Functional and neurochemical characterization of angiotensin type 1A receptor-expressing neurons in the nucleus of the solitary tract of the mouse


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Angiotensin II acts via two main receptors within the central nervous system, with the type 1A receptor (AT1AR) most widely expressed in adult neurons. Activation of the AT1, a receptor-expressing neurons in the nucleus of the solitary tract (NTS), the principal nucleus receiving central synapses of viscerosensory afferents, modulates cardiovascular reflexes. Expression of the AT1R occurs in high density within the NTS of most mammals, including humans, but the fundamental electrophysiological and neurochemical characteristics of the AT1AR-expressing NTS neurons are not known. To address this, we have used a transgenic mouse, in which the AT1AR promoter drives expression of green fluorescent protein (GFP). Approximately one-third of AT1AR-expressing neurons express the catecholamine-synthetic enzyme tyrosine hydroxylase (TH), and a subpopulation of these stained for the transcription factor paired-like homeobox 2b (Phox2b). A third group, comprising approximately two-thirds of the AT1AR-expressing NTS neurons, showed Phox2b immunoreactivity alone. A fourth group in the ventral subnucleus expressed neither TH nor Phox2b. In whole cell recordings from slices in vitro, AT1AR-GFP neurons exhibited voltage-activated potassium currents, including the transient outward current and the M-type potassium current. In two different mouse strains, both AT1AR-GFP neurons and TH-GFP neurons exhibited voltage-activated potassium currents, including the transient outward current and the M-type potassium current. In two different mouse strains, both AT1AR-GFP neurons and TH-GFP neurons showed similar AT1AR-mediated depolarizing responses to superfusion with angiotensin II. These data provide a comprehensive description of AT1AR-expressing neurons in the NTS and increase our understanding of the complex actions of this neuropeptide in the modulation of viscerosensory processing.

Viscerosensory reflexes provide rapid adjustments in autonomic activity to maintain homeostasis of multiple physiological systems in response to behavioral requirements. This critical function is essential for a range of activities, including simple daily activities such as changing posture from lying to standing or modulating food intake. Inhibition, or decreased sensitivity of some reflexes, such as the baroreceptor reflex, is associated with poor health outcomes in a wide range of cardiovascular diseases (34). Interestingly, aberrant baroreceptor reflex function is also observed early in the progression of many neurological diseases, including depression, neurodegenerative diseases, and schizophrenia (9, 12, 62).

The central terminals of viscerosensory afferents synapse in the nucleus of the solitary tract (NTS). The primary transmitter employed by viscerosensory afferent neurons is glutamate, but within the NTS many other neurotransmitters and neuromodulators interact to influence reflex function (4). A key modulator for the baroreceptor reflex, which is also known to play a role in aberrant baroreceptor reflex function in cardiovascular diseases, is angiotensin II (ANG II).

Acting via its type 1 receptor (AT1R) in the NTS, ANG II inhibits the baroreceptor-heart rate (HR) reflex (11, 13). Bilateral microinjection of the AT1R antagonist, CV-11974 [Candesartan (Cand)], into the NTS increases the sensitivity of the baroreceptor-HR reflex in both normotensive and hypertensive rats (46), suggesting a tonic influence of endogenous ANG II. Although microinjections of ANG II into the NTS have dose-dependent effects on blood pressure and sympathetic nerve activity (14, 15, 57), these specific parameters are not affected by microinjection of Cand alone (46). These results indicate that different neuronal populations in the NTS might underlie tonic baroreceptor inhibitory and sympathetic vasomotor responses.

Determining the neuronal circuitry involved in the actions of ANG II in the NTS is complicated by the fact the AT1R is expressed by multiple cell types. Receptor binding sites for ANG II occur in the somata of viscerosensory neurons in the nodose ganglion, and ~40% of the ANG II receptor binding sites in the NTS are located on the terminals of these sensory neurons (3, 25, 30, 40). In the human medulla oblongata, ANG II receptor binding sites occur on vagal afferent fibers coursing from the lateral surface of the medulla to the solitary tract (1). The majority of the nonafferent AT1AR expression in the NTS appears to be associated with neurons (19, 28), although some data support the expression of AT1R on vascular endothelial cells in this region (11, 53). Studies on transgenic reporter mice in which the AT1AR promoter drives expression of green fluorescent protein (GFP) have demonstrated colocalization of the AT1AR and the catecholamine-synthetic enzyme tyrosine hydroxylase (TH; 28). However, this global study did not attempt further characterization of the AT1AR-expressing neurons. Expression of the AT1R is extensive throughout the
rostral-caudal extent of the NTS, suggesting involvement of ANG II in modulating a greater range of viscerosensory inputs than just those related to cardiovascular function.

