

NMDA Receptors Formed by NR1 in *Xenopus laevis* Oocytes Do Not Contain the Endogenous Subunit XenU1

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ABSTRACT

Activation of N-methyl-D-aspartate-selective ionotropic glutamate receptors (NMDA receptors) requires two agonists, glutamate and glycine. These ligands are thought to bind to the NR2 and NR1 subunits, respectively, apparently ruling out the formation of functional homomeric receptors. However, NMDA-mediated currents are observed when the mammalian NR1 subunit is expressed alone in *Xenopus laevis* oocytes. These currents have been generally ascribed to a functional association between NR1 and the endogenous glutamate receptor subunit XenU1. To determine whether such a functional association does in fact occur, we have isolated cDNAs for both XenU1 and XenU1a, a presumed nonallelic counterpart. We investigated whether the coexpression of either XenU1 or

XenU1a with NR1 in either *X. laevis* oocytes and human embryonic kidney (HEK) 293 cells had any effect on the observed NMDA receptor responses. In oocytes, coinjection of XenU1 with NR1 did not increase the observed currents compared with injection of NR1 alone; similarly, in HEK 293 cells, coexpression of XenU1 and NR1 did not result in the formation of functional channels. We also found no pharmacological or biochemical evidence for interaction between the two subunits. We conclude, therefore, that XenU1 does not associate with the NR1 subunit and that an alternative explanation must be sought for the channels observed when NR1 is expressed alone in oocytes.

Ionotropic glutamate receptors selective for NMDA are hetero-oligomers of NR1, NR2 and, in some instances, NR3 subunits (Dingledine et al., 1999). They are unique in that they require two agonists, glutamate and glycine, to open (Kleckner and Dingledine, 1988; Mayer et al., 1989). The binding sites for these two ligands have been localized by site-directed mutagenesis to the NR2 and NR1 subunits, respectively (Kuryatov et al., 1994; Laube et al., 1997; Anson et al., 1998). Homomeric NMDA receptors should therefore be nonfunctional, but small currents are in fact observed when glutamate and glycine are coapplied to *Xenopus laevis* oocytes injected with the NR1 subunit mRNA alone (Moriyoshi et al., 1991). There are two possible explanations for this observation. First, the NR1 subunit may be forming functional homomeric channels in oocytes. This would require that glutamate can bind to and activate NR1, either at a currently unidentified native site or at an ectopic site (for example the glycine binding site). Alternatively, the NR1 subunit may be combining with an endogenous *X. laevis* subunit to form the observed channels. Such combinations of

recombinant and endogenous *X. laevis* proteins have been observed with other channel subunits expressed in oocytes (Buller and White, 1990; Hedin et al., 1996). The involvement of an endogenous *X. laevis* protein would also explain why the NR1 "homomers" are seen only in *X. laevis* oocytes and not in mammalian cells (Monyer et al., 1992).

The endogenous subunit hypothesis seems to have been generally accepted after it was reported that the mRNA for XenU1, a *X. laevis* glutamate receptor subunit, was expressed at low levels in oocytes (Soloviev and Barnard, 1997). The XenU1 subunit was first classified as a non-NMDA receptor subunit (Ishimaru et al., 1996), sharing the greatest sequence similarity with kainate binding proteins. It was later shown that XenU1 could form functional glutamate-gated channels in association with the *X. laevis* NMDA receptor subunit XenNR1 (Soloviev et al., 1996). These channels had unusual properties, opening in response to kainate and α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid as well as glutamate and requiring glycine as a coagonist (Soloviev et al., 1996). When XenU1 mRNA was identified in oocytes, it was therefore suggested that XenU1 was assembling with the mammalian NR1 subunit to form the observed "homomeric" NR1 channels (Soloviev and Barnard, 1997). A

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ABBREVIATIONS: NMDA, N-methyl-D-aspartic acid; HEK, human embryonic kidney; PCR, polymerase chain reaction; PEI, polyethyleneimine; CGP 39653, D,L-(E)-2-amino-4-propyl-5-phosphoro-3-pentanoic acid; MDL 105,519, (E)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1H-indole-2-carboxylic acid; MK-801, (-)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; 7-CKA, 7-chlorokynurenic acid.

low level of endogenous XenU1 expression would then perhaps account for the relatively small current sizes observed.

To test the validity of this hypothesis, we looked at the effect of coexpressing XenU1 with rat NR1. We expected that if a heteromeric receptor containing XenU1 were responsible for the "homomeric NR1" currents, then it should be possible to recreate these channels heterologously in both oocytes and mammalian cells. We therefore isolated cDNA clones of XenU1 and a 95% identical variant, XenU1a, and looked at the effect of their coexpression with the rat NR1-1a subunit in both *X. laevis* oocytes and human embryonic kidney (HEK) 293 cells.

Materials and Methods

Isolation of the XenU1a cDNA Clone and Generation of the XenU1 Expression Plasmid. The oligonucleotide primers used by Soloviev and Barnard to identify XenU1 mRNA in *X. laevis* oocytes (Soloviev and Barnard, 1997), U and U_{rev}, were used to PCR-amplify a 560-bp fragment beginning at position +50 of XenU1 from a stage 28 to 30 tailbud tadpole head library (Hemmati-Brivanlou et al., 1991). The sequence of the PCR product was confirmed after subcloning into pBSK(-), and showed several discrepancies from the published sequence (Ishimaru et al., 1996). The fragment was subsequently radiolabeled with [α -³²P]ATP and used as a probe to screen the same tadpole library. A total of 1×10^6 plaques were screened from the library, which contained 825,000 independent clones (Hemmati-Brivanlou et al., 1991). Filters containing recombinant plaques were hybridized with the probe at high stringency (1 M NaCl, 50 mM Tris, pH 8.3, 2 \times Denhardt's solution, 0.5% SDS, 100 μ g/ml salmon sperm DNA, and 0.1% sodium pyrophosphate, 50°C). The filters were sequentially washed in 2 \times standard saline/phosphate/EDTA and 0.5% SDS at 60°C, so cDNAs with homology to XenU1 could be identified. Three clones were selected for further study and were plaque-purified, rescued as pBluescript plasmids, and analyzed by restriction endonuclease digestion and sequencing. All three cDNAs represented partial overlapping clones, with 94% identity (89 differences in 1439 bp), to XenU1 (Ishimaru et al., 1996). The cDNA represented by the 3 clones was denoted XenU1a. The first 259 bp of the published XenU1 cDNA open reading frame was not present in any of the isolated clones. This missing 5' segment was obtained by PCR using *Pyrococcus furiosus* polymerase (Stratagene, La Jolla CA), from the tadpole library. The forward primer extended from position +1 to +29 of the XenU1 sequence (this region might therefore not correspond exactly to the actual XenU1a sequence but is entirely within the signal peptide), and the reverse primer was from position +524 to +501 of XenU1a. This fragment was combined with the partial clone using nested-PCR to obtain a full-length clone in the *Kpn*I and *Xho*I sites of pcDNA 3.1(-) (Invitrogen, Carlsbad, CA). The XenU1a cDNA sequence has been deposited in GenBank (accession number AF441126).

