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Targeting angiotensin type-2 receptors located on pressor neurons in the nucleus of the solitary tract to relieve hypertension in mice

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Aims These studies evaluate whether angiotensin type-2 receptors (AT2Rs) that are expressed on γ-aminobutyric acid (GABA) neurons in the nucleus of the solitary tract (NTS) represent a novel endogenous blood pressure-lowering mechanism.

Methods and results Experiments combined advanced genetic and neuroanatomical techniques, pharmacology, electrophysiology, and optogenetics in mice to define the structure and cardiovascular-related function of NTS neurons that contain AT2R. Using mice with Cre-recombinase directed to the AT2R gene, we discovered that optogenetic stimulation of AT2R-expressing neurons in the NTS increases GABA release and blood pressure. To evaluate the role of the receptor, *per se*, in cardiovascular regulation, we chronically delivered C21, a selective AT2R agonist, into the brains of normotensive mice and found that central AT2R activation reduces GABA-related gene expression and blunts the pressor responses induced by optogenetic excitation of NTS AT2R neurons. Next, using *in situ* hybridization, we found that the levels of *Agtr2* mRNAs in GABAergic NTS neurons rise during experimentally induced hypertension, and we hypothesized that this increased expression may be exploited to ameliorate the disease. Consistent with this, final experiments revealed that central administration of C21 attenuates hypertension, an effect that is abolished in mice lacking AT2R in GABAergic NTS neurons.

Conclusion These studies unveil novel hindbrain circuits that maintain arterial blood pressure, and reveal a specific population of AT2R that can be engaged to alleviate hypertension. The implication is that these discrete receptors may serve as an access point for activating an endogenous depressor circuit.

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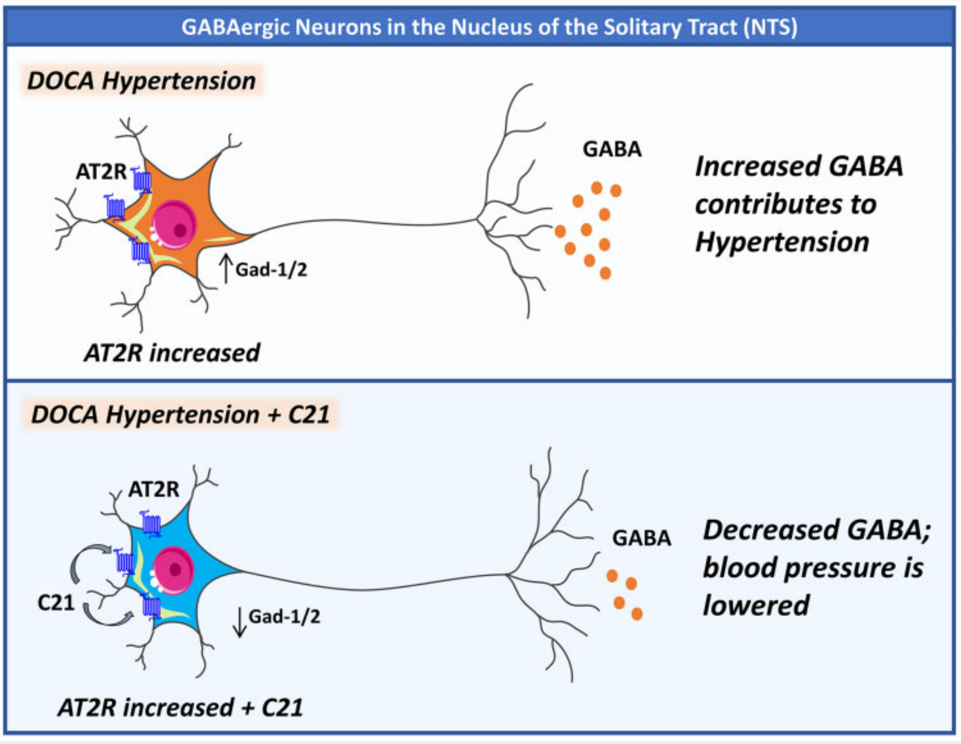
Graphical Abstract



Keywords Baroreflex • GABA • Blood pressure • Hindbrain • RAS

1. Introduction

Hypertension is the leading risk factor for the development of cardiovascular disease and stroke, which are the first and fifth leading causes of death in the USA.[1] Despite lifestyle changes and multi-drug based therapies, nearly 20% of all hypertensive patients in the USA remain with high blood pressure.[2] The brain controls fluid consumption, neuroendocrine secretion, and autonomic responses that maintain blood pressure and osmolality at levels optimal for survival. Dysregulation of these responses is requisite for the blood volume expansion and/or vasoconstriction that promotes hypertension, and as such, it has been proposed that the brain is complicit in the aetiology of the disease.[3] Accordingly, understanding the neuronal plasticity that underlies the onset of hypertension may provide insight towards new and effective treatments.

