et al. (2009), where no dependence of C-terminal cassettes were observed with NR1/NR3 receptors in oocytes. The basis for this discrepancy is unknown, but could be due to efficient trafficking of all NR1 splice variants with the oocyte-specific subunit XenU1 (Sloviev and Barnard, 1997) that may obscure C-terminal domain properties. The NR1 splice variant-dependent profile that we observed is consistent with other studies, showing the effect of NR1 C-terminal domain properties on subunit trafficking (Okabe et al., 1999). Cross-linking experiments in the present study showed that a greater percentage of NR1-4a/NR3A receptors were surface-labeled versus that of NR1-1a/NR3A receptors, suggesting that at least some of difference between glycine responses observed in the present study

Fig. 6. Effects of NMDA receptor glycine-site antagonists. **A**, representative traces of glycine activation of NR1-1a/NR3A and NR1-4a/NR3A receptors in the absence and presence of 100 μ M 7-CK. Scale bars, 5 s. **B**, summary graph of 7-CK potentiation of NR1-1a- and NR1-4a-containing receptors. Data represent the mean $\pm$ S.E.M. of the steady-state current from NR1-1a ($n = 3$ cells) and NR1-4a ($n = 7$ cells) containing NR3 receptors. **C**, traces of glycine activation of mutant NR1-1a D732N- and NR1-4a D732N-containing NR3A receptors in the absence and presence of 100 μ M 7-CK. Inset shows glycine activation of NR1-1a D732N/NR3A current. **D**, summary graph of 7-CK potentiation of NR1-1a D732N- and NR1-4a D732N-containing receptors. Data represent the mean $\pm$ S.E.M. of the steady-state current from NR1-1a D732N ($n = 7$ cells) and NR1-4a D732N ($n = 10$ cells) containing NR3 receptors.

are due to differences in trafficking of functional NR1/NR3 receptors.

The lack of functional expression with the NR1-1a subunit combined with the results from cross-linking experiments suggests that few NR1-1a/NR3 receptors are expressed at the surface. However, other studies have shown that NR1-1a/NR3 receptors are expressed at the surface in HEK293 cells (Perez-Otano et al., 2001). It is possible that this lack of functional expression could be due to the rapidly desensitizing nature of these receptors. In a study by Awobuluyi et al. (2007) disruption of NR1 glycine binding enhanced currents by reducing or eliminating agonist-dependent desensitization of NR1/NR3 receptor current. We found that mutation of a phenylalanine (Phe484) that reduces glycine binding to the NR1 subunit resulted in robust currents that showed little to no desensitization. Introduction of the F484A mutation into the NR1-4a splice variant produced a similar increase in current amplitude without altering the difference in the cur-

rent density relationship between NR1-1a and NR1-4a splice variants. This finding suggests that, under normal conditions, wild-type NR1-1a/NR3 receptors are in a highly desensitized state and are effectively undetectable at physiological glycine levels.

Although agonist-dependent desensitization plays an important role in determining the magnitude of glycine-induced currents of NR1/NR3 receptors, no differences in glycine sensitivity between splice were noted as the glycine EC_{50} was similar across all NR1 splice variants. Likewise, the splice variant effect was not due to the presence of the N1 cassette because results were similar whether NR1-1a or NR1-1b variants were used. This finding is consistent with results obtained in oocytes reported by Cavara et al. (2009) and indicates that the expression of NR1/NR3 receptors is independent of inclusion of the N1 cassette.

