Characterization and Comparison of the NR3A Subunit of the NMDA Receptor in Recombinant Systems and Primary Cortical Neurons

YASNORY F. SASAKI, THOMAS ROTHE, LOUIS S. PREMKUMAR, SAUMYA DAS, JIANKUN CUI, MARIA V. TALANTOVA, HON-KIT WONG, XIANDI GONG, SHING FAI CHAN, DONGXIAN ZHANG, NOBUKI NAKANISHI, NIKOLAUS J. SUCHER, AND STUART A. LIPTON.

INTRODUCTION

We and others recently reported the identification of a new N-methyl-D-aspartate receptor (NMDAR) subunit, NR3A (Ciabarra et al. 1995; Sucher et al. 1995). In recombinant systems, NR3A expressed alone does not form functional channels. However, the inclusion of NR3A with NR1 and NR2 in heteromultimeric channels results in a smaller unitary conductance than NR1/NR2 channels (Das et al. 1998). Consistent with this finding, in NR3A-deficient mice, NMDA-evoked currents of cortical neurons are larger than in wild-type littermates. Interestingly, NR3A knock-out mice manifest an increase in the number of dendritic spines in layer V of the cerebral cortex, possibly because of the increased NMDA-mediated current (Das et al. 1998). Several questions, however, remained unanswered in previous studies of this new subunit. For example, the biochemical experiments on the rodent brain did not allow us to determine whether NR3A could associate individually with NR1 and NR2 subunits; only experiments on transfected cell lines would allow this level of sophistication. Second, a more detailed kinetic and permeation analysis to determine how NR3A affects single-channel activity is needed to begin to decipher the mechanism of action of this subunit. Third, we knew that NR3A mRNA was expressed mainly in young postnatal rodents (Ciabarra et al. 1995; Sucher et al. 1995), but a detailed developmental time course of NR3A at the protein level has been lacking until now. Moreover, a comparison of protein levels of NR3A with functional expression by monitoring NMDA-evoked currents at various developmental stages is necessary to begin to characterize the putative role of NR3A in synaptogenesis. Finally, the question of other isoforms of NR3A has not been adequately addressed to date. Here we attempt to answer these outstanding questions with regard to NR3A to offer a more definitive characterization of this recently described NMDAR subunit.

METHODS

Polyclonal and monoclonal anti-NR3A antibodies

The polyclonal NR3A antibody was previously described (Das et al. 1998). In addition, we used a monoclonal antibody generated against an NR3A–glutathione-S-transferase (GST) fusion protein, as described elsewhere (H.-K. Wong, X.-B. Liu, M. F. Matos, S. F. Chan, J. S. Trimmer, N. Nakanishi, I. Pérez-Otaño, S. A. Lipton, E. G. Jones, and N. J. Sucher, unpublished observations).

COS-7 cell expression

A myc epitope (EQKLISEEDL) was inserted into NR3A cDNA at residue 80 in the coding region in order for the tagged molecule to be translated in COS-7 cells.
recognized by an additional antibody against myc (Santa Cruz). myc-NR3A was cloned into the mammalian expression vector GW1-CMV (British Biotechnology). Expression vectors containing NR1–1a and NR2B cDNAs were obtained from Dr. M. Sheng. COS-7 cell transfection and lystate preparation were performed as described (Sheng et al. 1994).

### Immunoprecipitation and immunoblotting

Immunoprecipitation was carried out by incubating lysates (600 μg of synaptic membranes) with the primary polyclonal NR3A antibody (10 μg/ml) at 4°C for 1 h (Das et al. 1998). Immunoprecipitates were eluted from protein A sepharose in SDS sample buffer, and immunoblotting was performed as we have described using peroxidase-conjugated anti-rabbit IgG (Jackson Immunochemical) and enhanced chemiluminescent reagents (Amersham) (Das et al. 1998). The same amount of protein was applied to each lane.

