Angiotensin Type-2 Receptors Influence the Activity of Vasopressin Neurons in the Paraventricular Nucleus of the Hypothalamus in Male Mice

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It is known that angiotensin-II acts at its type-1 receptor to stimulate vasopressin (AVP) secretion, which may contribute to angiotensin-II-induced hypertension. Less well known is the impact of angiotensin type-2 receptor (AT2R) activation on these processes. Studies conducted in a transgenic AT2R enhanced green fluorescent protein reporter mouse revealed that although AT2R are not themselves localized to AVP neurons within the paraventricular nucleus of the hypothalamus (PVN), they are localized to neurons that extend processes into the PVN. In the present set of studies, we set out to characterize the origin, phenotype, and function of nerve terminals within the PVN that arise from AT2R-enhanced green fluorescent protein-positive neurons and synapse onto AVP neurons. Initial experiments combined genetic and neuroanatomical techniques to determine that GABAergic neurons derived from the peri-PVN area containing AT2R make appositions onto AVP neurons within the PVN, thereby positioning AT2R to negatively regulate neuroendocrine secretion. Subsequent patch-clamp electrophysiological experiments revealed that selective activation of AT2R in the peri-PVN area using compound 21 facilitates inhibitory (ie, GABAergic) neurotransmission and leads to reduced activity of AVP neurons within the PVN. Final experiments determined the functional impact of AT2R activation by testing the effects of compound 21 on plasma AVP levels. Collectively, these experiments revealed that AT2R expressing neurons make GABAergic synapses onto AVP neurons that inhibit AVP neuronal activity and suppress baseline systemic AVP levels. These findings have direct implications in the targeting of AT2R for disorders of AVP secretion and also for the alleviation of high blood pressure. (Endocrinology 157: 3167–3180, 2016)
blood pressure (BP) (1–9). One important action of Ang-II at its AT1R is its ability to stimulate vasopressin (AVP) secretion subsequent to hypotension or decreases in blood volume. Administration of AT1R antagonists systemically or into the brain reduces AVP secretion induced by Ang-II (1, 7, 8, 10, 11). It has been further postulated that the enhancement of AVP secretion by the activation of brain AT1R contributes to the development of hypertension, a widespread health problem and a major risk factor for the development of cardiovascular disease, the leading cause of death in the United States. Less well known is the impact of central angiotensin type-2 receptor (AT2R) activation on AVP secretion.

It has been widely hypothesized that the balance between AT1R and AT2R governs the responses of tissues to Ang-II, and it is possible that selective activation of AT2R may antagonize the neuroendocrine secretion associated with hypothalamic AT1R stimulation. That being said, the therapeutic utility of targeting these receptors within the brain has often been overlooked due to the inability to effectively localize central AT2R, leading to the false assumption that AT2R are not highly expressed in or near central nervous system nuclei that regulate neuroendocrine responses.

Using recent advances in molecular and genetic techniques to localize AT2R within the brain with a high degree of sensitivity and resolution, we have previously determined that AT2R-positive cell bodies and nerve terminals are well positioned to influence brain regions that regulate neuroendocrine axes (12). One critical site of Ang-II actions within the brain is the paraventricular nucleus of the hypothalamus (PVN), which is an integrative region for the control of several homeostatic systems and densely expresses the AT1R (13). The PVN can be divided into a number of functionally distinct subgroups, which collectively transmit and receive signals to and from brain regions regulating body fluid homeostasis. Parvocellular PVN neurons, many of which express AT1R (13), can be neurosecretory (eg, those expressing CRH to influence the hypothalamic pituitary adrenal axis) or preautonomic (eg, those projecting to the hindbrain to influence sympathetic outflow and BP) (14–16). Magnocellular PVN neurons express AVP or oxytocin (OT) (17), and their activation results in the release of AVP and OT from the posterior pituitary into the circulation. Of relevance, although the PVN itself does not contain any AT2R-positive neuronal cell bodies, nerve fibers and/or terminals arising from AT2R-positive cells are densely localized to the parvocellular and magnocellular portions of the PVN. Further, we have previously determined that these AT2R-positive fibers/terminals come into close proximity to AVP neurons within this area and that many of these AT2R-positive neurons originate from brain regions that send inhibitory projections to the PVN, including the preoptic area and γ-aminobutyric acid (GABA)ergic neurons that lie adjacent to the PVN (12).

