AVR/NAVR deficiency lowers blood pressure and differentially affects urinary concentrating ability, cognition, and anxiety-like behavior in male and female mice

Victoria L. M. Herrera, Pia Bagamasbad, Julius L. Decano, and Nelson Ruiz-Opazo

Section of Molecular Medicine, Department of Medicine, and Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, Massachusetts

Submitted 11 August 2010; accepted in final form 2 October 2010

Herrera VL, Bagamasbad P, Decano JL, Ruiz-Opazo N. AVR/NAVR deficiency lowers blood pressure and differentially affects urinary concentrating ability, cognition, and anxiety-like behavior in male and female mice. Physiol Genomics 43: 32–42, 2011. First published October 5, 2010; doi:10.1152/physiolgenomics.00154.2010.—Arginine vasopressin (AVP) and angiotensin II (ANG II) are two peptidergic hormones that differentially modulate distinct physiological functions in the brain, kidney, and blood vessels. Vasopressin, produced in hypothalamic (supraoptic and paraventricular nuclei) and extrahypothalamic (locus coeruleus, stria terminalis) structures, is released and acts on peripheral organs, as well as on the brain through paracrine/autocrine loops and through vasopressinergic neuronal innervation of several regions of the brain (22). Very briefly, in the periphery, AVP modulates water balance, vascular vasoressor activity, blood pressure (BP), and body temperature (14) and modulates complex neurobiological functions such as anxiety, learning and memory, and social behavior (2, 9–11). Likewise, ANG II regulates BP, vascular wall biology, and renal salt-water balance (13), as well as modulates learning and memory functions (42).

Previous studies have identified several “canonical” AVP- and ANG II-specific receptors classified pharmacologically as V1a, V1b, and V2-type of receptors for AVP (39) and AT1 and AT2-type receptors for ANG II (42). However, studies of knockout mice of said “canonical” AVP receptors, V1aR (11, 29), V1bR (32), V2R (54), and ANG II receptors, AT1–(7) and AT2-type (16) receptors do not completely account for established vasopressin- and/or ANG II-induced functions in the brain, kidney, and blood vessels. More specifically, noninbred V1aR−/− knockout male mice exhibited decreased anxiety-like behavior in the elevated plus maze test but normal spatial memory performance in the Morris water maze task (11) and lower BP levels compared with wild-type controls (29). Noninbred V1bR−/− knockout mice did not show differences in anxiety-like behavior or in spatial learning and memory when compared with wild-type controls; BP was not measured (11). However, since these knockout mouse studies were done on mixed genetic backgrounds, ambiguity due to potential founders from genetic heterogeneity remains. Female mice heterozygous for a nonsense mutation in V2R exhibited polyuria and reduced urinary concentrating ability when compared with wild-type controls, resembling X-linked nephrogenic diabetes insipidus (54). Likewise, in the ANG II receptor system, AT1AR−/− (angiotensin II type-1A receptor) and AT2R−/− (angiotensin II type-2A receptor) knockout mouse models have also been developed. No BP effects were observed in AT2R−/− mice (16). In AT1AR−/− knockout mice, males demonstrated lower BPs compared with wild-type controls (7); however, studies performed in AT1AR−/− mice backcrossed more than six generations onto a C57BL/6 genetic background did not detect differences in BP nor in sodium excretion (34). These observations suggest that AT1AR is not a key regulator of BP or renal sodium balance in C57BL/6 (34) and that some other “noncanonical” ANG II receptor is or that there is redundancy in BP and renal sodium regulation that is provided by other noncanonical ANG II receptors. Collectively, these observations would suggest that a greater complexity of AVP and ANG II receptors is evident, thus necessitating the need for study of noncanonical AVP and ANG II receptors.

We have elucidated a noncanonical AVP receptor system contained in two overlapping AVP receptors, AVR (a dual angiotensin-vasopressin receptor) and NAVR (a nonangioten-
sin, vasopressin receptor) both expressed from a single gene by alternative promoter usage and upregulated synergistically by testosterone and estrogen (17). The mouse AVR sequence is contained within NAVR’s amino acid residues 424–878. NAVR binds AVP exclusively, while AVR portrays high-affinity binding sites for both ANG II and AVP (17). AVR and NAVR are abundantly expressed in kidney and to a lesser extent in brain, liver, lung, adrenal gland, heart, and aorta (17, 47). In situ hybridization studies detected prominent AVR/NAVR expression in neurons throughout the brain wherein V1-type AVP and AT1/AT2-type ANG II receptors are detected (22). Additionally, the AVR/NAVR is detected in vasopressinergic magnocellular neurons in the supraoptic and paraventricular nuclei of the hypothalamus, along with another noncanonical AVP receptor, VACM-1 (vasopressin-activated calcium-mobilizing) receptor, whereas the canonical V2R AVR receptor is not present (22). Moreover, given our recent genetic studies in Dahl S × Dahl R rat intercrosses that elucidated two chromosome 1 overlapping QTLs (quantitative trait loci), BP-m2 at chr1–208.8 Mb affecting BP (19), and Nav-3 at chr1–197.7 Mb influencing spatial learning and memory (49) and that in close proximity at chr1–201.0 Mb resides the AVR/NAVR locus suggest that it may underlie both QTLs influencing BP and spatial navigation performance. Altogether, these observations suggest the hypothesis that AVR/NAVR mediates known renal, vascular, and brain AVP/ANG II functions, not accounted for by canonical AVP and ANG II receptors respectively.

