N-methyl-D-aspartate receptor subunit NR3a expression and function in principal cells of the collecting duct

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Sproul A, Steele SL, Thai TL, Yu S, Klein JD, Sands JM, Bell PD. N-methyl-d-aspartate receptor subunit NR3a expression and function in principal cells of the collecting duct. Am J Physiol Renal Physiol 301: F44–F54, 2011. First published March 23, 2011; doi:10.1152/ajprenal.00666.2010.—N-methyl-d-aspartate receptors (NMDARs) are Ca2+-permeable, ligand-gated, nonselective cation channels that function as neuronal synaptic receptors but which are also expressed in multiple peripheral tissues. Here, we show for the first time that NMDAR subunits NR3a and NR3b are highly expressed in the neonatal kidney and that there is continued expression of NR3a in the renal medulla and papilla of the adult mouse. NR3a was also expressed in mIMCD-3 cells, where it was found that hypoxia and hypertonicity upregulated NR3a expression. Using short-hairpin (sh) RNA-based knockdown, a stable inner medullary collecting duct (IMCD) cell line was established that had ~80% decrease in NR3a. Knockdown cells exhibited an increased basal intracellular calcium concentration, reduced cell proliferation, and increased cell death. In addition, NR3a knockdown cells exhibited reduced water transport in response to the addition of vasopressin, suggesting an alteration in aquaporin-2 (AQP2) expression/function. Consistent with this notion, we demonstrate decreased surface expression of glycosylated AQP2 in IMCD cells transfected with NR3a shRNA. To determine whether this also occurred in vivo, we compared AQP2 levels in wild-type vs. in NR3a−/− mice. Total AQP2 protein levels in the outer and inner medulla were significantly reduced in knockout mice compared with control mice. Finally, NR3a−/− mice showed a significant delay in their ability to increase urine osmolality during water restriction. Thus NR3a may play a renoprotective role in collecting duct cells. Therefore, under conditions that are associated with high vasopressin levels, NR3a, by maintaining low intracellular calcium levels, protects the function of the principal cells to reabsorb water and thereby increase medullary osmolality.

cytosolic calcium; aquaporin 2; medullary tonicity

N-METHYL-D-ASPARTATE RECEPTORS (NMDARs) are a class of Ca2+-permeable ligand-gated nonselective cation channels that have been extensively characterized in neurons, but which are also expressed in multiple peripheral tissues (15). NMDARs are composed of NR1 in combination with NR2 or NR3 subunits (34, 44). Previous studies of NMDARs in the kidney have demonstrated expression of NR1 and NR2 subunits in both the cortex and medulla and have localized NR1 to the basolateral surface of the proximal convoluted tubule (11). Expression of NR1 and NR2 has also been reported in renal epithelial cell lines (22). Evidence of receptor function has been shown via intravenous infusion of NMDA that leads to a decrease in glomerular filtration rate and urine flow, effects which were reversed by coapplication of a competitive NMDAR antagonist, activator protein 5 (AP-5) (47).

At the present time, there is no evidence for expression of NR3 subunits (NR3a and NR3b) in the kidney. One confounding issue with regard to NR3 expression, at least in the brain under normal conditions, is that NR3 is highly expressed in the neonate with expression levels substantially decreasing shortly after birth. It is not, however, entirely absent in the adult: for instance, there is persistent expression of NR3a in the retina (43) and in layer V pyramidal neurons of the cerebral cortex (46).

Currently, NR3 is considered to be a modulator of NMDA receptor function (5, 45). Specifically, in heterologous expression systems, NR3-containing NMDARs exhibit a substantially lower Ca2+ permeability compared with canonical NR1/NR2 NMDARs (7, 14, 34, 39). This decrease in NMDAR Ca2+-permeability conferred to the NMDA receptor by expression of NR3 appears to play a role in orchestrating the correct timing of neuronal development and also in preventing neuronal damage. Perez-Otaño et al. (33) have shown that NR3a mediates activity-dependent internalization of NMDA receptors via coupling to clathrin-dependent endocytic machinery, which, in turn, allows NR3a to prevent synaptic NMDARs from prematurely activating the Ca2+-dependent pathways that lead to strengthening of specific synaptic connections (36, 40). NR3a may also be neuroprotective: Ca2+ influx through NMDARs is well characterized as a mediator of neuronal cell death (8), and NMDAR-mediated potassium efflux has been shown to result in additional neuronal damage (49). Nakanishi et al. (30) demonstrated that knockdown of NR3a enhanced neuronal excitotoxic insult, whereas NR3a overexpression was protective, and additionally found NR3a to be neuroprotective in a mouse model of brain hypoxia (30).