An HA tag (sequence YPYDVPDYA) was added to the XenU1a sequence using a tailed PCR primer (coding for the HA tag and a terminal *Xho*I site), paired with a primer 5' to the internal *Eco*RI site. The resulting fragment was digested with *Eco*RI and *Xho*I, and used to replace the equivalent fragment from the XenU1a clone to form XenU1a-HA. Clones with the same sequence as the published XenU1 cDNA were not obtained from the screened *X. laevis* library. A clone coding for the same predicted protein sequence as XenU1 was therefore generated from XenU1a-HA by serial mutagenesis of the 23 sites where XenU1 and XenU1a differed at the amino acid level (but excluding two differences in the putative signal peptide). PCR-based mutagenesis using *P. furiosus* polymerase and digestion with the *Dpn*I restriction endonuclease was used, and the resulting mutations verified by DNA sequencing. For the copurification experiments, hexa-histidine tags were inserted after the HA tag using PCR.

Electrophysiological Studies in *X. laevis* Oocytes and HEK Cells. *X. laevis* oocytes were prepared and maintained as described previously (Hollmann et al., 1993). mRNA was prepared using the T7 mMessage mMachine kit (Ambion, Austin TX). Oocytes were injected with 50 nl of mRNA at a concentration of 0.2 μ g/ μ l. Two to three days after mRNA injection, oocytes were placed individually in a recording chamber and perfused at a rate of \sim 3 ml/min with calcium-free Barth's solution composed of 88 mM NaCl, 1.1 mM KCl, 2.4 mM NaHCO₃, 1.8 mM BaCl₂, and 15 mM HEPES, pH 7.4. Two-electrode voltage clamp recordings were performed using an Axoclamp 2A amplifier (Axon Instruments, Union City, CA). Oocytes were voltage-clamped at -70 mV, and data were acquired and analyzed using software from Axon Instruments.

Standard calcium phosphate coprecipitation techniques were used to transiently transfect HEK 293 cells with plasmids containing NR1-1a, XenU1, and XenU1a as appropriate, and a plasmid carrying the coding sequence for the CD8 surface antigen. 48 h after transfections, cells were labeled with anti-CD8 antibody-coated beads (Dynal, Lake Success NY). Cells were constantly perfused with HEPES-buffered extracellular solution composed of 135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 20 mM sucrose, 10 mM glucose, and 5 mM HEPES, pH 7.4. Whole-cell patch-clamp recordings were made from cells that were labeled with beads. The pipette solution for whole-cell recording was composed of 95 mM CsF, 25 mM CsCl, 10 mM Cs-EGTA, 10 mM QX-314, 5 mM TEA, 5 mM 4-AP, 2 mM NaCl, 2 mM Mg-ATP, and 10 mM Cs-HEPES, pH adjusted to 7.3 with CsOH.

CoPurification Experiments with XenU1, XenU1a, and NR1. These experiments were carried out using XenU1-HA-His and XenU1a-HA-His constructs and rat NR1-1a expressed alone and in combination. Crude membrane fractions were prepared from HEK 293-T cells 48 h after transient transfection using three cycles of homogenization and low speed centrifugation (840g, 15 min, 4°C), the supernatants being pooled and membranes recovered by ultracentrifugation (50,000g, 30 min, 4°C). All further manipulations were carried out at 4°C unless stated. Membranes were solubilized using 1.5% Triton X-100 and 50 mM HEPES, pH 8.0, for 1 h. Insoluble material was removed by centrifugation (100,000g, 60 min), and imidazole added to a final concentration of 20 mM. The samples were then incubated with 1/10th volume Ni-NTA agarose (QIAGEN, Valencia CA), pre-equilibrated in the same buffer, for 1 h with gentle mixing on a rocking table. The beads were then recovered by centrifugation (2000 rpm, 1 min) and washed (4 \times) using the same buffer. Proteins binding to the beads were then eluted by addition of 1.5% Triton X-100, 50 mM HEPES, pH 8.0, and 400 mM imidazole. The eluates were then separated by SDS-polyacrylamide gel electrophoresis and electro-blotted onto Immobilon-P (Millipore, Bedford MA). Immuno-blotting was carried out using a monoclonal α -NR1 antibody (used at 1:500; Chemicon International, Temecula CA) and a monoclonal α -HA antibody (1:1000; Covance Research Products, Berkeley CA). Blots were developed using biotinylated secondary antibodies, the Vectastain ABC kit (Vector Labs, Burlingame, CA) and Sigma Fast DAB peroxidase substrate tablets (Sigma, St Louis, MO).

Radioligand Binding Assays. Membranes for binding assays were prepared as described for the copurification assay, with the addition of three washes in HEPES buffer (40 mM, pH 8.0), followed by centrifugation (50,000g, 30 min). Membranes were resuspended in HEPES buffer containing Triton X-100 (0.05%). All binding assays were performed in triplicate on ice, in a total volume of 500 μ l of binding buffer (10 mM HEPES, pH 8.0), and equilibrated for 2 h before rapid vacuum filtration and 2 \times 3.5-ml washes with cold HEPES buffer (10 mM, pH 8.0, 4°C). Saturation binding experiments with [³H]kainate (58 Ci/mmol; PerkinElmer Life Sciences, Boston MA) were performed using kainate concentrations in the range of 0.05–25 nM. Nonspecific binding was defined by the inclusion of 1 mM glutamate. Separation was onto GF/C filters (Whatman, Maidstone, UK), presoaked for 1 h in polyethyleneimine (PEI; Sigma/RBI, Natick, MA). Displacement of [³H]kainate binding by other ligands

over a range of concentrations was determined using duplicates. Binding experiments with other tritiated ligands were performed using the following ligands and conditions: [^3H]CGP 39653 (48.9 Ci/mmol; PerkinElmer), concentration ~ 30 nM, nonspecific counts defined in glutamate (1 mM), separated on GF/C filters; [^3H]MDL 105,519 (72 Ci/mmol; Amersham Biosciences, Piscataway, NJ), concentration ~ 15 nM, nonspecific counts defined in unlabeled MDL 105,519 (13 μM), separated on GF/C filters; [^3H]MK-801 (23.9 Ci/mmol; PerkinElmer), concentration ~ 50 nM, glutamate (0.1 mM) and glycine (0.1 mM) added to assay, nonspecific counts defined in cold MK-801 (50 μM), incubation for 5 h at 23°C , separated on GF/B filters (Whatman) presoaked for 1 h in PEI.