To test this hypothesis, we developed AVR/NAVR knockout mice by targeted disruption and investigated whether null deficiency of AVR/NAVR protein could differentially affect BP, cognitive performance and/or renal salt-water balance, deficiency of AVR/NAVR protein could differentially affect out mice by targeted disruption and investigated whether null mutations respectively. Not accounted for by canonical AVP and ANG II receptors.

RT-PCR of Mouse Kidney RNA

RNA from mouse kidneys was extracted with TRIzol reagent (Invitrogen) as described (18). We performed RT-PCR analysis of mouse kidney total RNAs using the following primers: for AVR/NAVR, upstream primer: 5'-AACAGAAGACCTGGTTGAAGC-3', downstream primer: 5'-GCATCCTGAGTGTCTTCACC-3', yielding a 297 bp RT-PCR product indicative of intact AVR/NAVR mRNA. We note that the forward primer encompasses amino acids K685KSLVK690 of NAVR, a region that was deleted to generate null mutants, hence not present in AVR/NAVR— kidney RNA (no 297 bp RT-PCR product). A β-actin primer pair (Promega; 285 bp RT-PCR product) was used as internal control to ascertain presence of equivalent amounts of RNA in all samples tested.

Animals

Animals utilized in the experiments described below are BC10 (backcross 10), ascertained to be >99.98% of C57BL/6 genetic background. Wild-type and AVR/NAVR— littermate mice were produced for the studies from BC10 (+/+−) × (+/+−) intercrosses. All mice were individually housed 1 wk prior beginning of testing. The first group consisted of 14 (−/−) males, 14 (−/−) females, 14 (+/+−) males, and 14 (+/+−) females. Testing was done in the following consecutive order and corresponding ages for this group: elevated plus maze (EPM) at 3 mo of age, social transmission of food preference (STFP) at 4 mo of age, and BP measurements at 6 mo of age (a 4 wk resting period was observed between the STFP and BP measurements). A second group of 12 (−/−) males, 11 (−/−) females, 11 (+/+−) males, and 11 (+/+−) females was utilized for Morris water maze (MWM) testing at 3 mo of age. A third group of 6 (−/−) males, 6 (−/−) females, 6 (+/+−) males, and 6 (+/+−) females was utilized for metabolic cage studies at 3 mo of age.

BP Measurements

We measured systolic BP in 6 mo old mice by tail-cuff sphygmanometer (Visitech BP 2000, Visitech CA) under mild anesthesia (Ketamine/Xylazine), ascertaining equivalent physiological state by limiting systolic BP measurements to periods with heart rate ranging from 350 to 550 beats/min (bpm) and equivalent duration on warming platform. For males: Ketamine at 0.075 mg/g body wt and Xylazine at 0.013 mg/g body wt. For females: Ketamine at 0.056 mg/g body wt and Xylazine at 0.01 mg/g body wt. We obtained three sets of 10 consecutive readings per mouse over a period of 30 min. We took an average of 21.2 readings within the 350–550 heart rate range. Systolic BP readings with heart rates <350 bpm were excluded.

Metabolic Cage Studies

Mice were maintained in pathogen-free, temperature-controlled environment under a 12 h light/dark cycle. Mice were individually
housed in metabolic cages (Lab Products, Seaford DE) for 3 days prior to measurements (habituation period). Water and powdered food (Harlan 2018 rodent chow) were made available ad libitum during the 3-day habituation period. We measured 24 h water consumption and 24 h urine volume at 2 h into the light cycle for 4 consecutive days. On the 4th day of measurement, mice were deprived of water and urine output was measured 24 h later. Maximal urinary osmolality after the 24 h water restriction was measured on a Micro Osmometer (Precision Systems, Natick, MA). The cages were cleaned daily, and the urine collection apparatus was lined with mineral oil to facilitate urine collection.

MWM Task

The MWM task was performed as described (18). Subjects were individually housed for 1 wk prior to the experiment. The MWM system (San Diego Instruments, San Diego, CA) consisted of a white plastic circular pool (70 cm in diameter) and a movable square platform (8 cm × 8 cm). The pool was filled with water (26 ± 1°C) and rendered opaque by the addition of 1 quart of 2% fat-reduced milk. The mouse’s path was monitored and recorded with the SMART computer tracking system (San Diego Instruments). The water maze was divided into four imaginary quadrants. Each animal was subject to 2 days of visible platform followed by 4 days of hidden platform test.

**Visible platform version.** The water maze system was located in a small observation room with blank walls. The platform was raised 1 cm above the water and was cued by attaching two 8.3 cm green cylinders to two opposing corners. Testing was performed for 2 consecutive days. Prior to the first trial of day 1, the mouse was dropped in the water and allowed to swim and led to the platform three times. The mouse was given 10 s rests on the platform in between the practice swims. A trial was initiated by placing the mouse in the water facing the pool perimeter in one of the chosen quadrants. The platform position randomly changed for each trial to avoid habituation to a particular quadrant. Six trials or swims per day were done from one of the four randomized start positions, which were located adjacent to the wall in the center of the four quadrants. The six trials were structured such that each mouse went through two sessions of three trials. There was a 10 min interval between trials in a session and a rest period in between sessions. Animals were counterbalanced with respect to subject groups. Latency to escape the water was recorded, and a 60 s limit was imposed on each trial. The mouse was guided to the platform and allowed to rest for 10 s if it failed to locate it in the specified time.