In the kidney the medullary region is an area of high tonicity and low oxygenation. Numerous studies have shown that renal epithelial cells within this area of the kidney synthesize specific organic osmolytes that protect these cells from this harsh environment and allow principal cells of the collecting duct to undergo vasopressin-dependent salt and water transport (4). This is accomplished, in large part, via increased transcription of synthetic enzymes and transporters under control of the tonicity-dependent transcription factor tonicity-responsive enhancer binding protein (TonEBP) (3, 4, 42). In assessing the human genome of NR3a and NR3b, in the region immediately upstream of coding exons, we identified a potential transcription factor binding site upstream of NR3a, which matched the known consensus binding sequence for the tonicity-dependent transcription factor TonEBP. This raised the possibility that NR3a might be constitutively expressed in cells that were...
chronically exposed to a hypertonic environment, such as exists in the renal medulla. Thus the purpose of this study was to determine whether the NR3a subunit is expressed in the medullary region of the kidney and to determine whether it plays a functional role in collecting duct cells.

METHODS

Materials

Reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Cell Culture

Inner medullary collecting duct (IMCD) cells (ATCC, Manassas, VA) (35) were cultured in DMEM/F12 (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Media was changed every 2–3 days, and cells were passaged at a 1:4 ratio after reaching confluence.

Stable Knockdown of NR3a in IMCD Cells

Four sets of predesigned short-hairpin (sh) RNA targeted against mouse NR3a in the pSM2c vector (Open Biosystems, Huntsville, AL) as well as the pSM2c control vector were amplified from bacterial stocks, and isolated with a QIAfilter plasmid midi kit (Qiagen). Integrity and purity of the plasmid amplifications were verified via agarose gel electrophoresis and the ratio of 260/280 UV absorbances. Constructs were transfected into IMCD cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and selection of stable clones was achieved via addition of puromycin to the culture media. Knockdown of NR3a, relative to control-transfected cells, was verified by Western blotting. Of the four clones tested, the first (V2mm_125088) exhibited ~80% knockdown and was selected for further characterization. shRNA sequence for this clone was CGGA-

ANIMALS

NR3a+/− and wild-type (WT) control mice of the same genetic background (sv129/ Black Swiss) were a generous gift of Dr. ShanPing Yu and were initially characterized by Das et al. (10). Mice were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal care was supervised by the Emory University Division of Animal Research, and protocols were approved by the Institutional Animal Care and Use Committee.

RT-PCR. Total RNA was isolated from confluent IMCD cultures or total lysates of postnatal day 1 (P1) mouse kidneys using an RNEasy Plus RNA isolation kit (Qiagen). cDNA was then transcribed from total RNA with an AMV reverse transcriptase (Promega, Madison, WI) and subsequently amplified with PCR Platinum Supermix (Invitrogen) or RedTaq (Sigma) in an Eppendorf Mastercycler gradient (model 5331, Eppendorf, Westbury, NY). PCR conditions are available upon request. Primer sequences for NMDA receptor subunits were ordered from Integrated DNA Technologies (Coralville, IA) or Eurfins MWG Operon (Huntsville, AL) and sequences were as follows: NR1 forward ACACAGAAGGGCTTTGTCCA (hairpin region underlined). Animals. NR3a+/− and wild-type (WT) control mice of the same genetic background (sv129/ Black Swiss) were a generous gift of Dr. ShanPing Yu and were initially characterized by Das et al. (10). Mice were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal care was supervised by the Emory University Division of Animal Research, and protocols were approved by the Institutional Animal Care and Use Committee.

Western Blot Analysis

Tissue preparation. Cortical, medullary, and papillary tissue fractions were isolated and homogenized as previously described (19–21).

Cell culture preparation. Confluent IMCDs cultured in 10-cm dishes were washed with PBS, transferred to 1.5-ml tubes with a rubber policeman, and centrifuged for 5 min at 1,000 g. The supernatant was removed, and RIPA buffer (Thermo-Fisher, Waltham, MA) was added to each sample.