The brain senses increased arterial pressure via baroreceptors that transduce stretch exerted on the vasculature into neural signals that excite second order neurons in the nucleus of the solitary tract (NTS). These second order neurons integrate baroreceptor inputs with other neural, humoral, and chemosensory signals, and subsequently, reduce cardiac output and vascular resistance to lower blood pressure.[4,5] Collectively, this is known as the baroreflex. Chronically elevated blood pressure, as with hypertension, requires its resetting such that higher pressures are maintained.[6] The activity of second order neurons is modulated by γ-aminobutyric acid (GABA) neurons also localized to the NTS and experimentally induced hypertension is associated with enhanced GABAergic signalling that causes baroreflex resetting and increased blood pressure.[7–9] The inference is that GABA actions within the NTS contribute to the onset of hypertension; however, GABA and its receptors are poor therapeutic targets because they are ubiquitous in the CNS. Consequently, characterization of discrete NTS neurons that influence arterial blood pressure may allow selective manipulation of GABAergic neurons to relieve hypertension.

The intriguing presence of the angiotensin type-2 receptor (AT2R) on a subset of GABAergic neurons in the NTS,[10] in conjunction with reports that central AT2R stimulation attenuates experimentally induced hypertension in rodents,[11,12] led us to hypothesize that the AT2R is a phenotypic marker for GABAergic neurons in the NTS whose function is coupled to the development of hypertension. Here, we implemented an integrated circuit mapping approach to characterize the structure and cardiovascular-related function of neurons in the NTS that express AT2Rs. First, we generated a novel mouse line with the expression of Cre-recombinase directed to the AT2R gene (*Agtr2*), which we used in experiments designed to determine the impact of optogenetic manipulation of neurons in the NTS that synthesize AT2R on GABA release and arterial blood pressure. To evaluate the role of the receptor, *per se*, in these processes, we chronically delivered Compound 21 (C21), a selective AT2R agonist,[13] into the brains of normotensive and hypertensive mice and assessed indices of GABA synthesis in the NTS, as well as cardiovascular responses to optogenetic stimulation. Final experiments examined the necessity of AT2Rs on GABAergic neurons in the NTS for the antihypertensive effects of C21. Taken together, our results suggest that neurons within the NTS that express AT2Rs can be targeted to reverse the development of hypertension.

2. Methods

Full details of Section 2 are described in the Supplementary material online.

2.1 Animals

Studies were conducted in adult male wild-type C57BL/6J-, AT2R-eGFP reporter- (Mutant Mouse Resource and Research Centers), $Agtr2^{Cre/y}$ (AT2R-Cre)- or $Agtr2^{loxp/y}$ (AT2R-flox; Welcome Trust Sanger Institute) x ROSA-stop-flox-tdTomato (Ai9; Jackson Laboratory Stock # 007909) mice that were 10–12 weeks old at the initiation of the experiments and were approved by the University of Florida Animal Care and Use committee. The principles governing the care and treatment of animals, as stated in the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences (eighth ed., 2011), were followed at all times during this study.

2.2 Anaesthesia and analgesia

For all surgical procedures, anaesthesia was induced using 100% oxygen/4% isoflurane, USP (Patterson Veterinary; 1.5 L/min), and was maintained throughout the surgeries by the administration of 100% oxygen/1.5–2% isoflurane (1.5 L/min). During these surgeries/procedures, the level of anaesthesia was monitored by checking the eye blink reflex and a reaction to paw pinch, and was adjusted if necessary. For recovery surgery, buprenorphine (0.1 mg/kg, sc) was administered immediately prior to the surgical procedure and also during the post-surgical recovery period (every 12 h for 48 h).

2.3 Euthanasia

For euthanasia, mice were administered a lethal dose of ketamine (ip, 10 mg/0.1 mL, KetaVed), followed either by decapitation or by transcardial perfusion with isotonic saline and 4% paraformaldehyde.

2.4 Administration of viral vectors

Transfer of light-sensitive channel-2 rhodopsin (ChR2) or the control eYFP into AT2R-containing NTS neurons was achieved through stereotaxic microinjection of Cre-dependent AAVs into AT2R-Cre mice. Deletion of AT2R from neurons within the dorsal vagal complex (DVC) was achieved by stereotaxic microinjection of AAV8-VGATp-iCre into AT2R-flox x Ai9 mice; controls expressed only the Ai9 gene.

2.5 *In vitro* patch-clamp electrophysiology

Horizontal brain slices (300 μm) were prepared using a vibratome and maintained in a recording chamber. Experiments were performed using a MultiClamp 700B amplifier paired with a Digidata 1440 A digitizer (Axon Instruments) and pCLAMP 10 software. Real-time and offline analysis of electrophysiological data were performed using custom software. Optogenetic activation evoked synaptic currents were recorded from ChR2 negative NTS and dorsal motor nucleus of the vagus (DMNX) neurons in the presence of bath applied ionotropic glutmate receptor antagonists [6,7-dinitroquinoxaline-2,3-dion (DNQX; 20 μM) and (2 R)-amino-5-phosphonovaleric acid; (2 R)-amino-5-phosphonopentanoate (AP5; 40 μM)], and using a high chloride K-gluconate internal solution, were considered likely to be mediated by synaptic activation of GABAergic receptors. A subset of such responses (*n*=7) was challenged with bath application of the GABA receptor antagonists picrotoxin (PTX; 100 μM) and CGP55845 (10 μM).