**Hidden platform version.** The experiment was done in the same room but with numerous salient visual cues placed in predefined locations in the vicinity (0.7–1.0 m) of the pool. The pool was filled until the platform was submerged 1 cm below the surface of the water.
Testing was done for 4 consecutive days. The trials were initiated in the same manner as the visible platform version, but this time the platform was maintained in one position for all the trials, and the mouse was randomly dropped in one of the three quadrants that did not contain the platform. Six trials were done per day and structured in the same manner as the visible platform version.

**Probe trial.** At the end of the 6th trial of day 4, the platform was removed and the mouse was dropped onto the quadrant diagonally opposite the platform's position. This was repeated for the 6th trial of day 5, 6, and 7.

---

**Table 1. Genomic organization of exons encoding mouse AVR/NAVR**

<table>
<thead>
<tr>
<th>Exon Number</th>
<th>Intron 3’ Seq</th>
<th>Exon Termini</th>
<th>Intron 5’ Seq</th>
<th>Exon Size, bp</th>
<th>Intron Size, bp</th>
<th>Domain Within Exon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ATGGATG</td>
<td>GCTCAG</td>
<td>gtaggt</td>
<td>291</td>
<td>554</td>
<td>AVP bd/Gs</td>
</tr>
<tr>
<td>2</td>
<td>cc ccc c</td>
<td>cc ccc c</td>
<td>cc ccc c</td>
<td>272</td>
<td>245</td>
<td>AVP bd/Gs</td>
</tr>
<tr>
<td>3</td>
<td>cc ccc c</td>
<td>cc ccc c</td>
<td>cc ccc c</td>
<td>56</td>
<td>216</td>
<td>AVP bd/Gs</td>
</tr>
<tr>
<td>4</td>
<td>cc ccc c</td>
<td>cc ccc c</td>
<td>cc ccc c</td>
<td>1747</td>
<td>959</td>
<td>AVP bd/Gs</td>
</tr>
<tr>
<td>5</td>
<td>cc ccc c</td>
<td>cc ccc c</td>
<td>cc ccc c</td>
<td>8153</td>
<td>959</td>
<td>AVP bd/Gs</td>
</tr>
<tr>
<td>6</td>
<td>cc ccc c</td>
<td>cc ccc c</td>
<td>cc ccc c</td>
<td>93</td>
<td>1156</td>
<td>AVP bd/Gs</td>
</tr>
<tr>
<td>7</td>
<td>cc ccc c</td>
<td>cc ccc c</td>
<td>cc ccc c</td>
<td>171</td>
<td>76</td>
<td>AVP bd/Gs</td>
</tr>
<tr>
<td>8</td>
<td>cc ccc c</td>
<td>cc ccc c</td>
<td>cc ccc c</td>
<td>168</td>
<td>549</td>
<td>AVP bd/Gs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>737</td>
<td></td>
<td>Poly-A</td>
</tr>
</tbody>
</table>

*Exons accounting for AVR; †NAVR exon 1 size determined from initiation Methionine (ATG); ††AVR exon 1 size determined from initiation Methionine (ATG); NAVR, nonangiotensin II/vasopressin receptor; AVR, angiotensin II/vasopressin receptor; AVR bd, angiotensin II/vasopressin binding domain; NAVR bd, angiotensin II binding domain; Gs, Gs activator domain.*
opposite to that which contained the platform. During this probe trial the mouse was allowed to swim for 60 s.

**STFP Task**

The STFP task was performed as described (18). Mice were individually housed for 1 wk prior to the experiment. Mice were trained or shaped to consume powdered food (Harlan 2018 rodent chow) for 3 days prior to testing. During the shaping period, mice were subject to a 23.5 h food-deprivation schedule and offered powdered food in their home cage for 30 min per day for 3 consecutive days. Powdered food was presented in 4 oz powdered food feeders and jar holders (Allentown caging equipment, Allentown, NJ). The animals were given 1/3 rodent chow pellet immediately following the 30 min shaping session. Food consumption was measured during the 3 shaping days, and an animal was considered “shaped” when it consumed at least 0.5 g of powdered food. Shaped subjects were deprived of food for 24 h. An anesthetized 6 wk old CD-1 mice (Harlan) of the same sex as the test subject was used as demonstrator. A specific scented food was sprinkled on top of the head of the demonstrator. The demonstrator was placed in the test subject’s cage and left there for 20 min for the subject to inspect. At the end of the exploratory period, the demonstrator was removed, and a 5 min and 24 h time delay was enforced for females and a 24 h and 72 h delay for males. At the end of the time delay, the test subject was offered two scented foods of a predetermined odor pairing in separate jars (50 mm apart), one of which was identical to the scented food presented by the demonstrator. The food was presented in a counterbalanced manner in the absence of water. To avoid inherited food preference as a confounder we used paired odors previously ascertained to be of equivalent baseline preference for male and female C57BL/6j mice (18). Preassigned odor pairings for the male subjects were 1% anise vs. 0.4% cumin for the 24 h retention time, and 0.25% clove vs. 0.25% sage for the 72 h retention time. Odor pairings for female subjects were 1% thyme vs. 0.25% curry for the 5 min retention time and 0.25% clove and 0.25% sage for the 24 h retention time.