Results

Preliminary screens for the XenU1 subunit cDNA were carried out using a probe obtained by PCR from a *X. laevis* tadpole head cDNA library (Hemmati-Brivanlou et al., 1991), amplified with the same primers used by Soloviev and Barnard to obtain reverse-transcribed XenU1 mRNA from *X. laevis* oocytes (Soloviev and Barnard, 1997). Using this probe three overlapping clones were identified, corresponding to a single XenU1-like cDNA, designated XenU1a. These clones were missing sequence corresponding to the first 259 bp of the published XenU1 open reading frame. This was subsequently isolated by PCR from the same library, as described under *Materials and Methods*. The complete XenU1a cDNA was 94% identical at the nucleotide level (90 differences in 1437 bp; Fig. 1) to the published XenU1 sequence (Ishimaru et al., 1996). The predicted mature polypeptides were 95% identical (23 differences in 462 residues; Fig. 2). We also generated a clone corresponding to the published sequence of the XenU1 cDNA using PCR mutagenesis and characterized this in parallel with XenU1a (Fig. 1).

We first characterized the binding of various radioligands to combinations of XenU1, XenU1a and rat NR1-1a. The XenU1 subunit has been reported to bind [^3H]kainate with nanomolar affinity, and to bind the NMDA glycine site antagonist [^3H]dichlorokynurenic acid with undefined affinity (Soloviev et al., 1998). Membranes were prepared from HEK 293-T cells transiently transfected with XenU1 alone, XenU1a alone, NR1-1a alone, and XenU1 together with NR1-1a. Ligand binding to these various subunit combinations was first tested with single concentrations of [^3H]kainate (10–30 nM; a non-NMDA receptor agonist), [^3H]CGP 39653 (30 nM; an NMDA receptor glutamate-site antagonist), [^3H]MDL 105,519 (5–15 nM; an NMDA receptor glycine site antagonist), and [^3H]MK-801 (50 nM; an NMDA receptor open-channel blocker). Of the four ligands, no binding was observed in any of the samples with either MK-801 or CGP 39653 (data not shown). Significant levels of [^3H]MDL 105,519 binding were only observed in samples containing the NR1-1a subunit (Fig. 3A). This binding was displaced by glycine (1 mM) and MDL 105,519 (2 μM), but not 100 μM kainate (Fig. 3A).

Similarly, [^3H]kainate binding was observed only in samples containing XenU1, but surprisingly not those containing XenU1a (Fig. 3A). [^3H]kainate binding to XenU1 and XenU1+NR1 was displaced by domoate (2 μM), glutamate (1 mM), and MDL 105,519 (2 μM), but not 1 mM glycine (Fig. 3A). The affinity of kainate binding to XenU1 was determined in saturation binding experiments to be 0.47 ± 0.1 nM ($n = 3$; Fig. 3B). This is similar to the figure of 1.2 nM

reported for XenU1 expressed in COS-7 cells (Soloviev et al., 1998). No significant difference in kainate binding affinity was observed when XenU1 was coexpressed with NR1-1a ($K_d = 0.35 \pm 0.1$ nM, $n = 3$; Fig. 3B). The affinities of glutamate and MDL 105,519 for XenU1 and the XenU1 coexpressed with NR1 were estimated using competition assays. The K_i values for binding to XenU1 alone and XenU1 coexpressed with NR1 were 21 ± 4 and 17 ± 2 nM, respectively, for glutamate ($n = 3$) and 31 ± 10 and 24 ± 8 nM, respectively, for MDL 105,519 ($n = 3$) (Fig. 3C). The lack of detectable [^3H]MDL binding to XenU1 probably reflects differences between the apparent K_i and the actual K_d values. Overall, the coexpression of NR1 and XenU1 seemed to have no effect on the binding of any of the ligands tested.

We next looked at the properties of channels formed from various subunit combinations in *X. laevis* oocytes using injection of in vitro transcribed mRNAs. We first looked at whether the NMDA receptor currents resulting from NR1 expression alone and NR1 coexpression with NR2A differed significantly in their responses to glycine and glutamate site antagonists. Responses to the coapplication of glutamate (100 μM) and glycine (10 μM) were measured in the presence and absence of the NMDA receptor glutamate site antagonist DL-2-amino-5-phosphonopentanoic acid and the NMDA receptor glycine site antagonist 7-chlorokynurenic acid

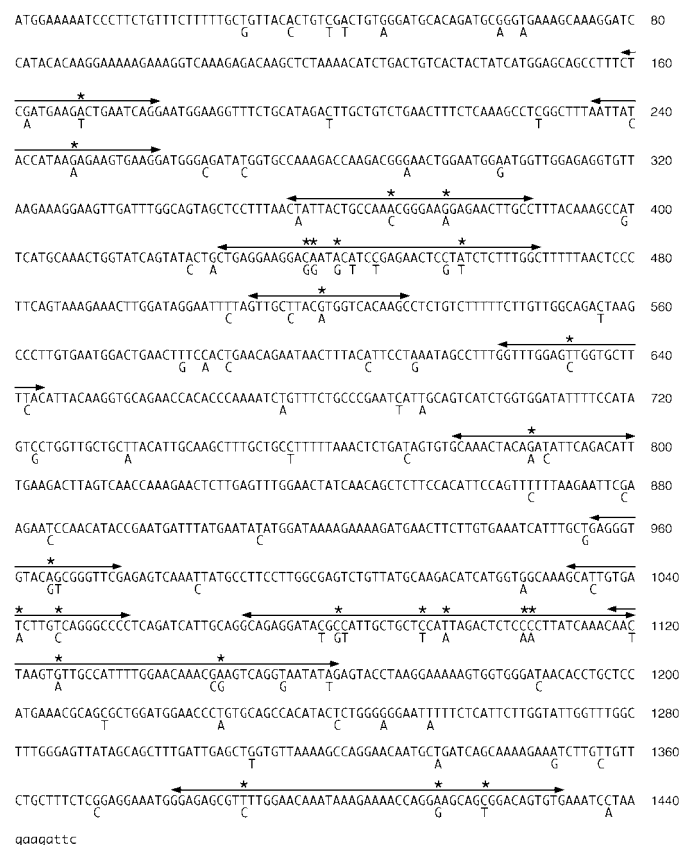


Fig. 1. Nucleotide sequence of XenU1a cDNA. The XenU1 sequence is shown below where this differs from the XenU1 sequence. Double-headed arrows indicate the positions of the PCR primers used in the stepwise mutation of the XenU1a sequence to XenU1, with the changed nucleotides indicated by asterisks. All other nucleotide changes were silent, except for changes affecting two codons within the signal peptide, which were not changed. Nucleotides within the open reading frame are shown in uppercase, and those outside in lowercase.

