Revisiting the Postulated "Unitary Glutamate Receptor":
Electrophysiological and Pharmacological Analysis in Two
Heterologous Expression Systems Fails to Detect Evidence for
Its Existence

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ABSTRACT

Several years ago evidence for a so-called "unitary glutamate receptor" was published. This unique type of glutamate receptor was reported to be activated by the traditional agonists of all three major glutamate receptor subfamilies [i.e., α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainate, and N-methyl-D-aspartate (NMDA)] in a glycine-dependent as well as magnesium-blockable manner and was reported to consist of an NR1 subunit coexpressed with the kainate binding protein (KBP) from *Xenopus laevis*, XenU1. To re-examine the existence of such a receptor, we cloned two splice variants of the *X. laevis* NMDA receptor subunit NR1, XenNR1-4a and XenNR1-4b, and expressed them in *X. laevis* oocytes as well as in human embryonic kidney (HEK) 293 cells, either alone or with the *X. laevis* KBP subunit XenU1. In addition, we coexpressed XenU1 separately with all eight splice variants of the rat NR1 subunit. In no case did we see evidence of a unitary glutamate receptor pharmacology. In HEK293 cells, we did not get receptor response unless an NR2 subunit was coexpressed. In *X. laevis* oocytes, we did observe responses to glutamate/glycine as well as small responses to glycine alone, but these were independent of coexpressed XenU1. Neither AMPA nor kainate ever elicited significant responses. Because we verified that XenU1 is expressed and inserted into the plasma membrane of HEK293 cells, we conclude that XenU1 and NR1 do not form the postulated unitary glutamate receptor. Furthermore, successful amplification of a fragment of a *X. laevis* NR2 subunit indicates that *X. laevis* uses NR2 subunits and not XenU1 to form heteromeric complexes with NR1.

Ionotropic glutamate receptors (iGluRs) constitute by far the most abundant excitatory neurotransmitter receptor system in the central nervous system (CNS). Therefore, it was perhaps not surprising that long before the cloning of the first glutamate receptor subunit, GluR1 (Hollmann et al., 1989), a subdivision of the iGluRs into the functionally distinct groups of NMDA and non-NMDA receptors had been proposed based on pharmacological evidence (Watkins and Evans, 1981; Mayer and Westbrook, 1987). Thereafter, additional pharmacologically distinguishable receptor subtypes were identified, which eventually were confirmed at the molecular level when recombinantly expressed cDNAs allowed specific functional analysis of their electrophysiological and pharmacological properties (for review, see Hollmann and Heinemann, 1994; Dingledine et al., 1999; Hollmann, 1999). In addition to the 16 subunits classified as components of either AMPA receptors (GluR1 to GluR4), KA receptors (GluR5 to GluR7, KA1, and KA2) or NMDA receptors (NR1, NR2A to NR2D, NR3A, and NR3B), several additional subunits were identified, which did not assemble into functional ion channels. These included the δ subunits δ1 and δ2 and the KBPs, which so far have been found exclusively in non-mammalian vertebrates such as amphibians, birds, and fish (Henley, 1994; Wo and Oswald, 1995; Hollmann, 1999), with up to two different subunits identified in any one species (goldfish; Wo and Oswald, 1994). The KBPs have the distinction of being only half the size of all other iGluRs (~50 kDa), which sets them aside as a structurally distinct group among the iGluRs.

ABBREVIATIONS: iGluR, ionotropic glutamate receptor; CNS, central nervous system; NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; KA, kainate; KBP, kainate binding protein; HEK, human embryonic kidney; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); EGFP, enhanced green fluorescent protein; NFR, normal frog Ringer; I/V, current-voltage; MK-801, (−)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; DNQX, 6,7-dinitroquinoxaline-2,3-dione.
Materials and Methods

Isolation of the XenNR1-4a cDNA and Construction of the XenNR1-4b and XenNR1-4b(E166G) cDNAs. For the cloning of X. laevis glutamate receptor subunits, three degenerate oligonucleotides were designed as PCR primers based on a sequence alignment of GluK1 (GenBank accession no. X17184), KAT1 (U08257), GluR6 (Z11548), NR1-1a (U08261), and XenNR1 (X94081). These primers, 5'-GGCTWYTGRTSGACCTG-3' (alternatively, 5'-TGGAAYGG-MATGRTKGGMG-3') and 5'-GAARGCWGCARGTTRGC-3' (with K = G/T, M = A/C, R = A/G, S = G/C, Y = C/T, and W = A/T), were used in an RT-PCR of RNA extracted from adult female X. laevis brain as template. A 594-bp fragment of XenNR1 (Soloviev et al., 1996) beginning at position +1425 was amplified. The PCR product was radiolabeled with [α-32P]dCTP using HexaLabel DNA labeling kit (MBI Fermentas, St. Leon-Rot, Germany) and used as a probe to screen X. laevis embryo cDNA library (Stratagene, La Jolla, CA) at high-stringency conditions (125 mM NaCl, 7.5 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 5 × Denhardt’s solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA at 65°C). The nylon filters (Roche Diagnostics, Mannheim, Germany) were washed in washing buffer (50 mM NaCl, 3 mM Tris-HCl, pH 7.4, 0.2 mM EDTA, and 0.1% SDS) at 65°C. One clone was selected for further study and was plaque-purified, rescued as pBluescript plasmid, and analyzed by restriction endonuclease digestion and sequencing. We identified the isolated clone as a full-length NMDA receptor subunit from X. laevis lacking a 63-bp sequence (= exon 5 in rat) in the region encoding the T-terminus, with the published XenNR1 sequence. Further differences to XenNR1 are six single base deviations (positions 120, 557, 756, 2130, 2202, and 2223 of the XenNR1 coding region) of which five do not alter the encoded amino acid. Only the deviation at position 557 alters the amino acid sequence of the encoded protein by replacing glycine 166 of the mature protein by glutamate. Because of the homology of the cloned X. laevis glutamate receptor subunit compared with the NR1-4a splice variant from rat, we called the cloned subunit XenNR1-4a. The XenNR1-4a cDNA sequence has been deposited in GenBank (accession no. DQ666918).

The 63-bp sequence that XenNR1-4a is missing compared with the published XenNR1 sequence (which is the XenNR1-4b splice variant) was confirmed to be expressed in frog brain by performing an RT-PCR on adult female X. laevis brain RNA using primers flanking the 63-bp sequence (5'-ATGCCATCCAGTGCTCTATCTG-3' and 5'-GAGAGATTAATACCTGGTCTCAGT-3'). Fragments of interest were isolated, subcloned into the EcoRV site of pSGEM, and analyzed by sequencing.