**EPM**

The EPM system (San Diego Instruments, San Diego, CA) consisted of a white cross-shaped maze (660 mm × 660 mm, 400 mm high) with two opposing arms (50 mm wide) open and the two opposing arms (50 mm wide) bordered by walls (150 mm in height). The EPM was placed in a small observation room with blank walls. Lamp lights were placed just above the end of the open arms. Mice were placed in the room 1 h prior to the start of the experiment to adapt to the room. The mouse was tested by placing it in the middle of the maze facing one of the four arms. The mouse was allowed to explore the maze for 5 min, and its path was tracked and recorded using the SMART computer tracking system (San Diego Instruments). Animals were counterbalanced with respect to four arms of the maze at which they were dropped. With the use of the SMART computer tracking system, the maze was visually divided into five quadrants. Four quadrants corresponded to the four maze arms and central square quadrant that covered the central part of the four maze arms a distance equivalent to half the length of the mouse. Analysis was done by counting the number and time of visits to the closed arms and open arms. An animal was considered inside a maze arm when all its four paws were within the borders of the arm.

**Analysis of Signaling Proteins Modulated by AVR and NAVR**

Analysis of ligand-dependent modulation of different signaling proteins by AVR and NAVR was done in Cos1-avr and Cos1-NAVR permanent cell transfectants expressing rat AVR and rat NAVR, respectively (17). We used a single time point of 30 min and a single dose for AVR and NAVR ligands (ANG II, 10 nmol/l; AVP, 10 nmol/l). For multiplex analysis we utilized the Kinex Antibody Microarray System (Kinexus) spanning 506 phosphoprotein-specific antibodies in duplicates or multiple replicates, as well as 740 pan-specific antibodies of signaling proteins. For signaling proteins shown in Table 2 data are presented as percentage change from control (% CFC), or change detected after 30 minutes of AVP or ANG II treatment compared with nontreated transfectant-matched controls, respectively. The %CFC = [TreatedAve − ControlAve]/ControlAve × 100. %CFC > 25% is suggested as a significant difference. We present values exhibiting >25% CFC and with % error range between duplicates <20%. The % error range = [Duplicate − Average]/ Average. For quantitative analysis of signaling proteins presented in Fig. 8, phospho-site specific antibodies were utilized in Kinetworks protein kinase multiblot analysis (custom performed by Kinexus) for quantitative measures. All fluorescent signals were normalized to background, and a coefficient (1/actual scan time/60 s) was used as the correcting factor to standardize trace quantity measurements (actual trace quantity × coefficient) for all samples. Data are presented as percent change from control, or change detected after 30 min of ANG II or AVP treatment compared with nontreated transfectant-matched controls, respectively.

**Statistical Analyses**

Statistical tests (ANOVA or t-tests) were performed with the SigmaStat 2.03 software package. P < 0.05 was considered statistically significant.

**RESULTS**

**BP and Urinary Concentrating Ability of AVR/NAVR−/− Mice**

We generated AVR/NAVR−/− mice, deficient in both AVR and NAVR expression (Fig. 1). AVR/NAVR−/− mice were born at expected Mendelian frequencies from AVR/NAVR−/− parent's (genotype distribution from 171 pups produced: 38 +/+ , 97 +/− , 36 −/− ) with no morphological abnormalities. However, AVR/NAVR−/− male and female mice show reduced systolic BP (Fig. 3A, P < 0.01 and 3G, P < 0.01 for males and females respectively) without affecting heart rate (Fig. 3, B and H, for males and females, respectively) compared with age-matched, wild-type male and female littermates. Because of the prominent AVR/NAVR renal expression (17) and the well-established role of AVP in water homeostasis (4), we analyzed the urinary concentrating ability of AVR/NAVR−/− mice. Under basal conditions with free access to drinking water, AVR/NAVR−/− male mice showed significantly greater urine flow (Fig. 3D, P < 0.05) than wild-type male mice. Moreover, after a 24 h water restriction, AVR/NAVR−/− male mice exhibited impaired ability to decrease urine flow (Fig. 3E, P < 0.05) and showed significantly lower maximal urinary osmolality compared with wild-type littermates (Fig. 3F, P < 0.05). In contrast, AVR/NAVR−/− female mice did not differ from wild-type female mice in urinary concentrating function (Fig. 3, J−L).

**Spatial Navigational Performance of AVR/NAVR−/− Mice**

To explore the role of AVR/NAVR in brain function we investigated the effect of AVR/NAVR−/− deficiency on representative paradigms of cognitive behaviors. For cognition mice were first tested on the MWM task that evaluates visuospatial cognition (37). Measurements of spatial learning on the MWM revealed impaired performance in AVR/NAVR−/− male mice.
SEX-SPECIFIC EFFECTS OF AVR/NAVR DEFICIENCY

Table 2. Additional signaling proteins detected by ab-microarray involved in learning and memory phosphorylated/dephosphorylated by AVR and NAVR upon ANG II and AVP stimulation