In the present set of studies, we set out to further characterize the origin, phenotype, and function of nerve terminals within the PVN that arise from AT2R-eGFP-positive neurons. Initial experiments combined genetic and neuroanatomical techniques to test the hypothesis that GABAergic neurons containing AT2R make appositions onto AVP neurons within the PVN, thereby positioning AT2R to negatively regulate neuroendocrine secretion and BP. Subsequent experiments used patch-clamp electrophysiological techniques to test the hypothesis that activation of AT2R on neurons that project to the PVN will facilitate inhibitory (ie, GABAergic) neurotransmission and that this will lead to reduced activity of AVP neurons within the PVN. Final experiments assessed the functional impact of AT2R activation on plasma AVP levels. Collectively, these experiments tested the overall hypothesis that AT2R expressing neurons which make GABAergic synapses onto AVP neurons, inhibit AVP neuronal activity, and suppress baseline systemic AVP levels.

### Materials and Methods

#### Animals

Animal studies were performed at the University of Florida in Gainesville or at Augusta University. In all cases, experimental protocols were approved by the Institutional Animal Care and Use Committees at the University of Florida or Augusta University and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male mice (10–12 wk old) or rats (4–6 wk old) were maintained in temperature and humidity-controlled rooms on a 12-hour light, 12-hour dark cycle with food and water available ad libitum.

Some experiments used a mouse line that expresses enhanced green fluorescent protein (eGFP) in all cells that express the AT2R (ie, eGFP expression is driven by all of the regulatory sequences of the AT2R bacterial artificial chromosome [BAC] gene) to determine the precise localization of neurons that produce the AT2R (AT2R-eGFP; Mutant Mouse Regional Resource Centers; 030278). Briefly, the eGFP reporter gene and subsequent polyadenylation sequence were inserted into the AT2R BAC clone at the start codon of the first coding exon of the AT2R gene. This construct was then used to produce the transgenic mouse and results in eGFP expression driven by all of the regulatory sequences of the AT2R BAC gene. For additional details, see the vendor’s website (www.mmrrc.org). This mouse line was previously characterized (12), and it was determined that the distribution of AT2R-eGFP in the mouse brain is comparable with the AT2R binding observed using autoradiography (18). Mice used for the studies were backcrossed onto a C57BL/6J

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background for more than 5 generations and were hemizygous for the transgene.

In one study, AT2R-eGFP mice were crossed with mice that express both CRH-Cre (stock 012704; The Jackson Laboratory) and tdTomato-Stop-Flox (stock 007914; The Jackson Laboratory) to generate male double reporter mice in which AT2R-positive cells fluoresce green and CRH-positive cells fluoresce red. Importantly, we have previously determined that there is a high degree of colocalization of red fluorescent protein and CRH mRNA in mice expressing both CRH-Cre and tdTomato-Stop-Flox (19). For anatomical studies, a total of 20 AT2R-eGFP and 4 dual AT2R-eGFP/CRH-tdTomato reporter mice were used. For measurements of plasma AVP levels, 39 C57BL/6J mice were used. For in vitro electrophysiology, 6 AT2R-eGFP mice and 6 heterozygous transgenic eGFP-AVP Wistar rats were used (20).

**Retrograde tracer injections**

AT2R-eGFP reporter mice were anesthetized using isoflurane, administered analgescic (Buprenorphine) and prepared for stereotaxic surgery. Some mice received unilateral stereotaxic microiontophoretic injections of FluoroGold (FG) (2% wt/vol in 0.9% NaCl; 5 μA; 7-s on/off for 8 min) into the rostral ventrolateral medulla (RVLM), using glass micropipettes (inside diameter of ~30 μm) and the following coordinates from [Lambda]: anteroposterior −1.57 mm and mediolateral +1.30 mm; and from the surface of the brain: dorsoventral −5.07 mm. Others received such injections into the PVN (coordinates from bregma: anteroposterior 0.00 mm, mediolateral +0.25 mm, and dorsoventral −4.75 mm). Seven to 10 days after surgery, mice were anesthetized with pentobarbital and perfused and brain tissue was processed for immunohistochemistry (IHC) as described below. In all cases, injection sites were analyzed and missed injections served as controls for neuroanatomical specificity. Retrograde tracing studies were performed in a total of 10 mice, 6 of which received injections into the RVLM and 4 of which received injections into the PVN. This yielded 3 “hits” in the PVN and 4 hits in the RVLM, all of which were used for the IHC studies described below.

**Tissue collection and sectioning**

In order to collect brain tissue for in situ hybridization (ISH) and/or IHC studies, mice were anesthetized with pentobarbital and perfused transcardially with 0.15M NaCl followed by 4% paraformaldehyde. Brains were then postfixed for 3–4 hours, after which they were stored in 30% sucrose until sectioning using a Leica CM3050S cryostat (Leica). For IHC studies that did not also incorporate ISH, perfused mouse brains were sectioned at 30 μm into 6 serial sections and immediately mounted onto SuperFrost Plus Gold Microscope Slides. After air-drying at room temperature for 20–30 minutes, slides were stored at −80°C until further processing. All solutions were prepared with diethylpyrocarbonate-treated water and filtered using a 0.22-μm filter and tissue collection and sectioning were performed in ribonuclease-free conditions.

