Expression and developmental regulation of the NMDA receptor subunits in the kidney and cardiovascular system

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Department of Pediatrics, Divisions of 1Neonatology, 2Nephrology, and 4Cardiology and 5Departments of Pediatrics, Microbiology and the Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, New Orleans, Louisiana 70112; and 3Department of Medicine, Veterans Administration Medical Center, University of Florida, Gainesville, Florida 32611

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Leung, Jocelyn C., Brett R. Travis, Jill W. Verlander, Satinder K. Sandhu, Song-Gui Yang, Arnold H. Zea, I. David Weiner, and Douglas M. Silverstein. Expression and developmental regulation of the NMDA receptor subunits in the kidney and cardiovascular system. Am J Physiol Regul Integr Comp Physiol 283: R964–R971, 2002. First published June 13, 2002; 10.1152/ajpregu.00629.2001.—An- tagonists to the N-methyl-d-aspartate (NMDA) receptor bind to various extraneuronal tissues. We therefore assessed the expression of the main NMDA subunit, NR1, in various tissues. We demonstrate that NR1 appears to be most abundant in the rat kidney and heart. NR1 is present in total rat kidney, cortex, and medulla. Of the NR2 subunits, only the NR2C subunit protein is present in the kidney. The abundance of the NR1 subunit protein increases with kidney development. Both NR1 and NR2C are present in opossum kidney, Madin-Darby canine kidney, and LLC-PK1 cells. Immunohistochemistry studies show that the NR1 subunit is present in the renal proximal tubule. NR1 is abundant in the atrium and ventricle but is also expressed in the aorta and pulmonary artery. The NR2 subunits are not expressed in the heart. NR1 subunit protein expression is constant throughout heart development. Finally, the NR1 subunit protein is expressed in heart cells (H9c2) grown in culture. These studies reveal the presence of the NMDA receptor in the kidney and the cardiovascular system.

N-methyl-d-aspartate receptor; development

The NR1 subunit is the main subunit of the NMDA receptor. The NR1 subunit is essential for channel activity, whereas the NR2 subunits, although not essential for function, can confer modulatory properties (6). Indeed, studies reveal that homooligomeric assembly of two NR1 subunits results in a complete functioning channel (13). Although the channel linked to the receptor is permeable to sodium, potassium, cesium, and other cations, its main function is to transmit calcium (10). Its main role in the central nervous system is to play a role in neuroexcitatory pathways. It is distinct from various types of calcium channels (L, T, N, P, and Q) that exist in various tissue types, although studies reveal that it can induce processing of L-type calcium channels in hippocampal neurons (4). The channel is regulated by glutamate, magnesium, and sodium (3, 9). It operates as a single-channel current, characterized by bursts of short openings, interrupted by brief closures. Its main conductance state is 40–50 pS, with occasional smaller ones (1).

There is recent evidence that the receptor-channel complex may exist outside the central nervous system. Studies of Nasstrom et al. (14) indicate that antagonists of the NMDA receptor bind to various tissues outside the central nervous system, including the heart, stomach, pancreas, and kidney. Furthermore, the NR2C subunit is present in the adult rat pancreas (7), and the NR2B subunit is transiently expressed in the newborn rat heart (17). In addition, yotiao, a protein of the neuromuscular junction that colocalizes with the NMDA receptor, is present within the central nervous system but also in various extraneuronal tissues, including the heart, placenta, and skeletal muscle (8). Finally, kidney tubules are sensitive to strychnine, glycine, ACPC, d-alanine, and d-serine, all specific agonists of the NMDA receptor found in the brain (5, 11). On the basis of the above studies, we hypothesized that these tissues may also express an NMDA-like receptor.