2.6 Millar catheter cardiovascular recording

In anaesthetized mice, blood pressure and heart rate were measured using a Millar catheter (Model SPR1000) implanted into the aortic arch and connected to a PowerLab signal transduction unit (AD Instruments). Data were analysed using Labchart8 software (AD Instruments).

2.7 Telemetric cardiovascular recording

Catheters of the radiotelemetry transmitters (PAC-10; Data Sciences International) were implanted into the distal left carotid artery. Subsequently, the device itself was positioned subcutaneously in the left flank region. Upon completion of the surgical procedure, mice recovered for 2 weeks before initiating cardiovascular recordings that were performed using DataQuest ART or Ponemah software (Data Sciences International).

2.8 DOCA-Salt hypertension

Mice were rendered hypertensive via subcutaneous implantation of 100 mg DOCA pellets (Innovative Research of America) in the interscapular region and subsequent *ad libitum* access to isotonic saline drink.[14]

2.9 Chronic icv administration of C21

Some mice underwent stereotaxic surgery to implant osmotic minipumps and brain infusion kits (Alzet, DURECT Corporation) to chronically deliver the selective AT2R agonist, C21 (7.5 ng/kg/h) or aCSF vehicle into the lateral cerebral ventricle (icv). The dose of C21 used for these studies is based on experience and on published studies that have revealed selectivity of this non-peptide agonist for AT2R at comparable doses.[15,16] As seen in Supplementary *Figure S2*, this dose of C21 is indeed AT2R-selective.

2.10 *In vivo* optogenetics

Mice injected with *AAV-ChR2* and *AAV-eYFP* into NTS and/or implanted with osmotic minipumps and brain infusion kits for the icv delivery of C21 or saline were implanted with a Millar catheter and then fixed to the stereotaxic frame. Fibre optic posts were positioned immediately dorsal to the NTS. After a period of stable baseline recording, blue laser light was turned on for 1 min at 1, 5, 10, and 15 Hz (20 ms pulses; 10 mW).

2.11 Tissue collection

For real-time (RT)–PCR studies, mice were euthanized and whole brains were removed and flash frozen in dry ice-cooled isopentane for gene expression analysis. For *in situ* hybridization (ISH) and immunohistochemistry studies, mice were anaesthetized and perfused transcardially with isotonic saline followed by 4% paraformaldehyde. Brains were then post-fixed for 3–4 h, after which they were stored in 30% sucrose until sectioning.

2.12 RNA isolation, cDNA synthesis, and semi-quantitative RT–PCR

RNA extraction and DNase treatment were performed using RNeasy columns (Qiagen) according to the manufacturer's instructions. Subsequently, iScript (Bio-Rad) was used to synthesize cDNA from 200 ng of total RNA. Finally, gene expression was assessed by RT–PCR using a StepOne RT–PCR system, TaqMan Gene Expression Master Mix, and validated TaqMan probes (Applied Biosystems).

2.13 ISH (RNAscope)

Fluorescent RNAscope ISH studies were performed on brains collected from reporter or AAV-injected mice as per the manufacturer's instructions, using the same modifications that allow for visualization of mRNA transcripts in the presence of preserved eGFP, eYFP, or tdTomato protein.[10] Immunohistochemistry for reporter genes was conducted after completion of ISH.

2.14 Immunohistochemistry

Immunohistochemistry was performed using standard procedures[10] with Chicken IgY Anti-GFP (1:500) or anti-HuC/D (1:1000) as primary antibodies.

2.15 Image capture and processing

Images were captured and processed using Axiovision 4.8.2 software and a Zeiss AxioImager fluorescent Apotome microscope. For dual immunohistochemistry/RNAscope ISH, z-stacks of the proteins and transcripts of interest were captured at 20× and 40× magnifications throughout the NTS identified using neuroanatomical landmarks found in a mouse brain atlas.[17]

For immunohistochemistry studies, that did not incorporate ISH, images were captured at lower magnification (5–10×). Projection images of the z-stacks were generated and all final figures were then prepared using Adobe Photoshop, where brightness and contrast were adjusted to provide optimal visualization.

2.16 Image analysis

The 20× magnification z-stacks of ROIs were used for the manual determination of the percentage of eGFP or eYFP neurons that contain mRNAs of interest and also to quantify the total *Agtr2* mRNA within the intermediate NTS using ImageJ. Importantly, reporter gene-expressing neurons were considered to contain the mRNA if at least three visible transcripts, defined as an individual punctate dot, were observed within the volume of the eGFP, eYFP, or tdTomato fluorescence. Data are reported as the percentage of eGFP, eYFP, or tdTomato cells that contain the mRNA within the NTS. ImageJ was used to ascertain the average integrated density of *Agtr2* mRNA within the entire intermediate NTS. Zen Lite image analysis software (Zeiss) was used to determine the average quantity of *Agtr2*-mRNA transcripts per AT2R-eGFP + Gad1 mRNA-containing cell.