Application of ANG II to slices of rat NTS induces depolarization in ~15% of randomly recorded neurons during synaptic blockade, suggesting that it is acting via a postsynaptic receptor (10). This result is consistent with the response to ANG II of neurons in other autonomic nuclei of the central nervous system, when recorded during synaptic blockade (39, 42). The relatively low proportion of ANG II-responsive neurons makes it difficult to determine the mechanisms inducing this depolarization and increased excitability. This task is made more complicated by the presynaptic effects of AT1AR activation, which mediate an increase in excitatory neurotransmission (10, 37). Direct application of ANG II to NTS neurons during excitatory synaptic blockade induces depolarization, increased action potential frequency, and a decrease in input resistance (10, 56). Others observe an increase in inhibitory GABAergic input and a decrease in the excitatory input to NTS neurons following ANG II application under these conditions. These changes occur despite a failure to demonstrate a change in membrane resistance (37, 52).

The aim of the studies reported here was to define the neurochemical phenotype(s) of AT1AR-expressing NTS neurons and their electrophysiological properties. We used the AT1AR-GFP mouse to overcome many of the technical limitations that have previously hindered our understanding of ANG II function in the NTS. We also directly compared the functional characteristics of the AT1AR-expressing NTS neurons with those of NTS neurons that express TH, using a transgenic TH-GFP mouse.

METHODS

All procedures were conducted in accordance with the Australian National Health and Medical Research Council’s Code of Practice for the Use of Animals in Research, and experiments were approved by the University of Melbourne Animal Ethics Committee (AEC no. 1112208) or Florey Neuroscience Institute Animal Ethics Committee (FNI AEC nos. 12-089, 13-107, 13-114, and 14-105).

Animals. The AT1AR-GFP mice were obtained from the GENSAT project (Rockefeller Institute, New York, NY) as described previously (19) and bred and housed at the Biological Research Facility, The University of Melbourne (Melbourne, VIC, Australia). The TH-GFP mice (59) were bred and housed in the Florey Institute of Neuroscience and Mental Health. Mice of both sexes were housed at 20–22°C and 40–45% relative humidity, with 12-h light-dark cycle and access to standard mouse chow and water ad libitum.

Immunohistochemistry. Immunohistochemical staining was performed as described previously with modifications as outlined (18, 43). Mice (n = 8) were injected with ketamine (200 mg/kg) and xylazine (20 mg/kg) intraperitoneally and perfused with saline followed by 4% formaldehyde. The brains were removed and immersed in 4% formaldehyde (3–5 h at room temperature) and then 20% sucrose overnight. The brains were frozen, and serial 40-μm-thick sections were cut in the coronal plane at a cryostat and stored at −20°C in cryoprotectant. Sections were incubated with primary antibodies for 2–3 days on a shaker at room temperature. The primary antibodies used were chicken anti-ANG II (1:4,000, Millipore Bioscience Research Reagents, for fluorescent detection; and 1:50,000, Aves Laboratories, for peroxidase detection), rabbit anti-TH (1:4,000, Millipore Bioscience Research Reagents), sheep anti-TH (1:5,000, Millipore Bioscience Research Reagents), rabbit anti-γ-glutamyl fibrillar acidic protein (GFAP, 1:5,000, Dako Agilent Pathology Solutions), human anti-Hu (1:5,000, a generous gift from Prof. V. Lennon, Mayo Clinic), and guinea pig anti-parvalbumin (1:50,000, Millipore Bioscience Research Reagents). Immunohistochemical detection was performed if series resistance and leak current were determined to be more than 30 MΩ and 100 pA, respectively. Voltage or current data were sampled at 50–200 kHz (protocol dependent) and filtered to 10 kHz using Digidata 1440 (Molecular Devices) and pClamp software (version 10.3; Molecular Devices).
Whole cell configuration was obtained for 2 min to allow for the recording to stabilize. Then, current clamp protocols were employed to record the voltage-gated transient K+ current (I_v), the steady-state voltage-activated transient K+ current (I_Ks), and the M-type K+ current (I_KM) as described below. Presynaptic release of glutamate was measured by examining frequency and amplitude of spontaneous excitatory post-synaptic potentials (sEPSPs). The resting membrane potential was then recorded in current-clamp mode, and in the first 10 s of recording the reservoir supplying the bath was changed to a separate reservoir containing either ACSF alone or ACSF in combination with the AT1R antagonist, Candid (10 μM; Astra Hassle), the AT2R antagonist, PD-122319 (PD; 10 μM; P186; Sigma-Aldrich), or the voltage-gated Na+ channel blocker, tetrodotoxin (TTX; 100 nM; BN0517; Biotrend). After a 5-min recording time, the reservoir supplying the bath was changed to ACSF with 2 mM ANG II (2078 AusPEP) only or 2 mM ANG II in combination with 10 mM Candid, 10 mM PD, or 100 mM TTX, respectively, and a further 5 min were recorded. The traces in Fig. 6 are truncated to show the period of maximal change. Changes to membrane potential and sEPSPs were analyzed by averaging a 40-s period before and after the bath content was replaced with the new solution. Only a single neuron was recorded from each slice, and changes in bath temperature were compensated using an in-line preheater (Analog TC2BIP; Cell Micro Controls, Norfolk, VA).