For the introduction of the 63-bp sequence into XenNR1-4a, two overlapping tail primers were synthesized: 5'-CTCTGACCGCTATTCTCTATATCTCCTTGCTGCTG-3' and 5'-AGGAAAGTTGGTCGAGGTTCTCATGTTCCTTTTGTGTGCACTTCTCTCTTA-3'. These tail primers were used together with the oligonucleotides 5'-GCTTGAGGCTGAGAGCACAAGCACAAGG-3' and 5'-CATATACACAAAGGTGTGCTGAG-3' as primers in an overlap expansion PCR. The resulting 1354-bp PCR product and XenNR1-4a were digested with BglII and Bpu10I and ligated to produce XenNR1-4b.

Because our XenNR1-4b contained a glutamate at position 166 instead of the glycine reported for the cDNA clone of Soloviev et al. (1996), we introduced a glycine by PCR-mediated mutagenesis with the mutations primers 5'-TTAATCTTTTGACCTTCTTCCCTCTCTAAACAGGTCTCCTCCTATGAGAGGAAAAGAGACTCAAGCACAAGT-3' and antisense primers located in the exons 21 (5'-TCTCTTGAAGGTCGAGAAGGGGAAAGAGTTCTCCTTCTTTTTTACCCAGGTCTCCTCCTATGAGAGGAAAAGAGACTCAAGCACAAGT-3'). The resulting 1354-bp PCR product, which contained the E166G mutation, and XenNR1-4b were digested with BglII and Bpu10I and ligated to produce XenNR1-4b(E166G).

To screen for C-terminally alternatively spliced forms of X. laevis NR1, we performed several RT-PCRs. First, we used oligonucleotides flanking the putative alternatively spliced regions (5'-GAACTCAGCATATGACAGAACACAAGT-3' and 5'-CCAATGATTTAGGGGAAAAGACTGACAG-3'). The resulting 1354-bp PCR product, which contained the E166G mutation, and XenNR1-4b were digested with BglII and Bpu10I and ligated to produce XenNR1-4b(E166G).

For electrophysiological investigation of the isolated XenNR1-4b subunits in X. laevis oocytes and HEK293 cells, the cDNAs were subcloned into the expression vectors pSGEM-KS and pcDNA3 (Invitrogen, Karlsruhe, Germany), respectively.

Tagging of Receptor Subunits. For Western blot analysis, XenU1 (GenBank accession no. DQ073428, isolated in our laboratory previously; Vimmann et al., 1997) was C-terminally tagged with a
myc epitope-encoding sequence and a polyhistidin tag. To create XenU1-myc-His, the complete XenU1 coding region was amplified by PCR using the primers 5′-CCACGGTGCTTGTTCCTTT-3′ and 5′-GAATACTCGTGGCTTTCTACG-3′, replacing the native stop codon by an XhoI restriction site. The PCR product was digested with EcoRV and XhoI and ligated into the EcoRV/XhoI-cut pcDNA4/TTO/myc-His vector (Invitrogen).

For the analysis of the subcellular localization of X. laevis NR1 receptor subunits and XenU1 in HEK293 cells by confocal microscopy, the subunits were C-terminally tagged with EGFP and DsRed2, respectively. The cDNAs of the XenNR1 subunits were subcloned into the XhoI and SacII restriction sites of pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA). The stop codon was deleted by performing a PCR using the primers 5′-GAACCTGACACTGTA-CAGACACA-3′ and 5′-TGATGAGCCGTGCCACATGTTAATACAGAAG-3′, after restriction digest of the PCR product (578 bp) with AgeI and ligation of the fragment into the AgeI-cut XenNR1-4a/4b-pEGFP-N1, producing XenNR1-4a-EGFP and XenNR1-4b-EGFP. To create XenU1-DsRed2, the XenU1 cDNA was isolated by a restriction digest with XbaI and XhoI and ligated into the Nhel/XhoI-cut pDsRed2-N1 vector (BD Biosciences Clontech). The XenU1 C terminus was amplified by PCR using the primers 5′-GATGACACTTCTTGTTAAATC-3′ and 5′-CAGAGATGGGGCTCCATTTGCTAGGCACT-3′. The PCR product (523 bp) was digested with AgeI and ligated into AgeI-cut XenU1/pDsRed2-N2.

cRNA Synthesis. cRNA synthesis was done as described previously (Hollmann et al., 1994). In brief, template DNA was linearized with NheI. cRNA was synthesized from 1 μg of linearized DNA using an in vitro transcription kit (MBI Fermentas) with a modified protocol that uses 800 μM GpppG (MBI Fermentas) for capping and an extended reaction time of 3 h with T7 polymerase. Trace labeling was performed with [α-35S]UTP to allow calculation of yields and evaluation of transcript quality by agarose gel electrophoresis.

Electrophysiological Studies in X. laevis Oocytes. Frog oocytes of stages V or VI were surgically removed from the ovaries of X. laevis (Nasco, Fort Atkinson, WI) anesthetized with 3-amino-benzoic acid ethylster (1.5 g/l; Sigma, Taufkirchen, Germany). Lumps of ~20 oocytes were incubated with 784 U/ml (4 mg/ml) collagenase acid ethylester (1.5 g/l; Sigma, Taufkirchen, Germany). Lumps of oocytes were incubated in free Barth’s solution (88 mM NaCl, 1.1 mM KCl, 2.4 mM NaHCO3, 0.8 mM MgSO4, and 15 mM HEPES, pH adjusted to 7.6 with NaOH) giving a time resolution for equilibration of 10 to 30 ms (Udgaonkar and Hess, 1987). The external buffer consisted of 140 mM NaCl, 4 mM KCl, 2.5 mM CaCl2, and 10 mM HEPES, pH adjusted to 7.2 with NaOH.

HEK293 Cell Transfection. HEK293 cells were transfected with recombinant vector DNA using a modified calcium phosphate precipitation technique (Chen and Okayama, 1987). Exponentially growing cells in polystyrene-coated 35-mm dishes were transfected with 2 to 5 μg of DNA. For precipitation, a mixture of 2 μg of each DNA, 10 μl of CaCl2 (2.5 M), and water (ad 100 μl) was incubated for 20 min at room temperature. Next, 100 μl of 2× HEPES-buffered saline (280 mM NaCl, 1.5 mM Na2HPO4., and 40 mM HEPES, pH adjusted to 7.1 with NaOH) was added, and the mixture was incubated for 20 min at room temperature. Then, 500 μl of cell culture medium (Dulbecco’s modified Eagle’s medium and 10% fetal bovine serum) was added in droplets. The whole mixture was transferred to a 35-mm dish, and the cells were incubated for 8 h at 37°C with 3% CO2. After incubation, the cells were washed twice with 2 ml of phosphate-buffered saline before growth medium was added (for patch clamp and Western blots, minimal essential medium; for confocal microscopy, Earle’s minimal essential medium). After transfection, HEK293 cells were allowed to express receptor for 48 to 96 h at 37°C with 5% CO2.

If the transfected cells were analyzed by the patch-clamp technique, 1 μg of EGFP/pDNA3 was added to the precipitation mixture. Thus, transfected cells could be identified by their green fluorescence when excited at 488 nm.

For Western blot analysis, 85-mm dishes were used. Therefore, the amount of all ingredients of the precipitation mixture was increased 6-fold.