### Single-channel recordings and analysis in the oocyte expression system

Single-channel activity of NMDA receptors was recorded with the patch-clamp technique in the outside-out configuration from excised patches of *Xenopus laevis* oocytes, as detailed elsewhere (Das et al. 1998; Premkumar and Auerbach 1997a,b). In brief, oocytes were injected with 50 nl of cRNA (1 μg/ml) for NR1–1a, NR2A, or NR3A (or NR3A-2, the longer splice variant) at ratios varying from 1.1:1 to 1:1.5, as we previously described in Das et al. (1998). Approximately 4 days after injection, oocytes were exposed to a hypertonic solution to remove the vitelline layer. Currents were activated by rapid (≤2-ms exchange time) superfusion of 10–20 μM NMDA plus 10 μM glycine. Patch pipettes contained (in mM) 90 Na gluconate, 10 NaCl, 4 HEPES, 0.25 GTP, and 10 bis-/H11011/-aminophenoxy) /H9262/-KCl, pH 7.3. The extracellular solution was comprised of (in mM) 100 NaCl, 2.5 KCl, 5 HEPES, and 1.5 EGTA, with no added CaCl2; pH 7.3. EGTA was omitted when [Ca2+]0 was raised to 1–10 mM for assessment of Ca2+ permeability ratios. In some experiments, 1 mM Mg2+ was added. All electrophysiology experiments were performed at room temperature. Recordings were made with an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). Patches were superfused with the agonist solution for several minutes before recording to establish a steady-state level of activity. The currents were digitized at 94–100 kHz and stored in video format. For analysis, currents were played back later in pClamp version 6.0.3 (Axon Instruments, Foster City, CA), filtered at 2 kHz (≤3 dB frequency with an 8-pole low-pass Bessel filter; Frequency Devices, Haverhill, MA), and digitized at 10 kHz. All points amplitude histograms were generated for each continuous segment of data to be analyzed (≤5 min duration) and fitted with Gaussian functions using Origin software. Typically, stretches with ≤1,000 openings were analyzed. Single-channel data were analyzed with a β-test version of the TAC program (TAC X4.0.1; created in the HHMI/Yale University laboratory of Dr. Fred J. Sigworth by Dr. Lalitha Venkataramanan, available through Bruxton Corp.). The amplitude histogram of single-channel openings was constructed by an event-detecting program (TAC) using a fixed threshold of ≥2 times the SD of the noise, which by serendipity was virtually identical to the 50% threshold value. Channel kinetics were analyzed using log-likelihood algorithms (the Yale Hidden Markov Model program of TAC), which directly fits a kinetic model to acquired data. The advantage of this method is that possible signal distortion by filtering is digitally corrected by an inverse filter using an averaged step response obtained under the same recording conditions. Resolved channel events were examined first by a stability test, and their durations were corrected according to the methods of Colquhoun and Sigworth (incorporated in the TAC program). Events shorter than 200 μs (≤1.5 × rise time) were excluded before constructing open- and closed-time histograms. These histograms were fitted in the TACfit program with one or more exponential components to the maximum likelihood values. Closed times were analyzed in patches in which no double openings were observed over at least a 10-min recording period. For the assessment of Ca2+ permeability, single-channel mean current amplitudes in response to NMDA were determined from all-points histograms constructed for each holding potential using several patches. These values were used to plot each point of the current-voltage (I-V) relationship in low Ca2+ (2 mM CaCl2) and high Ca2+ (10 mM CaCl2) extracellular solutions in the absence of calcium chelators or magnesium ions. Calculations were corrected for a liquid junction potential of 3.5 mV. Using the change in reversal potential observed in the presence of different concentrations of Ca2+, the permeability ratio of Ca2+ to the monovalent ions Na+ and K+ (Pc/Pos) was calculated with an equation derived from the extended Goldman-Hodgkin-Katz equation, assuming

\[
Pc/Pos = \frac{M_o(1 - [exp(Ei - Eo)/(RT/F)])}{Ei - Eo}/[4Ca_o - 4Ca_i]
\]

where \( M_o \) is the concentration of extracellular Na+ and K+ (the only monovalent cations present under these conditions), \( E_i \) and \( E_o \) are the reversal potentials measured in extracellular Ca2+ concentrations \( C_{a_o} \) and \( C_{a_i} \), \( R \) is the Universal Gas Constant, \( T \) is absolute temperature, and \( F \) is Faraday’s Constant.

### Whole cell recordings of transfected HEK 293 cells

For expression in HEK 293 cells, we used an NR3A-enhanced green fluorescent protein (GFP) cDNA construct to identify cells that were expressing high levels of this NMDAR subunit. NR3A cDNA was amplified by PCR using Pfu DNA polymerase (Stratagene, La Jolla, CA) and inserted into the mammalian expression vector pEGFP (Clontech, Palo Alto, CA) with Sal I and Smal I. The construct was verified by DNA sequencing using an AutoRead Sequencing Kit (Amersham Pharmacia Biotech). This NR3A construct was co-transfected into HEK 293 cells by calcium phosphate precipitation with NR1 and NR2A or NR2B. The brightest GFP-positive cells were assessed for Mg2+ sensitivity since it was most likely that they had the greatest fraction of NR3A-containing NMDA receptors. In cases in which NR1 and NR2 were expressed in the absence of NR3A, an NR1-GFP construct was utilized to visualize transfected cells. To avoid excitotoxicity, 500 μM 2-amino-5-phosphonovalerate (APV) or 200 μM 7-chlorokynurenate were included in the culture medium. Of note, either of these drugs blocked the current of NR3A-containing channels in these experiments. Two to 3 days posttransfection, cells were transferred to a recording chamber and superfused with HEPES-buffered extracellular solution, pH 7.4, containing (in mM) 135 NaCl, 5 KCl, 1 CaCl2, 10 sucrose, 10 glucose, and 5 HEPES (Sigma, St. Louis, MO). Patch electrodes were made from borosilicate glass and fire polished to a final tip resistance of 5–7 MΩ. Whole cell recordings were made from fluorescently labeled cells using an Axopatch 200B amplifier (Axon Instruments). The pipette solution for whole cell recording contained (in mM) 120 CsCl, 20 tetraethylammonium chloride (TEA-Cl), 10 HEPES, and 2.25 EGTA, adjusted to 7.4 with CsOH. NMDA (200 μM) and glycine (10 μM) were administered in the external solution with either no added Mg2+ (“0 mM”), 1 mM Mg2+, or 10 mM Mg2+. The drugs were applied by a linear array of seven plastic pipes connected to solution reservoirs. Solution flow through each pipe was individually controlled using the pClamp 7 software program. Cells were continuously bathed in a stream of solution from the flow pipe (0.5 ml per minute); only one barrel was used for superfusion at a given time. I-V relationships were constructed by performing voltage ramps generated by pClamp 6.0.3 software. Each trial consisted of three 4-s ramps from −80 to +40 mV. Ramps were performed before, during, and after drug application, and each set was averaged. The net I-V plot was constructed by...
subtracting the averages of the trials before and after drug application from the average during drug application. Corrections were made for liquid junction potentials (Neher 1992).