Sample preparation (tissue and cell culture). Sample homogenates were sonicated with a sonic dismembrator set at 1.5 W (Fisher Scientific, Pittsburgh, PA), incubated on ice for 30 min, and centrifuged for 30 min at 10,000 g at 4°C. The supernatant was collected, and sample protein concentrations were determined via DC protein assay (Bio-Rad, Richmond, CA). Samples were subsequently denatured via incubation with LDS NuPage 4× (Invitrogen) containing 100 mM dithiothreitol for 5 min at 95°C or 30 min at 37°C. and run on protein TGX gels (Bio-Rad) in Tris/glycine/SDS running buffer (Bio-Rad) before being transferred to nitrocellulose (Invitrogen). Nitrocellulose was blocked for 30–60 min with 3–5% of blocking grade nonfat dry milk (Bio-Rad) before overnight incubation with primary antibodies for NR3a, NR3b, NR1, NR2a, or NR2b (catalog nos. 07–356, 07–351, AB9864, 06–313, and 06–600, respectively, Millipore, Billerica, MA), or β-actin (catalog no. sc-7777, Santa Cruz Biotechnology, Santa Cruz, CA). AQP2 primary antibody was a generous gift of Roger Fenton (31). All primary antibodies were used at a concentration of 1:1,000, except that for β-actin, which was 1:2,000. The following day, blots were washed, incubated for 1.5 h with 1:2,000 of horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG as appropriate (Millipore), and visualized with enhanced chemiluminescence by transferring membranes for 5 min with ECL Plus (GE Healthcare/Amersham, Buckinghamshire, UK) before exposure and development of CL-XPosure film (Thermo-Fisher).

Assessment of In Vitro Hypoxic Upregulation of NR3a

Confluent IMCDs were placed in an airtight modular chamber (Billups-Rothenberg, Del Mar, CA) which was perfused for 10 min with a gas mixture containing 95% nitrogen and 5% CO2. The

Fig. 1. Expression of N-methyl-d-aspartate receptor (NMDAR) NR3 subunits in the neonatal mouse kidney. A: PCR amplification of NR3a and NR3b mRNA from P1 and older mouse kidney lysates. B: Western blot for expression of NR3a and NR3b in lysates of P1 and older mouse kidney.
chamber was then incubated for 24 h at 37°C, after which cells were immediately collected and processed for Western blotting, as described above.

**Assessment of In Vivo Osmotic Upregulation of NR3a**

Drinking water was removed from the cages of four male WT (sv129/Black Swiss) mice aged >6 wk for 20 h, after which the mice were euthanized and nephrectomized. Outer and inner medullary fractions were isolated and processed for Western blotting, as described above. Medullary fractions from five age- and sex-matched control mice with continuous ad libitum water access were processed analogously.

**Immunocytochemistry**

IMCDs were grown to confluence on permeable supports and deprived of serum for 24 h before addition of 5 nM dDAVP or vehicle for 30 min. After 30 min, cells were fixed with 2% paraformaldehyde for 10 min. Filters were then washed 3× with PBS, permeabilized with 0.2% Triton-X 100, washed again 3× with PBS, and incubated for ≥1 h in 5% BSA in PBS before overnight incubation at 4°C with a primary antibody directed against the C terminus of aquaporin-2 (AQP2), previously characterized by Nielsen et al. (2, 31). The following day, filters were washed 3× with PBS and incubated with Dylight-488-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature. Cells were then washed 3× with PBS and incubated overnight with an additional primary antibody directed against ZO-1 (catalog no. 40–2200, Invitrogen), washed 3× with PBS and incubated with goat anti-rabbit conjugated to Alexa-Fluor 594 secondary antibody (Invitrogen), and washed with 3× with PBS. Membranes were excised with a scalpel and mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). All antibodies were applied in a blocking solution containing 5% BSA in PBS. Slides were subsequently imaged with a Leica TCS SPF Con-
focal system running Leica Application Suite Advanced Fluorescence software version 2.3.1. (Leica Microsystems, Bannockburn, IL). Immunocytochemistry for NR3a was prepared analogously, except that cultures were prepared in chamber slides (Lab-Tek/Cole-Parmer, Vernon Hills, IL).