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The Strychnine-insensitive N-methyl-d-aspartate (NMDA) receptor, part of the glutamate receptor family, was originally identified in the central nervous system. This receptor is linked to a calcium channel. Its agonists include magnesium, d-serine, d-alanine, and L-amino-cyclopropane carboxylic acid (ACPC). The NMDA receptor is comprised of various subunits. The main subunits are NR1, NR2A, NR2B, NR2C, and NR2D. Each receptor contains at least one NR1 subunit, linked to either another NR1 subunit or any of the NR2 subunits (1, 12, 13).
METHODS

Protein Isolation

_Tissue._ Four- and twelve-day-old and adult (3 mo old, 200–250 g) female Sprague-Dawley rats were anesthetized with 65 mg/kg ip pentobarbital sodium. The brain, heart, lung, kidney, thymus, stomach, and pancreas were excised and the tissues were rapidly placed in a solution containing 250 mM sucrose, 1 mM EDTA, 5 mM Tris, 0.5 mM benzamidine HCl, 1 μM leupeptin, 1 μM pepstatin A, and 0.3 mM PMSF, and homogenized.

_Culture cells._ MATERIALS. A cell lysis buffer base was made containing 50 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate (Na3OV4), 0.5% Triton X, and distilled water to 10 ml. To this, proteinase inhibitors were added (1 μM leupeptin, 1 μM pepstatin A, and 0.3 mM PMSF).

_PROTOCOL._ Cells were scraped from tissue culture flasks and centrifuged for 10 min at 12,000 rpm. The supernatant was discarded, and at least 10 μl of lysis buffer was added to the cells. This was incubated on ice for 7 min and subsequently centrifuged at 4°C for 10 min. The supernatant was removed and saved for immunoblot analysis.

Western Blot Analysis

_Samples._ Samples containing equivalent amounts (20 μg) of protein were boiled for 5 min at 95°C in 2× Laemmlli sample buffer (BioRad, Hercules, CA) consisting of 62.5 mM Tris·HCl, pH 6.8, 2% SDS, 25% glycerol, and 0.01% bromophenol blue with 5% (vol/vol) 2-mercaptoethanol. Proteins were electrophoretically separated using 4–15% linear gradient Tris·HCl ready gels (BioRad). The proteins from these gels were then transferred to PVDF membranes (Invitrogen, Carlsbad, CA) using transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol, and dH2O). Nonspecific binding to PVDF membranes was blocked with 5% (wt/vol) nonfat milk powder in TBST (20 mM Tris base, 500 mM NaCl, 0.01% Tween 20, and dH2O). Membranes were incubated for 1 h at room temperature with the NMDA antibodies diluted in TBST containing 5% (wt/vol) nonfat milk powder. The NR1 antibody (added in concentration 1:100) is a mouse anti-rat monoclonal antibody obtained from Upstate Biotechnology (Lake Placid, NY). The size of the NR1 subunit protein is 130 kDa. The NR2A antibody (added in concentration 1:200) is a rabbit anti-human polyclonal antibody obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The NR2B, NR2C, and NR2D antibodies are goat anti-mouse polyclonal antibodies also obtained from Santa Cruz Biotechnology. The NR2 subunit proteins are NR2A 176 kDa, NR2B 178 kDa, NR2C 149 kDa, and NR2D 158 kDa. The membranes were then washed with three changes of TBST after which a 1:20,000 dilution of horseradish peroxidase (HRP)-linked secondary antibody was incubated with the membranes for 20 min at room temperature. The secondary antibody used for the NR1 subunit was an anti-mouse antibody. The secondary antibody used for the NR2A subunit was an anti-rabbit antibody, whereas the secondary antibody used for the NR2B, NR2C, and NR2D subunits was an anti-goat antibody. The membranes were then washed again with three changes of TBST and then processed for chemiluminescence detection using the ECL Plus detection system (Amersham, Upsala, Sweden). The membranes were then exposed to the autoradiograph.
Kodak Biomax ML film (Eastman Kodak, Rochester, NY) for up to 5 min.

For each blot assessing the NR2 antibodies, loading was assessed by immunoblot with antisera to a monoclonal mouse anti-rabbit antibody directed against GAPDH (Research Diagnostic, Flanders, NJ). For GAPDH, the secondary IgG antibody was linked to HRP and added at a concentration of 1:20,000. Detection was achieved by use of ECL Plus.