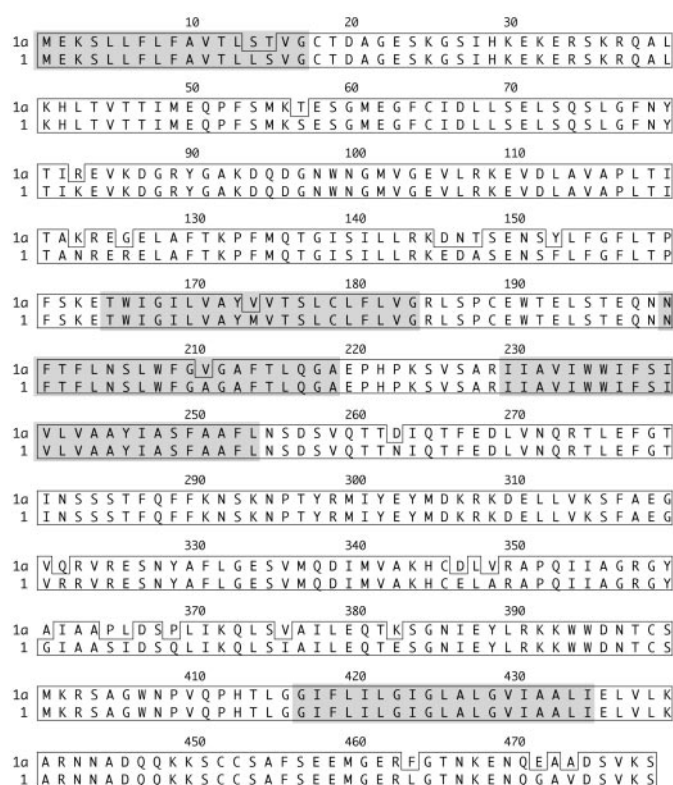


Fig. 2. Alignment of the deduced amino acid sequences of XenU1a (labeled 1a) and XenU1 (labeled 1; GenBank accession X93491). The locations of the predicted signal peptide and the membrane domains M1-M4 are shaded gray according to the assignment of Ishimaru et al. (1996).

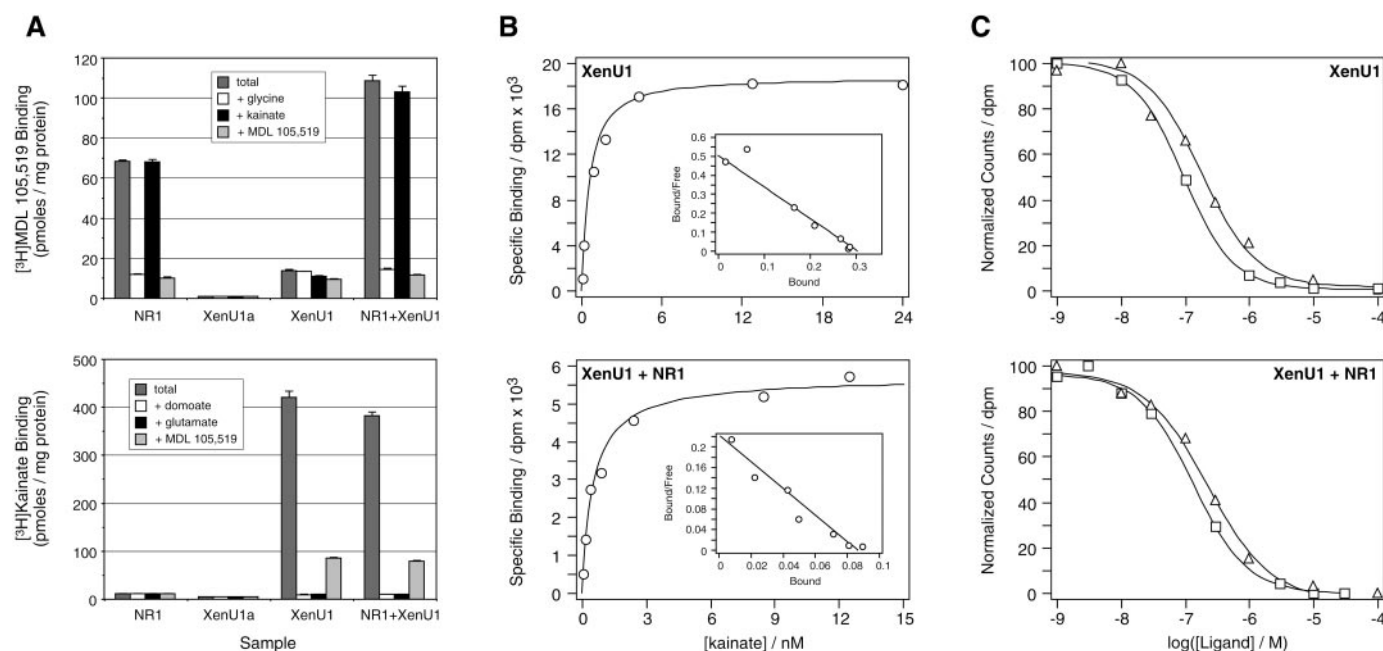


Fig. 3. Binding of [^3H]kainate to membranes isolated from HEK cells transiently transfected with NR1, XenU1a and XenU1 alone, and XenU1 with rat NR1-1a. A, binding of [^3H]MDL 105,519 and [^3H]kainate to isolated HEK cell membranes. The two tritiated ligands were at concentrations of 4.4 and 10.3 nM, respectively, and the concentrations of the ligands used for displacement were 1 mM glycine, 100 μM kainate, 2 μM MDL 105,519, 2 μM domoate, and 1 mM glutamate. Plotted values are the mean \pm S.E.M. from a single experiment with each condition tested in triplicate. B, representative saturation binding curves, with inset Scatchard transformations. Affinities were determined using nonlinear curve fitting to the Hill equation. In the examples shown, the K_d values were 0.59 and 0.51 nM for XenU1 and XenU1+NR1, respectively. C, representative curves showing displacement of [^3H]kainate by glutamate (\square) and MDL 105,519 (\triangle). In the examples shown, the nanomolar IC_{50} values (estimated K_i values in parentheses) for glutamate displacement were 76 (14) and 130 (21) for XenU1 and XenU1+NR1, respectively. The nanomolar IC_{50} values (estimated K_i values) for MDL 105,519 displacement were 210 (50) and 200 (38) for XenU1 and XenU1+NR1, respectively.

(7-CKA) (Fig. 4, A and B). As expected, both antagonists inhibited responses in oocytes injected with NR1 and NR2a with IC_{50} values of 45 μM and 3.4 μM , respectively. Strikingly, in oocytes injected with NR1 alone, DL-2-amino-5-phosphopentanoic acid at concentrations up to 100 μM had no effect, whereas 7-CKA still inhibited the responses to glycine and glutamate coapplication with an IC_{50} of 40 μM (Fig. 4, A and B).