**Immunohistochemistry**

Three-month-old mice were anesthetized with isoflurane administered at >5% until breathing ceased. A midline abdominal incision was performed, and the aorta was transected. Kidneys were removed, preserved in 10% buffered formalin, embedded in paraffin, and cut into 5-μm sagittal sections. Tissue sections were deparaffinized and rehydrated by treatment with xylene and ethanol and subsequently incubated for 30 min at 55°C in a freshly boiled antigen-unmasking solution (10 mM Tris, 1 mM EDTA, 0.05% Tween 20 at pH 9.0). Sections were then washed with water and PBS-T and incubated for 30–60 min in a blocking solution containing 5% BSA in PBS with 5% serum matched to the species of the secondary antibody. Sections were subsequently incubated overnight at 4°C with 20 g/ml rhodamine-conjugated Dolichos biflorus agglutinin (Vector Laboratories) and primary antibody directed against NR3a (Millipore) diluted 1:1,000 in PBS with 5% BSA. The following day, sections were washed for 3 × 5 min with PBS-T, incubated with fluorescein-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch) in PBS with 5% BSA, and counterstained with Hoechst nuclear stain (Invitrogen) before mounting with Vectashield mounting medium (Vector Laboratories).

**Osmolality Measurements**

IMCD cells were cultured to confluence on permeable supports with 0.4-μm pores (Costar/Corning, Corning, NY) and deprived of serum for 24 h before experiments were performed. Determination of the transepithelial osmotic gradient was determined by separate measurements of the osmolality of the basolateral and apical compartments of the Transwell chambers and is expressed as the difference between these values (basolateral — apical osmolality). Osmolality was determined from 10-μl samples of apical and basolateral culture media in a Vapro vapor-pressure osmometer (Wescor, Logan, UT). After measurement of baseline osmolalities, 5 nM vasopressin was added to the basolateral compartment, and additional measurements were made every 15 min for 45 min, with maximal readings used to determine transepithelial osmotic gradients. Samples were maintained at 37°C, 5% CO₂ between readings, and calibration of the osmometer to 290, 1,000, and 100 mmol/kgH₂O standards was performed before each experiment.

**Labeling and Isolation of Cell Surface Proteins**

For assessment of surface-expressed proteins, cells were cultured to confluency on 10-cm dishes. Cells were then treated with NHS-SS-biotin, and biotinylated surface proteins were isolated through binding to an avidin-containing column using a cell surface protein isolation kit (Thermo-Fisher), according to the manufacturer’s protocol. Concentration of the isolated surface fraction as well as the eluted non-surface fraction from the same preparation were determined via DC protein assay and subsequently analyzed via Western blotting as described above. For surface labeling of AQP2, dishes were incubated overnight in serum-free DMEM/F12 with 10 nM AVP and additionally exposed to 10 nM AVP for 30 min before biotinylation.

**Calcium Imaging Experiments**

Fura 2-AM and fluo 4-AM loading. Fifty micrograms of fura 2-AM or fluo 4-AM dye (Invitrogen) was dissolved in 10 μl Pluronic F127, 20% wt/vol in DMSO (TefLabs, Austin, TX) and added to 10 ml of phenol-free DMEM/F12 (Mediatech). Cells were then incubated in this solution for 45 min, washed two to three times in PBS, and incubated for an additional 20 min in phenol-free DMEM/F12 containing 0.5–1.0 mM water-soluble probenicid (Invitrogen).

**Comparison of intracellular Ca²⁺.** Differences in intracellular Ca²⁺ concentration ([Ca²⁺]i) between shRNA and control-transfected IMCD cells were determined as follows. Cells were cultured to

![Fig. 3. Expression of NMDAR subunits in inner medullary collecting duct (IMCD) cell line. A: PCR amplification of NMDAR subunits present in IMCD cell line. B: Western blotting for NMDAR subunits in IMCD cells. C: representative immunocytochemical staining of NR3a (green) in IMCD cells. Nuclei were counterstained in blue with Hoechst dye.](http://ajprenal.physiology.org/)
confluence in black clear bottom 96-well plates (Costar/Corning) and incubated with fura 2 or fluo 4 as described above. Determination of fluo 4-AM fluorescence intensity was accomplished by exciting at 480 nm with emission at 510 nm in a Fluoroskan Ascent Fluorescence plate reader (Labsystems/Thermo, Hudson, NH). Background fluorescence was determined by exciting unloaded cells at the same wavelengths. Fura 2-AM ratios were determined by exciting at 340 and 380 nm with emission at 510 nm in a NOVOstar microplate reader (BMG Labtech, Cary, NC) with cells maintained at 37°C. After subtracting background fluorescence values, an estimation of intracellular Ca²⁺ concentration was calculated according to the formula

$$[\text{Ca}^{2+}]_{\text{free}} = K_d \times \left[\frac{(R - R_{\text{min}})/(R_{\text{max}} - R)}{F_{\text{380}}} / F_{\text{min}}\right]$$