**Immunoprecipitation**

Affinity-purified antibody to NR1 (4 μg) was attached to 30 μl of Gamma Bind-G Sepharose beads (Amersham) by incubating overnight at 4°C with gentle agitation, followed by three washes in a solution containing 250 mM sucrose, 1 mM EDTA, 5 mM Tris, 0.5 mM benzamidine HCl, 1 μM leupeptin, 1 μM pepstatin A, and 0.3 mM PMSF. Before incubation of protein with NR1, the protein (0.5 mg) was incubated with purified mouse IgG1 (0.5 μg) (Becton Dickinson, San Jose, CA) to minimize nonspecific binding. The supernatant from this reaction was captured and washed gently. The protein was then added to a tube containing the beads coated with NR1 and the mixture was incubated for 1 h at 4°C with gentle agitation. Afterward, the beads were washed three times and resuspended in 30 μl 2× Laemmli buffer. The samples were boiled for 5 min, and Western blot analysis was performed on the supernatant as described above.

**Immunohistochemistry**

All experiments were performed on 5-μm-thick sections of rat brain or kidney preserved by in vivo perfusion with 2% paraformaldehyde-lysine-periodate and embedded in polyester wax. NR1 immunoreactivity was detected by immunoperoxidase histochemistry using the mouse anti-NR1 antibody diluted 1:25 and a biotinylated anti-mouse IgG secondary antibody. No counterstain was used.

**Culture Cells**

All cells were purchased from American Type Culture Collection.

Opossum kidney cells. Media used was MEM (Eagle) with Earle’s balanced salt solution (BSS) adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. On arrival, the cells were warmed to 37°C and then added to culture media. After centrifugation, the media was discarded and the process was repeated. The cells were then placed in 50-ml flasks and incubated at 37°C until confluence. For propagation of cells, the medium was removed, and 10 ml 0.25% trypsin-0.03% EDTA solution was added briefly and then removed and fresh trypsin-EDTA was added for 5 min.

The flask was placed at 37°C until the cells detached. Fresh growing media were added, aspirated, and dispensed into new flasks. The cells were grown at 37°C and 5% CO₂ in air atmosphere.

**Madin-Darby canine kidney cells.** Handling and propagation of cells on arrival was identical to that used for opossum kidney (OK) cells (see above), including the media used. The cells were incubated in 37°C and 5% CO₂ in air atmosphere.

**Pig kidney cells (LLC-PK₁ cells).** Handling and propagation of cells on arrival was identical to that used for OK cells (see above), except that the media used was medium 199 adjusted to contain 1.5 g/l sodium bicarbonate and 3% fetal bovine serum. The cells were grown at 37°C and 5% CO₂ in air atmosphere.

**Heart cells (H9c2).** Handling and propagation of cells on arrival was identical to that used for OK cells (see above), except that cells were grown in DMEM with 2 mM L-glutamine and Earle’s BSS adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. The cells were grown at 37°C and 5% CO₂ in air atmosphere.

**Statistical Analysis**

Densitometric values from scanned immunoblots were determined and the data are expressed as means ± SE. Groups were compared by one-way ANOVA with Bonferroni’s multiple comparison test. Significance is defined as P < 0.05.

**RESULTS**

**NR1 Subunit Protein Expression in the Brain**

NR1 and all of the NR2 subunits are expressed in the brain (1, 6, 12, 13). To establish the efficacy of our Western blot studies for the NMDA receptor subunits in extraneuronal tissues, we first assessed the expression of the NMDA subunits in the adult rat brain. Our results confirm that NR1 and all of the NR2 subunits are expressed in the adult rat brain (Fig. 1).