Voltage-activated currents were recorded using published protocols (49). Recordings of I_Ks activation kinetics were made during a preconditioning command voltage (−90 mV, 400 ms) followed by a +10-mV, 800-ms step protocol for 11 steps (from −100 to 0 mV). Conversely, I_Ks inactivation involved an incremental −10-mV, 400-ms preconditioning pulse step (from −100 to 0 mV) followed by a depolarization command potential (−10 mV, 800 ms). I_VK amplitude was measured by subtracting the baseline outward current (≥600 mV after step) from the peak of the transient (<150 ms after step). The amplitude of I_Ks was measured by averaging the current for a period of 200 ms >600 ms after the command step. The passive leak current was then subtracted. I_Ka was recorded by increasing the command potential to 0 mV for 500 ms before reducing it in 10-mV increments over five steps (from −10 to −60 mV). The transient outward current (<150 ms after step) was subtracted from the steady-state hyperpolarization (>300 ms).

Spontaneous and voltage-activated current responses were measured using Clampfit 10.34 (Molecular Devices). All measurements and analysis were performed on raw traces, and a low-pass eighth-pole Bessel filter (3 kHz) was applied to traces for representation in figures. Membrane potential was averaged across a 40-s period. Normalized current activation and inactivation were transferred to Origin 2016 (OriginLab, Northampton, MA) for fitting using a Boltzmann-Sax function as per the following equation:

\[
\frac{I}{I_{\text{Max}}} = 1 \left[ 1 + e^{-\left(\frac{V_{\text{m}} - V_{\text{h}}}{V_{\text{s}} - V_{\text{h}}} \right)} \right]
\]

where I is the current at a given membrane voltage, I_max is the maximum current recorded at the highest voltage step, V_m is membrane voltage with the command potential or preconditioning pulse, V_h is the membrane potential at half-maximum current, and V_s is a slope factor. The sEPSPs were detected using minianalysis (Synaptosoft, Fort Lee, NJ).

The distribution of all recorded neurons was mapped by comparing their photographed location relative to brain microstructures, including the area postrema (AP), obex, fourth ventricle, and central canal, using a stereotaxic atlas of the mouse brain as a reference (54).

Electrophysiology data analysis and statistics. As defined previously for electrophysiology experiments involving superfusion of brain slices with ANG II (16, 41), a change in membrane potential was defined as one >2 mV and/or twice the standard deviation of the baseline period. A two-way repeated-measures ANOVA was used to compare the change that occurred between groups with ANG II treatment. If an interaction was observed between groups (ANG II alone, Candid, PD, and TTX) and treatment (before and after ANG II application), a post hoc Holm-Sidak multiple comparison was performed. A Kruskal-Wallis one-way analysis of variance on ranks was performed on the change in membrane potential, between groups (ACSF, Candid, PD, and TTX), before and during bath application of ANG II.

The frequency and amplitude of EPSPs for individual neurons, before and after each treatment, was compared using a Kolmogorov-Smirnov test within each cell (Minianalysis; Synaptosoft). Average EPSP frequency and amplitude were analyzed within treatment groups during baseline and ANG II application using a Student’s paired t-test. If the values were not normally distributed, a Wilcoxon signed-rank test was performed (SigmaStat 3.5).

The magnitude of I_Ks, I_Ka, and I_Km currents and the hyperpolarization step were determined using Clampfit (Molecular Devices) and normalized to cell capacitance. Inactivation currents were measured relative to the preconditioning command potential, whereas activation currents were measured relative to the test command potential, each plotted against potential. Off-line leak subtraction was performed using interlaced current injections. Frequency histograms of current magnitude were fit with a Gaussian curve for comparisons. The most common currents are reported as the maximum of the fit Gaussian function.

RESULTS

Heterogeneous distribution of AT1R-expressing neurons in the NTS. To map the cellular expression of AT1R within the dorsal vagal complex of the brain stem, we examined the distribution of AT1R-GFP immunoreactivity throughout the rostrocaudal extent of the NTS, AP, and dorsal motor nucleus of the vagus (DMX) in AT1R-GFP mice. Previous work has demonstrated that AT1R-GFP expression provides a faithful representation of the distribution of AT1R-expressing cells in this model (19, 28). The maps in Fig. 1 are from one mouse but represent a consistent distribution observed in all mice. Other figures are taken from several other animals. The AT1R-expressing cells have a neuronal morphology, with multipolar somata, and multiple processes. Restriction of AT1R-GFP expression to neurons is supported by the coexpression with the neuronal lineage marker, Hu, and the absence of AT1R-GFP colocalization with GFAP (Fig. 2; 28). The solitary tract also contained AT1R-GFP-labeled axons (Fig. 1K) consistent with AT1R expression in vescerosensory neurons (3). The entire dorsal vagal complex (i.e., the NTS, AP, and DMX) contained networks of AT1R-GFP-stained fibers. Within the AP, this network appeared as a haze of AT1R-GFP immunoreactivity, suggestive of AT1R predominately in fine nerve processes and possibly in other nonglial cellular elements. A few scattered AT1R-GFP-immunoreactive neurons occurred in the AP and DMX (Fig. 1). The majority of GFP-labeled neurons occurred in the NTS, with obvious differences in the numbers present in different NTS subnuclei. At caudal levels, AT1R-GFP-immunoreactive neurons were most numerous in the medial subnucleus of the NTS (Fig. 1E) and appeared to form a continuous column with AT1R-GFP-positive cells in the dorsomedial subnucleus at more rostral levels (Fig. 1N). Many GFP-stained neurons were also observed in the ventral subnucleus surrounding the solitary tract (Fig. 1E). At the level of the AP, the dorsolateral subnucleus contained scattered AT1R-GFP-positive neurons (Fig. 1, E and H), which had smaller somata than those in
either the medial or ventral subnuclei. Similarly, the interstitial subnucleus of the caudal NTS contained many small somata showing AT1R-GFP immunoreactivity (Fig. 1E). The central (Fig. 1K) and gelatinous subnuclei (Fig. 1H) were conspicuous for the absence of AT1R-GFP-labeled neurons. The distribution of neuronal AT1R-GFP immunoreactivity in different subnuclei of the NTS and the variation in the morphology of immunoreactive neurons suggest that AT1R-expressing neurons in the NTS are likely to be functionally heterogeneous.