Electrophysiological Studies in HEK293 Cells. Whole cell recordings were performed using a HEKA EPC-9 amplifier (HEKA) controlled by Pulse software (HEKA). Recombinant piptettes were pulled from borosilicate glass (GC150TTL-10; Clarke Electromedical Instruments, Pambourne, UK) using a PIP5 pipette vertical puller (HEKA). Ligand was applied using a theta glass capillary (Hilgenberg) that bathed the suspended cell in a laminar flow of solution, giving a time resolution for equilibration of 10 to 30 ms (Udgaonkar and Hess, 1987). The external buffer consisted of 140 mM NaCl, 4 mM KCl, 2 mM CaCl2, and 10 mM HEPES, pH adjusted to 7.3 with NaOH; the internal buffer was 110 mM Cs gluconate, 20 mM CsCl, 4 mM MgCl2, 1 mM MgSO4, 0.5 mM CaCl2, 5 mM EGTA, and 10 mM HEPES, pH adjusted to 7.4 with KOH. The current responses were measured at room temperature at a holding potential of −60 mV.

Subunit Expression Analysis by Western Blotting. Western blot analysis was performed on HEK293 cells transfected with XenU1-myc-His DNA 48 h after transfection. Cells were washed in phosphate-buffered saline and swelling buffer [10 mM HEPES (pH adjusted to 7.9 with KOH), 1.5 mM MgCl2, 10 mM KCl, and 0.5 mM dithiothreitol] and incubated on ice in swelling buffer afterward. Next, cells were homogenized using a douncer, and then 1/10 volume of stabilization buffer [300 mM HEPES (pH adjusted to 7.9 with KOH), 30 mM MgCl2, and 1.4 M KCl] was added. Nuclei and crude cell fragments were removed by low-speed centrifugation (2500g; 1 min; 4°C). The supernatant was used in an ultracentrifugation (100,000g; 1 h; 4°C), and to the resulting membrane pellet urea buffer (8 M urea, 375 mM Tris-HCl, pH 6.8, and 0.1% SDS) and SDS-polyacrylamide gel electrophoresis loading buffer (25 mM Tris-HCl, pH 6.8, 6% SDS, 800 mM β-mercaptoethanol, 20% glycerol, 0.1% bromphenol blue, and 8 M urea) were added. Then, the sample was incubated in boiling water (10 min). Proteins were separated by SDS-polyacrylamide gel electrophoresis on an 8% gel and electrophoretically transferred on nitrocellulose membranes (GE Healthcare, Little Chalfont.
Buckinghamshire, UK). The nitrocellulose membranes were blocked with 4% nonfat dry milk (Glueckske, Muenchen, Germany) in Tris-buffered saline/Tween 20 (140 mM NaCl, 20 mM Tris-HCl, pH 7.6, and 0.1% Tween 20), and detection of proteins was carried out using mouse anti-my (gift from B. J. Benecke, Ruhr University, Bochum, Germany) and donkey anti-mouse (Dianova, Hamburg, Germany) antibodies. Blots were developed using enhanced chemiluminescence solutions (Pierce Chemical, Rockford, IL).

**Analysis of Subcellular Localization by Confocal Microscopy of Fluorescently Labeled Subunits.** Confocal microscopy was performed on a Leica TCS SP2 (Leica, Mannheim, Germany) laser scanning confocal microscope using a Leica 63×, 1.3 numerical aperture, water immersion lens. HEK293 cells expressing *X. laevis* glutamate receptor subunits were plated on 35-mm glass-bottomed culture dishes and were kept in minimal essential medium with Earl’s salts and 10% fetal calf serum but without phenol red (Sigma). During measurement, the dishes were preserved in a heated microscope chamber (H. Saur, Reutlingen, Germany), which adjusted the temperature to 37°C and the CO2 percentage to 7% permanently. Earle's salts and 10% fetal calf serum but without phenol red (Sigma). Analysis of Subcellular Localization by Confocal Microscopy of Fluorescently Labeled Subunits. Confocal microscopy was performed on a Leica TCS SP2 (Leica, Mannheim, Germany) laser scanning confocal microscope using a Leica 63×, 1.3 numerical aperture, water immersion lens. HEK293 cells expressing *X. laevis* glutamate receptor subunits were plated on 35-mm glass-bottomed culture dishes and were kept in minimal essential medium with Earl's salts and 10% fetal calf serum but without phenol red (Sigma). During measurement, the dishes were preserved in a heated microscope chamber (H. Saur, Reutlingen, Germany), which adjusted the temperature to 37°C and the CO2 percentage to 7% permanently. Colocalization studies were performed using dual excitation and emission filter sets. Specificity of labeling was established by examination of single labeled samples, and signal detection was optimized to ensure absence of signal crossover. For the analysis of the laser scanning confocal micrographs (including identification of colocalization), the Leica software was used.

**Results**

We initially set out to test whether coexpression of the *X. laevis* KBP XenU1 with any of the eight known rat NR1 splice variants (Hollmann et al., 1993) can generate the reported unitary glutamate receptor. For electrophysiological investigation, the eight rat NR1 splice variants were expressed alone and together with XenU1 in *X. laevis* oocytes. To test whether functional glutamate receptors were present, we applied glutamate (100 μM) together with the coagonist glycine (10 μM). Furthermore, we tested for the reported unique unitary glutamate receptor pharmacology by application of kainate (100 μM) or AMPA (100 μM), each with and without glycine. Because it is known that glycine applied alone can generate currents from NMDA receptors (Kleckner and Dingledine, 1988; Moriyoshi et al., 1991; Laube et al., 1993), we additionally applied glycine alone in all experiments. In all oocytes injected with NR1 with or without XenU1, we found significant current responses upon application of glutamate/glycine. Some oocytes showed small currents also upon application of kainate/glycine, AMPA/glycine, or glycine alone, which were all equal in size. By contrast, we never obtained a current response upon application of kainate or AMPA alone (Table 1). We therefore conclude that these small currents were induced by glycine alone without any effect exerted by AMPA or kainate. In addition, we found no significant difference in current amplitudes between oocytes expressing NR1 alone or together with XenU1. This finding was independent of the NR1 splice variant and included the NR1-4b splice variant (Fig. 1), which originally was reported to form ion channels of the unitary glutamate receptor type, at least for the *X. laevis* homolog of NR1-4b (Soloviev et al., 1996).