**ISH (RNAscope)**

Studies using RNAscope ISH (Advanced Cell Diagnostics) were performed on brain tissue collected from AT2R-eGFP mice as per the manufacturer’s instructions and as previously described (12). Briefly, after allowing tissue sections to dry at 25°C for 30 minutes, they were incubated with pretreatment 4 (a protease) and then underwent the RNAscope Multiplex Fluorescent ISH protocol. Specific details of the RNAscope ISH technique are outlined on the vendor’s website. Target probes are designed using the proprietary ACD RNAscope Probe Design pipeline, and contain 20 short double-Z oligonucleotide probe pairs that are gene specific. For amplification and visualization, both Z-probes must bind to the mRNA of interest. RNAscope detection reagents are then sequentially hybridized to amplify the signal and subsequently color-label the individual mRNA transcripts. For these experiments, the color label was assigned to either FAR RED (excitation, 647 nm; emission, 690 ± 10 nm) or ORANGE (excitation, 550 nm; emission, 580 ± 10 nm). Using this technique, each punctate dot represents a single mRNA target molecule.

Probes for glutamic acid decarboxylase-1 (Gad1) and vesicular glutamate transporter-2 (vGlut2) were used to determine the percentage of AT2R-eGFP neurons in the brain regions of interest (ROIs) that are GABAergic or glutamatergic, respectively. These areas included the following: the median preoptic nucleus (MnPO), the area directly surrounding the PVN (peri-PVN), the lateral ventral septum (LVS), and the bed nucleus of the stria terminalis (BNST). Each slide contained 4 sections. For each ROI the following probe combinations were used: 1) negative control probe, 2) positive control probe, 3) Gad1, and 4) vGlut2. Sections that were hybridized with the negative and positive control probes were used to determine the exposure time and image processing necessary to provide optimal visualization of RNA signal and also to control for possible RNA degradation for each brain.

**Immunohistochemistry**

All primary antibodies were characterized by the manufacturers and in previously published studies (12, 21, 22) and are

<table>
<thead>
<tr>
<th>Peptide/Protein Target</th>
<th>Antigen Sequence (if Known)</th>
<th>Name of Antibody</th>
<th>Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody</th>
<th>Species Raised in; Monoclonal or Polyclonal</th>
<th>Dilution Used</th>
</tr>
</thead>
<tbody>
<tr>
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<td>GFP</td>
<td>GFP</td>
<td>Life Technologies, A10262 Dr H. Gainer, National Institutes of Health, PS-38</td>
<td>Chicken; IgY Mouse; monoclonal</td>
<td>1:1000</td>
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<td>AVP-NP</td>
<td>Dr H. Gainer, National Institutes of Health, PS-41 Millipore, AB153</td>
<td>Mouse; monoclonal</td>
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<td>Chicken; IgY Mouse; monoclonal</td>
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Table 1. Primary Antibody Table
listed in Table 1. First, the antibody directed toward GFP, was previously validated in Refs. 12, 22, and its specificity was substantiated for the present study by the lack of staining on sections originating from mice that did not express GFP. Next, both anti-OT-neurophysin (NP) (PS-38) and anti-AVP-NP (PS-41) were validated in Refs. 21, 23, in which it was determined these antibodies appropriately and specifically labeled the posterior pituitary, the paraventricular and supraoptic nuclei. Additionally, liquid-phase RIA was used to determine that anti-OT-NP (PS-38) is highly specific for the OT-NP complex and does not cross-react with AVP-NP, whereas the anti-AVP-NP (PS-41) reacts with AVP-NP exclusively. Furthermore, as in Refs. 21, 24, the specificity of the rabbit polyclonal antibody against FG was corroborated by the lack of staining on sections originating from mice without FG treatment. All secondary antibodies were purchased from Jackson ImmunoResearch, raised in donkey, and used at a 1:500 dilution.

In general, the IHC procedures were as follows: brain sections were removed from cryoprotectant solution and rinsed 5 times for 5 minutes in 50mM potassium phosphate buffered saline (KPBS) and then incubated in blocking solution (2% normal donkey serum and 0.2% Triton X-100 in 50mM KPBS) for 2 hours at 25°C. This was followed by incubation with the primary antibody in blocking solution for 18 hours at 4°C. Sections were again rinsed 5 times for 5 minutes in 50mM KPBS and then incubated in the secondary antibody for 2 hours at 25°C. After a final series of rinses (5 × 5 min), sections were mounted onto slides, allowed to air dry, and then cover slipped using polyvinyl alcohol mounting medium. For double-label IHC, sections were incubated with both primary antibodies simultaneously and, subsequently, both secondary antibodies simultaneously. Importantly, all qualitative IHC studies were performed in 3–4 separate mice (the specific group sizes are included in the figure legends).