AT1R is expressed in TH-immunoreactive NTS neurons. It is not known whether AT1R expression is a neurochemical signature for a unique neuronal phenotype within the NTS. We therefore determined the degree of coexpression of AT1R-GFP with TH in the NTS by double-labeling immunofluorescence. Table 1 shows the proportion of AT1R-GFP neurons that are TH-immunoreactive at different rostrocaudal planes from four mice. One group of TH-immunoreactive neurons that spanned the lateral part of the medial subnucleus and the medial part of the ventral subnucleus expressed AT1R-GFP (Fig. 3, A and B). These formed a continuous column extending from the commissural NTS to the most rostral levels of the NTS (Fig. 3C). At the most rostral level, the TH-positive neurons are probably part of the C2 cell group, although we did not formally test this presumption. Compared with other AT1R-GFP-stained neurons at the same rostrocaudal levels, the TH+ /AT1R-GFP neurons tended to have large somata. Only a subset of TH-immunoreactive neurons were AT1R-GFP positive. The AT1R-GFP-negative TH population was particularly striking in the gelatinous/commissural subnuclei, bordering the AP, where all TH-positive neurons were AT1R-GFP negative (Fig. 3, Ba and Bb). As a group throughout the NTS, 32% of TH-immunoreactive NTS neurons expressed AT1R-GFP. Two groups of AT1R-GFP neurons were also obvious for their lack of TH immunoreactivity. One TH-negative, AT1R-GFP-positive group was located within the medial part of the medial subnucleus between the level of the commissural NTS caudally and mid-AP rostrally (Fig. 3B). The other group occurred in the ventral subnucleus (Fig. 2, A and B). The small number of AT1R-GFP-labeled neurons scattered through the DMX also lacked TH immunoreactivity.
Noncatecholaminergic, AT1AR-expressing NTS neurons are Phox2b immunoreactive. The transcription factor Phox2 is present in many neurons of the NTS and occurs in barosensitive neurons (35). We therefore tested whether AT1AR-expressing neurons might also contain Phox2b. We observed Phox2b immunostaining in many NTS neurons throughout the rostrocaudal length of the nucleus and in all subnuclei. Although most Phox2b-positive neurons did not express AT1AR-GFP, the majority of AT1AR-GFP neurons in most subregions throughout the rostral-caudal length of the NTS were stained for Phox2b (Fig. 3, D–F). The major, and prominent, exception was the lateral part of the ventral subnucleus of the NTS where AT1AR-GFP neurons did not contain Phox2b (Fig. 3E).

Throughout the NTS, most of the TH neurons also stained for Phox2b. Similar to the AT1AR-expressing neurons, there were many neurons that were Phox2b positive but TH negative. Triple immunofluorescent labeling revealed that within the medial subnucleus of the NTS, some neurons colabeled for AT1AR-GFP, Phox2b and TH. Within the dorsolateral subnucleus and the dorsal part of the medial subnucleus of the NTS, however, the majority of AT1AR-GFP neurons contained Phox2b immunoreactivity but were not labeled for TH (Fig. 4).

Electrophysiological characteristics of AT1AR-GFP and TH-GFP neurons. Patch pipettes were visually guided to record from GFP-expressing cells in the NTS in slices from either AT1AR-GFP or TH-GFP mice. The expression of GFP was confirmed before, and during, whole cell recordings using fluorescence microscopy. The AT1AR-GFP and TH-GFP neurons in the NTS exhibited similar basal characteristics (capacitance: AT1AR, 18 ± 1 pF; TH, 22 ± 3 pF; input resistance: AT1AR, 389 ± 41 MΩ; TH, 293 ± 50 MΩ; membrane potential: AT1AR, −59.8 ± 0.8 mV; TH, −57.3 ± 2.6 mV). Neurons were predominantly sampled from the medial and ventral subnuclei at different rostrocaudal levels of the NTS (see Figs. 6 and 7).