2.17 Statistics

Statistical analyses were performed using GraphPad Prism Software. In all cases, statistical significance was set at $P < 0.05$. Specific details of the tests performed are included in the Supplementary tables.

3. Results

3.1 Development and validation of an AT2R-Cre knock-in mouse line

Prior anatomical studies from our group and others determined that the NTS contains a discrete population of neurons that express AT2Rs.[10,18] To ascertain the function of these neurons, we engineered mice with the expression of Cre-recombinase directed to the *Agtr2* gene (AT2R-Cre) (*Figure 1A*). Initial studies used a Cre-inducible AAV to direct eYFP to the cells in the NTS that express AT2R (*Figure 1B*). After allowing 3 weeks for stable transfection of the virus, we conducted RNAscope *ISH* for

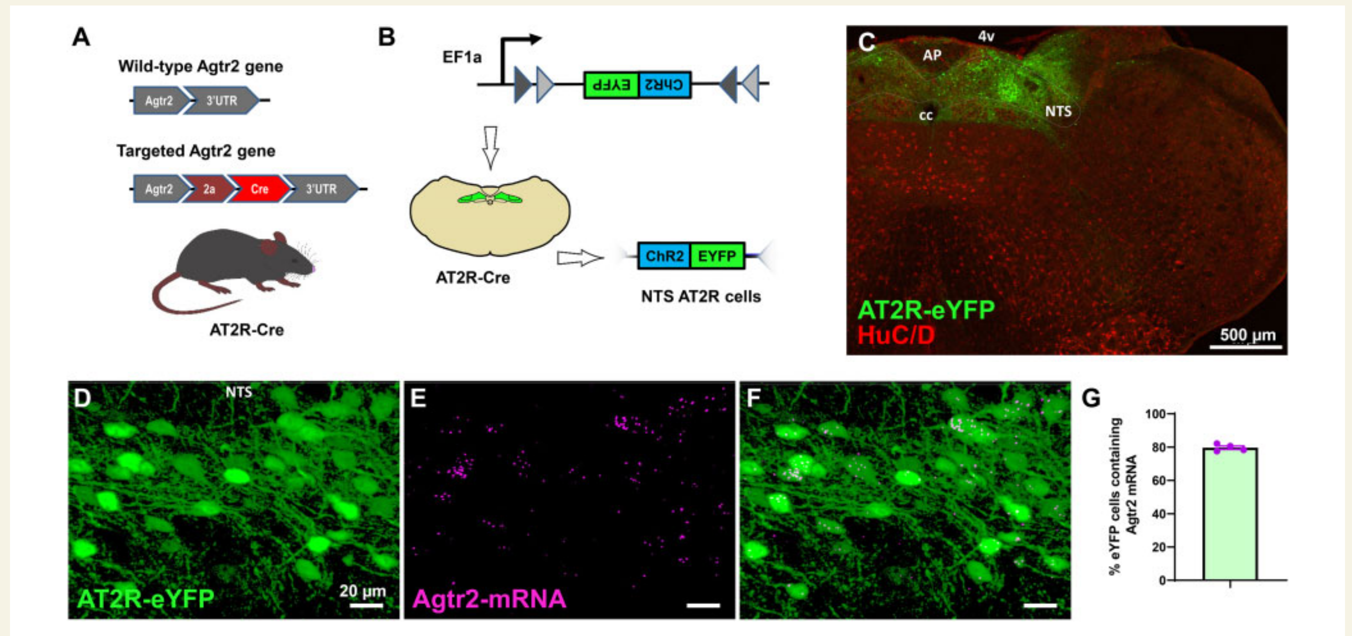


Figure 1 Development and validation of AT2R-Cre knock-in mice. (*A*) AT2R-Cre mouse line schematic. (*B*) Procedure used to generate experimental subjects that express eYFP in AT2R cells of the NTS. (*C*) Representative image of AT2R-containing cells expressing eYFP (green); the neuronal marker, HuC/D is depicted in red. AP, area postrema; 4v, fourth cerebral ventricle; cc, central canal. (*D–F*) Representative images of (*D*) eYFP, (*E*) *Agtr2* mRNAs, and (*F*) their co-localization in the NTS of an AT2R-Cre mouse injected with the Cre-dependent AAV-eYFP. (*G*) Quantification of co-localization of eYFP and *Agtr2*-mRNA (*n*=4). Bars=SEM.