**NR1 Subunit Protein Expression in Peripheral Tissues**

The NR1 is the main and essential subunit of the NMDA (6, 13). Therefore, we screened various extraneuronal tissues that bind agonists to the NMDA receptor to ascertain which tissues express the NR1 subunit. The protein for the NR1 subunit of the NMDA receptor was evaluated by Western blot analysis. Immunoprecipitation and subsequent immunoblotting
studies reveal that the NR1 protein subunit is detectable in all tissues, except the pancreas (Fig. 2A). On the basis of the apparent abundant expression of the NR1 subunit of the NMDA receptor in the kidney and heart, we focused our molecular studies on NMDA receptor expression in these tissues.

Immunoprecipitation and subsequent immunoblotting studies reveal that the NR1 protein subunit is expressed in the total adult rat kidney, renal cortex, and medulla (Fig. 2B). The NR2A, NR2B, and NR2D subunit proteins are not detectable in the kidney. Alternatively, the NR2C subunit protein (149 kDa) is plentiful in total adult rat kidney, renal cortex, and medulla (Fig. 3).

Developmental Expression of the NR1 and NR2C Subunit Proteins in the Kidney

NR1 subunit protein expression is low in the 4-day-old rat kidney but increases with development. Analysis by one-way ANOVA with Bonferroni’s multiple comparison test reveals a significant (P = 0.03) difference in NR1 abundance between the 4-day-old and adult kidney (Fig. 4A). Although there is a modest increase of NR2C subunit expression throughout renal development (Fig. 4B) this did not reach statistical significance (P = 0.2). We did not detect any of the other NR2 subunits in immature rat kidneys (data not shown).

Immunohistochemistry of NR1 in the Brain and Kidney

As a control, we first performed immunohistochemistry studies of NR1 in the rat brain. Our studies reveal that NR1 is abundantly expressed in neurons within the rat brain (Fig. 5a) and absent in the negative control (Fig. 5b). The staining is similar to that seen in prior studies. NR1 is localized to the proximal tubule (S1-S3) of the kidney (Fig. 6, a and b), and absent in the negative control (Fig. 6c).

Expression of the NR1 and NR2C Subunit Proteins in Renal Culture Cells

To more precisely establish which regions of the kidney express the NMDA receptor, we assessed the expression of the NR1 and NR2 subunits in three kidney cell culture lines: LLC-PK1 cells, which are proximal tubule epithelial cells but also possess some distal tubule-like properties; 2) Madin-Darby canine kidney (MDCK) cells, with properties representative of the distal tubule; and 3) OK cells, a proximal tubule-like cell line. Western blot analysis indicates that the NR1 (Fig. 7A) and NR2C (Fig. 7B) subunit proteins are present in all three cell lines. Specifically, the NR1 subunit appears to be most abundant in LLC-PK1 and OK cells, whereas the NR2C subunit seems most plentiful in MDCK cells.

NR1 and NR2 Subunit Protein Expression Within the Cardiovascular System

Immunoprecipitation and subsequent immunoblotting studies show that the NR1 protein subunit is present in the atrium and ventricle, with more subtle expression in the aorta and pulmonary artery (Fig. 8). The NR2A, NR2B, NR2C, and NR2D subunit proteins are not detectable in heart tissue, which was specifi-
cally excised to include the aorta and the pulmonary artery (Fig. 9), whereas the ubiquitous gene GAPDH is present in these tissues.

**Developmental Expression of the NR1 Subunit Protein in the Heart**

Western blot (Fig. 10A) and the accompanying densitometric analysis (Fig. 10B) reveal that NR1 subunit protein expression is constant throughout heart development ($P = 0.9$ among the groups). We did not detect any of the NR2 subunits in immature rat hearts.

**Expression of the NR1 Subunit Protein in Heart Culture Cells**

We assessed NR1 subunit protein expression in an established heart cell line, H9c2. Our studies demonstrate that the NR1 subunit is expressed in these heart cells (Fig. 11).