### Image capture and processing

All images were captured and processed using Axiovision 4.8.2 software and a Zeiss Axiosmager fluorescent Apotome microscope. For RNAscope ISH and dual IHC/RNAscope ISH, z-stacks of the proteins and transcripts of interest were captured at ×40 magnification throughout the ROIs using neuroanatomical landmarks found in a mouse brain atlas (25). In all cases, z-steps were set at 0.5 μm, with an average of 20 optical sections per image. For each experiment, sections hybridized with the positive control probes were used to determine the exposure time and image processing required to provide optimal visualization of RNA signal. These same parameters were then used for visualization of mRNAs of interest in experimental sections, to assess background fluorescence in sections hybridized with the negative control probe (dihydrodipicolinate reductase) and to determine the specificity of the probes using tissue obtained from knockout mice. Importantly, using these exposure times and image processing parameters there was minimal or no fluorescence in sections hybridized with the negative control probe and in sections obtained from knockout mice. All final figures were then prepared using Adobe Photoshop 7.0 where the brightness and contrast was adjusted to provide optimal visualization.

### Image analysis

Analysis of colocalization of mRNA transcripts with eGFP fluorescence was performed on selected brain regions in 4 separate AT2R-eGFP mouse brains; ×40 magnification z-stacks of ROIs were used to determine the percentage of eGFP neurons that contain Gad1 or vGlut2 mRNA. An average of 2–4 z-stacks were captured for each ROI, depending on the rostrocaudal length of the particular ROI. eGFP neurons were considered to contain the RNA of interest if at least 3 visible transcripts, defined as an individual punctate dot, were observed within the volume of the eGFP fluorescence. Data are reported as the percentage of AT2R-eGFP cells that contain the RNA for each gene within each ROI.

In order to determine appositions between eGFP nerve terminals and AVP neurons within the PVN, ×40 z-stacks (with an average of 20 optical sections;
0.5 μm/section) were assessed. Appositions were verified by a lack of separation between eGFP-positive boutons and the cell type-specific marker (AVP).

**Slice preparation for electrophysiology**

Hypothalamic brain slices were prepared according to methods previously described (26, 27). Briefly, mice (n = 6) rats (n = 6) were anesthetized with pentobarbital (50 mg/kg ip); brains dissected out and hypothalamic coronal slices (210 μm) containing the PVN were cut in an oxygenated ice-cold artificial cerebrospinal fluid (aCSF), containing 119 mM NaCl, 2.5 mM KCl, 1 mM MgSO4, 26 mM NaHCO3, 1.25 mM Na2HPO4, 20 mM D-glucose, 0.4 mM ascorbic acid, 2 mM CaCl2, and 2 mM pyruvic acid (pH 7.3; 295 mOsm). Slices were placed in a holding chamber containing aCSF and kept at room temperature until use.

**Electrophysiology**

Hypothalamic slices were transferred to a recording chamber and superfused with continuously bubbled (95% O2-5% CO2) aCSF (30°C–32°C) at a flow rate of approximately 3.0 mL/min. Thin-walled (1.5-mm outer diameter, 1.17-mm inner diameter) borosilicate glass (G150TF-3; Warner Instruments) was used to pull patch pipettes (3–5 MΩ) from horizontal micropipette puller (P-97; Sutter Instruments). The internal solution contained the following: 135 mM potassium gluconate, 0.2 mM EGTA, 10 mM HEPES, 10 mM KCl, 0.9 mM MgCl2, 4 mM Mg2+ ATP, 0.3 mM Na+ GTP, and 20 mM phosphocreatine (Na+); pH was adjusted to 7.2–7.3 with KOH. For voltage-clamp experiments, patch pipettes were filled with a high Cl-containing solution: 140 mM KCl, 10 mM HEPES, 0.2 mM EGTA, 4 mM Mg2+ ATP, 0.3 mM Na+ GTP, and 20 mM phosphocreatine (Na+). When noted, neurons were intracellularly labeled with Alexa Fluor 555 (50 μM). Recordings were obtained from fluorescently labeled AT2R-eGFP cells and from eGFP-AVP neurons with an Axopatch 200B amplifier (Axon Instruments), using a combination of fluorescence illumination and infrared differential interference contrast videomicroscopy. The voltage output was digitized at 16-bit resolution, 10 kHz, and was filtered at 2 kHz (Digidata 1440A; Axon Instruments). Data were discarded if the series resistance was not stable throughout the entire recording (>20% change) (26, 27). Drugs were applied via a single bolus injection into the bath (0.5 mL). Mean firing activity and membrane potential values were calculated from a 1-minute period before drug application and a 1-minute period around the peak effect, using Clampfit (Axon Instruments) or MiniAnalysis (Synaptosoft) software. Spontaneous inhibitory postsynaptic currents (IPSCs) (recorded at −70 mV) were detected and analyzed using MiniAnalysis. A detection threshold was set at −75 pA for IPSCs peak, to extract IPSCs without contamination with glutamate-mediated excitatory postsynaptic current (28).