Agtr2 mRNA and immunohistochemistry for eYFP protein to assess the fidelity of Cre direction to AT2R-expressing cells. *Figure 1C* depicts the location of virus transfected neurons in a representative coronal section through the intermediate NTS. We determined that *Agtr2* mRNA was present in ∼80% of eYFP-labelled NTS neurons that were surveyed, indicating that Cre-recombinase activity faithfully follows *Agtr2* mRNA expression within the NTS (536 of 670 neurons sampled from 4 mice) (*Figure 1D–G*).

3.2 AT2R-expressing neurons in the NTS increase arterial blood pressure and release GABA onto post-synaptic neurons

To evaluate whether the activity of AT2R-containing neurons in the NTS affects cardiovascular function, we used a Cre-inducible AAV to direct the expression of the light-sensitive excitatory opsin, ChR2, and the fluorophore, eYFP, to these cells (*Figure 2A*). Live brain slices through the NTS were used for *in vitro* whole-cell patch-clamp recordings (*Figure 2B and C*). All eYFP-expressing cells tested (*n*=9) demonstrated strong inward currents in response to continuous blue light exposure in voltage-clamp configuration (611.80±78.55 pA) and fired action potentials when exposed to pulses of blue light in current-clamp configuration (*Figure 2C*). These results validate functional opsin expression within AT2R-eYFP-expressing neurons.[19]

To test the hypothesis that the activity of neurons in the NTS that express the AT2R is coupled to changes in vascular resistance and cardiac output, we injected AAV-eYFP or AAV-ChR2-eYFP into the NTS of AT2R-Cre mice. Three weeks later, mice were anaesthetized and a Millar catheter was inserted into the carotid artery. Mice were then placed into a stereotaxic frame, a micro-craniotomy was performed and a fibre optic connected to a laser-light source was positioned dorsal to the NTS, thereby allowing for optogenetic stimulation during cardiovascular recordings. In this way, we were able to assess the effects of intrinsic (i.e. non-AT2R-mediated) activation of the AT2R-expressing neurons on blood pressure. Relative to control mice given AAV-eYFP, mice with ChR2 directed to AT2R-expressing neurons exhibited significant elevations in blood pressure, heart rate, and heart rate variability (HRV) upon exposure to blue light (10 Hz, 1 min) (*Figure 2D*) that persisted after the cessation of optical stimulation. That is, there was significant effect of AAV condition, time, and a time by condition interaction for the cardiovascular parameters assessed (see Supplementary *Tables S1 and S2* for ANOVA results and absolute values for cardiovascular parameters, respectively). Interestingly, these elevations in blood pressure were frequency-dependent (*Figure 2E* and Supplementary *Table S3*). These results suggest that intrinsic excitation of neurons in the NTS that express AT2Rs is sufficient to elevate blood pressure, and based on the HRV data, the mechanism includes sympathoexcitation.

We next conducted additional neuroanatomical and electrophysiological experiments to better understand how excitation of AT2R-expressing neurons in the NTS affects blood pressure and heart rate. Initial experiments investigated the phenotype of neurons in the NTS that express AT2R. To accomplish this, AT2R-Cre mice that had received the AAV-ChR2-eYFP were perfused, and then their brains were extracted and processed for eYFP immunohistochemistry with RNAscope *ISH* for vesicular GABA transporter (*VGAT*). *Figure 2F–H* depicts the co-localization of *VGAT* mRNAs to AT2R-eYFP cells within the NTS. Quantitative analysis of images obtained from four mice revealed that ∼83% of the AT2R-eYFP neurons in the NTS express *VGAT* mRNAs (556 of 665 cells from 4 mice) (*Figure 2I*). These results are consistent with our published data, which demonstrate that AT2R-expressing neurons in the NTS are by and large GABAergic.[10] Further examination of these images (*Figure 2F–H*; Supplementary *Figure S1* for additional representative images) reveals that although the majority of AT2R-eYFP neurons express *VGAT* mRNA, not all neurons expressing *VGAT* mRNAs also express AT2R-eYFP. The implication is that AT2Rs are expressed on a subset of GABAergic neurons in the NTS.

Follow-up experiments evaluated the connectivity of neurons in the NTS that express AT2R. AT2R-Cre mice were injected with AAV-ChR2-eYFP into the NTS and, 3 weeks later, horizontal brain slices through the NTS were used for *in vitro* whole-cell patch-clamp recordings. A combination of epifluorescence and differential interference contrast microscopy was used to record from neurons that were in close proximity to eYFP-labelled fibres but that were themselves devoid of eYFP-labelling (*Figure 2J–L*). These NTS neurons were voltage-clamped (-70 mV) in the presence of glutamate receptor antagonists (DNQX and AP5) and subjected to a high chloride internal solution. Notably, 18 of 19 non-eYFP-expressing NTS neurons tested under these conditions demonstrated clear light-evoked inward currents (*Figure 2K*), suggesting synaptic activation of $GABA_A$ receptors. Consistent with this interpretation, light-evoked inward currents were effectively eliminated in all NTS neurons (3/3) directly challenged by bath application of the GABA receptor antagonists (PTX and CGP55845, *P*=0.001) (*Figure 2L*). Overall, these data indicate that AT2R-expressing NTS neurons predominantly release GABA, and that they make synaptic contacts with other NTS neurons.