**DISCUSSION**

Recent studies suggest that the NMDA receptor may exist outside the central nervous system. Specifically, studies indicate that agonists to the NMDA receptor bind various tissues outside the central nervous system, including the cardiovascular system, stomach, pancreas, and kidney (14). Furthermore, the NR2C subunit is present in the pancreas (7), and the NR2B subunit is transiently expressed in the newborn rat heart (17). Finally, studies reveal that kidney tubules are sensitive to strychnine, glycine, ACPC, D-alanine, and D-serine, all specific agonists of the NMDA receptor found in the brain (5, 11). On the basis of the above studies, we hypothesized that these tissues may also express an NMDA-like receptor.

We initially began our studies by aiming to confirm the presence of the NR1 and NR2 subunits in the rat brain. Our studies reveal that NR1 and all of the NR2 subunits are expressed in the rat brain, consistent with various other studies (1, 6, 12, 13). The main subunit of the NMDA receptor is NR1. Our studies demonstrate that the NR1 subunit is present in various extraneuronal tissues and appears to be most abundant in the rat kidney and heart. We, therefore, narrowed our focus to explore the expression and developmental pat-
tern of the NMDA receptor subunits in the kidney and cardiovascular system.

The NR1 and NR2C subunits are present in the rat renal cortex and medulla. The other NR2 subunit proteins are not expressed in the kidney. NR1 subunit protein abundance increases with renal development. NR2C subunit protein expression appears to increase modestly throughout renal development, but the increase was not statistically significant. To localize the expression of the NR1 and NR2C subunits in the kidney, we assessed their expression in renal culture cells. NR1 appears to be most abundant in LLC-PK1 and OK cells, both proximal tubule cell lines, whereas the NR2C subunit appears to be most plentiful in MDCK cells, a distal tubule cell line. Finally, immunohistochemistry studies reveal that the NR1 subunit is abundant in the apical region of the proximal tubule (S1-S3). It should be noted Deng et al. (2) recently showed that, similar to our data, the NR1 protein subunit is expressed in the kidney. However, their studies did not...
assess the expression of the NR2 subunits, and they did not explore the developmental expression of any of the NMDA subunits.

Our current studies also show that NR1 is expressed in the adult rat heart. It is present in the atrium and ventricle and, although not abundant, visible in the pulmonary artery and descending aorta. We found no evidence that any of the NR2 subunits are present in the adult rat heart. Regarding the NR2 subunits, our data are in agreement with the recent studies by Seeber et al. (17), who demonstrated that none of the NR2 subunits are present in the adult rat heart. Thus we speculate that the adult rat heart NMDA receptor consists of homooligomeric NR1 subunits. There is relatively constant expression of the NR1 subunit in the developing rat heart. Finally, the NR1 subunit is expressed in H9c2 cells, a heart cell culture line, indicating its expression in heart parenchyma.

One important question remains: are the peripheral NMDA receptors located within the parenchyma of the kidney and heart, the vasculature of these tissues, or within the neuronal innervation located within these organs? The ample expression of the NR1 and NR2C subunits in three renal culture cell lines implies that the NMDA receptor is expressed within the renal parenchyma. Furthermore, the expression of the NR1 subunit in heart culture cells also suggests that it is present in heart parenchymal tissue.

Interestingly, the receptor has been localized in the parenchyma of at least one other peripheral tissue. Said et al. (15) identified NMDA receptors within the rat lung, consistent with our finding of some NR1 expression in the lung. Furthermore, their elegant studies showed that the receptor subunits are present in lung cells and colocalize with other neurotransmitters, suggesting that there is a functional NMDA receptor in the lung (16).

In summary, our studies show that NR1, the main subunit of the NMDA receptor, is located in various extraneuronal tissues, including the rat kidney and heart. There is also some expression of the NR1 subunit in the lung, thymus, and stomach, suggesting that the NMDA receptor may play a much wider role than previously speculated.

There may be at least two forms (NR1-NR1 and NR1-NR2C) of the rat renal NMDA receptor, whereas the rat heart NMDA receptor is likely comprised of homooligomeric NR1 subunits. Given the wide variety of conditions affected by the NMDA receptor in the central nervous system, significant work is ahead to ascertain the function of the NMDA receptor in the rat kidney and cardiovascular system.

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