**Osmotic minipump implantation**

ALZET osmotic minipumps and Brain Infusion kits (DURECT Corp) for the chronic delivery of C21 (7.5 ng/kg/h for 1 wk) or aCSF control into the lateral cerebral ventricle intracerebroventricular (icv) were prepared according to the manufacturer’s instructions. After allowing the pumps to prime, by incubating them in a 37°C water bath for a minimum of 48 hours, pumps and infusion kits were implanted using a Kopf stereotaxic device as described (29). The coordinates from bregma were as follows: 0.2 mm posterior, 1.0 mm lateral, and 2.5 mm ventral.

**Analysis of plasma AVP levels**

For the assessment of plasma AVP levels, trunk blood was collected from unanesthetized mice given either C21 or saline vehicle icv for 1 week. Blood samples were collected during the circadian nadir of AVP secretion and kept on ice for a maximum of 45 minutes before centrifugation and plasma collection for AVP analysis using a commercially available RIA kit (Phoenix Pharmaceuticals).

**Statistical analysis**

All values are expressed as mean ± SE. For electrophysiological studies, Student’s paired t tests were used to compare the effects of drug treatment. Differences were considered significant at P < .05, and n refers to the number of cells. Similarly, for the analysis of plasma AVP levels a Student’s t test was performed, with the threshold for consideration as statistically significant being set at P < .05. All statistical analyses were conducted using GraphPad Prism (GraphPad Software).
Results

AT2R-eGFP does not colocalize with OT, AVP, or CRH neurons in the PVN or with RVLM-projecting PVN neurons

We have previously determined that AT2R-eGFP neuronal cell bodies are likely not localized to the PVN and observed no overlap between AT2R-eGFP and AVP immunoreactivity within the cell bodies of the PVN (12). Initial studies in the present manuscript confirmed and built upon these findings by evaluating the degree of colocalization between AT2R-eGFP and various neuronal phenotypes within the PVN. Figure 1 depicts the lack of colocalization between AT2R-eGFP and AVP (Figure 1, A–C), OT (Figure 1, D–F), and CRH (Figure 1, G–I) neurons within the PVN. Furthermore, examination of brains from mice that received injections of the retrograde neuronal tract tracer into the RVLM revealed that AT2R-eGFP was similarly not expressed on RVLM-projecting preautonomic neurons within the area (Figure 1, J–L). It is also evident from the images, that rather, AT2R-eGFP-expressing neurons were located in regions surrounding the proper PVN.

AT2R-eGFP is localized to terminals within the PVN that make appositions onto AVP neurons

Despite the lack of localization of AT2R-eGFP to the cell bodies within the PVN, we have previously observed an abundance of AT2R-eGFP neuronal fibers/terminals that come in apparent close contact to AVP neurons within the area (12). As in this previous publication (12), Figure 2 highlights the observation that AT2R-eGFP terminals are near AVP neurons and make apparent appositions onto these neurons. The implication is that AT2R-eGFP cells are positioned to regulate the activity of AVP neurons via impacting neurotransmitter release onto these neurons. This finding led to experiments designed to determine the origin and phenotype of the AT2R-eGFP neurons that project to the PVN, and to test the hypothesis that the activation of AT2R impacts neurotransmitter release onto AVP neurons within the PVN.

The origin of the AT2R-eGFP projections into the PVN

In the next set of studies, we exploited the use of the retrograde neuronal tract tracer, FG, to determine the localization of the AT2R-eGFP...
neurons that send projections into the PVN (Figure 3). Figure 3A depicts a coronal atlas section through the center of the injection site within the PVN and highlights the localization and spread of the FG administration in the 3 mice used for this study. One representative missed injection is also included in this figure and delineated in red. Figure 3B illustrates a representative injection site from one of these mice (mouse number 2). Areas that were examined for colocalization of FG and AT2R-eGFP included the neurons directly surrounding the PVN (peri-PVN), which have previously been determined to exhibit a high degree of colocalization with the GABAergic marker, Gad1 (12), as well as the following regions: the MnPO, the BNST, the LVS, the zona incerta, and the nucleus of the solitary tract. Of these areas, the highest degree of colocalization was observed within the MnPO (Figure 3, C–E) and within the peri-PVN neurons (Figure 3, F–H), suggesting that the predominant source of AT2R-eGFP inputs to the PVN originate from these brain regions. There were also scatterings of AT2R-eGFP/FG-coloabeled cells observed in the BNST, LVS, and the zona incerta (data not shown).