NR3A-deficient and wild-type mice

NR3A(−/−) mice were generated in our laboratory as previously described (Das et al. 1998). Homozygous NR3A(−/−) and wild-type littermate mice were investigated between postnatal days 5 and 16 (P5 and P16). After performing electrophysiological experiments and their evaluation in a masked fashion, the exact genotype of each tested mouse was determined by RT-PCR analysis of the tails, as previously described (Das et al. 1998; Sucher and Deitche 1995). Mice of both genotypes developed normally and were not obviously distinguishable by body weight or behavior.

Isolation of NR3A-deficient and wild-type neurons for electrophysiological recordings

Acutely isolated cortical neurons were obtained using a combination of established methods (Baughman et al. 1991; Das et al. 1998; Kay and Wong 1986). Mice pups were decapitated, and the brains were rapidly removed and placed in a cooled storage vessel with Hanks’ balanced salt solution (HBSS), pH 7.22. Standard HBSS contained (in mM) 137 NaCl, 1 NaHCO₃, 0.35 Na₂HPO₄, 5.4 KCl, 0.45 KH₂PO₄, 1.25 CaCl₂, 0.5 MgSO₄, 0.5 MgCl₂, 5 HEPES, and 20 glucose. pH was adjusted to 7.22 with 0.3 N NaOH. Blocks of parietooccipital cortex from both hemispheres were cut with a scalpel, the dura removed, and a series of partial cuts were made to aid penetration of enzyme. Blocks were transferred to 15-ml conical polystyrene tubes containing 3.5 ml HBSS (pH 6.5) to which 3.5 U/ml papain, 1.7 mM DL-cysteine, 20 µg/ml bovine serum albumin, and NMDA receptor antagonists (100 µM dl-APV, 1 mM kynurenic acid, and 10 mM MgCl₂) were added. The vials were placed in an incubator (37°C, 10% CO₂) and periodically agitated. After 20 min, the enzyme solution was replaced with a fresh 3.5-ml aliquot (40 min total in enzyme solution). The tissue was rinsed two times (7 ml each) with Dulbecco’s modified Eagle’s medium containing 25 mM HEPES, 25 mM glucose, 100 µM glutamine, 1 mM kynurenic acid, and 10 mM MgCl₂, pH 7.22 (termed “triturating medium”), followed by a 5-min incubation (37°C, 10% CO₂) in triturating medium containing 0.1 mg/ml DNase and 100 µM dl-APV. After two rinses the tissue was gently triturated in 1 ml of medium with a glass serological pipette for 10 passes. The supernatant was then plated onto glass cover slips by body weight or behavior.

The latter was calculated assuming a standard liquid junction potentials (Neher 1992).

Brain membrane preparations

Fractions of crude brain membranes (CMs), synaptic plasma membranes (SPMs), and postsynaptic densities (PSDs) were prepared from Sprague-Dawley rats. In all experiments, the membrane fractions were prepared from the forebrain only. For the crude membrane preparation, 9 volumes of cold dissection buffer (50 mM Tris-acetate (TA), pH 7.4, 10% sucrose, 5 mM EDTA), and containing a freshly added protease inhibitor cocktail consisting of 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 µg/ml benzamidine, 10 µM leupeptin, and 1 µM pepstatin were added to 1 volume of dissected brain tissue. The brain tissue in the dissection buffer was homogenized in a Potter homogenizer. Subsequently, the homogenates were centrifuged at 10,000 x g for 10 min at 4°C. The supernatants were employed as described previously (Rogers et al. 1991). The pellets were resuspended in 2% SDS in IP buffer, rocked overnight, and subsequently frozen for storage at −80°C.

Cloning and sequencing of a new NR3A isoform (NR3A-2)

A rat brain cDNA library was screened using a rat NR3A cDNA fragment as the probe and following our published methods (Das et al. 1998; Sucher et al. 1995). Restriction mapping and nucleotide sequence analysis were used to confirm the presence of an apparent splice variant of NR3A. Electrophysiological recordings were obtained with both isoforms, and NR3A is used to designate the original shorter subunit (NR3A-1), and NR3A-2, the new longer splice variant.
In situ hybridization of NR3A-2 mRNA

In situ hybridizations were performed on mouse brains as previously described (Cui et al. 1999) using cRNA encoding the antisense sequence of the unique NR3A-2 exon. Control experiments using the sense sequence probe were performed in parallel and produced no signal. To investigate developmental changes in the expression pattern of mRNA containing the unique NR3A-2 exon, we processed tissue simultaneously from all age groups and quantified the signals in three areas of the brain in P7, P14, and adult (10 wk) mice. We outlined the area of the cerebral cortex, hippocampus, and thalamus and scanned the autoradiograms with a digital scanner (Scan Maker, Microtex) using Scan Wizard PPC 3.24 software. We then quantified the signal density using the National Institutes of Health Image 1.61 computer program. The values for each brain area at P14 and adult were compared with that of P7 with an unpaired Student’s t-test.