In contrast to the large currents observed when NR1 and NR2A were coinjected into oocytes (720 ± 150 nA, $n = 3$; Fig. 4A), injection of NR1 alone resulted in small currents (5–7 nA, Fig. 4C, Table 1). No currents were ever observed in response to coapplication of glutamate and glycine in oocytes injected with either XenU1 or XenU1a alone ($n > 30$; data not shown), consistent with the data in the original XenU1 cloning article (Ishimaru et al., 1996). If the small currents observed after NR1 injection were the result of association with low levels of endogenous XenU1 or XenU1a, recombinant expression of these subunits should increase the observed currents. No increases in current size were seen, however, when the XenU1 or XenU1a subunits were coinjected with NR1-1a, compared with the currents observed when NR1-1a was injected alone in the same batches of oocytes (Table 1). We also observed no currents in response to coapplication of 50 μM glycine with 200 μM kainate ($n = 5$, data not shown), a combination that activates receptors composed of XenU1 and XenNR1 (Soloviev et al., 1996). To further characterize the observed responses and check for more subtle variations, we constructed dose response curves for both NR1-1a alone, and NR1-1a in combination with XenU1 (Fig. 4D). No significant changes in the EC_{50} of either glutamate or glycine

were observed when the XenU1 subunit was present (Table 1). No currents were ever observed in oocytes expressing NR1 (alone or in combination with XenU1 or XenU1a), when glutamate (100 μM) or glycine (50 μM) were added in the absence of the other coagonist.

Cotransfection of XenU1 and rat NR1 in HEK 293 cells was then tested to determine whether it would result in measurable NMDA receptor responses using whole-cell patch-clamp recordings. The expression of NR1-1a, XenU1, or XenU1a alone gave no identifiable currents in response to coapplication of glycine (50 μM) with either glutamate (100 μM) or

NMDA (100 μM), as expected from previous reports. Coexpression of either XenU1 or XenU1a with NR1-1a similarly gave no measurable responses (data not shown), in a total of nine transfection-positive cells.

Because there are other potential reasons for the absence of currents in HEK cells beyond a simple failure of the subunits to associate, copurification experiments were then conducted using HEK-T cells transiently transfected with NR1 and histidine-tagged XenU1 and XenU1a in combination (Fig. 5). Crude membranes were prepared from the cells, and the receptor subunits solubilized in Triton X-100 as described