**Phenotype of AT2R-eGFP neurons in selected regions that provide inputs into the PVN**

The MnPO and peri-PVN AT2R-eGFP neurons were assessed using ISH for the GABAergic marker, Gad1, and the glutamatergic marker, vGlut2, in order to further ascertain whether AT2R are positioned to impact excitatory and/or inhibitory neurotransmission onto PVN neurons. These studies revealed that within the MnPO, AT2R-eGFP neurons are exclusively glutamatergic (Figure 4, A–D), whereas those localized to the peri-PVN express the GABAergic marker, Gad1 (Figure 4, E–H), consistent again with the prediction that AT2R are positioned to impact both inhibitory and excitatory transmitter release onto PVN neurons. Also of relevance, the AT2R-eGFP neurons in the BNST and LVS contained exclusively Gad1 mRNA, indicating that the AT2R-eGFP neurons within these areas are exclusively GABAergic (data not shown).

**General electrophysiological properties of AT2R-eGFP cells**

To determine the functional relevance of AT2R expressed in peri-PVN GABAergic neurons, we obtained whole-cell patch clamp recordings from 16 identified AT2R-eGFP neurons located within the peri-PVN area (see Figure 5 for representative samples). Recorded AT2R-eGFP neurons had a mean resting membrane potential of $-56.8 \pm 2.4$ mV, a mean input resistance of $847.8 \pm$
remained relatively constant during repetitive stimulation (Figure 6, C and F). Moreover, the interspike intervals (0.5–5 pA) (Figure 6, B and E) or duration (150–1500 ms) were not significantly affected (P > .9). To determine whether C21’s effect on PVN eGFP-AVP GABAergic synaptic activity was action potential-dependent, experiments were repeated in the presence of 0.5μM tetrodotoxin (TTX). Under these conditions, none of the recorded AVP neurons (n = 5) showed changes in either the frequency (basal, 4.3 ± 1.5 Hz vs C21, 3.5 ± 1.5 Hz; P > .09) (Figure 8F), or the amplitude (P > .5) (data not shown) of the GABA_A-mediated IPSCs.

Impact of icv AT2R activation using C21 on AVP secretion in vivo

Based on previously published studies examining the impact of AT2R on AVP secretion (30–32), as well as the above evidence that AT2R are positioned to negatively regulate AVP neurons, we predicted that chronic central AT2R activation in vivo, would attenuate baseline systemic AVP. Consistent with this prediction, chronic icv C21 administration (7.5 ng/kg · h for 1 wk) led to a significant reduction in plasma AVP levels (Figure 9), again, consistent with an inhibitory influence of the AT2R over
AVP secretion. Of relevance, this dose of C21 did not impact plasma osmolality (C21, 314.3 ± 9004 H11006 1.28 vs CON, 316.6 ± H11006 0.97; n = 7/group), body mass (C21, 26.9 ± 0.55 g vs CON, 27.5 ± 0.64 g; n = 9/group), mean daily food intake (C21, 3.5 ± 0.39 g/d vs CON, 3.7 ± 0.46 g/d standard chow; n = 9/group), or water intake (C21, 3.9 ± 0.56 mL/d vs CON, 4.1 ± 0.65 mL/d; n = 9/group) relative to vehicle-treated control mice.

**Discussion**

Ang-II acts at AT1R to stimulate AVP secretion (1), which influences hydromineral balance and cardiovascular function by promoting the reabsorption of water in the kidneys and eliciting vasoconstriction. Although there are many lines of evidence suggesting that AT2R largely opposes AT1R activation, the mechanisms underlying this apparent opposition within the brain have largely been unexplored, as has AT2R’s influence over AVP secretion. There has been speculation that AT1R and AT2R stimulate opposing intracellular signaling cascades within the same neurons; however, evidence that these receptors are localized to the same cells within the brain is lacking. In fact, previous studies in the AT2R-eGFP reporter mouse indicate that AT1R and AT2R are largely localized to separate populations of neurons, and that AT2R-eGFP neurons surrounding the PVN do not contain AT1R (12). In the present study, we took advantage of the recently characterized AT2R-eGFP reporter mouse to determine the localization of the AT2R neurons relative to AVP neurons within the PVN. We then used the selective AT2R agonist, C21, to determine the impact of the activation of this receptor on the activity of
both AT2R-eGFP neurons that project to the PVN and on inhibitory neurotransmitter release onto these AVP neurons. The key findings are that AT2R expressed on GABAergic interneurons residing in the peri-PVN region synapse onto AVP neurons within the PVN and activation of these AT2R increases GABAergic synaptic activity, resulting in enhanced inhibitory neurotransmitter release onto AVP neurons and reduced baseline AVP levels.