RESULTS

COS cell immunoprecipitation

We first sought to examine whether NR3A could form a stable biochemical complex with either NR1 or NR2. This information will contribute to our evolving knowledge of the stoichiometry of NMDARs with respect to determining whether NR3A can associate with either of the other two major subfamilies of NMDAR subunits. This type of experiment can only be performed in a cell line transfected in a pair-wise fashion with each clone. To this end, NMDAR proteins were transiently expressed in COS-7 cells, and lysates of transfected cells were analyzed for the presence of stable protein-protein complexes. Figure 1A shows analysis of lysates prepared from cells transfected with NR1, myc-NR3A, and both of these two genes. These lysates were immunoprecipitated with NR1 antibody, and the immunoprecipitates were subjected to immunoblotting using NR1 and NR3A antibodies (Fig. 1A, 3 right-hand lanes). NR3A protein co-precipitated with NR1 from cells expressing both of these subunits (Fig. 1A, right-most lane). A reverse experiment in which the myc antibody was used for immunoprecipitation also detected a stable complex between NR3A and NR1 (data not shown). Similar experiments were performed for NR3A and NR2B (Fig. 1B), the major NR2 subunit expressed in immature cortical neurons (Das et al. 1998). Like NR1, NR2B coprecipitated with NR3A from the lysate of cells expressing both proteins (Fig. 1B, right-most lane). These data indicate that, when expressed in COS-7 cells, NR3A stably binds to NR1 and to NR2B proteins.

Conductance and kinetics of recombinant NR3A-containing single channels

Previously, we reported that co-injection of NR3A cRNA along with NR1/NR2A (or NR2B) resulted in the appearance of a small conductance channel in addition to the large conductance observed after injection of NR1/NR2A (or NR2B) alone (Das et al. 1998). In the present study, we monitored single-channel activity in 30 outside-out patches after co-injection of NR1/NR2A/NR3A and 9 patches after only NR1/NR2A co-injection. After injection of a 1:1:2 ratio of NR1/NR2A/NR3A cRNAs, we recorded from 8 patches that had both the small and large conductance channels; of the 1:1:5 ratio NR1/NR2A/NR3A co-injections, 14 patches had small and large conductance channels, 4 patches had only the large conductance, and 4 patches manifest only the small conductance channels. Additionally, we recorded from three patches containing the longer carboxy terminal splice variant, NR3A-2, in conjunction with NR1 and NR2A. In all of these recordings, the Po, the probability of a channel being open, was very low. In fact, many additional patches were pulled that had no activity at all, far in excess to those showing activity.

For recombinant receptors under very low extracellular [Ca2+] recording conditions (1.5 mM EGTA and no added Ca2+) and as confirmed with all-points histograms, we observed only one major conductance in oocytes injected with NR1/NR2A (or NR1/NR2B) but two distinct conductance states with NR1/NR2A/NR3A (or NR1/NR2B/NR3A) (Fig. 2A) (see also Das et al. 1998). Under these low Ca2+ conditions, subconductance states are minimized (Das et al. 1998;
Stern et al. 1992), facilitating the analysis of the small conductance state in the presence of the large conductance. The smaller conductance channel did not represent a subconductance state because analysis of the conductances revealed that the smaller and larger states appeared to be independent, as observed in \( n = 30 \) patches each containing at least hundreds of transitions. Consistent with our previous report, we found from total amplitude histograms that the large channel had a conductance of 75 \( \pm 2.5 \) pS and the smaller channel, 35 \( \pm 3.4 \) pS (mean \( \pm \) SD, \( n = 30 \)). In the presence of 1 mM extracellular \( \text{Ca}^{2+} \), the larger channel had a slope conductance of 47.9 \( \pm 1.5 \) pS and the smaller channel, 26.1 \( \pm 1.4 \) pS (\( n = 4 \)). Additional recordings made under these physiological \( \text{Ca}^{2+} \) conditions after co-injection of cRNA encoding the longer splice variant of NR3A (NR1/NR2A/NR3A-2) yielded similar results (large conductance, 54.2 \( \pm 1.9 \) pS; small conductance, 27.8 \( \pm 0.9 \) pS, \( n = 3 \)). The larger conductance was consistent with that reported previously for channels composed of recombinant NR1/NR2A subunits when recorded in physiological extracellular \( [\text{Ca}^{2+}] \) (McBain and Mayer 1994; Stern et al. 1992, 1994).

We focused our kinetic analysis particularly on the smaller conductance channels that presumably represent heteromers containing NR1/NR2A/NR3A (Das et al. 1998). For ease of analysis, we selected for kinetic analysis the data from patches in which we observed only a single small conductance channel during the entire epoch of recording, lasting tens of minutes (Fig. 2B). The small channel had an apparent mean open time of 4.3 \( \pm 0.2 \) ms (mean \( \pm \) SE) and a mean closed time of 185 \( \pm 11 \) ms.