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Protein Symbol</th>
<th>Phosph Site</th>
<th>Role in Learning and Memory</th>
<th>AVR+ANG II %CFC</th>
<th>AVR+AVP %CFC</th>
<th>NAVR+AVP %CFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium/calmodulin-dependent protein-serine kinase 2 alpha</td>
<td>CaMK2a</td>
<td>T286</td>
<td>NMDAR2B phosphorylation (52), Synapsin 1 phosphorylation (8), E-LTP expression (30), metaplasticity (55)</td>
<td>−32</td>
<td>−26</td>
<td>−57</td>
</tr>
<tr>
<td>cAMP-dependent protein-serine kinase catalytic subunit alpha/beta</td>
<td>PKA Ca/b</td>
<td>T197</td>
<td>PKA: Synapsin 1 phosphorylation (8), E-LTP expression (30), L-LTP expression (1), &quot;Priming&quot; LTP (40)</td>
<td>23</td>
<td>−18</td>
<td>42</td>
</tr>
<tr>
<td>cAMP-dependent protein-serine kinase catalytic subunit beta</td>
<td>PKA Cb</td>
<td>S338</td>
<td>Synapsin 1 isoform Ia Synapsin 1 S9 phosphorylation (39), MARCKS phosphorylation (31), LTP induction (21)</td>
<td>108</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>cAMP-dependent protein-serine kinase regulatory type 2 subunit beta</td>
<td>PKA R2b</td>
<td>S114</td>
<td>PKC: Synapsin 1 phosphorylation (43), LTP induction (21)</td>
<td>39</td>
<td>43</td>
<td>6</td>
</tr>
<tr>
<td>Protein-serine kinase C beta 2</td>
<td>PKC b2</td>
<td>T641</td>
<td>PKC: NMDAR2B phosphorylation (43), LTP induction (20)</td>
<td>56</td>
<td>36</td>
<td>35</td>
</tr>
<tr>
<td>Protein-serine kinase C delta</td>
<td>PKC d</td>
<td>Y313</td>
<td>MARCKS phosphorylation (31), LTP induction (21)</td>
<td>23</td>
<td>21</td>
<td>50</td>
</tr>
<tr>
<td>Protein-serine kinase C gamma</td>
<td>PKC g</td>
<td>T674</td>
<td>Tau S712 Neurite outgrowth (51), involvement in Alzheimer’s disease (51)</td>
<td>15</td>
<td>90</td>
<td>−6</td>
</tr>
<tr>
<td>Protein-serine kinase C eta</td>
<td>PKC h</td>
<td>T655</td>
<td>Tau S716 Neurite outgrowth (51), involvement in Alzheimer’s disease (51)</td>
<td>5</td>
<td>50</td>
<td>−3</td>
</tr>
<tr>
<td>Protein-serine kinase C lambda/ iota</td>
<td>PKC l</td>
<td>T555</td>
<td>Tau S716 Neurite outgrowth (51), involvement in Alzheimer’s disease (51)</td>
<td>15</td>
<td>90</td>
<td>−6</td>
</tr>
<tr>
<td>Protein-serine kinase C mu</td>
<td>PKC m</td>
<td>S738+</td>
<td>Tau S716 Neurite outgrowth (51), involvement in Alzheimer’s disease (51)</td>
<td>5</td>
<td>50</td>
<td>−3</td>
</tr>
<tr>
<td>(Protein kinase D)</td>
<td>PKC d</td>
<td>Y313</td>
<td>PKC: Synapsin 1 phosphorylation (43), LTP induction (20)</td>
<td>39</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>Protein-serine kinase C theta</td>
<td>PKC q</td>
<td>T638</td>
<td>PKC: Synapsin 1 phosphorylation (43), LTP induction (20)</td>
<td>5</td>
<td>21</td>
<td>66</td>
</tr>
<tr>
<td>zeta/lambda</td>
<td>PKC z/l</td>
<td>T410/ T403</td>
<td>PKC: Synapsin 1 phosphorylation (43), LTP induction (20)</td>
<td>4</td>
<td>51</td>
<td>43</td>
</tr>
<tr>
<td>Src proto-oncogene-encoded protein-tyrosine kinase</td>
<td>Src</td>
<td>Y418</td>
<td>NMDAR2B phosphorylation (43), LTP induction (20)</td>
<td>38</td>
<td>33</td>
<td>30</td>
</tr>
<tr>
<td>Synapsin 1 isoform Ia</td>
<td>Synapsin 1</td>
<td>S605</td>
<td>Regulation of neurotransmitter release (56)</td>
<td>−42</td>
<td>44</td>
<td>8</td>
</tr>
<tr>
<td>Synapsin 1 isoform Ia</td>
<td>Synapsin 1</td>
<td>S9</td>
<td>Tau S720 Neurite outgrowth (51), involvement in Alzheimer’s disease (51)</td>
<td>−30</td>
<td>26</td>
<td>−9</td>
</tr>
<tr>
<td>Microtubule-associated protein tau</td>
<td>Tau</td>
<td>S720</td>
<td>Tau S738 Neurite outgrowth (51), involvement in Alzheimer’s disease (51)</td>
<td>15</td>
<td>90</td>
<td>−6</td>
</tr>
<tr>
<td>Microtubule-associated protein tau</td>
<td>Tau</td>
<td>T547</td>
<td>Tau S530 Increased latency to target counter when compared with wild-control (4, C, P &lt; 0.05)</td>
<td>15</td>
<td>140</td>
<td>16</td>
</tr>
<tr>
<td>Microtubule-associated protein tau</td>
<td>Tau</td>
<td>T547</td>
<td>Tau S530 Increased latency to target counter when compared with wild-control (4, C, P &lt; 0.05)</td>
<td>−23</td>
<td>50</td>
<td>37</td>
</tr>
<tr>
<td>Microtubule-associated protein tau</td>
<td>Tau</td>
<td>S716</td>
<td>Tau S716 Increased latency to target counter when compared with wild-control (4, C, P &lt; 0.05)</td>
<td>9</td>
<td>46</td>
<td>−4</td>
</tr>
<tr>
<td>Microtubule-associated protein tau</td>
<td>Tau</td>
<td>S716</td>
<td>Tau S716 Increased latency to target counter when compared with wild-control (4, C, P &lt; 0.05)</td>
<td>14</td>
<td>28</td>
<td>8</td>
</tr>
<tr>
<td>Microtubule-associated protein tau</td>
<td>Tau</td>
<td>S515</td>
<td>Tau S716 Increased latency to target counter when compared with wild-control (4, C, P &lt; 0.05)</td>
<td>−35</td>
<td>35</td>
<td>−16</td>
</tr>
<tr>
<td>EribB2 (neu) receptor-tyrosine kinase</td>
<td>ErbB2</td>
<td>Y1248</td>
<td>Tau S716 Increased latency to target counter when compared with wild-control (4, C, P &lt; 0.05)</td>
<td>121</td>
<td>−15</td>
<td>9</td>
</tr>
<tr>
<td>Mammalian target of rapamycin</td>
<td>mTOR</td>
<td>S2448</td>
<td>Tau S716 Increased latency to target counter when compared with wild-control (4, C, P &lt; 0.05)</td>
<td>−6</td>
<td>−52</td>
<td>−9</td>
</tr>
</tbody>
</table>