Although we found no evidence of a unitary glutamate receptor when we combined rat NR1 subunits with XenU1, we could not rule out its existence because Soloviev et al. (1996) had used the *X. laevis* homolog of NR1-4b, XenNR1-4b, in combination with XenU1 when they observed the unitary glutamate receptor properties. To be able to analyze the exact same subunit combination as Soloviev et al. (1996) in their original description of the unitary glutamate receptor, we screened a cDNA library from *X. laevis* embryo for XenNR1 cDNAs using as a probe a 594-bp fragment of a

**Table 1**

<table>
<thead>
<tr>
<th>Expressed Subunit</th>
<th>Gly/Gly (100 μM/10 μM)</th>
<th>KA/Gly (100 μM/10 μM)</th>
<th>AMPA/Gly (100 μM/10 μM)</th>
<th>Gly (10 μM)</th>
<th>KA (100 μM)</th>
<th>AMPA (100 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1-1a + XenU1</td>
<td>6.6 ± 1.1 (10)</td>
<td>0.9 ± 0.3 (6)</td>
<td>0.7 ± 0.3 (6)</td>
<td>0.5 ± 0.2 (6)</td>
<td>0 (6)</td>
<td>0 (6)</td>
</tr>
<tr>
<td>− XenU1</td>
<td>6.6 ± 0.6 (10)</td>
<td>0.2 ± 0.2 (6)</td>
<td>0.3 ± 0.2 (6)</td>
<td>0.2 ± 0.2 (6)</td>
<td>0 (6)</td>
<td>0 (6)</td>
</tr>
<tr>
<td>NR1-1b + XenU1</td>
<td>21.2 ± 2.8 (16)</td>
<td>0.5 ± 0.2 (6)</td>
<td>0.5 ± 0.2 (6)</td>
<td>0.4 ± 0.2 (6)</td>
<td>0 (6)</td>
<td>0 (6)</td>
</tr>
<tr>
<td>− XenU1</td>
<td>17.2 ± 2.4 (17)</td>
<td>0.2 ± 0.2 (7)</td>
<td>0.3 ± 0.3 (7)</td>
<td>0.5 ± 0.4 (9)</td>
<td>0 (6)</td>
<td>0 (6)</td>
</tr>
<tr>
<td>NR1-2a + XenU1</td>
<td>12.2 ± 3.0 (6)</td>
<td>0 (4)</td>
<td>0 (4)</td>
<td>0 (4)</td>
<td>0 (5)</td>
<td>0 (5)</td>
</tr>
<tr>
<td>− XenU1</td>
<td>12.0 ± 3.1 (8)</td>
<td>0.7 ± 0.6 (5)</td>
<td>0.2 ± 0.2 (5)</td>
<td>0.3 ± 0.3 (5)</td>
<td>0 (5)</td>
<td>0 (5)</td>
</tr>
<tr>
<td>NR1-2b + XenU1</td>
<td>7.4 ± 1.4 (10)</td>
<td>0.1 ± 0.1 (6)</td>
<td>0.2 ± 0.2 (6)</td>
<td>0.1 ± 0.1 (7)</td>
<td>0 (6)</td>
<td>0 (6)</td>
</tr>
<tr>
<td>− XenU1</td>
<td>6.7 ± 0.8 (11)</td>
<td>0 (6)</td>
<td>0 (6)</td>
<td>0 (6)</td>
<td>0 (6)</td>
<td>0 (6)</td>
</tr>
<tr>
<td>NR1-3a + XenU1</td>
<td>67.8 ± 9.9 (8)</td>
<td>1.4 ± 0.4 (6)</td>
<td>1.6 ± 0.5 (6)</td>
<td>1.4 ± 0.5 (6)</td>
<td>0 (6)</td>
<td>0 (6)</td>
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<tr>
<td>− XenU1</td>
<td>81.4 ± 7.4 (10)</td>
<td>2.4 ± 0.6 (6)</td>
<td>2.5 ± 0.9 (6)</td>
<td>2.6 ± 0.7 (6)</td>
<td>0 (6)</td>
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<tr>
<td>NR1-3b + XenU1</td>
<td>102.7 ± 23.9 (9)</td>
<td>0.8 ± 0.5 (6)</td>
<td>1.4 ± 1.0 (6)</td>
<td>1.5 ± 1.0 (6)</td>
<td>0 (6)</td>
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<tr>
<td>− XenU1</td>
<td>134.9 ± 26.5 (13)</td>
<td>3.0 ± 1.9 (6)</td>
<td>2.4 ± 1.2 (6)</td>
<td>2.6 ± 1.4 (6)</td>
<td>0 (6)</td>
<td>0 (6)</td>
</tr>
<tr>
<td>NR1-4a + XenU1</td>
<td>10.2 ± 1.7 (8)</td>
<td>1.2 ± 0.6 (7)</td>
<td>0.9 ± 0.5 (7)</td>
<td>1.0 ± 0.5 (7)</td>
<td>0 (6)</td>
<td>0 (6)</td>
</tr>
<tr>
<td>− XenU1</td>
<td>8.9 ± 1.1 (8)</td>
<td>0 (6)</td>
<td>0 (6)</td>
<td>0 (6)</td>
<td>0 (6)</td>
<td>0 (6)</td>
</tr>
<tr>
<td>NR1-4b + XenU1</td>
<td>22.0 ± 1.6 (9)</td>
<td>0.5 ± 0.4 (6)</td>
<td>0.4 ± 0.4 (6)</td>
<td>0.4 ± 0.3 (6)</td>
<td>0 (6)</td>
<td>0 (6)</td>
</tr>
<tr>
<td>− XenU1</td>
<td>19.9 ± 5.5 (11)</td>
<td>0 (6)</td>
<td>0 (6)</td>
<td>0 (6)</td>
<td>0 (6)</td>
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X. laevis NR1 subunit amplified by PCR from X. laevis brain reverse-transcribed total RNA. One clone was identified corresponding to a full-length X. laevis NMDA receptor cDNA. The cDNA lacked a 63-nucleotide sequence in the N-terminal domain compared with the published XenNR1; additionally, six nucleotides differed. Five of these differences did not alter the encoded amino acids (see Materials and Methods), whereas one alternate nucleotide replaced glycine 166 of the mature protein reported by Soloviev et al. (1996) by glutamate. Sequence comparison with the rat NR1 splice variants showed our clone to be the X. laevis homolog of the rat NR1-4a subunit, XenNR1-4a. To obtain the X. laevis NR1 subunit XenNR1-4b, we introduced the missing 63-bp sequence (=exon 5 in rat), which is characteristic for b splice variants (Hollmann et al., 1993), by overlap extension PCR. In addition, we amplified the sequence around the X. laevis exon 5 homolog by RT-PCR from X. laevis brain mRNA and obtained two specific bands (455 and 518 bp), which prove that a and b splice variants were expressed in adult female X. laevis brain. The fact that the 455-bp band was much stronger than the 518-bp band indicates that NR1 a splice variants are more common in X. laevis brain than b splice variants.

To check the X. laevis exon 5-homologous sequence, we subcloned the 518-bp fragment and analyzed it by sequencing. We found our exon 5-homologous sequence to be identical to that reported by Soloviev et al. (1996). However, we confirmed that the G166E amino acid deviation mentioned above was genuine. To screen for further XenNR1 splice variants, we performed several PCRs with oligonucleotides recognizing the sequences upstream and downstream of the C-terminally spliced exons 21 and 22 known from rat NR1 (Hollmann et al., 1993), but we did not obtain evidence for the existence of more alternatively spliced forms of XenNR1. By contrast, if we used a sense primer located upstream of exon 5 homolog by RT-PCR from X. laevis brain mRNA and obtained no specific bands. These results are evidence that in addition to NR1-4, at least the NR1-3 splice variants exist in X. laevis.