Resolved channel events were used for constructing open- and closed-time histograms (Fig. 2, C and D). The open-time histogram could be best fit by the log likelihood method with one exponential component having a time constant of 4.4 ms after implementation of a 200-\( \mu \)s threshold. The closed-time histogram could be best fit with two exponential components with time constants of 193.5 and 0.4 ms. The existence of two components in the closed-time histogram suggested that kinetic models of the receptor must contain at least two closed states. Overall, the small conductance had a low open probability (0.03), a consequence of the channel remaining predominantly in the longer closed state. However, a very prolonged closed

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**FIG. 2.** Kinetic analysis of the recombinant small conductance channel expressed in oocytes injected with NR1/NR2A/NR3A cRNAs. A: representative single-channel recordings from an outside-out patch illustrating a large and a small conductance in response to \( \text{N}-\text{methyl}-D-\text{aspartate} \) (NMDA; cRNA of NR1/NR2A/NR3A subunits injected into the oocyte at a 1:1:2 ratio). B: single-channel recordings of NMDA-evoked activity in an outside-out patch that contained only the small conductance channel (cRNA of NR1/NR2A/NR3A injected into the oocyte at a 1:1:5 ratio). The bottom traces represent an expanded portion of the top traces. The conductance of the channel was 35 pS, and the membrane potential was held at \( -80 \) mV. C and D: for the smaller channel, after fitting with a 2-state kinetic model, resolved channel events (considering events of duration longer than 200 \( \mu \)s) were used to construct open-time (C, total events = 494) and closed-time histograms (D, total events = 536). The open-time histogram was best fit with one exponential component having an apparent mean open time of 4.4 ms. The closed-time histogram was best fit with 2 exponential components with time constants of 193.5 and 0.4 ms. E: open-time histogram for the larger channel (total events = 1,116), which could be best fit with one exponential component and had an apparent mean open time of 3.5 ms. It is possible that a faster component of the open time was present for both the small and large conductances, but attempts at fitting these components did not increase the maximum likelihood value.
state (>1,000 ms), similar to that observed between superclusters of NR1-a/NR2A or NR1-a/NR2D recombinant channels (Wyllie et al. 1998) was not observed.

We also analyzed single-channel data obtained from patches excised from oocytes injected with only NR1/NR2A cRNA and which therefore displayed only the large conductance channel. More than one channel was observed in each of these patches, and hence only the mean open time was measured and analyzed (Fig. 2E). Log likelihood fitting indicated that the mean open time was 3.5 ± 0.2 ms in low Ca²⁺ and 3.3 ± 0.3 ms in physiological Ca²⁺ (n = 3–5 in each case), consistent with previously reported values (Antonov et al. 1998; Stern et al. 1992, 1994).

**Calcium permeability of recombinant channels in oocytes containing NR3A**

The ratio of permeability of single channels to Ca²⁺ and Na⁺ was computed using excised patches obtained from oocytes injected with NR1/NR2A cRNAs as well as oocytes injected with NR1/NR2A/NR3A cRNAs. *I-V* relationships (Fig. 3) were constructed for large conductance channels (representing NR1/NR2A subunits) and small conductance channels (representing NR1/NR2A/NR3A subunits) in high and low extracellular [Ca²⁺] (see METHODS for details). The reversal potentials of the *I*-V curves were used to calculate the relative permeability of Ca²⁺ and monovalent ions (P_{Ca}/P_M) from the extended Goldman-Hodgkin-Katz equation. The larger conductance had a P_{Ca}/P_M of 6.8, while the smaller conductance had a P_{Ca}/P_M of 0.8. Therefore the addition of NR3A decreased the permeability to Ca²⁺ by almost 90%.

**Magnesium insensitivity of recombinant channels in oocytes containing NR3A**

An important characteristic of NMDAR-operated channels is their sensitivity to blockade by extracellular Mg²⁺ (Mayer et al. 1984; Nowak et al. 1984). To assess Mg²⁺ inhibition in NR3A-containing channels, we performed the following series of experiments. Single channels in outside-out excised patches from oocytes injected with NR1/NR2A/NR3A cRNA were assessed for their susceptibility to blockade by Mg²⁺. As previously shown, the large conductance channel was presumably composed of NR1/NR2A subunits, while the small conductance channel putatively contained NR1/NR2A/NR3A subunits. In single patches displaying both the large and small conductance in response to NMDA/glycine stimulation, we observed that only the large conductance channel, but not the smaller, was blocked by 1 mM extracellular MgCl₂ at −80 mV (Fig. 4, A and B, n = 4 patches). These results are consistent with the notion that the large conductance, representing NR1/NR2A channels, is sensitive to Mg²⁺ block, as expected, but NR1/NR2A/NR3A channels are relatively insensitive to Mg²⁺ block.

**Whole cell HEK 293 cell Mg²⁺ insensitivity**

Mg²⁺ sensitivity of recombinant NMDA receptor/channels containing the NR3A subunit versus channels without the NR3A subunit was determined from I-V curves of transfected HEK 293 cells. In an attempt to ensure that the maximal number of NMDA receptors contained NR3A, we transfected an NR3A construct containing GFP so that the brightest fluorescent cells contained the greatest amount of NR3A, and we chose to record from these cells. Whole cell recordings from HEK 293 cells were made, and net *I-V* relationships were used to monitor Mg²⁺ sensitivity in either nominally "0 Mg²⁺" (no added Mg²⁺), 1 mM Mg²⁺, or 10 mM external Mg²⁺ concentrations. Unlike cells transfected only with NR1/NR2B constructs, those also transfected with NR3A were relatively insensitive to Mg²⁺ block (Fig. 4, C and D).