Recorded neurons were assigned a treatment group at random and had cell bodies equally distributed across the medial NTS cell column. There were no differences in baseline electrophysiological parameters observed between neurons that were assigned to different drug assay protocols. As sex differences in AT1R signaling have been reported previously (55, 66), differences in basal characteristics, or the response to ANG II, of neurons from male and female mice were assessed. The resting membrane potential of AT1AR neurons from female animals was less negative (female, −56.6 mV; male, −62.2 mV; P < 0.05, Holm-Sidak multiple comparisons). No other differences were observed, and neurons from all male and female mice were grouped for further analysis.

Because there is heterogeneity in the expression of the Ik, current in the total population of NTS neurons (8, 23), we sought to determine whether the presence of specific K+ conductances defined populations of AT1AR- or TH-expressing neurons. We recorded Ik, IkA (AT1AR only), and Ik, currents using a standard series of command potentials (Fig. 5). All TH-expressing (n = 14) and all except 2 out of 53 AT1AR-expressing NTS neurons displayed Ik, currents. The most common range of current magnitudes in the activation protocols was similar between TH neurons (47.4 ± 5.1 pA/pF) compared with the most common range for AT1AR neurons (43.0 ± 2.2 pA/pF; P = 0.24, Mann-Whitney rank sum test). Activation and inactivation of Ik, were assessed by comparing the mean Vh and Vc between neuron populations. There was no difference in Vh between AT1AR-GFP (activation, −35.3 ± 0.8 mV; inactivation, −70.6 ± 3.1 mV) and TH-GFP neurons (activation, −36.6 ± 1.5; inactivation, −67.3 ± 4.9) or Vc in AT1AR-GFP (activation, 8.0 ± 0.9; inactivation, 7.2 ± 0.6) compared with TH-GFP neurons (activation, 7.3 ± 0.6; inactivation, 8.0 ± 0.7; Mann-Whitney rank sum test, not significant). Thus few differences were observed in voltage-activated current expression between the two neurochemical phenotypes (Fig. 5C).

Similar to Ik, IkA showed voltage-dependent characteristics in NTS neurons (Fig. 5D). Like the Ik, there was no difference in the most common magnitude of IkA between AT1AR neurons (34.7 ± 2.1 pA/pF) compared with TH neurons (27.6 ± 2.0 pA/pF; P = 0.12, Mann-Whitney rank sum test). In a subset of 30 AT1AR neurons the presence of an IkA was assessed using a series of depolarization steps after a conditioning depolarization (Fig. 5G). The resulting transient current was

Table 1. Proportion of AT1AR-GFP-expressing neurons in the NTS that contain TH immunoreactivity

<table>
<thead>
<tr>
<th>Bregma, mm</th>
<th>Subnucleus</th>
<th>AT1AR-GFP</th>
<th>TH/AT1AR-GFP</th>
<th>Double Labeled, %</th>
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<tbody>
<tr>
<td>−7.92</td>
<td>Medial</td>
<td>68 ± 5</td>
<td>16 ± 1</td>
<td>24</td>
</tr>
<tr>
<td>−7.76</td>
<td>Medial</td>
<td>102 ± 7</td>
<td>27 ± 2</td>
<td>27</td>
</tr>
<tr>
<td>Dorsolateral</td>
<td>Medial</td>
<td>25 ± 3</td>
<td>1 ± 1</td>
<td>4</td>
</tr>
<tr>
<td>Ventral</td>
<td>21 ± 3</td>
<td>1 ± 0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>−7.56</td>
<td>Medial</td>
<td>94 ± 8</td>
<td>14 ± 3</td>
<td>14</td>
</tr>
<tr>
<td>Dorsolateral</td>
<td>Medial</td>
<td>20 ± 2</td>
<td>2 ± 1</td>
<td>8</td>
</tr>
<tr>
<td>Ventral</td>
<td>8 ± 2</td>
<td>0</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>−7.28</td>
<td>Medial</td>
<td>98 ± 5</td>
<td>8 ± 2</td>
<td>8</td>
</tr>
<tr>
<td>Ventral</td>
<td>13 ± 3</td>
<td>0</td>
<td>8</td>
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</table>

Values are means ± SE. The total number of type 1A receptor (AT1AR)-expressing neurons, as well as the number of those also immunoreactive for tyrosine hydroxylase (TH), was counted at different rostrocaudal levels of the nucleus of the solitary tract (NTS) subnuclei in four representative mice. From these counts the proportion of all AT1AR-expressing neurons that contain TH immunoreactivity was calculated.
Fig. 3. Some AT1AR-expressing neurons also express tyrosine hydroxylase (TH) and paired-like homeobox 2b (Phox2b). Fluorescence photomicrographs of coronal sections of the NTS at 7.9 mm (A and D), 7.6 mm (B and E), and 7.3 mm (C and F) caudal to bregma. The micrographs in A–C show double immunofluorescent staining for tyrosine hydroxylase (TH; red) and GFP (green) with the channels merged. The associated panels immediately to the right show the areas in the hatched boxes at higher magnification and with color channels separated (e.g., Aa shows TH alone and Ab shows GFP alone). Arrowheads indicate double-labeled neurons. The photomicrographs in D and E show fluorescence immunohistochemistry for Phox2b (red) plus GFP (green) with the channels merged. The associated panels immediately to the right show the areas in the hatched boxes at higher magnification and with color channels separated (e.g., Da shows Phox2b alone and Db shows GFP alone). Solid arrowheads indicate double-labeled neurons; the open arrowheads indicate a neuron that only expresses the AT1AR. The positions of the area postrema (AP) and dorsal motor nucleus of the vagus (DMX) are shown in relevant sections. Scale bar, 100 μm.

