**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 subunit XenU1 and 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 (Kleckenr 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 (Busser 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
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, 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 (Hemmatti-Briavanlou 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 [γ-32P]ATP and used as a probe to screen the same tadpole library. A total of 1 × 10⁶ plaques were screened from the library, which contained 825,000 independent clones (Hemmatti-Briavanlou 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 × Denhardt’s solution, 0.5% SDS, 100 μg/ml salmon sperm DNA, and 0.1% sodium pyrophosphate, 50°C). The filters were sequentially washed in 2 × standard saline/sodium 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 P. furiosus was obtained by PCR using Pyrococcus furiosus, La Jolla CA), from the tadpole library. The forward primer was from position /H11003, and showed several discrepancies from the published sequence (Ishimaru et al., 1996). The fragment was subsequently radiolabeled with [γ-32P]ATP and used as a probe to screen the same tadpole library. A total of 1 × 10⁶ plaques were screened from the library, which contained 825,000 independent clones (Hemmatti-Briavanlou 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 × Denhardt’s solution, 0.5% SDS, 100 μg/ml salmon sperm DNA, and 0.1% sodium pyrophosphate, 50°C). The filters were sequentially washed in 2 × standard saline/sodium 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.

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An HA tag (sequence VYPDVPDYA) was added to the XenU1a sequence using a tailed PCR primer (coding for the HA tag and a terminal XhoI site), paired with a primer 5′ to the internal EcoRI site. The resulting fragment was digested with EcoRI and XhoI, 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 DpnI 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 μg/μl. Two to three days after mRNA injection, oocytes were placed individually in a recording chamber and perfused at a rate of ~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 (Dy neat, 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 CsEGTA, 10 mM QX-314, 5 mM 4-AP, 2 mM MgATP, 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 (840, 15 min, 4°C), the supernatants being pooled and membranes recovered by ultra-centrifugation (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×) 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-polycrylamide 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 μl of binding buffer (10 mM HEPES, pH 8.0, and equilibrated for 2 h before rapid vacuum filtration and 2× 3.5-m1 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 μM 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

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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 receptor 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 ± 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_d 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_d 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 absence and presence of the NMDA receptor glutamate site antagonist DL-2-amino-5-phosphonopentanoic acid and the NMDA receptor glycine site antagonist 7-chlorokynurenine acid.
(7-CKA) (Fig. 4, A and B). As expected, both antagonists inhibited responses in oocytes injected with NR1 and NR2a with IC50 values of 45 μM and 3.4 μM, respectively. Strikingly, in oocytes injected with NR1 alone, dl-2-amino-5-phosphonopentanoic acid at concentrations up to 100 μM had no effect, whereas 7-CKA still inhibited the responses to glycine and glutamate coapplication with an IC50 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 cojected 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 EC50 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 NR1–1α, XenU1, or XenU1α 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 XenU1α with NR1–1α 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 XenU1α 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 ± 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 (●) or glutamate (○) concentrations were varied whereas the coagonist concentrations were maintained at 100 μM glutamate and 50 μM glycine, respectively. Values were plotted as the mean ± 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

<table>
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<tr>
<th>Experiment</th>
<th>NR1 Alone</th>
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<tr>
<td>XenU1a</td>
<td></td>
<td></td>
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<tr>
<td>Current Sizes (nA)</td>
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<td>7.5 ± 0.8</td>
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<tr>
<td>XenU1</td>
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<tr>
<td>Current Sizes (nA)</td>
<td>5.1 ± 0.4</td>
<td>19</td>
<td>5.1 ± 0.4</td>
<td>18</td>
</tr>
<tr>
<td>Glycine EC50 (nM)</td>
<td>180 ± 30</td>
<td>10</td>
<td>260 ± 40</td>
<td>5</td>
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<tr>
<td>Glutamate EC50 (nM)</td>
<td>810 ± 90</td>
<td>8</td>
<td>650 ± 100</td>
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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, ristituate glutamate binds to the NR1 subunit expressed in mammalian cells with a \( K_v \) value of \( \sim 270 \) nM (Laurie and Seeburg, 1994), although this binding was not displaced by other NMDA receptor ligands. Other groups using \(^3H\)CGP 39653 or a lower (10 nM) concentration of \(^3H\)glutamate reported no binding (Grinwood 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.

**Acknowledgments**

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