AVR, angiotensin-vasopressin receptor; NAVR, nonangiotensin-vasopressin receptor; ANG II, angiotensin II; AVP, arginine-vasopressin; %CFC, percentage change from control; Phosph Site, phosphorylation site (tracked with specified phospho-site-specific antibodies); LTP, long-term potentiation; %CFC > 25 (boldface) and < −25 (italics) are highlighted.

compared with wild-type male mice. On the probe trial for spatial memory, AVR/NAVR−/− male mice did not show enhanced preference for the target quadrant over other quadrants, while wild-type male mice exhibited target selectivity [Fig. 4A, wild-type male mice: P < 0.01; Fig. 4B, AVR/NAVR−/− male mice: P = not significant (n.s.)]. Consistently, AVR/NAVR−/− male mice showed increased latency to target counter when compared with wild-type controls (Fig. 4C, P < 0.05), corroborating their inferior search accuracy for the hidden platform. In contrast, AVR/NAVR−/− females performed better by showing target selectivity compared with wild-type females, which were unable to locate the hidden platform (Fig. 4D, wild-type female mice: P = n.s.; Fig. 4E, AVR/NAVR−/− female mice: P < 0.01). Concordantly, wild-type female mice demonstrated increased latency to target counter when compared with AVR/NAV−/− females (Fig. 4F, P < 0.05). Notably, AVR/NAV−/− and wild-type mice exhibited comparable ability for cue learning (Fig. 5), indicating absence of sensory motor deficits among the group tested, thus validating the learning differences detected in the MWM test. These results demonstrate diametrical sex-specific effects of AVR/NAV deficiency on spatial learning: impairment in males but improvement in females.

Associative Learning Performance of AVR/NAV−/− Mice

To further substantiate the findings on the MWM we tested mice on another cognitive task that is natural and nonspatial, the social transmission of food preference, STFP, test (5). This task evaluates associative learning by utilizing an odor-odor
pair-choice paradigm, a natural task in rodents. On STFP testing, wild-type male mice showed efficient performance in this task, recognizing the trained odor at 24 h (Fig. 6A, P < 0.001) and 72 h (Fig. 6C, P < 0.001) retention times. However, AVR/NAVR−/− deficient-male mice did not recognize the trained odor at 72 h retention time (Fig. 6D, P = n.s.). In contrast, AVR/NAVR−/− females demonstrated efficient performance of this task by recognizing the trained odor at 24 h retention time (Fig. 6H, P < 0.001), while wild-type females were unable to distinguish trained from nontrained odors (Fig. 6G, P = n.s.). Results of the STFP test parallel the findings in the MWM task, demonstrating diatometric effects of AVR/NAVR deficiency in cognitive performance between male and females, therefore confirming sex-specific functionality for AVR/NAVR in cognition.

Testing Anxiety-like Behavior in AVR/NAVR−/− Mice

Because AVP has been associated in anxiety disorders (11) we then tested AVR/NAVR−/− deficient and wild-type mice in the EPM task, an experimental paradigm broadly used for measurements of anxiety-like behavior in rodents (25). AVR/NAVR−/− deficient and wild-type males did not differ in the percentage of entries on open arms (Fig. 7A, P = n.s.). However, the percentage of entries on open arms of the AVR/NAVR−/− deficient female group was significantly increased compared with wild-type female littermates (Fig. 7C, P < 0.05). Entries on enclosed arms were similar in both male and female contrasting groups, thus indicating equivalent levels of overall locomotor activity. These results show that AVR/NAVR deficiency produces a female-specific anxiolytic-like effect in the EPM task.