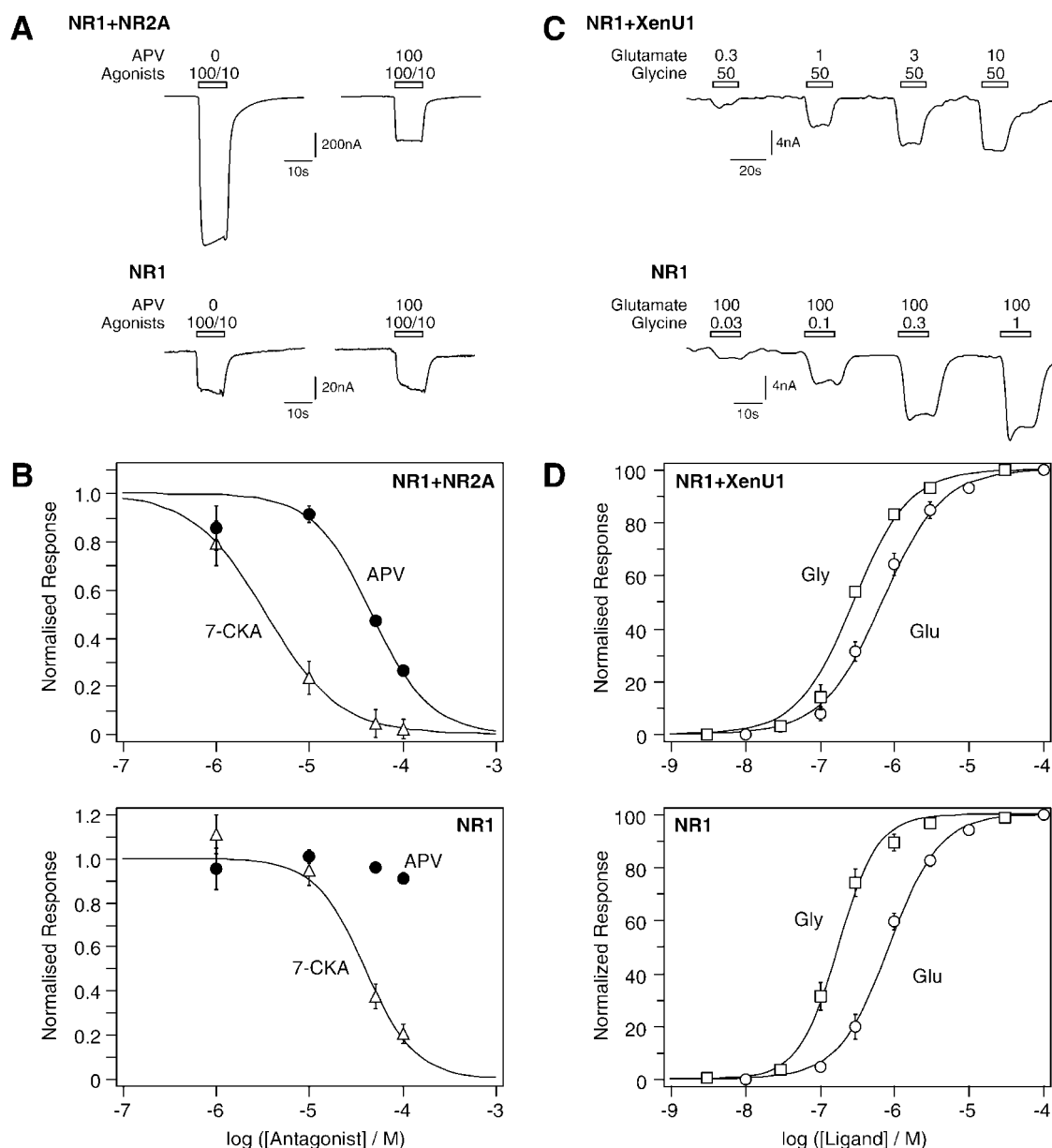


Fig. 4. Properties of currents recorded from *X. laevis* oocytes injected with combinations of subunit mRNAs. **A**, representative recordings from oocytes injected with rat NR1 and NR2A (upper trace) and NR1 alone (lower trace), as described under *Materials and Methods*. Coapplication of agonists (glutamate/glycine, respectively) and APV is indicated by gray bars (concentrations in μM). **B**, inhibition curves for 7-CKA and APV obtained from oocytes injected with rat NR1 and NR2A (upper graph) and NR1 alone (lower graph). Values are the mean \pm S.E.M. of three experiments, normalized to the response to 100 μM glutamate/10 μM glycine in the absence of inhibitors. **C**, representative recordings of voltage-clamped oocytes injected with NR1 coinjected with XenU1 (upper trace) and NR1 alone (lower trace). Coapplication of glutamate and glycine (gray bars) at the indicated concentrations (in nanomolar) resulted in inward currents. **D**, glutamate and glycine dose response curves determined from oocytes injected with NR1 and XenU1 (upper graph) and NR1 alone (lower graph). Glycine (\square) or glutamate (\circ) concentrations were varied whereas the coagonist concentrations were maintained at 100 μM glutamate and 50 μM glycine, respectively. Values were plotted as the mean \pm S.E.M. for n determinations. The EC_{50} values are given in Table 1.

under *Materials and Methods*. Ni-NTA agarose beads were used to purify the His-tagged subunits, along with any associated proteins. The proteins copurified in this way were identified by immunoblot using antibodies specific for NR1 and the HA tag (Fig. 5). Similar results were observed for both XenU1 (Fig. 5A) and XenU1a (Fig. 5B). Whereas XenU1-HA-His, XenU1a-HA-His, and NR1 were all efficiently solubilized (Fig. 5, lanes 1–3), only the His-tagged XenU1 and XenU1a subunits bound to the Ni-NTA resin (Fig. 5, lanes 4–6). The NR1 subunit did not bind to the Ni-NTA beads (Fig. 5, lane 4) and was not copurified when coexpressed with either XenU1 or XenU1a (Fig. 5, A and B, lane 6).

Discussion

Glycine and Glutamate Interact with Different NMDA Receptor Subunits. The original isolation of the NR1 subunit cDNA by expression cloning in *X. laevis* oocytes (Moriyoshi et al., 1991) led to the assumption that the sites for both glutamate and glycine were physically located on NR1. This view was not changed by the subsequent cloning of the NR2 subunits (Meguro et al., 1992; Monyer et al., 1992; Ishii et al., 1993), which were initially viewed as structural

subunits playing a primarily modulatory role (Monyer et al., 1992; Ishii et al., 1993). However, mutations made to the agonist binding domain in NR1 affected receptor activation by glycine but not glutamate (Kuryatov et al., 1994). This suggested that glycine occupied the site on NR1 equivalent to where glutamate was known to bind in non-NMDA receptor subunits (Uchino et al., 1992; Stern-Bach et al., 1994). Glutamate would therefore have to be binding elsewhere in the NMDA receptor complex.

Mutagenesis of the NR2A and NR2B subunits showed that glutamate in fact binds to the homologous site on NR2 subunits (Laube et al., 1997; Anson et al., 1998). Ironically, although clarifying the role of the two subunits in NMDA receptor function, these data called into question the identity of the receptor observed when NR1 is expressed alone in *X. laevis* oocytes. Several lines of indirect evidence favored an association between NR1 and an endogenous oocyte protein. First, the *X. laevis* glutamate receptor subunit XenU1 was proposed to be the endogenous partner to NR1 on the basis of PCR amplification of XenU1 mRNA from oocytes (Soloviev and Barnard, 1997). Second, although initially identified as a non-NMDA receptor subunit, XenU1 was reported to form functional channels in association with the *X. laevis* NMDA subunit, XenNR1 (Soloviev et al., 1996). XenNR1 has been reported to associate with mammalian NR2 subunits to form functional NMDA receptors (Soloviev et al., 1996), and is currently the only identified *X. laevis* NMDA receptor subunit. This strengthens the apparent similarities between XenU1 and NR2, making an association between XenU1 and mammalian NR1 more plausible.

The Ligand Binding Properties of XenU1 and NR1 Are Unaffected by Coexpression. In this study, we set out to determine whether such an association actually occurs. The cDNA clone we originally isolated, XenU1a, was 95% identical to the published clone, XenU1. *X. laevis* is pseudo-tetraploid from an ancient chromosome duplication event and therefore has nonallelic variants of many of its genes (Graf and Fischberg, 1986; Scavo et al., 1991; Bergwitz et al., 1998). It is likely that XenU1 and XenU1a represent such a nonallelic pair. Because either or both of these genes may

TABLE 1

Properties of channels formed in *X. laevis* oocytes by injection of NR1 alone, NR1 co-injected with XenU1 and NR1 co-injected with XenU1a

In each case, a total of 10 ng of mRNA was injected, and recordings were made 2 to 3 days later. Maximal currents were determined using 100 μ M glutamate and 50 μ M glycine. The current sizes for NR1 injected alone were obtained using from the same batches of oocytes as the respective coinjected samples, injected and recorded contemporaneously. Values for half-maximal activation EC_{50} by glycine were determined using 100 μ M glutamate and varied glycine concentrations in the range 3 nM to 30 μ M. EC_{50} values for glutamate were determined using 50 μ M glycine and varied glutamate concentrations in the range 10 nM to 100 μ M. The properties of oocytes injected with NR1 alone or coinjected with a XenU subunit were not significantly different in any instance *t* test.

Experiment	NR1 Alone	<i>n</i>	NR1 + XenU	<i>n</i>
XenU1a				
Current Sizes (nA)	7.3 \pm 0.4	17	7.5 \pm 0.8	19
XenU1				
Current Sizes (nA)	5.1 \pm 0.4	19	5.1 \pm 0.4	18
Glycine EC_{50} (nM)	180 \pm 30	10	260 \pm 40	5
Glutamate EC_{50} (nM)	810 \pm 90	8	650 \pm 100	7