Voltage dependent (Fig. 5H) with the most common voltage magnitude being 3.83 ± 0.19 pA/µF. Taken together, these data show a range of current magnitudes within each neurochemical phenotype and few differences in voltage-activated currents between AT1AR-GFP and TH-GFP neurons.

Response of AT1AR-GFP and TH-GFP neurons to ANG II. Superfusion with 2 µM ANG II depolarized 14/17 AT1AR-GFP neurons (Fig. 6A) and 7/14 TH-GFP neurons (Fig. 7A). In nine AT1AR-GFP neurons the depolarization reached threshold and initiated action potentials in neurons that had previously been silent (Fig. 6). Input resistance did not change with ANG II in either group (P > 0.05, Student’s t-test). To negate influences of desensitization on the response to ANG II, separate groups of neurons were superfused with Cand, PD, or TTX. These compounds did not alter membrane potential (before ANG II alone, −52.6 ± 1.5 mV; during Cand, −53.1 ± 2.2 mV; during PD, −52.6 ± 1.8 mV; during TTX, −54.9 ± 1.3 mV; P = 0.724, 1-way ANOVA; Fig. 6A). Superfusion with ANG II (2 µM) in the presence of Cand blocked membrane depolarization in 11/14 neurons; the remaining 3 neurons depolarized and reached action potential threshold (Fig. 6). In neurons treated with PD, ANG II depolarized 10/11 neurons (Fig. 6). Superfusion of ANG II plus TTX resulted in depolarization in 9/11 neurons tested (Fig. 6). No consistent differences were observed in resting membrane potential between any groups (P > 0.05, 2-way repeated-measures ANOVA), and there were no correlations between the starting Vm and the change in Vm elicited by ANG II in any group (P > 0.05), indicating that the magnitude of depolarization was not dependent on resting membrane potential of a neuron. Interestingly, there was no effect of ANG II on input resistance regardless of treatment group (Wilcoxon signed-rank test, not significant). These results support a direct effect of ANG II acting via the AT1AR to excite AT1AR-GFP neurons.

The proportion of TH-GFP neurons depolarized by ANG II was less than that for the AT1AR-GFP neurons (Fig. 7). In half the TH-GFP neurons, ANG II either did not change membrane potential, 4 neurons, or induced hyperpolarization, 3 neurons (Fig. 7B). This effect was independent of the subnucleus in which the neuron was recorded (Fig. 7C) but presumably reflects the anatomical observation that only a proportion of AT1AR-GFP express TH in any NTS subnucleus.

The AT1AR is expressed in sensory neurons in nodose ganglia (3), and ANG II is reported to potentiate glutamatergic EPSPs induced by solitary tract stimulation in some NTS.

Table 2. Proportion of AT1AR-GFP-expressing neurons in the NTS that contain Phox2b immunoreactivity.

<table>
<thead>
<tr>
<th>Bregma, mm</th>
<th>Subnucleus</th>
<th>AT1AR-GFP</th>
<th>Phox2b+/AT1AR-GFP</th>
<th>Double Labeled, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>−7.92</td>
<td>Medial</td>
<td>68 ± 5</td>
<td>23 ± 3</td>
<td>34</td>
</tr>
<tr>
<td>−7.76</td>
<td>Medial</td>
<td>102 ± 7</td>
<td>55 ± 4</td>
<td>55</td>
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<td></td>
<td>Dorsalateral</td>
<td>25 ± 3</td>
<td>10 ± 2</td>
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<td>Ventral</td>
<td>21 ± 3</td>
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<td>5</td>
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<td>−7.56</td>
<td>Medial</td>
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<td>Dorsalateral</td>
<td>20 ± 2</td>
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<td>Ventral</td>
<td>8 ± 2</td>
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<td>−7.28</td>
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<td></td>
<td>Ventral</td>
<td>13 ± 3</td>
<td>4 ± 2</td>
<td>31</td>
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</table>

Values are means ± SE. The total number of AT1AR-expressing neurons, as well as the number of those also immunoreactive for paired-like homeobox 2b (Phox2b), was counted at different rostrocaudal levels of the NTS subnuclei in four representative mice. From these counts the proportion of all AT1AR-expressing neurons that contain Phox2b-immunoreactivity was calculated.
neurons (10). We examined sEPSP frequency and amplitude to determine whether ANG II was acting via a presynaptic site. We saw no difference in baseline sEPSP frequency or amplitude between AT1AR-GFP and TH-GFP neurons (AT1AR-GFP, 3.0 ± 0.4 Hz and 1.7 ± 0.1 mV; TH-GFP, 2.9 ± 0.8 Hz and 2.5 ± 0.5 mV). Superfusion with ANG II did not alter sEPSP frequency or amplitude (post-ANG II compared with pre-ANG II: AT1AR-GFP, 2.3 ± 0.7 Hz and 1.75 ± 0.2 mV; TH-GFP, 3.2 ± 1.0 Hz and 3.6 ± 0.8 mV; not significant). Combined, these data indicate a postsynaptic site of action for ANG II on AT1AR-GFP and TH-GFP NTS neurons, which results in depolarization, as the spontaneous activity of afferents innervating these NTS neurons is not modulated by ANG II. It is possible that AT1AR-expressing viscerosensory neurons target non-AT1AR-expressing neurons, although this requires further direct examination.