A number of studies have shown that NR1 glycine-site antagonists can potentiate currents of NR1/NR3 receptors

A NR1-4a/NR2A/NR3A

B NR1-4a/NR2A/NR3B

C

Fig. 7. Activation of NR1/NR3A receptors in the presence of NR2A. A, representative traces from HEK293 cells transfected with NR1-4a/NR2A/NR3A or B, NR1-4a/NR2A/NR3B and activated with 10 μ M glutamate (Glu) + 100 μ M glycine (Gly), Glu alone, Gly alone, Glu + Gly + 100 μ M APV, Gly + 100 μ M 7-CK, and Gly + 10 μ M 5,7-DCK. Traces showing the effect of 7-CK and 5,7-DCK from each respective subunit combination are increased in scale to better show drug action. C, summary of change in steady-state current by 7-CK and 5,7-DCK on NR1-4a/NR2A/NR3A and NR1-4a/NR2A/NR3B recombinant receptors. Data represent the mean $\pm$ S.E.M. of the steady-state current measurement from NR1-4a/NR2A/NR3A ($n = 4$ cells 7-CK; 4 cells 5,7-DCK) and NR1-4a/NR2A/NR3B ($n = 6$ cells 7-CK; 3 cells 5,7-DCK) receptors.

(Awobuluyi et al., 2007; Madry et al., 2007, 2008). In HEK293 cells the NR1 glycine site antagonist, 7-CK, potentiated steady-state currents from NR1-1a and NR1-4a-containing NR1/NR3 receptors to a similar extent. In NR1-4a/NR3A receptors, the potentiation of steady-state current by 7-CK was accompanied by a corresponding block of peak current. A similar action of 7-CK has been reported previously in NR1/NR3A/NR3B receptors (Smothers and Woodward, 2007). This suggests a preferential action of 7-CK to inhibit the NR1 subunit of the NR1/NR3 receptor and is consistent with studies showing that glycine binding to the NR3 subunit results in activation, whereas glycine binding to the NR1 subunit contributes to peak current and promotes desensitization (Awobuluyi et al., 2007; Madry et al., 2007, 2008). Exactly how 7-CK potentiates the currents of NR1/NR3 receptors is currently unknown. When glycine binding was disrupted by mutation of Asp732 residue in the NR1 subunit, we observed large potentiation of the glycine-activated current by 7-CK. Activation of the NR1(D732N)/NR3 receptor is presumably via glycine binding to the NR3 subunit because binding to NR1 is disrupted by the mutation (Furukawa and Gouaux, 2003). The potentiation that we

observed was similar to that shown in oocytes by Madry et al. (2008) who reported a large potentiation of NR1/NR3A currents by coapplication of zinc and the NR1 glycine site antagonist, MDL. Our findings indicate that 7-CK probably acts at a location different from the NR1 glycine binding site to produce potentiation and suggests that other sites are present on the NR1 subunit that can modulate NR1/NR3 receptor properties. This view is consistent with other studies showing zinc and MDL potentiation of NR1/NR3 receptors (Madry et al., 2008) and indicates that these receptors possess unique gating mechanisms with NR1 glycine-site antagonists promoting receptor activation.

In a previous study we reported that expression of NR3 subunits along with NR1-1a and NR2 subunits in HEK293 cells resulted in the appearance of only conventional NMDA receptors (Smothers and Woodward, 2007). However, in this present study, we found that inclusion of the NR1-4a splice variant generated both NR1/NR3 receptor currents and those from conventional NMDA receptors. These NR1/NR3 currents were insensitive to the glutamate antagonist APV, potentiated by 7-CK, and were blocked by the NR1 glycine-site antagonist, 5,7-DCK. This finding is in agreement with

that of a recent study showing that separate populations of NR1/NR3 and NR1/NR2 receptors can form in oocytes (Ulbrich and Isacoff, 2008).

Despite the robust expression of glycine-activated currents in NR1/NR3 transfected HEK293 cells or oocytes, the function and identification of native NR1/NR3 receptors is still questionable (Tong et al., 2008). The results from the present study demonstrate that NR1 splice variants play a prominent role in the functional expression of these receptors and suggest that insults that alter NR1 splice variant expression or brain areas high in NR1 C2' splice variants may promote formation of these receptors (Nagy et al., 2003; Zhang and Diamond, 2009). Demonstration and elucidation of the role of NR1/NR3 receptors in native systems could have important implications for various neurological functions and disease states (Salter and Fern, 2005; Henson et al., 2008).

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