It had been reported that the sequence encoding the N-terminal domain of XenNR1 showed significant differences compared with rat NR1, and it had been suggested that these differences alter the receptor properties (Soloviev et al., 1996). Because an extensive characterization of the X. laevis NR1 subunit had never been reported, we tested whether the two isolated XenNR1-4 splice variants are functional subunits and behave similar to mammalian NR1 subunits. We initially expressed XenNR1-4a and XenNR1-4b alone or with the rat NR2B subunit in X. laevis oocytes as well as in HEK293 cells. In oocytes, both homomerically expressed X. laevis NR1 subunits showed small currents upon application of 100 μM glutamate with 10 μM glycine (XenNR1-4a, 3.5 ± 0.5 nA, n = 8; XenNR1-4b, 12.0 ± 1.6 nA, n = 5) and 100 μM NMDA with 10 μM glycine (XenNR1-4a, 1.3 ± 0.4 nA, n = 8; XenNR1-4b, 2.6 ± 1.3 nA, n = 5; Fig. 2A). When we expressed the X. laevis NR1 splice variants together with rat NR2B, the current amplitudes were greatly increased as is found with rat NR1 subunits when coexpressed with NR2B (Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Ishii et al., 1993). We obtained steady-state currents of 1100 ± 218 nA (n = 16) and 5917 ± 166 nA (n = 7) upon application of glutamate/glycine for XenNR1-4a/NR2B and XenNR1-4b/NR2B, respectively, and 411 ± 77 nA (n = 16) and 826 ± 65 nA (n = 7) upon application of NMDA/glycine (Fig. 2B). We then recorded dose-response curves for glutamate and NMDA, of XenNR1-4a/NR2B and XenNR1-4b/NR2B (Fig. 2D). We calculated EC50 values of 2.0 ± 0.3 μM (glutamate) and 60 ± 13 μM (NMDA) for XenNR1-4a/NR2B and 2.8 ± 0.5 μM (glutamate) and 240 ± 25 μM (NMDA) for XenNR1-4a/NR2B. Next, we investigated the NMDA receptor-specific glycine dependence of glutamate-induced currents known from mammalian NMDA receptors (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). We found that XenNR1-4a/NR2B as well as XenNR1-4b/NR2B were activated by glutamate applied alone, but the steady-state currents obtained were relatively small, 10 ± 3 nA (n = 6) and 94 ± 20 nA (n = 9), respectively. Likewise, upon application of glycine alone, small currents were obtained for XenNR1-4a/NR2B (17 ± 5 nA; n = 6) and XenNR1-4b/NR2B (211 ± 74 nA; n = 9). Coapplication of glutamate and glycine increased the current amplitudes for XenNR1-4a/NR2B by approximately 136-fold (1361 ± 446 nA; n = 6) compared with glutamate-induced currents, and for XenNR1-4b/NR2B by approximately 77-fold (7283 ± 658 nA; n = 9; Fig. 2C). Another NMDA receptor-specific property of the X. laevis NR1 splice variants could be shown as we analyzed the glutamate/glycine-induced current flow in the presence and absence of extracellular Mg2+. We found that IV curves recorded in Mg2+-free NFR were linear for both XenNR1 splice variants investigated, whereas in the presence of 1.8 mM extracellular Mg2+ ions the X. laevis NMDA receptor complexes showed rectifying IV with no significant current flow at negative membrane potentials, but linearly increasing currents at positive membrane potentials (Fig. 2E). Further experiments showed that the receptors are not blocked by divalent cations in general but that the receptor complexes containing X. laevis NR1 subunits are highly permeable for Ca2+ ions (data not shown). In addition to the Mg2+ block, X.

**Fig. 1.** Comparison of agonist-induced current responses recorded from voltage-clamped X. laevis oocytes injected with cRNAs of the rat NR1 splice variants NR1-4a and NR1-4b alone and together with XenU1. The experimental details were as described under Materials and Methods. The applied agonist concentrations were 100 μM glutamate, 100 μM KA, 100 μM AMPA, and 10 μM glycine. The application of agonists is indicated by black bars.
laevis NMRA receptors can be blocked by MK-801. A concentration of 1 μM MK-801 was sufficient to reduce the glutamate/glycine-induced current response of XenNR1-4a/NR2B and XenNR1-4b/NR2B to 7 ± 2% (n = 9) and 5 ± 3% (n = 6), of the maximal currents, respectively. All these experiments demonstrate that X. laevis NR1 subunits are functionally equivalent to the homologous subunits from R. norvegicus.

After the functional analysis of the isolated X. laevis NR1 splice variants in X. laevis oocytes, we tested whether the XenNR1 subunits form functional glutamate-gated ion channels also in HEK293 cells. We subcloned XenNR1-4a and XenNR1-4b into the expression vector pcDNA3 and transfected each subunit into HEK293 cells, alone as well as together with rat NR2B. The cells were analyzed 2 to 3 days after transfection by patch-clamp recordings using the whole cell recording mode. In cells transfected only with XenNR1-4a (n = 16) or XenNR1-4b (n = 15), we never detected significant current in response to the application of 100 μM glutamate plus 10 μM glycine. As expected, NR2B alone also did not form functional receptor complexes (Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Ishii et al., 1993). However, when we coexpressed any of the X. laevis NR1 splice variants and rat NR2B, approximately one-third of the patched cells showed a current response upon application of glutamate/glycine. The current amplitudes measured were 71 ± 16 pA for XenNR1-4a/NR2B (n = 13) and 94 ± 29 pA for XenNR1-4b/NR2B (n = 11; Fig. 3).

We then analyzed the electrophysiologic properties of XenNR1-4 splice variants coexpressed with XenU1, the putative unitary glutamate receptor, in X. laevis oocytes as well as in HEK293 cells. As a control, we also expressed the XenNR1 subunits alone. In oocytes, we performed applications of glutamate (100 μM), KA (100 μM), and AMPA (10 μM), each alone and together with 10 μM glycine. In addition, glycine was applied alone (Fig. 4A). We found that XenNR1-4a never showed current responses to KA (with or without glycine), AMPA (with or without glycine), or glycine, neither when expressed alone nor when coexpressed with XenU1. Only application of glutamate/glycine induced small currents in oocytes expressing XenNR1-4a or XenNR1-4a plus XenU1. However, comparing the current amplitudes recorded from XenNR1-4a with those obtained from XenNR1-4a plus XenU1, no significant differences were obtained (Table 2). When we investigated XenNR1-4b, we similarly found no significant current responses upon application of KA (with or without glycine), AMPA (with or without glycine), or glycine. Exclusively glutamate was able to induce currents in the presence of glycine. When we coexpressed XenNR1-4b and XenU1, which is the reported subunit combination of the unitary glutamate receptor (Soloviev et al., 1996, 1998), we found in one of five oocytes tiny currents upon application of KA/glycine (1.4 nA) and AMPA/ glycine (1.5 nA). However, glycine alone induced currents that were equal in size (1.0 nA), whereas application of KA or AMPA alone never induced current responses. Therefore, we conclude these currents to be induced by glycine alone, as