where $K_d$ is the dissociation constant of fura 2 for Ca²⁺, R is the 510-nm emission ratio of excitation at 340 and 380 nm, $R_{\text{min}}$ is the ratio with zero-free Ca²⁺, $R_{\text{max}}$ is the ratio with saturating free Ca²⁺, $F_{\text{380}}$ is the 510-nm emission at 380 excitation with zero-free Ca²⁺, and $F_{\text{380 min}}$ is the 510-nm emission at 380 excitation with saturating Ca²⁺. Experimentally determined values for $R_{\text{min}}$ and $R_{\text{max}}$ were 0.485 and 23.966, respectively. $K_d$ was based on the manufacturer’s value with adjustment for temperature and pH of the actual experimental conditions.

**Characterization of d-serine agonist effect on $[Ca^{2+}]_i$.** IMCD cells were plated on black-walled Equiglass 24 well plates (Genetix, Hampshire, UK) or black-walled 96-well plates (Costar/Corning) and grown to confluence before loading with fura 2-AM, as described above. After measurement of baseline emissions at 510 nm with 340- and 380-nm excitation, either d-serine or vehicle was added to wells and mixed via pipetting before repeating measurements of fluorescence emission.

**Assessment of Urine Concentrating Ability in WT and NR3a⁻/⁻ Mice**

Four male NR3a⁻/⁻ mice and four WT controls of the same age and background strain (sv129/Black Swiss) were placed in metabolic cages (Tecniplast, Exton, PA) with free access to food and water. After a 24-h equilibration period, normal urine was collected for 24 h. After this initial equilibration period, water bottles were removed from the cages and urine was collected hourly for 18 h before euthanization. The osmolality of collected urine samples was determined via vapor-pressure osmometry, as described above for in vitro osmolality measurements. Osmolalities for samples exceeding the linear range of the osmometer were determined by measuring dilutions of the original samples. For assessment of statistical significance, osmolalities collected during water restriction were pooled by 6-h intervals.

**Statistical Analysis**

Differences between groups were determined by Student’s t-test or by one-way ANOVA, in conjunction with Tukey’s test for honestly significant differences, as appropriate. $P$ values $<0.05$ were considered significant. Data are shown as means ± SE.

**RESULTS**

**Renal NR3a Expression**

Because NR3 subunits are known to be expressed at high levels relatively early in development (33), we initially used whole cell lysates of neonatal mouse kidney to determine whether NR3a and NR3b are present in kidney tissue. PCR amplification of mRNA from total kidney lysates of neonatal
mice produced bands of the expected size which, when sequenced, matched the known nucleotide sequences for NR3a and NR3b (Fig. 1A). Expression of NR3a and NR3b protein was also clearly evident in the neonate, but was observed to rapidly decline with increasing age (Fig. 1B). However, it should be noted that there was still a faint band at the expected molecular weight for NR3a in the adult mouse.

Immunofluorescence using an antibody directed against NR3a was performed to determine whether there might be a specific renal localization of NR3a in the adult kidney. Using confocal imaging, very little staining was found in the renal cortex, whereas we found intense staining for NR3a that was localized to tubules within the inner medulla and papilla (Fig. 2, A–D). Because this localization was consistent with expression in collecting duct cells, we next assessed colocalization of NR3a with D. biflorus agglutinin, a surface lectin which binds selectively to principal cells of the collecting duct (16). As shown in (Fig. 2E), NR3a localizes to the basolateral membrane of collecting duct cells, a nephron segment not previously known to express NMDA receptors.

NR3a Expression in a Collecting Duct Cell Line

To further characterize NMDA receptors in this nephron segment, we examined the expression of NMDAR subunits in a cell line (mIMCD-3, IMCD hereafter) known to recapitulate key functional properties of the collecting duct (35). PCR amplification of total RNA isolated from confluent IMCD cultures yielded bands of the expected product size for all NMDAR subunits except NR2b (Fig. 3A), and the identity of amplified products was verified by DNA sequencing. Western blot analysis confirmed expression of subunits NR1, NR2a, NR3a, and NR3b (Fig. 3B), while immunocytochemical staining further confirmed expression of NR3a (Fig. 3C).