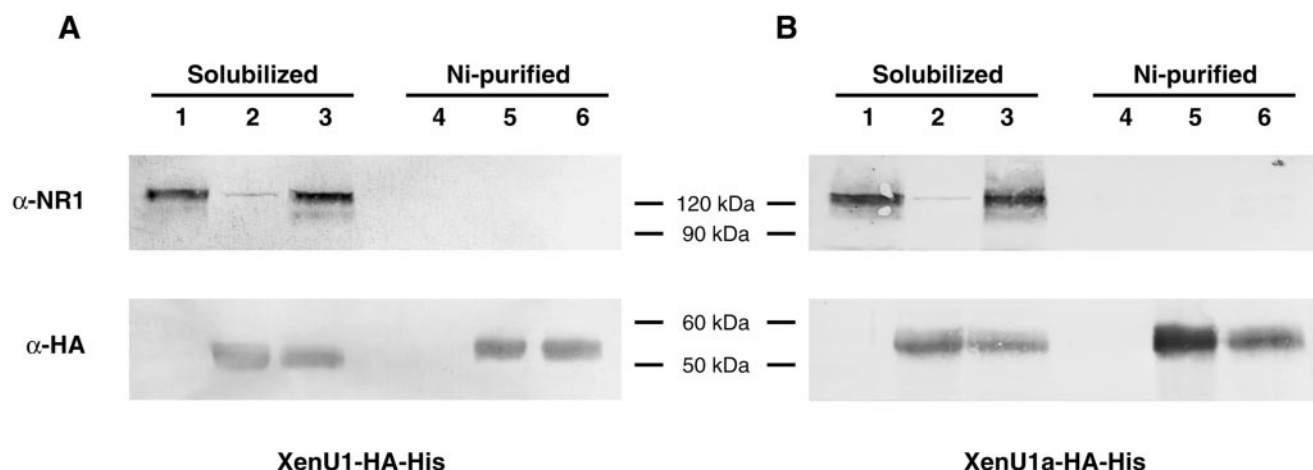


Fig. 5. Copurification assays of XenU1, XenU1a, and NR1 expressed in HEK cells. Experimental details are described under *Materials and Methods*. A, copurification assay with NR1-1a and XenU1-HA-His. Samples after solubilization and before loading on the Ni-NTA agarose were loaded in lanes 1 to 3. Samples eluted from the Ni-NTA agarose were loaded in lanes 4 to 6. Samples were loaded as follows: 1 and 4, NR1-1a alone; 2 and 5, XenU1 alone; 3 and 6, NR1-1a and XenU1-HA-His. The upper blot was developed with α -NR1 antibody, and the lower blot with α -HA antibody. B, copurification assay with NR1-1a and XenU1a-HA-His. Samples were loaded as in A. The position of molecular mass standards is indicated.

represent functional *X. laevis* glutamate receptor subunits, we conducted experiments in parallel with XenU1 and XenU1a where possible. We first characterized the ligand binding properties of XenU1, XenU1a and rat NR1-1a, expressed in HEK 293-T cells both alone and in combination. It is common for heteromeric receptors to display binding profiles that are distinct from their constituent subunits expressed alone. We tested a number of ligands; where binding was observed, we characterized the ability of other ligands to displace it. The NMDA receptor glycine-site antagonist MDL 105,519 bound to membranes containing the NR1 subunit, but the coexpression of XenU1 or XenU1a had no effect on the level of binding, or the ability of kainate, glycine or MDL 105,519 itself to displace this binding. Similarly, kainate bound with high affinity to membranes containing the XenU1 subunit, but coexpression of NR1 had no detectable effect. Both the affinity of the receptor for kainate and the apparent affinities of the receptor for glutamate and MDL 105,519 remained unchanged. Binding profiles therefore gave no indication of an association between NR1 and XenU1.

XenU1/NR1 Heteromers Could Not Be Identified Electrophysiologically or Biochemically. Although changes to ligand binding properties would have demonstrated an association between XenU1 and NR1, the lack of changes does not rule out such an association. We therefore looked for currents in oocytes or HEK cells that could be identified as responses from XenU1/NR1 heteromers. Low-level expression of XenU1 in oocytes has been cited as the reason for the relatively small currents observed when NR1 is expressed (Soloviev and Barnard, 1997). Similarly, the absence of XenU1 in mammalian cell lines has been used to explain the failure of NR1 expression to produce currents (Soloviev and Barnard, 1997). If these explanations are correct, then over-expression of both subunits in oocytes should lead to significantly larger currents than those observed with NR1 expression alone, whereas their coexpression in mammalian cells should also produce functional receptors. No changes in current sizes were seen in oocytes, however, and no currents were observed in HEK293 cells expressing both subunits. We could therefore not identify any currents or changes to currents as a result of coexpressing XenU1 (or XenU1a) and NR1, as would be predicted if XenU1 were the "endogenous subunit".

In a final experiment to identify a physical association between XenU1 and NR1, we used a copurification assay based on the purification of His-tagged XenU1 and XenU1a with nickel-NTA agarose to look for complexes containing both subunits. The NR1 subunit did not bind to the beads when expressed alone, nor did it copurify with XenU1 or XenU1a when it was coexpressed with these subunits. This again suggests that XenU1 and XenU1a do not associate with NR1, functionally or otherwise. Taken together, our data strongly suggest that the currents observed when NR1 is expressed alone in oocytes are not the result of a functional association with endogenous XenU1 or XenU1a.

Homomeric Channels versus Endogenous Subunits. If XenU1 is not the endogenous subunit, then the initial question of the identity of NR1 "homomers" remains. The fact that XenU1 does not associate with NR1 does not necessarily rule out a role for an endogenous *X. laevis* subunit. XenNR1 is the only NMDA subunit identified so far, but it would not

be expected to associate with NR1 because it has already been reported to form functional receptors with mammalian NR2 subunits. There is, however, probably a *X. laevis* counterpart to NR2. A portion of NMDA receptors in *X. laevis* has the unusual pharmacology of XenU1/XenNR1 heteromers, but the rest have a more traditional agonist profile (Soloviev et al., 1996). However, as we have shown with XenU1, if and when other *X. laevis* NMDA subunits are identified, their existence should not be taken as a demonstration of their role in the "homomeric" NR1 receptors.

In the meantime, it is intriguing to speculate whether NR1 may indeed form functional homomeric channels in oocytes as originally supposed. This would require that not just glycine but also glutamate could bind to and gate NR1. There is some evidence suggesting that glutamate can bind to the NR1 subunit. When the NMDA receptor glutamate-site antagonist CGP 55802A was used as a photoaffinity label, polypeptides of approximately 115 and 160 kDa were labeled (Marti et al., 1993). These are the sizes of the NR1 and NR2 subunits, respectively, suggesting that this compound binds to both subunits. Also, tritiated glutamate binds to the NR1 subunit expressed in mammalian cells with a K_d value of ~270 nM (Laurie and Seeburg, 1994), although this binding was not displaced by other NMDA receptor ligands. Other groups using [3 H]CGP 39653 or a lower (10 nM) concentration of [3 H]glutamate reported no binding (Grimwood et al., 1995; Kawamoto et al., 1995), but these reports are not necessarily contradictory. If glutamate binds to NR1, then functional homomeric NR1 receptors are theoretically possible. Glutamate could conceivably be binding to either the same site in the S1S2 domain as glycine, or to a second unidentified binding site (for example in the N-terminal leucine-isoleucine-valine binding protein homology domain). Binding of radiolabeled glycine to NMDA receptors is not detectable, so it cannot be determined whether glutamate can displace glycine from the NR1 subunit. Ultimately, it remains to be determined whether homomeric NR1 receptors do in fact form or whether an endogenous *X. laevis* subunit other than XenU1 is involved.