Elevations in brain Ang-II lead to activation of AVP neurons causing a rise in systemic AVP levels that can be attenuated by administration of AT1R blockers either via the intracerebroventricular route or specifically within magnocellular neuron containing areas of the brain (33–35). Further, several recent studies have indicated that Ang-II may play an important role in renin-angiotensin system-dependent hypertension (36). Based on data from BAC transgenic reporter mice, it is unlikely that magnocellular neurons of the PVN (or supraoptic nucleus) contain AT1R or AT2R (12, 37), and the impact of Ang-II on AVP secretion may therefore be mediated by Ang-II receptor-positive neural connections arising from upstream brain nuclei, such as the organum vasculosum of the lamina terminalis and subfornical organ (38, 39). Although the stimulatory effects of Ang-II on AVP secretion are likely mediated by the AT1R (34, 35, 40), several lines of evidence support the notion that activation of AT2R acts in opposition to this neurosecretory effect. For example, whole-body genetic deletion or pharmacological blockade of AT2R (using PD123219) in mice augment Ang-II induced AVP release, reflected by increased plasma AVP levels (31, 32) and decreased pituitary AVP content (30). That being said, the therapeutic utility of AT2R agonists has only recently started to be evaluated in this regard.

It is well established that GABA is a dominant inhibitory neurotransmitter in the hypothalamus (41), and that GABAergic inputs to the PVN arise from various intrahypothalamic regions, including the MnPO and the peri-PVN area (42–45). However, whether these inhibitory inputs, particularly those impinging on AVP neurons, express AT2R is at present unknown. Moreover, whether activation of AT2R in these terminals would impact GABAergic function, neuronal activity and release onto AVP neurons is also unknown. Thus, one of the critical questions addressed in the neuroanatomical studies presented here was whether AT2R are anatomically positioned to influence GABAergic inhibitory neurotransmitter release within the PVN. Based on our previous data indicating that AT2R are localized to GABAergic neurons and to brain regions that are known to exert inhibitory control over PVN neurons (46), we hypothesized AT2R-positive connections within the PVN would be mostly GABAergic. Consistent with this prediction, Gad1 mRNA and AT2R-eGFP were indeed colocalized in the neurons that directly surround the PVN and project to the PVN. The positioning of AT2R-eGFP to inhibitory inputs to the PVN, however, was not exclusive as there was also a prevalence of AT2R-eGFP localization to excitatory glutamatergic inputs to the PVN, including MnPO neurons that were exclusively glutamatergic. Thus, although the AT2R-eGFP neurons surrounding the PVN are indeed positioned to influence inhibitory (GABAergic) neurotransmission onto AVP neurons, the AT2R-positive MnPO to PVN connection may exert an excitatory influence over AVP secretion.

![Figure 7](image-url)
Figure 8. Activation of AT2R increases GABAergic synaptic activity in PVN eGFP-AVP neurons. A, Representative trace of IPSCs in voltage-clamp mode (holding potential, −70mV) of a PVN eGFP-AVP neuron before and after a transient bolus of C21 (arrow). B, Representative recording segments of IPSCs before and after C21 application are shown at an expanded time scale visualization. C, Summary plot of mean IPSC frequency vs time from the example shown in A (time bin, 20). D, Cumulative histograms of interevent interval (left) and amplitude (right) of GABA<sub>A</sub> IPSCs.
Subsequent studies focused on how AT2R agonism with C21 influenced the activity of AT2R-eGFP neurons residing in the peri-PVN, and how this in turn, affected the activity of AVP neurons within the PVN. Of relevance, several studies have used either the AT2R antagonist, PD123319, or AT2R knockout mice to demonstrate that, at the doses used in the present study, C21 exerts AT2R-specific effects (47–50). Initial patch-clamp electrophysiological studies revealed that AT2R-expressing neurons in the peri-PVN shared similar general electrophysiological properties to previously described hypothalamic interneurons, including GABAergic ones, such as the expression of a rebound low-threshold spike and a mostly linear current/voltage relationship (51, 52). Importantly, we found that activation of AT2R with the selective agonist C21 excites AT2R-eGFP neurons in the peri-PVN area, evoking and/or increasing their firing discharge. These findings are in agreement with previously published studies that have indicated a stimulatory effect of AT2R activation on the firing rates of neurons, but are in contrast to some previous results indicating that AT2R activation can be hyperpolarizing (53–57). It is important to note however, that these previous studies have largely been conducted in cultured neurons, and the impact of AT2R activation in a neural network that involves the PVN had not previously been assessed.