Regulation of Renal NR3a

Hypoxia and increased osmolality are inherent features of the inner medullary and papillary regions, and both have been identified as factors that drive expression of specific genes in mice...
vivo via induction of the transcription factors HIF-1α and TonEBP, respectively (4, 24, 41). To determine whether either of these factors might account for the specific medullary and papillary expression of NR3a, we evaluated the expression of NR3a in IMCDs under conditions of decreased oxygen or increased osmolality. After 24-h incubation of IMCDs in 0.1% oxygen, there was a fourfold increase in the expression of total NR3a protein (Fig. 4C). In terms of elevated osmolality, confocal assessment of NR3a fluorescence in IMCDs exposed to gradated increases in media NaCl concentration suggested that media tonicity might additionally increase expression of NR3a (Fig. 4A). To assess this possibility in vivo, we subjected mice to water restriction for 18 h and found that this maneuver resulted in a significant increase in NR3a protein in medullary/papillary regions of the kidney (Fig. 4B).

**Physiological Significance of NR3a in Collecting Duct Cells**

To further assess the functional role of NR3a in the collecting duct cells, we stably transfected IMCD cells with shRNA constructs directed against NR3a. Of four constructs utilized, one exhibited an 80% decrease in Western blot expression of NR3a compared with control-transfected cells (Fig. 5, A and B). As shown in (Fig. 5D), IMCD cells with NR3a responded to the glycine-site agonist D-serine (27) with an increase in \([\text{Ca}^{2+}]_i\) (as assessed by the fura 2 excitation ratio) while NR3a knockdown cells failed to respond to this agonist. In these studies, we noted that baseline \([\text{Ca}^{2+}]_i\) tended to be higher in NR3a knockdown cells. To verify the elevated \([\text{Ca}^{2+}]_i\) in knockdown cells, additional experiments were performed using the Ca2+-sensitive dye fluo 4. Although this is not a ratiometric dye, fluo 4 fluorescent intensity was significantly higher in knockdown cells compared with control-transfected cells \((n = 168 \text{ wells}, 4 \text{ separate experiments}) (P < 0.001). This suggested that knockdown of NR3a might result in increased cytosolic calcium, a finding which was confirmed with the ratiometric dye fura 2 (Fig. 5C). Thus with knockdown of NR3a there is a significant increase in \([\text{Ca}^{2+}]_i\) compared with cells that express normal levels of NR3a. The reason that knockdown resulted in elevations in \([\text{Ca}^{2+}]_i\) is not entirely clear. It should be noted that we were unable to obtain consis-

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**Fig. 7. Transepithelial osmotic gradient.** Transepithelial osmotic gradient in control- and shRNA-transfected IMCD cells before (bars 1 and 2) and after (bars 3 and 4) basolateral addition of 5 nM AVP. Last column demonstrates absence of effect when epithelial sodium channels are inhibited via pretreatment with amiloride \((n = 15–20 \text{ wells/group from 4–7 separate passages})\). \(*P < 0.05, **P < 0.01, \text{ANOVA.}\)

**Fig. 8. Surface expression of aquaporin-2 (AQP2) in control- and shRNA-transfected IMCD cells.** A: Western blot of biotinylated surface fraction (left) and nonbiotinylated intracellular fraction from control- and shRNA-transfected IMCD cells. Bottom: densitometry for expression of 40- to 46-kDa glycosylated AQP2 in surface (left) and intracellular (right) fractions from 4 separate experiments. \(*\*P = 0.0026, ***P < 0.0001, \text{Student’s} \text{t-test.}\) B and C: confocal assessment of AQP2 expression in shRNA- and control-transfected IMCD cells. B: confocal images taken through the apical plane of IMCDs grown on Transwell filters. Nuclei were stained with Hoescht (blue), red = tight junctions (ZO-1), and green = AQP2. C: sagittal view of confocal Z-stack demonstrating apical staining of AQP2 in control-transfected cells (white arrows). (Lower line of green staining along the bottom corresponds to nonspecific staining and autofluorescence of the Transwell filter which was also observed in the absence of primary antibody and on unstained filters.)

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tent and replicable increases in $[\text{Ca}^{2+}]$, with classic NMDAR agonists (NMDA or glutamate both at 100 μM–1 mM). One reason for this is that glutamate is present in the culture media. Thus there might be continuous activation of a NR1/NR2 receptors which in the absence of NR3a would lead to sustained increased $\text{Ca}^{2+}$ entry. On the other hand, it is also possible that NR3a is having a unique effect on $\text{Ca}^{2+}$ regulation independently of classic NMDAR signaling.