![Fig. 2. Properties of currents recorded from X. laevis oocytes injected with combinations of receptor subunit cRNAs. Experimental details are described under Materials and Methods. Glu, 100 μM glutamate; NMDA, 100 μM NMDA; Gly, 10 μM glycine, unless stated differently. Coapplication of agonists is indicated by black bars. A, representative recordings from oocytes injected with XenNR1-4a (top trace) or XenNR1-4b (bottom trace) cRNAs. B, representative recordings from voltage-clamped oocytes injected with rat NR2B and XenNR1-4a (top trace) or XenNR1-4b (bottom trace) cRNAs. C, representative recordings from oocytes injected with XenNR1-4a plus NR2B (top trace) or XenNR1-4b plus NR2B (bottom trace) cRNAs showing the glycine dependence of glutamate-induced currents. D, glutamate and NMDA dose-response curves determined from oocytes injected with NR2B and XenNR1-4a (top graph) or XenNR1-4b (bottom graph) cRNAs. Glutamate (●) and NMDA (■) concentrations were varied whereas the coagonist concentration was maintained at 10 μM glycine. Values are the means ± S.E.M. of four experiments, normalized to the responses to 100 μM glutamate/10 μM glycine or 1 mM NMDA/10 μM glycine. The EC₅₀ values are given in the text. E, IV curves recorded from oocytes injected with rat NR2B and XenNR1-4a (top graph) or XenNR1-4b (bottom graph) cRNAs. Recordings of all cells were done either in Mg²⁺-free NFR (black curves) or in Mg²⁺-free Ringer’s solution (gray curves). Note absence of outward rectification in Mg²⁺-free NFR.](image-url)

![Fig. 3. Representative current traces from patch-clamped HEK293 cells transfected with X. laevis NR1 splice variants alone and together with rat NR2B. The application of agonists (100 μM glutamate with 10 μM glycine) is indicated by black bars.](image-url)
was also shown above for rat NMDA receptor subunits with
and without XenU1. Glutamate/glycine induced larger cur-
rents (19.2 ± 4.6 nA; n = 9) that however, showed no signifi-
cant difference in their amplitudes compared with the cur-
rents recorded in oocytes expressing XenNR1-4b alone
(17.9 ± 3.1 nA; n = 7; Table 2).

However, as mentioned above, our XenNR1-4b showed a
one-amino acid difference in the N-terminal domain com-
pared with the XenNR1-4b of Soloviev et al. (1996). Because
we cannot rule out that this minor difference has an effect
on the receptor properties, we constructed Soloviev's XenNR1-
4b(E166G) cDNA by PCR-mediated mutagenesis (for details,
see Materials and Methods). This subunit was expressed
alone and together with XenU1 in X. laevis oocytes to test for
pharmacological properties that might indicate the existence
of a unitary glutamate receptor. Whereas XenNR1-
4b(E166G) forms perfectly functional glutamate/glycine-acti-
vated ion channels in oocytes, we could not find any of the
unique unitary glutamate receptor properties. As observed
for our original XenNR1-4b subunit, kainate or AMPA ap-
plied alone never elicited current response. Coapplication of
any of those agonists together with glycine in some oocytes
yielded tiny currents (1.0–1.8 nA), which in every case were
equal in size to currents induced by glycine applied alone
(Table 2). Therefore, we interpret these small responses to
represent pure glycine currents, as has been shown for all
other NMDA receptor subunits investigated. Thus, the
single amino acid deviation at position 166 of the mature
XenNR1-4b protein does not seem to alter the ion channel
properties.

Because we could not detect any of the unique non-NMDA
receptor agonist-induced currents that were reported to be
characteristic for the unitary glutamate receptor, we tried to
find other hints for its existence. However, the lack of AMPA-
duced currents minimized our chances to re-examine func-
tional properties described by Soloviev et al. (1996). Two
characteristics could still be tested: the reported inhibitory
effects of 6,7-dinitroquinoxaline-2,3-dione (DNQX) and
AMPA. We found that currents induced by coapplication of
glutamate (100 μM) and glycine (10 μM) surprisingly were
slightly inhibited by coapplied AMPA (100 μM). However,
this effect was entirely independent of coexpressed XenU1.
Thus, the glutamate/glycine-induced currents were reduced
to 70 ± 3% (n = 5) and 81 ± 6% (n = 5) for XenNR1-4a and
XenNR1-4b plus XenU1, respectively. Likewise, we found the
currents of XenNR1-4b and XenNR1-4b plus XenU1 to be
reduced by coapplication of AMPA to 81 ± 5% (n = 5) and
72 ± 6% (n = 5), respectively. To test whether this inhibitory
effect by AMPA is dependent on the coexpressed XenU1, we
coexpressed the XenNR1 subunits with rat NR2B. In this
experiment, we also found an AMPA inhibition, to 59 ± 4%
(n = 5) and 28 ± 7% (n = 4) for XenNR1-4a/NR2B and
XenNR1-4b/NR2B, respectively. Likewise, an inhibitory ef-
fect of DNQX (50 μM) on currents induced by application of
NMDA (500 μM) plus glycine (10 μM) was observed. Cur-
rents of oocytes expressing any XenNR1-4 splice variant,
with or without XenU1, were significantly decreased when
DNQX was coapplied (XenNR1-4a, 49 ± 7%, n = 5; 
XenNR1-4a + XenU1, 54 ± 4%, n = 5; XenNR1-4b, 63 ± 7%,
n = 5; and XenNR1-4b + XenU1, 62 ± 7%, n = 5). As for
AMPA, this effect was also observed in oocytes expressing
heteromeric NMDA receptor complexes (XenNR1-4a/NR2B,

![Table 2](image_url)

Steady-state currents of X. laevis NR1-4 splice variants alone and together with XenU1

The currents given are the means ± S.E.M. The numbers of oocytes measured are given in parentheses. Note that currents smaller than 1 nA are not detectable in the X. laevis oocyte expression system. Values smaller than 1 nA result from averaging cells showing currents ≥1 nA with cells showing no current response upon application of the same agonist(s). XenNR1-4b(E166G) has the exact same amino acid sequence as the clone used by Soloviev et al. (1996).
Therefore, the inhibitory effects of DNQX and AMPA do not seem to be unique unitary glutamate receptor properties as postulated (Soloviev et al., 1996) but rather are a normal feature of X. laevis NMDA receptors.