**Single-channel recordings in primary cortical neurons**

Single-channel recordings of primary mouse cortical neurons from *P8* animals in the presence of physiological extracellular Ca²⁺ also indicated two distinct populations of native NMDA receptors with a large conductance of 56.4 ± 8.8 pS and a small conductance of 20.0 ± 5.2 pS (Fig. 5A, n = 5 outside-out patches). Analysis of individual transitions showed that no direct transitions occurred between the 56- and 20-pS conductance levels, suggesting that the smaller channel was not a subconductance state. Similar values had previously been observed in patches from neonatal neurons by others (McBain and Mayer 1994; Strecker et al. 1994). Both the large and small conductances were inhibited by n-APV. Log likelihood fitting indicated that the mean open time of the larger conductance was 2.9 ± 0.2 ms and, for the smaller conductance, 3.6 ± 0.6 ms. In the same patch, the small conductance receptor opened.

![Image](http://jn.physiology.org/content/87/4/2057/F3.large.jpg)  
**Fig. 3.** Current-voltage (*I*-*V*) relationship for single-channel recordings in the presence of 2 and 10 mM extracellular Ca²⁺. In 2 mM Ca²⁺/*Mg²⁺*-free extracellular solution (see METHODS), the large and small conductance channels had reversal potentials (V_{rev}) of +3.2 and ±2.0 mV, respectively. However, in 10 mM extracellular Ca²⁺, the large conductance channel had a reversal potential of ±18.1 mV and the smaller conductance channel, ±5.0 mV. Hence the small conductance channel displayed less permeability to Ca²⁺ than the large conductance. Each data point was constructed using recordings from *n* = 4 patches; each symbol is the mean current amplitude from all-points histograms constructed from at least hundreds of openings. The SE was less than the size of the symbol for each data point. In each case, V_{rev} was calculated from a least-squares fit to the data.
FIG. 4. Incorporation of the NR3A subunit reduces the sensitivity to Mg2+ block of NMDA receptors. A: current traces recorded from an outside-out patch excised from an oocyte injected with NR1/NR2A/NR3A subunit cRNAs (holding potential, −80 mV). Note that the small and large conductance levels (representing NR1/NR2A/NR3A and NR1/NR2A receptors, respectively) were seen in divalent free (1.5 mM EGTA) extracellular solutions. Application of 1 mM Mg2+ did not block the current completely, with a small conductance channel remaining (note the unitary conductance appeared smaller in the presence of divalents due to enhanced surface charge screening and hence a smaller driving force). No activity was observed in the absence of NMDA (20 μM) and glycine (10 μM). Similar results were obtained from n = 4 patches. B: currents recorded under similar conditions from a patch excised from an oocyte injected with NR1/NR2A subunit cRNAs. In this case, channels with a single, large conductance level were activated by NMDA. Application of 1 mM Mg2+ blocked the channel activity virtually completely. Current traces shown at higher time resolution are marked with asterisks and vertical lines. Similar results were obtained from n = 4 patches. C and D: representative examples of NMDA-evoked currents examined in voltage ramps on HEK 293 cells transfected with NR1/NR2B alone or in combination with NR3A. Cells transfected with NR1/NR2B/NR3A compared with NR1/NR2B alone were relatively resistant to block by 1 mM extracellular Mg2+ (C). In fact, in HEK cells transfected with NR1/NR2B/NR3A cRNA, NMDA-evoked current was relatively resistant to both 1 and 10 mM Mg2+ (D). For each construct, similar results were obtained from n = 4 HEK cells.

Developmental expression of NR3A-containing channels in mouse cortical neurons

In wild-type mouse cortical neurons, NMDA-activated currents increase during the first two postnatal weeks (McDonald and Johnston 1990). To examine NMDA-activated currents during postnatal development, acutely isolated cortical neurons were tested for their response to application of 200 μM NMDA in the presence of 10 μM glycine at −60 mV. Our previous results had demonstrated that essentially all cortical neurons in these cultures expressed NR3A by both PCR and electrophysiological criteria (Das et al. 1998). A total of 131 neurons from wild-type mice were investigated between days P5 and P16. Of this number, 103 cells responded to NMDA, as illustrated in Fig. 6A, left-hand panel). Monitoring the peak whole-cell current, we found that the NMDA current density increased by 4.4-fold (Fig. 6, P16 to P1). A total of 131 neurons from wild-type mice were investigated between days P5 and P16. Of this number, 103 cells responded to NMDA, as illustrated in Fig. 6A, left-hand panel). Monitoring the peak whole-cell current, we found that the NMDA current density increased by 4.4-fold (Fig. 6, P16 to P1).
size of cortical neurons (Fig. 6B). Compared with wild-type, NR3A(−/−) neurons manifested statistically larger NMDA-evoked current densities at P5–P6 and P7–P8, with a subsequent decrease to normal at later ages. The time constant of desensitization of NMDA-evoked currents (τ) was not, however, different in wild-type and NR3A-deficient mice: NR3A(+/+) neurons, 1.69 ± 0.13 s, n = 45; NR3A(−/−) neurons, 1.62 ± 0.11 s, n = 64 (Fig. 6C).