Interestingly, two neurons exposed to ANG II alone and three neurons exposed to ANG II in combination with Cand displayed membrane potential hyperpolarization (Fig. 6C). This effect did not occur during coapplication of ANG II and either PD or TTX.

**DISCUSSION**

Employing a transgenic mouse, in which GFP expression is induced by the AT1R promoter, we have characterized the distribution, neurochemical phenotypes, and electrophysiological properties of AT1R-expressing cells throughout the dorsal vagal complex, with emphasis on the NTS. All AT1R expression in somata occurs in neurons, which are differentially distributed among NTS subnuclei. Within the NTS, we found no evidence for expression of AT1R in astrocytes.
Colocalization of GFP with the transcription factor Phox2b in AT1AR-GFP neurons was common, and a subset of the AT1AR/Phox2b neurons also contained immunoreactivity for TH. The AT1AR-expressing neurons in the ventral subnucleus of the NTS were conspicuous for their lack of both TH and Phox2b immunoreactivity. The transient voltage-activated $K^+$ current $I_A$ as well as the M-type $K^+$ current $I_M$ occurred in virtually all of the AT1AR-expressing NTS neurons. Most AT1AR-expressing neurons were excited by ANG II, which acts via postsynaptic AT1AR on the recorded neurons. Similar responses were observed in TH-GFP neurons; although in agreement with the observation that not all TH NTS neurons express the AT1AR, only ~50% of the TH neurons were responsive to ANG II.

Although binding studies in the 1980s demonstrated that the AT1R is expressed in high concentrations in the NTS of most mammals (2), including humans (1, 45), a comprehensive description of the distribution of NTS cells that express the AT1AR has not been possible because of technical limitations (e.g., 31). The production of a transgenic reporter mouse, in which faithful expression of GFP occurs in regions known to express the AT1AR, has finally allowed us to map these important neurons (19). Consistent with previous binding studies (1, 3), the AT1AR-GFP mouse demonstrates AT1AR throughout the NTS, in distinct populations of intrinsic NTS neurons and also in axons of viscerosensory afferents within the solitary tract. Although the distribution of the AT1AR within medial subnuclei supports the participation of ANG II in central modulation of cardiorespiratory functions (4), expression in rostral and lateral subnuclei of the NTS points to a potential involvement in noncardiovascular functions. A key finding of this study is that several different groups of neurons with distinct neurochemical phenotypes express the AT1AR.
including neurons that are immunoreactive for either TH or Phox2b and neurons that contain both of these markers. The diversity in the location and types of neurons that express the AT1AR presumably underlies the complex actions of ANG II within the NTS (14, 57), which should be unraveled by further studies examining the responses of the AT1AR-expressing neurons to activation of different visceral sensory modalities.

Knowledge of the function of Phox2-expressing neurons in the NTS in cardiovascular regulation is relatively rudimentary. Phox2-expressing NTS neurons are activated following systemic injection of phenylephrine to increase blood pressure (35). Genetic deletion of Phox2b profoundly alters the development of visceral sensory reflex pathways because NTS and visceral sensory afferent neurons fail to form (21). However, because of the widespread distribution of Phox2b in the NTS, its genetic deletion would halt development of both TH-positive and TH-negative neurons and thus does not shed further light on the functional role of these two different neuronal groups. Of interest is the coexpression of Phox2b and 11-β-hydroxysteroid dehydrogenase type 2 (HSD2) in some NTS neurons, and this may shed further light on the role of some AT1AR-expressing neurons in blood pressure regulation and fluid homeostasis (27).

The TH-immunoreactive neurons in the NTS form the A2/C2 cell groups, and the A2 neurons make up 85% of the NTS projection to paraventricular regions of paraventricular nucleus of the hypothalamus (PVN; 60). These two groups of catecholaminergic NTS neurons play an important role in visceral sensory function. Most catecholaminergic NTS neurons in the NTS receive direct, excitatory inputs from visceral sensory afferents (5). Hypoxia (38), various aversive psychological and physiological stressors (22), and noxious stimuli (33) activate subgroups of the A2/C2 neurons. Many visceral sensory stimuli, including but not limited to food ingestion or gastric distension (58, 67), cause increases in systemic blood pressure (35). However, the involvement of A2/C2 neurons in baroreceptor reflex modulation is more contentious. Lesioning A2/C2 neurons by microinjection of the catecholamine-selective neurotoxin, 6-hydroxydopamine, into the NTS either enhances bradycardic response to baroreceptor stimulation (29, 32) or has no effect (63). Chronic inhibition of A2 neurons, by viral transduction to express an inward rectifier potassium channel, also does not affect the gain of the baroreceptor-HR reflex (26).