In order to determine whether the AT2R-mediated increased firing activity of GABAergic interneurons residing in the peri-PVN elicited a change in GABAergic neurotransmission released onto AVP neurons, we directly monitored GABAergic receptor-mediated IPSCs in identified AVP neurons of the PVN. We found that C21 increased the frequency of GABA IPSCs (without altering IPSC amplitude) in AVP neurons and that this effect was completely eliminated when action potentials were blocked with the Na+ channel blocker TTX. These results are consistent with the notion that AT2R activation increases firing discharge of GABAergic interneurons, resulting in an action potential-dependent increase in GABAergic synaptic activity in targeted AVP neurons within the PVN. These results, in conjunction with our neuroanatomical studies, provide strong evidence for functional connectivity between AT2R-expressing GABAergic interneurons in the peri-PVN region, and AVP neurons within the PVN.

It is worth noting that previous studies have reported an AT1R-mediated inhibition of GABA IPSCs in RVLM-projecting PVN neurons (58, 59). In addition to these global opposing actions between AT2R and AT1R on GABAergic synaptic function within the PVN, there are important mechanistic differences as well. Although the AT2R-mediated enhancement of GABA function was dependent on evoking firing activity in GABAergic interneurons (ie, TTX dependent), the AT1R-mediated inhibition was independent of their firing activity (ie, TTX independent), and involved increases in IPSC miniature frequency but not amplitude (ie, presynaptic effect). Taken together, these results support a differential neuronal compartmentalization of AT2R and AT1R, with the former located in the soma of the GABAergic interneurons (this study), and the latter on their presynaptic axonal terminals within the PVN (59).

Based on the present results, along with previously published studies examining the impact of AT2R on AVP secretion (30–32), we predicted that chronic central AT2R activation in vivo, would negatively regulate AVP secretion. As expected, we found that chronic icv C21 administration reduced baseline plasma AVP levels. An important caveat to take into account when interpreting these in vivo results, however, is that our experimental approach results in the activation of AT2R throughout the brain and not specifically within the PVN. Thus, although our in vitro studies support that AT2R expressed in peri-PVN GABAergic interneurons are positioned to negatively regulate the activity of AVP neurons, the present studies do not entirely support or refute the hypothesis that the in vivo effects were indeed mediated by AT2R inputs to AVP neurons in the PVN. Rather, they indicate that brain AT2R activation reduces plasma AVP levels. Another important point to note is that although the AVP levels values presented here are higher than expected based on previous studies measuring plasma AVP concentration in rats (60), these values are comparable with, or slightly lower than, studies measuring plasma AVP in mice (33, 61).

**Figure 8.** (Continued). before and after C21 application. E, Summary data of mean frequency and amplitude of GABA EIPSCs in eGFP-AVP neurons before and after C21 exposure. F, Summary data of mean GABA EIPSCs frequency in eGFP-AVP neurons before and after C21 in the presence of TTX (0.5 μM). Bars, SEM; **, P < .01.
Collectively, these results are consistent with the overall hypothesis that functional AT2R are positioned to regulate inhibitory neurotransmission onto AVP neurons. That being said, the present studies also revealed that AT2R are additionally localized to glutamatergic inputs. The inference is perhaps that AT2R are positioned to both negatively and positively regulate the activity of AVP neurons and it is possible that the impact that the AT2R makes on AVP secretion is in large part dependent on the physiological condition, and which population of AT2R inputs become activated. As a consequence, the impact of activation of AT2R throughout the entirety of the brain on AVP secretion becomes less predictable. In this regard, it is possible that although AT2R within the peri-PVN likely inhibit AVP neurons, AT2R with glutamatergic inputs into the PVN (ie, MnPo) may excite AVP neurons. Therefore, the balance between these inputs may predict the impact of AT2R activation on AVP secretion.

In order to effectively be able to exploit the inhibitory influence of AT2R over fluid/electrolyte balance and BP regulation in future therapeutics, it is essential to develop a thorough understanding of the neural pathways and systems that AT2R impact. The present studies collectively demonstrate that functional AT2R are situated such that they can exert an inhibitory influence over AVP neurons within the PVN. These findings have direct implications, not only in using AT2R agonist in the alleviation of AVP-dependent hypertension, but also in disorders of AVP secretion. Future studies will determine how best to exploit this pathway.

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References


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