In the brain, NR3a has been suggested to be protective, possibly by preventing increases in $[\text{Ca}^{2+}]$. To determine whether reduction of NR3a was altering normal homeostatic mechanisms of collecting duct cells, we measured the rate of cell proliferation and the rate of cell death in control and NR3a knockdown cells. As shown in (Fig. 6, A and B) in nonconfluent cell cultures, the rate of cell proliferation was less in NR3a knockdown cells compared with control cells. This may be related to the higher rate of cell death in knockdown cells as assessed by lactate dehydrogenase release (Fig. 6, C and D) and trypan dye accumulation (Fig. 6E).

**NR3a Regulates AQP2-Induced Water Flow**

A primary function of the renal collecting duct is to reabsorb water from tubular fluid when the peptide hormone AVP is released under conditions of decreased water availability (12, 23, 26). To assess whether the increase in $[\text{Ca}^{2+}]$, induced by knockdown of NR3a might alter this process, we cultured IMCD cells on permeable supports with isomotic DMEM/F12 on both apical and basolateral surfaces. After 15–30 min of 10 nM AVP addition to the basolateral compartment, we measured osmolality in the apical and basolateral compartments. As shown in (Fig. 7), this resulted in the generation of an osmotic gradient in IMCD cells transfected with shRNA compared with control cells. In control cells, the lack of an osmotic gradient with the addition of vasopressin may be due to concomitant salt and water transport. In NR3a knockdown cells, AVP-stimulated salt transport (amiloride-sensitive epithelial sodium channel activity) led to a significant osmotic gradient, which could be due to a deficit in water movement across the epithelium. This suggested that there might be an alteration in AQP2 abundance/trafficking in NR3a knockdown cells. To assess this possibility, we biotinylated cell-surface proteins and compared the relative expression of surface and intracellular proteins in shRNA- and control-transfected IMCDs. This revealed a significant decrease in both surface and intracellular expression of the 40- to 46-kDa glycosylated form of AQP2 (Fig. 8A). The expression pattern of AQP2 was also assessed with confocal microscopy in IMCDs grown on permeable supports following 30-min exposure to the vasopressin receptor agonist dDAVP (10 nM): while both cell lines exhibited expression of AQP2, instances of apical expression of AQP2 were clearly evident in control cells, but not in shRNA-transfected IMCDs (Fig. 8, B and C).

**NR3a Alters Renal Concentrating Ability**

To determine whether AQP2 protein/trafficking is also altered in vivo, we used NR3a$^{-/-}$ mice and compared these to age- and sex-matched matched WT controls. Western blotting of tissue lysates revealed a significant decrease in AQP2 expression in the outer medulla and inner medulla of NR3a$^{-/-}$ mice in both glycosylated and unglycosylated forms of AQP2 (Fig. 9A).

To determine whether the observed decrease in AQP2 expression associated with knockdown or genetic deletion of NR3a had functional consequences in vivo, we assessed urine concentrating ability in control and NR3a$^{-/-}$ mice. After 6 h of water deprivation, WT mice exhibited a significantly increased urine osmolality compared with urine collected from NR3a$^{-/-}$ mice (Fig. 10).

**DISCUSSION**

The major finding of this study is that the NR3a subunit of the NMDAR is expressed in the kidney. This subunit was highly expressed in neonate mouse kidney, and its presence drastically diminished over a relatively short period of time following birth. This finding is consistent with what has been reported in neurons (28, 46). The explanation or significance for the high level of expression of NR3a in the neonate and its...
subsequent decline thereafter has not been fully explained, although in the brain it has been proposed to be involved in neuroprotection (30) and activity-dependent sculpting of synapses (33, 34, 36). The function on NR3a in the developing kidney is, at present, completely unknown. Perhaps it may play an important role in nephron development. Although NR3a declines with age, there are examples of sustained NR3a levels, for instance, in discrete regions of the central nervous system including the amygdala, layer V pyramidal neurons, mesencephalic trigeminal neurons, and retina (43, 46). The recognition that the genetic region upstream of exons coding NR3a contains a putative TonEBP binding site led us to determine whether there might be tonicity-driven expression of NR3a in the medullary region of the adult kidney.