A similar set of experiments was performed on neurons within the adjacent DMNX that also did not express AT2R-eYFP and that were identified by their large soma and location proximal to the caudal end of the fourth ventricle. Remarkably, 19 out of 19 DMNX motoneurons were also responsive to optogenetic stimulation identical to that used to test NTS neurons (*Figure 2M–O*). Also similar to results in the NTS, we found that light-evoked responses in putative DMNX neurons were effectively eliminated by bath application of GABAergic receptor antagonists in four of five cells tested (*P*=0.0001, *n*=4, *Figure 2O*). Collectively, these results establish that within the NTS, AT2Rs are expressed by GABAergic neurons that form functional inhibitory synapses onto neighbouring neurons residing in the NTS and DMNX.

3.3 Central delivery of C21 lowers basal and light-evoked elevations in blood pressure, effects that are accompanied by decreased GABA-related gene expression

While the previous experiments revealed that intrinsic activation of AT2R-expressing neurons in the NTS is coupled to increases in blood pressure and heart rate, they did not address whether stimulation of the AT2R itself using an AT2R agonist affects neuronal and cardiovascular function. Thus, our next experiments examined the effects of chronic central administration of the selective AT2R agonist, C21 (7.5 ng/kg/h, icv), on GABA-related gene expression in the NTS and on cardiovascular responses to optical stimulation. Normotensive mice were outfitted with brain infusion kits and osmotic minipumps that chronically infused aCSF control (CON) or C21 (icv) (*Figure 3A*). Two weeks after the initiation of the experiment, mice were euthanized, brains were extracted and the DVC, encompassing the NTS, area postrema, and DMNX, was micro-dissected for gene expression analyses. Relative to CON, chronic delivery of C21 significantly decreased mRNAs for GABA synthetic enzymes, glutamate decarboxylase 1 (*Gad1*) and glutamate decarboxylase 2 (*Gad2*), and $GABA_B$ receptor 2 (*Gabbr2*) in the DVC (*Figure 3B*). In contrast, the levels of mRNAs for *VGAT*,