Because we did not find any of the reported unique unitary glutamate receptor properties in oocytes, we turned to investigating the existence of this unique receptor type in a mammalian cell line that unlike X. laevis oocytes does not express XenU1 endogenously. We expressed XenNR1-4a as well as XenNR1-4b and XenNR1-4b(E166G) together with XenU1 in HEK293 cells. To rule out that XenU1 generates functional homomeric receptor complexes in HEK293 cells, we also expressed XenU1 alone. However, we never obtained significant current on any cell analyzed, regardless whether it expressed XenU1 alone (n = 13; Fig. 4B) or XenNR1-4a/ XenU1 (n = 30), XenNR1-4b/XenU1 (n = 34). Because Soloviev et al. (1996) based all their evidence for the existence of a unitary glutamate receptor on patch-clamp experiments in HEK293 cells, we additionally investigated electrophysiologically the XenNR1-4b(E166G)/XenU1 subunit combination in HEK293 cells. In none of the cells tested (n = 12) did we get a current response upon application of glutamate/glycine, although XenNR1-4b(E166G) forms functional ion channels in coexpression with rat NR2B (data not shown). Because the lack of current response may have been due to a lack of expression of XenU1 protein, expression was tested by Western blot analysis. Because there is no commercially available antibody against XenU1, we C-terminally tagged the protein with a myc-polysthisidine-tag (for details, see Materials and Methods). The cDNA encoding XenU1-myc-His was transfected into HEK293 cells and 40 h after transfection, we performed a membrane preparation. Membrane proteins were solubilized and separated by SDS-polyacrylamide gel electrophoresis, blotted onto a nitrocellulose membrane, and the presence of XenU1-myc-His was checked using a mouse anti-myc antibody. This experiment confirmed that XenU1-myc-His is expressed in HEK293 cells (Fig. 5). Although lack of XenU1 expression was thus ruled out, it was still possible that XenU1 was not located in the plasma membrane and therefore was unable to interact with XenNR1 to form a unitary glutamate receptor. We therefore investigated the intracellular localization of the X. laevis glutamate receptor subunits by means of confocal microscopy. We C-terminally tagged XenU1 with DsRed2 and the two XenNR1-4 splice variants with EGFP. cDNAs encoding these fusion proteins were transfected into HEK293 cells either separately or in combinations: XenNR1-4a-EGFP, XenNR1-4b-EGFP, XenU1-DsRed2, XenNR1-4a-EGFP plus XenU1-DsRed2, and XenNR1-4b-EGFP plus XenU1-DsRed2 (Fig. 6). We found that XenU1-DsRed2 was strongly expressed in the plasma membrane. When we expressed the EGFP-tagged X. laevis NR1-4 splice variants alone, we found green fluorescence in the plasma membrane as well as inside the cells (Fig. 6A). Upon coexpression of XenNR1 and XenU1, we found that each subunit was localized to the same regions as when it was expressed alone. XenU1 coexpression did not lead to an increase in XenNR1-4 plasma membrane expression or to a reduction in intracellular XenNR1-4 fluorescence (compare Fig. 6, A with B). XenNR1 and XenU1 subunits were colocalized in the plasma membrane (Fig. 6B). Thus, both subunits clearly are in a position to interact to form a heteromeric complex. Nevertheless, as described above, no indication of the unitary glutamate receptor pharmacology could be detected electrophysiologically.

If XenU1 does not interact with NR1 subunits, it is unlikely that XenU1 has the function of an NR2 substitute in X. laevis CNS as had been suggested previously (Soloviev and Barnard, 1997). This means that to generate functional NMDA receptors X. laevis has to express a functional equivalent to mammalian NR2 subunits. Therefore, we expected X. laevis to possess glutamate receptor subunits that are homologous to the rat NR2 subunits. To prove this hypothesis, we screened X. laevis brain cDNA for other glutamate receptors than XenNR1, particularly NR2 subunits. This was done by RT-PCR using the same degenerated primers we previously used for generating the XenNR1 probe. The amplified DNA fragments were subcloned and analyzed by sequencing. We identified fragments of X. laevis glutamate receptor subunits that were homologous to rat GluR1 (=XenGluR1; 489 bp), GluR2 (=XenGluR2; 492 bp), and NR2B (=XenNR2B; 480 bp). Compared with its rat homolog

![Fig. 5. Western blot analysis of a crude membrane preparation (for details, see Materials and Methods) of HEK293 cells expressing XenU1-myc-His. For protein detection, a mouse anti-myc antibody was used.](at_download://content/doi/10.1093/molp/126.4.126/molp12600021.png)

![Fig. 6. Subcellular localization of X. laevis glutamate receptor subunits in HEK293 cells. The white bars in the right corners of the pictures indicate 10 μm. A. representative laser scanning confocal pictures of HEK293 cells expressing XenU1-DsRed2, XenNR1-4a-EGFP, or XenNR1-4b-EGFP. B. representative laser scanning confocal pictures of HEK293 cells expressing the EGFP-tagged XenNR1 splice variants XenNR1-4a (top) or XenNR1-4b (bottom) together with XenU1-DsRed2. Colocalization is highlighted by white dots in the overlay pictures (right column).](at_download://content/doi/10.1093/molp/126.4.126/molp12600022.png)
XenNR2B shows 81.7% sequence identity at the nucleotide level (Fig. 7) and 98% at the amino acid level. These data show that X. laevis actually possesses NR2 subunits and thus does not require XenU1 as an NR2 substitute. Therefore, XenU1 has to be classified as a X. laevis kainate binding protein of unknown function.

Discussion

**Coexpression of the X. laevis KBP XenU1 with Rat NR1 Splice Variants in X. laevis Oocytes Does Not Generate a Unitary Glutamate Receptor.** When Green et al. (2002) re-examined the unitary glutamate receptor, they could not confirm its existence, although they used the same methods as Soloviev et al. (1996): ligand binding studies, electrophysiological analysis in HEK293 cells and additionally in X. laevis oocytes, and coimmunoprecipitation experiments. However, proponents of the unitary glutamate receptor concept could criticize the study by Green et al. (2002) based on the fact that they used exclusively rat NR1-1a, which is a different NR1 splice variant and originates from a different species compared with the one used in the original reports (Soloviev et al., 1996, 1998). To rule out the existence of any possible splice variant-dependent interaction between NR1 and XenU1, we tested eight rat NR1 splice variants (Hollmann et al., 1993) in our attempts to re-examine the postulated unitary glutamate receptor. However, none of the splice variants showed a hint of the reported unique unitary glutamate receptor pharmacology. We only confirmed the well-known glutamate/glycine-induced currents and additionally observed tiny currents induced by glycine alone, the latter of which were never observed by Soloviev et al. (1996).

Because we used rat NR1 subunits in this splice variant comparison, we could not rule out a possible species-dependent interaction at this point.