With immunofluorescence, it was possible to determine that there was very little expression of NR3a in the adult mouse renal cortex. This was not the case, however, in the medullary/papillary region, where we detected a high level of expression of this protein. The pattern of staining we observed in this region was consistent with collecting duct-specific localization, a finding that was confirmed by costaining with the lectin *D. biflorus* agglutinin. This finding was also supported by the robust detection of NR3a protein in an IMCD cell line. In addition, we were able to show in IMCD cells that NR3a protein was upregulated by hypoxia while confocal assessment suggested that increased media osmolality might also increase [Ca\(^{2+}\)]\(_i\). IMCD cells failed, however, to respond with changes in [Ca\(^{2+}\)]\(_i\) to classic NMDAR agonists and antagonists. This suggests, although it certainly does not prove, that NR3a has other effects on cell function and regulation of [Ca\(^{2+}\)]\(_i\) that may be independent of the classic role of NMDAR subunits as ligand-gated surface receptors.

Fig. 10. Urine osmolality in WT and NR3a\(^{-/-}\) mice during water restriction. Urine osmolality was measured in urine collected from 4 WT and 4 NR3a\(^{-/-}\) mice housed in metabolic cages during water restriction. Urine was collected hourly; 0 = initial osmolality, 6 = time points between 0 and 6 h, and 12 = time points between 6 and 12, 18 = time points between 12 and 18 h. **P < 0.01, ANOVA.

Fig. 11. Hypothetical model of impact of NR3a on collecting duct function. Top: under conditions of hypertonicity and hypoxia, expression of NR3a is increased, minimizing Ca\(^{2+}\) permeability of collecting NMDARs, and maintaining a low [Ca\(^{2+}\)]\(_i\). Bottom: in the absence of NR3a, hypoxic and hypertonic induction of NR3a cannot occur and [Ca\(^{2+}\)]\(_i\) becomes elevated, leading to impaired AQP2 synthesis and/or trafficking. This, in turn, leads to decreased AQP2-dependent water transport and delayed urinary concentrating ability. V2R, vasopressin V2 receptor.
In the brain, NR3a is thought to be neuroprotective. To determine whether NR3a may play a similar role in collecting duct cells, we assessed cell growth rates and the rate of cell death in IMCD cultures. In IMCD cells with reduced NR3a, cell growth was slower and cell death was higher compared with control-transfected cells. One plausible explanation for this finding is that [Ca\(^{2+}\)], was higher in knockdown cells, as it is well accepted that there are substantial pathological consequences to sustained elevations in cell Ca\(^{2+}\). While the finding of decreased cell growth might simply be a reflection of increased cell death, adjusting the observed growth rates for the apparent rate of cell death did not fully account for the observed difference, suggesting an effect of NR3a on cell growth independent of any effect on cell death. A difference in cell growth could be attributed to perturbations in cytosolic calcium (37); however, this effect could also conceivably reflect perturbation of the interaction between NR3a and its intracellular binding to protein phosphatase PP2a (6, 25), an enzyme which has been shown to impact cell cycle progression (18).

To determine whether diminished NR3a affected the normal function of collecting duct principal cells, we assessed vasopressin-mediated water transport. This was accomplished by placing confluent IMCD cells, grown on filters in a chamber, with separate apical and basolateral solutions. In the presence of isosmotic solutions on both sides, the addition of vasopressin led to the generation of a significant amiloride-sensitive osmotic gradient in knockdown cells, which was not observed in control-transfected cells. We interpret this to mean that salt transport in control cells was accompanied by AQP2-mediated water transport, whereas NR3a knockout cells retained functional salt transport but exhibited a deficit in AQP2-dependent water transport. This interpretation was supported by the finding that AQP2 total protein and vasopressin-induced surface expression of AQP2 were diminished in knockdown cells. This in vitro finding was directly supported in NR3a knockdown IMCD cells and NR3a\(^{-/-}\) knockout mice, however, there would be chronic elevation of [Ca\(^{2+}\)], Such chronic increases are known to evoke a number of deleterious effects (8, 32), which might well be expected to lead to a decrease in protein levels. Thus a subject for future investigation into whether reductions in AQP2 reflect altered trafficking or synthesis or both and whether regulation of AQP2 might constitute a direct function of collecting duct NMDARs.

In conclusion, specific renal expression of NR3a in the medullary and papillary collecting duct is likely driven both by decreases in oxygen and increases in osmolality that occur in this environment. One function of NR3a in this region appears to be the maintenance of low cytosolic calcium to enable functional calcium-dependent signaling, including vasopressin-induced trafficking of AQP2. Thus NR3a may be renoprotective in collecting duct cells, and, under conditions that are associated with high vasopressin levels, may be one mechanism that protects the function of the principal cells to reabsorb water, thereby helping to maintain the countercurrent multiplication system.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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