AVR- and NAVR-specific Signaling Pathways

We previously reported specific signal transduction pathways involved in renal physiology or in ANG II- and AVP-mediated renal salt/water balance that are activated/deactivated by AVP- and ANG II-specific stimulation of AVR and NAVR expressed in distinct permanent Cos1-cell transfectants respectively, using a signaling protein antibody array (17). To further explore AVR/NAVR roles in cognitive performance observed in AVR/NAVR−/− deficient mice, we analyzed whether AVR stimulation by AVP or ANG II, and NAVR stimulation by AVP would phosphorylate or dephosphorylate signaling proteins known to be involved in learning and memory. Quantitative Western blot analysis shows that 30 min AVP treatment of stable Cos1-NAVR transfectants (expressing NAVR) resulted in the phosphorylation of several upstream modulators of learning and memory: CREB1 (long-term memory formation) (36), PKA R2a (LTP expression) (8, 30, 55), Erk1, and Erk2 (Synapsin 1 phosphorylation and LTP expression) (12, 35) and dephosphorylation of others like NMDAR2B (LTP expression) (38) and eIF2a (regulation of translation initiation/modulation of synaptic strength) (27) (Fig. 8). AVP stimulation of stable Cos1-AVR transfectants (expressing AVR) resulted in increased phosphorylation of CREB1, Erk1, and Erk2 and concomitant dephosphorylation of PKA Cb, PKC b2, and NMDAR2B. ANG II stimulation of AVR moderately increased Erk1 and Erk2 phosphorylation without affecting the other signaling proteins tested (Fig. 8). Phosphorylation of other signaling proteins involved in learning and memory was detected on multiplex analysis of signaling pathways by signaling protein antibody microarray (Table 2), among others, Tau (microtubule-associated protein tau) known to be involved in Alzheimer’s disease (51). These data further support the
observation that AVR and/or NAVR are involved in modulation of cognitive performance.

DISCUSSION

Major AVP and ANG II Receptor Functions

Altogether, our results indicate that the AVR/NAVR locus regulates a number of vascular, renal, and brain physiological functions consistent with their respective tissue distribution and signaling pathways phosphorylated/dephosphorylated by AVP-specific stimulation of AVR and NAVR, respectively, and ANG II-specific stimulation of AVR. Although we cannot distinguish in vivo AVR from NAVR function in AVR/NAVR −/− knockout mice due to their overlapping transcripts, some receptor-specific functions can be deduced from the signaling pathway analysis of AVR- and NAVR-specific Cos1 cell transfectants and their respective differential tissue expression pattern. The effects of AVR/NAVR deficiency on BP and salt-water balance is likely due more to renal AVR, since AVR is abundantly expressed in the kidney and since its AVP- and ANG II-stimulated signaling transduction pathways phosphorylate signaling proteins known to be involved in salt/water balance like PKA Cb, JAK2, Jun, and PKC b2 more robustly than AVP-stimulated NAVR (17). The observed effects on cognition in AVR/NAVR −/− deficient mice imply its importance in the modulation of different aspects of learning and memory. This hypothesis is supported by the finding that AVR and NAVR can phosphorylate/dephosphorylate signaling proteins involved in learning and memory upon hormone stimulation in their respective Cos1 transfectants (Fig. 8). We further note that the observed sex-specific diametrical effects on cognitive performance in AVR/NAVR −/− deficient mice are concordant with the well-established concept of sex-specific determinants of cognition (45, 50). In contrast to canonical AVP and ANG II receptors, AVR/NAVR is involved more comprehensively in known AVP- and ANG II-mediated roles in regulation of blood pressure, salt-water balance, and various cognitive functions.

Molecular Mechanism for Sex-specific Functions, Sexual Diergism

In addition, the vasopressinergic hormonal system is known to underlie functional sex differences (sexual diergism) of the central nervous system (45), thus our findings suggest that AVR/NAVR might mediate some of the vasopressinergic-dependent sex-specific functional differences in the mammalian central nervous system observed across different species, including rats, mice (24), chimpanzees (33), and humans (15). Sex-specific behaviors are thought to involve neural pathways that express the androgen receptor (50). In addition, estrogen contributes to modulation of multiple memory systems (28). Thus, the findings that the AVR/NAVR transcription unit contains an androgen response element and two estrogen binding sites and that promoter analysis of AVR/NAVR S1-regulatory region detects synergistic transcriptional upregulation by testosterone and estrogen (17) are collectively consistent with the hypothesis that AVR and/or NAVR modulate sex-specific functions of the central nervous system.

Putative Molecular Mechanism for Association of Hypertension and Cognitive Decline

Cumulative evidence points toward shared etiologic mechanisms between hypertension and diseases exhibiting cognitive

Fig. 4. Testing of spatial learning and memory in AVR/NAVR −/− and WT mice. Percentage distance traveled in quadrants (A, B, D, E) and latency to target counter (C, F) during the probe trial after completion of Morris water maze training in WT (open bars), AVR/NAVR −/− (filled bars) male (A, B, C) and female (D, E, F) mice (WT male mice: n = 12; WT female mice: n = 11 and AVR/NAVR −/− male mice: n = 11; WT female mice: n = 11 and AVR/NAVR −/− female mice: n = 11). Quadrants are: target (T), opposite (O), adjacent right (AR), and adjacent left (AL). *P < 0.05; **P < 0.01. Data represent means ± SE (1-way ANOVA with genotype as between-subjects factor: AVR/NAVR −/− deficient mice are compared to WT mice for each gender, respectively).