**Brain immunoblotting**

Protein lysates were prepared from mouse or rat cerebral cortices at various ages, and immunoblotting was performed using antibodies against NR3A (Fig. 7). Developmental changes in levels of NR3A protein observed in this experiment correlate well with NR3A mRNA levels, which we have monitored previously (Sucher et al. 1995). Specifically, NR3A
protein was weakly expressed in newborn rodents, while its level increased during the first week of life. NR3A protein levels began to recede after P8 and, by P35, were barely detectable with these methods. We have previously shown that there are no compensatory changes in NR1 or NR2 subunit expression in the NR3A-deficient mice (Das et al. 1998).

Alternative splicing of NR3A message

To investigate the presence of other isoforms of NR3A mRNA, we screened a rat brain cDNA library using a rat NR3A cDNA fragment as the probe. Following restriction mapping and nucleotide sequence analysis, we found a variant form of NR3A. This NR3A variant (NR3A-2) appears to be a product of alternative splicing, and contains an additional 20 amino acid sequence between the fourth hydrophobic domain (M4) and the C-terminus (Fig. 8). According to the proposed membrane topology of glutamate-receptor subunits, this region is thought to be located on the cytoplasmic side of the plasma membrane. The primary amino acid sequence of this apparent exon suggests that a threonine residue positioned at H7 could serve as a substrate for phosphorylation by calcium calmodulin-dependent protein kinase-II (CamK), protein kinase A (PKA), and protein kinase C (PKC). Single-cell RT-PCR from dissociated cortical neurons at P7 revealed that virtually all cells express NR3A-1, while some cells express both NR3A-1 and NR3A-2 (asterisks in Fig. 8). As stated above, functional
expression of the NR3A-2 subunit in recombinant systems in conjunction with NR1 and NR2A resulted in similar conductances as NR1/NR2A/NR3A-1 subunit expression.

As shown by in situ hybridization of mouse brain (Fig. 9), mRNA containing NR3A-2 was broadly expressed in many regions, including cerebral cortex, hippocampus, and thalamus. The expression was particularly prominent in the hippocampus, piriform cortex, hypothalamus, habenular nuclei, and amygdala. The regional pattern of NR3A-2 expression was not drastically altered among P7, P14, and adult brains. However, the level of expression was significantly reduced in adult brains compared with P7 or P14 brains. These results show that NR3A-2 mRNA is primarily expressed during early development. This temporal and regional pattern of expression is similar to that previously observed for pan NR3A (NR3A-1 + NR3A-2) mRNAs in brain (Ciabarra et al. 1995; Sucher et al. 1995).

DISCUSSION

In the present study we report biochemical and electrophysiological data supporting the notion that NR3A is a component of the NMDAR complex. Our previous studies had shown that NR3A could be co-immunoprecipitated with NR1 and NR2B from rodent brain lysates (Das et al. 1998). However, that work could not address the question of whether or not NR3A could associate with NR1 or NR2 subunits independently, as opposed to, for example, binding to one subunit, such as NR1, which in turn bound to the other (i.e., NR2). In the current study, these NMDAR subunits were co-expressed in a pair-wise manner with NR3A subunits in COS-7 cells, and co-immunoprecipitation was performed from cell extracts. These results suggest that NR3A can associate independently with NR1 and NR2B, and the use of transfected cell lines facilitated this analysis.

Next, we further analyzed the functional expression of NR3A in a heterologous expression system. We knew from previous work that when injected alone or in combination with NR1 or NR2 into Xenopus oocytes, NR3A cRNA did not affect NMDA-evoked currents. However, NR1/NR2/NR3A co-injection resulted in smaller macroscopic currents during two-electrode voltage clamp and a smaller unitary conductance in outside-out patch recordings (Ciabarra et al. 1995; Das et al. 1998; Sucher et al. 1995). Here we further characterized these currents, demonstrating not only a smaller conductance in oocytes after injection of NR1/NR2A/NR3A cRNA but also a small conductance in primary cortical neurons from P8 wild-type mice but not in NR3A(-/-) mice. Additionally, the smaller (NR3A-containing) channels were of lower Ca²⁺ permeability with a PCa/Pm ratio of 0.8 compared with 6.8 for the larger (NR1/NR2A) channels. The latter value compares fa-
vorable with previous reports on primary neurons of channels most likely comprised of NR1 and NR2 subunits (Mayer and Westbrook 1987). Additionally, we and our colleagues recently reported similar decreases in calcium permeability for NR3A-containing recombinant NMDAR channels during whole cell recording from HEK 293 cells (Peréz-Otaño et al. 2001).