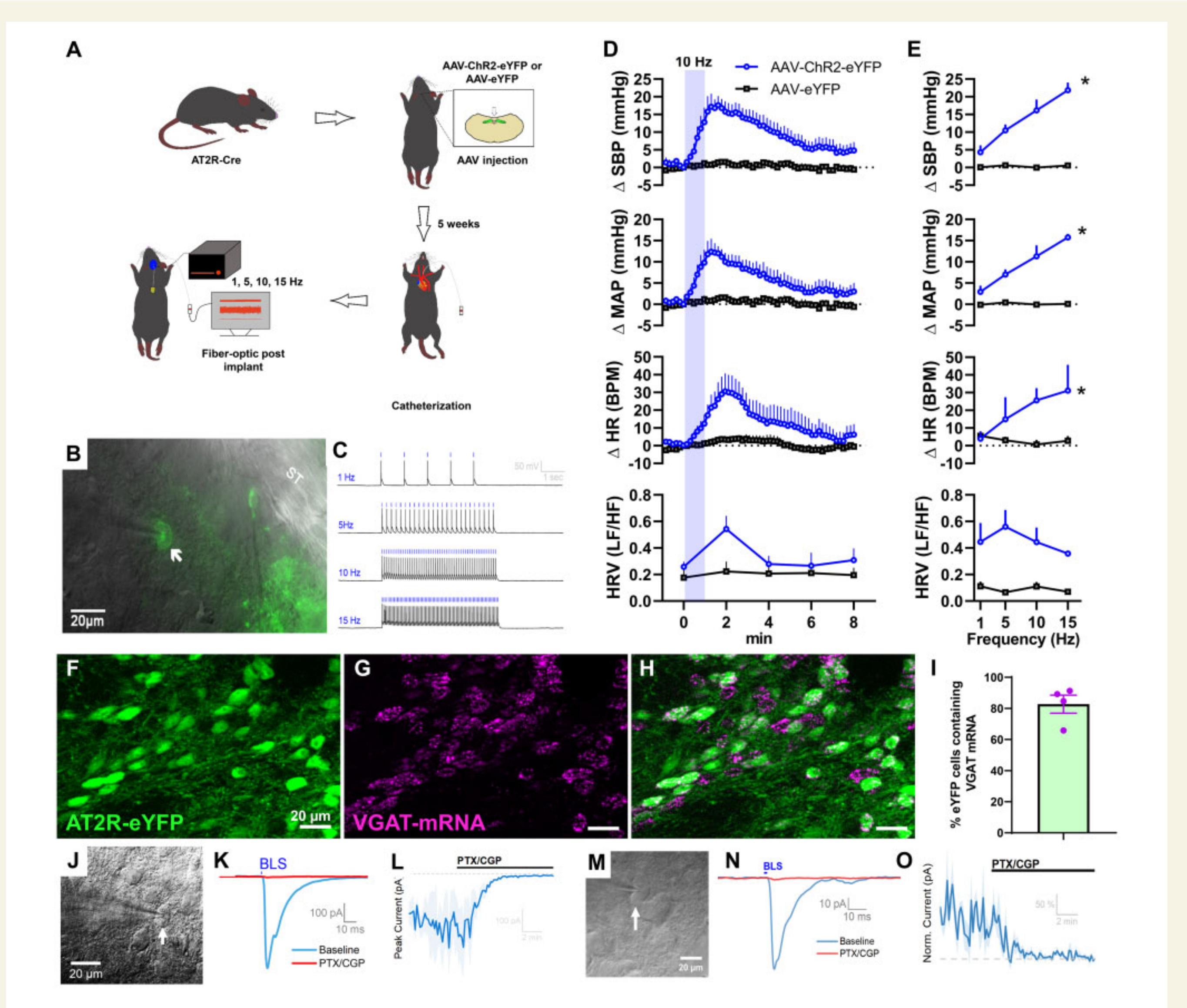


Figure 2 AT2R-expressing neurons increase blood pressure and release GABA onto post-synaptic neurons residing in the NTS. (*A*) Schematic depicting the experimental design. (*B*) ChR2-eYFP-containing AT2R neurons (arrow) were targeted for whole-cell recording using a combination of epifluorescence and differential interference contrast microscopy. (*C*) Blue light pulses elicited action potentials in all cells tested (*n*=9). (*D*) Time course of the impact of optogenetic stimulation (10 Hz; 10 mW; 20 ms pulses; 60 s) in the NTS of AT2R-Cre mice that received AAV-ChR2-eYFP or AAV-eYFP on changes in systolic blood pressure (ΔSBP), mean arterial pressure (ΔMAP), heart rate (ΔHR), and HRV; n=5/group. Bars=SEM. Two-way repeated measures ANOVA revealed an effect of AAV condition, time, and a time by condition interaction for the cardiovascular parameters assessed (see Supplementary *Table S1*). (*E*) Frequency-dependent impact of optogenetic stimulation of AT2R neurons on ΔSBP, ΔMAP, ΔHR, and HRV (1, 5, 10, and 15 Hz; 10 mW; 60 s); *n*=5/group; *=slope different than AAV-eYFP, $P<0.05$, linear regression analysis. Bars=SEM. (*F–H*) Representative images of coronal sections through the NTS of AAV-ChR2-eYFP mice that were processed for visualization of (*F*) eYFP immunofluorescence (green) and (*G*) *VGAT* mRNAs (magenta); (*H*) the merged image. (*I*) Number of eYFP neurons containing *VGAT* mRNA. Bar=SEM. (*J*) NTS neurons were targeted for whole-cell recordings (arrow). Scale bar=20 µm. (*K*) Light-evoked IPSC in a representative NTS neuron. (*L*) Light-evoked IPSCs in NTS were attenuated by exposure to PTX and CGP (*n*=3). (*M*) DMNX neurons were targeted for whole-cell recording (arrow). Scale bar=20 µm. (*N*) Light-evoked IPSC in a representative DMNX neuron. (*O*) Light-evoked IPSCs in DMNX were greatly attenuated by exposure to PTX and CGP (*n*=4). Solid and shaded lines in (*L*) and (*O*) depict mean and SEM. Effectiveness of bath applied antagonists tested against light-evoked synaptic responses observed *in vitro* was evaluated using a 1-sample Student's *t*-test (null hypothesis: mean =1, for data normalized to the baseline mean). See Supplementary *Tables S1–S3* for results of statistical analyses.

for subunits 1, 3, and 5 of the $GABA_A$ receptor (*Gabar1, Gabar3*, and *Gabar5*), for the $GABA_B$ receptor 1 (*Gabbr1*), for $GABA_A$ receptor gamma2 subunit (*Gabrg2*), and for the vesicular glutamate transporter 2 (*VGlut2*) within the DVC (*Figure 3B*) were not affected by C21. Central infusion of C21 affected neither the levels of mRNAs for *Gad1-*, *Gad2-*, and *$GABA_BR$* nor those of arginine vasopressin (*AVP*) and corticotrophin releasing