Interestingly, we have demonstrated here that not all TH neurons express the AT1AR and that only half the recorded TH-expressing neurons respond to ANG II. Given that TH neurons project to multiple different regions, it will be important to determine whether AT1AR expression identifies a subpopulation of TH neurons with specific projection or input characteristics.

The passive membrane properties of AT1AR-GFP neurons documented in this study, including resting membrane potential, \( I_{Ks} \), \( I_{Kv} \), and \( I_{Ka} \), are similar to those described previously in nonselective samples of NTS neurons (11, 17, 20, 61). Although we did not isolate currents using selective inhibitors, both inactivation and activation kinetics and current waveforms are consistent with previously described tetraethylammonium-sensitive, 4-aminopyridine-sensitive, and acetylcholine-sensitive voltage-activated potassium currents (17, 48). These currents are principally responsible for spike frequency adaptation and the hyperpolarization of neurons, respectively. Prominent \( I_{Ks} \) currents have been described in second-order NTS neurons projecting to the PVN, in A2 neurons, and in many second-order neurons receiving transient receptor potential V1-expressing visceral sensory input (5, 7, 8).

The majority of AT1AR-expressing NTS neurons recorded in this study (14/17) were excited by ANG II via the AT1R. It is not clear whether the three nonneurons did not have sufficient receptor expression to elicit a response or whether they represent an ectopic expression of GFP. The magnitude of the AT1AR-mediated depolarization observed in this study is much smaller than that reported in previous studies of NTS neurons (10). This difference may be due to the method by which

Fig. 7. ANG II evokes variable responses in TH-GFP NTS neurons. A: representative traces showing the membrane potential responses in TH-GFP neurons before and during bath application of ANG II (2 μM). The composition of the superfusate changes at the time indicated by black arrowheads. The top trace, indicated by a solid circle, is representative of neurons that hyperpolarized (open circle) to ANG II. The middle trace is a nonresponsive neuron (gray circle), and the bottom trace is representative of neurons excited by ANG II. B: ANG II caused variable responses in TH-GFP neurons that likely reflect heterogeneity of AT1AR expression within this population of NTS neurons. C: the position of each recorded neuron within the NTS did not seem to influence whether it responded to ANG II across 7.3, 7.6, and 7.9 mm caudal to bregma.
recordings were acquired and how the agonist was applied. Barnes et al. (10) applied ANG II directly to somata of recorded neurons, whereas in the present study, ANG II was bath applied. In experiments in which ANG II was bath applied at equivalent concentrations (1–3 μM) while recording from neurons in the rostral ventrolateral medulla the magnitude of the change in membrane potential was more consistent with the present study (56). The time course of the depolarization, between 1 and 5 min of ANG II application, in this study is also consistent with previous reports in neurons from other brain regions (6, 12, 50, 51). Though relatively small, a 4-mV depolarization significantly changes excitability as observed, for example, in the activation of individual neurons that express hM3-dsRed receptors exclusively activated by designer drugs (hM3-DREADD; 44). We conclude that ANG II would increase the responsiveness of NTS neurons to afferent input and thus potentiate throughput of visceral sensory information. Although ANG II induced depolarization in the presence of TTX, some membrane changes may also have occurred because of activation of presynaptic AT1Rs. However, the frequency and amplitude of sEPSPs in the same neurons did not change with ANG II in the presence of TTX despite the occurrence of depolarization, providing further evidence for ANG II acting at a postsynaptic site.

A small proportion of recorded NTS neurons exhibited hyperpolarization when exposed to ANG II alone or ANG II in the presence of Cand. This response did not occur in the ANG II plus PD or ANG II plus TTX groups. One possible explanation is that ANG II acts via the AT2 receptors to hyperpolarize neurons. There is evidence of AT2R expression in the NTS of the mouse, and AT2R activation in neurons has been shown to potentiate K+ conductance, potentially causing inhibition (24, 36). The lack of a hyperpolarization in the presence of TTX might also suggest that this response is not due to a direct postsynaptic effect on AT1R-expressing neurons, but involves NTS interneurons. Although this is an interesting observation, conclusions are difficult to draw from this data set because of the small number of neurons in which this response was observed.

**Perspectives and Significance**

These data show that AT1R-expressing neurons have a distinct distribution within the subnuclei of the NTS, suggesting that ANG II is involved in modulating a variety of autonomic reflex pathways. Because of this distribution, microinjection studies would require careful spatial restriction of agonist injections to clearly delineate the role(s) for ANG II within the NTS, such as that used to describe the renal sympathoinhibitory action of ANG II in the medial, commisural subnucleus of the NTS (64). The present study also describes the electrophysiological characteristics of AT1R-expressing NTS neurons. Activation of the AT1R depolarized these neurons and increased excitability, a response that was unaltered by TTX. In contrast, ANG II application had no effect on sEPSP frequency or amplitude, suggesting that activation of postreceptors mediated the depolarization. Together, these results represent an anatomical and physiological framework for beginning to unravel the involvement of ANG II in the multiple visceral sensory pathways and functions that are regulated by NTS neurons.

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