Fig. 5. Cued task and acquisition performance in male (A) and female (B) AVR/NAVR −/− and AVR/NAVR +/+ mice. Mean distance traveled on a visible version (blocks 1–4) and on the hidden version of the Morris water maze (blocks 5–12) were not different between male AVR/NAVR −/− and AVR/NAVR +/+ mice [1-way repeated-measures ANOVA with genotype as between-subjects factor: blocks 1–4, F(1,30) = 1.97, P = 0.19; blocks 5–12, F(1,70) = 0.077, P = 0.79] and between female AVR/NAVR −/− and AVR/NAVR +/+ mice [1-way repeated-measures ANOVA with genotype as between-subjects factor: blocks 1–4, F(1,30) = 0.014, P = 0.91; blocks 5–12, F(1,70) = 0.79, P = 0.39].
imperative (3, 23, 44) including Alzheimer’s disease (6, 26, 41). Furthermore, hypertension is known to be a powerful risk factor for Alzheimer’s disease (26, 41). Thus, our studies showing that AVR/NAVR deficiency causes pleiotropic effects on both BP and cognitive performance suggest that mutations or dysregulation of the AVR/NAVR locus, as a modulator of blood pressure and cognitive impairment, could be a contributory determinant of the observed aggregation of hypertension and risk of dementia and Alzheimer’s disease (3, 6, 23, 26, 41, 44). This notion is further supported by recent genetic experiments showing that the AVR/NAVR locus resides within two rat chromosome 1 QTLs: one affecting BP (BP-m2) (19) and one influencing spatial learning and memory (Nav-3) (49) in Dahl rats. These genetic observations, along with the elucidation of a Dahl S rat AVR/NAVR gene variant that exhibits sodium-induced dysfunction affecting ligand binding and Gs coupling (48) and our current AVR/NAVR/H11002 knocko ut mouse studies altogether suggest that AVR/NAVR might account for the observed effects of BP-m2 (19) and Nav-3 (49) on BP and navigational performance in the Dahl rat strain.

In summary, we have identified that AVR/NAVR plays key roles in sex-specific functions in the kidney (salt-water balance) and in the brain (anxiety-like behavior and cognitive performance), as well as modulates BP homeostasis in both sexes. Although AVR- and NAVR-specific roles in AVR/NAVR/H11002 mice remain to be delineated, the observed sex-specific effects demonstrate a molecular mechanism for sexual-diergic functions. Additionally the data suggest that pathological modulation or changes of AVR/NAVR could provide a molecular mechanism for the association of hypertension as a

Fig. 6. Testing of associative learning in AVR/NAVR−/− and WT mice. Social transmission of food preference measured as % odor preference in WT, AVR/NAVR−/− male (A, B, C, D), and female (E, F, G, H) observers at 5 min (E, F), 24 h (A, B, G, H), and 72 h (C, D) retention times (WT male mice: n = 14 mice; AVR/NAVR−/− male mice: n = 14; WT female mice: n = 14 and AVR/NAVR−/− female mice: n = 14). Tr, trained odor; Non-tr, nontrained odor. *p < 0.05. **p < 0.01, ***p < 0.001. Data represent means ± SE (2-sided t-test for group comparisons).

Fig. 7. Testing of anxiety-like behavior in AVR/NAVR−/− and WT mice. Anxiety-like behavior measured in the elevated plus maze in WT (open bars), AVR/NAVR−/− (filled bars) male (A, B) and female (C, D) mice showing percentage entries on open arms (A, C) and number of entries in closed arms (B, D). WT male mice: n = 14 mice; AVR/NAVR−/− male mice: n = 14; WT female mice: n = 13 and AVR/NAVR−/− female mice: n = 14. *p < 0.05. Data represent means ± SE (2-sided t-test for group comparisons).

Fig. 8. Quantitative analysis of signaling proteins modulated by AVR and NAVR in permanent Cos1 cell transfectants. The following phospho-site specific antibodies were utilized in Kinetworks protein kinase multiblot analysis for quantitative measures: cAMP response element binding protein 1 [S129/S133] (CREB 1); cAMP-dependent protein-serine kinase regulatory type 2 subunit alpha [S98] (PKA R2a); N-methyl-D-aspartate (NMDA) glutamate receptor 2B subunit [Y1474] (NMDAR2B); eukaryotic translation initiation factor 2 alpha [S51] (eIF2a); extracellular regulated protein-serine kinase 1 (p44 MAP kinase)[T202/Y204] (Erk1); extracellular regulated protein-serine kinase 2 (p42 MAP kinase)[T185/Y187] (Erk2). Data are presented as percent change from control after 30 min of ANG II or AVP treatment. AVR+AVP (black), AVR+ANG II (white), and NAVR+AVP (hatched). A change >25% may be considered a real change.
risk factor for vascular cognitive decline and/or risk factor for the exacerbation of Alzheimer’s disease. More studies are needed to test this hypothesis, which, if proven, would identify a new target for novel therapies aimed at attenuating hyper-tension-associated cognitive impairment.

GRANTS

This work was supported in part by National Heart, Lung, and Blood Institute Grant HL-086532 awarded to N. Ruiz-Opazo.

DISCLOSURES

No conflicts of interest (financial or otherwise) are declared by the author(s).

REFERENCES