**Structural, Functional, and Subcellular Localization Analysis of XenNR1-4a and XenNR1-4b with and without XenU1 Fails to Detect Evidence for the Existence of the Unitary Glutamate Receptor.** To investigate the exact same subunit combinations from the exact same species as used by Soloviev et al. (1996), we cloned two X. laevis NR1 splice variants: XenNR1-4a and XenNR1-4b. These subunits were identical to the published XenNR1 (Soloviev et al., 1996), except for one amino acid in position 166 in the N-terminal domain. The additional construction and electrophysiological investigation of XenNR1-4b/E166G, which is a clone encoding the exact same amino acid sequence as was used by Soloviev et al. (1996), showed that this single-amino acid deviation does not alter any pharmacological properties and did not lead to the formation of a unitary glutamate receptor when coexpressed with XenU1. Contrary to Soloviev et al. (1996), we found the a splice variant to be more abundant in X. laevis CNS than the b variant. A similar relationship had previously been shown for mammalian NR1 subunits (Sugihara et al., 1992; Hollmann and Heinemann, 1994). We confirmed the finding of Soloviev et al. (1996) that the N terminus of XenNR1 is 13% different from rat NR1. This sequence difference was suggested to alter the functional properties of the NR1 subunit, thus enabling XenNR1 to interact efficiently with XenU1 (Soloviev et al., 1996). Because a full characterization of XenNR1 has not been reported, we extensively characterized the functional properties of XenNR1-4a and XenNR1-4b. We found no functional differences between the X. laevis NR1 subunits and their rat homologs. The XenNR1 splice variants in our hands showed all the properties reported for rat NR1 subunits, such as activation by glutamate/glycine, NMDA/glycine, and glycine alone (Moriyoshi et al., 1991); glycine dependence of the induced currents (Kleckner and Dingledine, 1988; Laube et al., 1993); interaction with NR2 subunits (Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992); similar EC50 values; linear I/V curves in the absence of Mg2+; and block by MK-801 (Hollmann et al., 1993). In addition, we found two alleged unique unitary glutamate receptor properties (Soloviev et al., 1996) to occur in both X. laevis and rat NMDA receptors in the absence of XenU1: although AMPA and DNQX indeed antagonize agonist-induced currents at XenNR1 plus XenU1, the same effect can be demonstrated for XenNR1 expressed alone as well as in coexpression with rat NR2B. We never saw current upon application of non-NMDA receptor agonists in oocytes expressing XenNR1 with or without XenU1. In HEK293 cells, we never detected currents upon application of glutamate/glycine, although the cells were transfected with the same subunit combination, XenNR1-4b plus XenU1, which Soloviev et al. (1996) had used in their studies in HEK293 cells where they had observed currents. We also recorded from cells transfected separately with XenNR1 (n = 31) and XenU1 (n = 13) as negative controls and never obtained...
currents. By contrast, Soloviev et al. (1996) detected in one of 27 and two of 89 cells unexplained currents for “homomeric” XenNR1 and XenU1 receptors, respectively (Soloviev et al., 1996). This is surprising because NR1 subunits expressed homogeneously in mammalian cell lines in other studies have not been reported to form functional ion channels (Dingledine et al., 1999). Likewise, it had been reported that XenU1 does not form functional ion channels alone (Ishimaru et al., 1996), a property that is matched by the other five known KBPs (Henley, 1994; Hollmann, 1999). Using confocal microscopy, we showed that the lack of function in our experiments is not caused by a lack of expression. We demonstrated that XenNR1 and XenU1 are expressed in the plasma membrane and thus are in a position to interact to form the postulated unitary glutamate receptor. Therefore, as the subunit combinations XenNR1-4a plus XenU1 and XenNR1-4b plus XenU1 used by Soloviev et al. (1996) fail to generate a unitary glutamate receptor pharmacology despite proven membrane expression of all three subunits and proven functionality of XenNR1-4a and XenNR1-4b, we conclude that NR1 subunits do not form the postulated unitary glutamate receptor upon coexpression with XenU1.

The X. laevis KBP XenU1 Does Not Replace NR2 Subunits in the CNS of X. laevis. After Soloviev et al. (1996) postulated that XenU1 can interact with NR1 subunits to generate the unitary glutamate receptor, it was suggested that XenU1 may be a substitute for NR2 subunits in X. laevis CNS (Soloviev and Barnard, 1997). This hypothesis was supported by PCR-mediated screening experiments for X. laevis NR2 subunits, which did not lead to the identification of any such subunits (Soloviev and Barnard, 1997), despite evidence for their existence from the observed crossreactivity of an anti-rat NR2 antibody with X. laevis CNS proteins (Soloviev et al., 1996). However, when we used the same strategy as Soloviev and Barnard (1997) in screening experiments to identify an NR2 homolog in X. laevis brain, we did identify a fragment of a X. laevis NR2B subunit, which we termed XnNR2B. Likewise, in contrast to the report by Soloviev et al. (1996), our screening experiments revealed additional XenNR1 splice variants: a X. laevis homolog of the alternatively spliced exon 21 was identified, which proved the existence of XenNR1-3 splice variants. This indicates that X. laevis NR1 is likely to have the same gene structure and probably undergoes the same splicing events as known from rat NR1 (Hollmann et al., 1993). In addition, the existence of several other X. laevis glutamate receptor subunits has been verified: XenGluR1 and XenGluR2 (both in our laboratory; R. Trippe and M. Hollmann, unpublished observations); XenGluR5, and XenGluR6 (Ishimaru et al., 1996). Thus, it is possible to classify functional X. laevis glutamate receptor subunits just like mammalian receptors into AMPA, kainate, and NMDA receptor subfamilies (Hollmann, 1999). XenU1 belongs to the subfamily of the KBPs, known from nonmammalian vertebrates such as fish, birds, and amphibians. This is supported by several distinct properties that XenU1 shares with other KBPs (Henley, 1994), such as the high affinity for kainate and AMPA (Ishimaru et al., 1996; Soloviev et al., 1996), a glutamate receptor-untypical short N terminus (Ishimaru et al., 1996), low sequence homology with other glutamate receptor subunits (Hollmann, 1999), and functional ion pore domains (Villmann et al., 1997), despite the fact that full-length KBPs apparently do not form functional homomeric ion channels. The fact that sequence identity of XenU1 is only 67.9% compared with another amphibian KBP (from Rana pipiens berlandieri) is not surprising because the sequence identities of KBPs in general are very low; even the two KBPs known from one species (goldfish) only share 60.9% sequence identity (Wu and Oswald, 1994; Hollmann, 1999). Therefore, the low sequence homology of XenU1 compared with other KBPs is no reason to classify XenU1 differently as postulated in Ishimaru et al. (1996) and Soloviev et al. (1996). In addition, if a unitary glutamate receptor exists, it is very unlikely that XenU1 is involved in its formation because most experiments that provided evidence for such a receptor type were done in the mammalian CNS (Jahr and Stevens, 1987) where no KBPs or XenU1 homologs have ever been identified (Hollmann, 1999). Thus, although the X. laevis KBP XenU1 is definitely not involved in the formation of a unitary glutamate receptor, its physiological role still poses an interesting question and remains to be elucidated.

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