Another important feature of NMDAR-operated channels comprised of NR1 and NR2 subunits is their sensitivity to blockade by extracellular Mg$^{2+}$ (Mayer et al. 1984; Nowak et al. 1984). Since the pore loop (M2) region of NR3A is substantially different from that of NR1 and NR2 subunits, the question arose whether or not Mg$^{2+}$ blockade was similar in NR3A-containing channels. In the present study, we found that in outside-out patches from oocytes co-injected with NR1/NR2A/NR3A subunits the larger NMDA-activated conductance, similar to that observed in NR1/NR2A channels and presumably lacking NR3A (Das et al. 1998), was completely blocked by 1 mM Mg$^{2+}$ at −80 mV, as expected. In contrast, the small conductance, due to the presence of NR3A in addition to NR1 and NR2A (Das et al. 1998), was not blocked by 1 mM Mg$^{2+}$. This interesting finding suggests that during development, when most NR3A-containing channels are expressed, this conductance is not as susceptible to Mg$^{2+}$ block as NMDAR-evoked currents in the adult. Since the presence of NR3A not only reduces Mg$^{2+}$ sensitivity but also the Ca$^{2+}$ permeability of the unitary conductance, the overall effect is to produce an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)–like current after stimulation of these NMDAR channels. In fact, some parallels can be drawn between the effect of NR3A on NMDAR channels and the GluR2 subunit on AMPAR channels since GluR2 also drastically reduces Ca$^{2+}$ permeability.

When analyzed with the HHMI/Sigworth Laboratory implementation of the Hidden Markov Method (HMM) of single-channel data analysis, we found that the mean open time of channels comprised of NR1/NR2A/NR3A subunits was 4.4 ms compared with 2.9 ms for NR1/NR2A channels. This analysis assumes a Markovian process, i.e., that the ion channel makes instantaneous transitions between discrete states. The HHMI/Sigworth Laboratory implementation of the HMM software optimizes model parameters to produce the greatest likelihood fit. Previously, we had analyzed our data with the SUNY Buffalo implementation of the HMM, which fits a kinetic model to an idealized current trace and found a somewhat shorter open time for NR1/NR2A/NR3A channels. The present analysis with the HHMI/Sigworth embodiment of these programs fits a kinetic model directly to acquired data and offers a deconvolution correction for the noise bias of the recording amplifier. The illiterateness of poorly resolved transitions with this method is beneficial for accurate computation of mean open and closed times. Thus our findings with channels composed of NR1/NR2A/NR3A subunits include a low opening probability, smaller unitary conductance, and somewhat increased mean open time compared with NR1/NR2A channels (Fig. 2). Taken together, these results appear consistent with the smaller macroscopic current that we previously observed after co-injecting oocytes with cRNAs encoding the NR1/NR2/NR3A subunits (Sucher et al. 1995). Moreover, the lower Ca$^{2+}$ permeability suggests that channels containing the NR3A subunit may offer protection from excitotoxic injury during development, when this subunit is most prominently expressed.

In contrast, the relative insensitivity to Mg$^{2+}$ block might enhance the NMDAR component of synaptic activity during development. These hypotheses are currently being tested in NR3A-deficient and NR3A-transgenic mice. The presence of a large and a small conductance in primary cortical neurons from P8 wild-type mice, but only the larger conductance in NR3A-deficient mice, suggests that NR3A subunit underlies the smaller conductance under physiological conditions as well as in heterologous expression systems.

Immunoblotting experiments with NR3A antibody on rat and mouse brain extracts revealed that the temporal profile of NR3A protein expression is similar to that of NR3A mRNA expression (Sucher et al. 1995). We found that NR3A protein was expressed during the first 2 wk after birth, with peak expression at P5–P8. Consistent with this expression pattern, the electrophysiological phenotype of increased NMDA-evoked current in NR3A(−/−) mice was most evident at P5–P8. These correlated biochemical and physiological observations reinforce the notion that NR3A may function in the development of the immature brain.

In the present study, we also identified a longer splice variant of NR3A. An apparent exon encoding an additional 20 amino acid sequence is inserted at the cytoplasmic segment near the C-terminus. By in situ hybridization, NR3A-2 is expressed in areas where we and others had previously found pan NR3A mRNAs (Ciabarra et al. 1995; Sucher et al. 1995). Previous work had suggested the presence of a longer splice variant by RT-PCR (Sun et al. 1998), but Northern or in situ confirmation has not been reported prior to the present study. Our results show that the exon specific to NR3A is most highly expressed in piriform cortex, hypothalamus, and amygdala. The temporal pattern of NR3A-2 expression is also similar to that of pan NR3A mRNAs. Namely, NR3A-2 expression is high in developing brain, while expression in the adult brain is low. Functional expression of NR1/NR2A/NR3A-2 resulted in conductances similar in size to those of NR1/NR2A/NR3A-1. In other NMDAR subunits, this C-terminal segment has been shown to interact with various proteins in the postsynaptic cell. The C-terminus of the NR2 subunit contains alternatively spliced exons, and it interacts with calmodulin, α-actinin, and neuronal intermediate filaments in a manner specific to these splice variants (Ehlers et al. 1996, 1998; Wyszynsky et al. 1997). Therefore it is possible that this NR3A splice variant may also be involved in specific interactions with postsynaptic proteins. Moreover, since several molecules involved in cell signaling pathways, such as neuronal nitric oxide synthase, interact via PDZ domains either directly or indirectly with NMDAR subunits (Brenman et al. 1996), it will be important to elucidate these protein-protein interactions for NR3A.
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