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References

- Anson LC, Chen PE, Wyllie DAJ, Colquhoun D, and Schoeffer R (1998) Identification of amino acid residues of the NR2A subunit that control glutamate potency in recombinant NR1/NR2A NMDA receptors. *J Neurosci* **18**:581–589.
- Bergwitz C, Klein P, Kohno H, Forman SA, Lee K, Rubin D, and Juppner H (1998) Identification, functional characterization, and developmental expression of two nonallelic parathyroid hormone (PTH)/PTH-related peptide receptor isoforms in *Xenopus laevis* (Daudin). *Endocrinology* **139**:723–732.
- Buller AL and White MM (1990) Functional acetylcholine receptors expressed in *Xenopus* oocytes after injection of *Torpedo* β , γ , and δ subunit RNAs are a consequence of endogenous oocyte gene expression. *Mol Pharmacol* **37**:423–428.
- Dingledine R, Borges K, Bowie D, and Traynelis SF (1999) The glutamate receptor ion channels. *Pharmacol Rev* **51**:7–61.
- Graf JD and Fischberg M (1986) Albumin evolution in polyploid species of the genus *Xenopus*. *Biochem Genet* **24**:821–837.
- Grimwood S, Le Bourdellès B, and Whiting PJ (1995) Recombinant human NMDA homomeric NR1 receptors expressed in mammalian cells form a high affinity glycine antagonist binding site. *J Neurochem* **64**:525–530.
- Hedin KE, Lim NF, and Clapham DE (1996) Cloning of a *Xenopus laevis* inwardly

- rectifying K^+ channel subunit that permits GIRK1 expression of I_{KACH} currents in oocytes. *Neuron* **16**:423–429.
- Hemmati-Brivanlou A, de la Torre JR, Holt C and Harland RM (1991) Cephalic expression and molecular characterization of *Xenopus* En-2. *Development* **111**: 715–724.
- Hollmann M, Boulter J, Maron C, Beasley L, Sullivan J, Pecht G, and Heinemann S (1993) Zinc potentiates agonist-induced currents at certain splice variants of the NMDA receptor. *Neuron* **10**:943–954.
- Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M, Akazawa C, Shigemoto R, Mizuno N, Masu M, et al. (1993) Molecular characterization of the family of the *N*-methyl-D-aspartate receptor subunits. *J Biol Chem* **268**:2836–2843.
- Ishimaru H, Kamboj R, Ambrosini A, Henley JM, Soloviev MM, Sudan H, Rossier J, Abutidze K, Rampersad V, Userwood PNR, et al. (1996) A unitary non-NMDA receptor short subunit from *Xenopus*: DNA cloning and expression. *Recept Channels* **4**:31–49.
- Kawamoto S, Uchino S, Hattori S, Hamajima K, Mishina M, Nakajima-Iijima S, and Okuda K (1995) Expression and characterization of the $\zeta 1$ subunit of the *N*-methyl-D-aspartate (NMDA) receptor channel in a baculovirus system. *Mol Brain Res* **30**:137–148.
- Kleckner NW and Dingledine R (1988) Requirement for glycine in activation of NMDA receptors expressed in *Xenopus* oocytes. *Science (Wash DC)* **241**:835–837.
- Kuryatov A, Laube B, Betz H, and Kuhse J (1994) Mutational analysis of the glycine-binding site of the NMDA receptor: Structural similarity with bacterial amino acid-binding proteins. *Neuron* **12**:1291–1300.
- Laube B, Hirai H, Sturgess M, Betz H, and Kuhse J (1997) Molecular Determinants of agonist discrimination by NMDA receptor subunits: Analysis of the glutamate binding site on the NR2b subunit. *Neuron* **18**:493–503.
- Laurie DJ and Seeburg PH (1994) Ligand affinities at recombinant *N*-methyl-D-aspartate receptors depend on subunit composition. *Eur J Pharmacol* **268**:335–345.
- Marti T, Benke D, Mertens S, Heckendorn R, Pozza M, Allgeier H, Angst C, Laurie D, Seeburg P, and Mohler H (1993) Molecular distinction of three *N*-methyl-D-aspartate-receptor subtypes *in situ* and developmental receptor maturation demonstrated with the photoaffinity ligand ^{125}I -labeled CGP 55802A. *Proc Natl Acad Sci USA* **90**:8434–8438.
- Mayer ML, Vyklicky L, and Clements J (1989) Regulation of NMDA receptor desensitization in mouse hippocampal neurons by glycine. *Nature (Lond)* **338**:425–427.
- Meguro H, Mori H, Araki K, Kushiya E, Kutsuwada T, Yamazaki M, Kumanishi T, Arakawa M, Sakimura K, and Mishina M (1992) Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. *Nature (Lond)* **357**:70–74.
- Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, and Seeburg PH (1992) Heteromeric NMDA receptors: Molecular and functional distinction of subtypes. *Science (Wash DC)* **256**:1217–1221.
- Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N, and Nakanishi S (1991) Molecular cloning and characterization of the rat NMDA receptor. *Nature (Lond)* **354**:31–37.
- Scavo L, Shuldiner AR, Serrano J, Dashner R, Roth J, and de Pablo F (1991) Genes encoding receptors for insulin and insulin-like growth factor I are expressed in *Xenopus* oocytes and embryos. *Proc Natl Acad Sci USA* **88**, 6214–6218.
- Soloviev MM, Abutidze K, Mellor I, Streit P, Grishin EV, Usherwood PN, and Barnard EA (1998) Plasticity of agonist binding sites in hetero-oligomers of the unitary glutamate receptor subunit XenU1. *J Neurochem* **71**:991–1001.
- Soloviev MM and Barnard EA (1997) *Xenopus* oocytes express a unitary glutamate receptor endogenously. *J Mol Biol* **273**:14–18.
- Soloviev MM, Brierley MJ, Shao ZY, Mellor IR, Volkova TM, Kamboj R, Ishimaru H, Sudan H, Harris J, Foldes RL, et al. (1996) Functional Expression of a recombinant unitary glutamate receptor from *Xenopus*, which contains *N*-methyl-D-aspartate (NMDA and non-NMDA receptor subunits. *J Biol Chem* **271**:32572–32579.
- Stern-Bach Y, Bettler B, Hartley M, Sheppard PO, O'Hara PJ, and Heinemann SF (1994) Agonist selectivity of glutamate receptors is specified by two domains structurally related to bacterial amino acid-binding proteins. *Neuron* **13**:1345–1357.
- Uchino S, Sakimura K, Nagahari K, and Mishina M (1992) Mutations in a putative agonist binding region of the AMPA-selective glutamate receptor channel. *FEBS Lett* **308**:253–257.

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