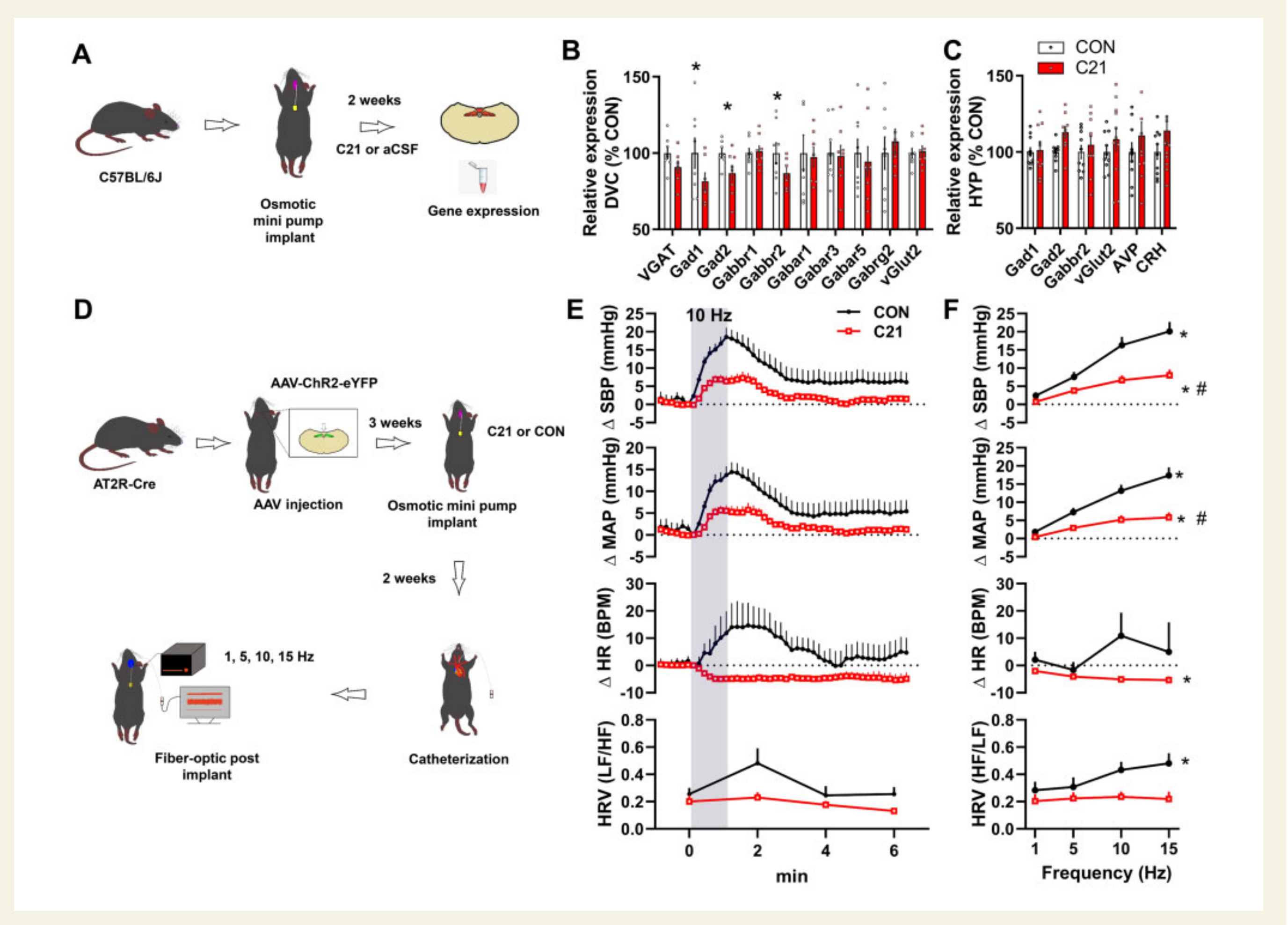


Figure 3 Stimulation of AT2R within the brain reduces blood pressure and indices of GABA release in the DVC. (*A*) Schematic depicting the experimental design used to evaluate the impact of chronic C21 administration (icv; 7.5 ng/kg/h; 2 weeks) on GABA-related gene expression. (*B* and *C*) Gene expression within the (*B*) DVC or (*C*) hypothalamus (HYP) as assessed by qRT–PCR in mice given chronic icv C21 or controls (CON) given icv aCSF; *VGAT, Gad1, Gad2, Gabbr1, Gabbr2*, $GABA_A$ receptor subunits 1, 3, and 5 (*Gabar1, Gabar3*, and *Gabar5*), $GABA_A$ receptor gamma2 subunit (*Gabrg2*), *VGlut2, AVP*, and *CRH*; *n*=8/group; *=*P*<0.05; *t*-test. (*D*) Schematic depicting the experimental design used to evaluate the impact of chronic icv C21 on optical stimulation of AT2R neurons within the NTS. (*E*) Time course of the impact of optogenetic stimulation of AT2R neurons in the NTS (10 Hz; 10 mW; 20 ms pulses; 60 s) on ΔSBP, ΔMAP, ΔHR, and HRV; *n*=7–8/group. Two-way repeated measures ANOVA revealed an effect of C21, time and an interaction between C21 and time for the cardiovascular parameters assessed. (*F*) Frequency-dependent impact of optogenetic stimulation of AT2R neurons on ΔSBP, ΔMAP, ΔHR, and HRV (1, 5, 10, and 15 Hz; 10 mW; 20 ms pulses; 60 s; *n*=5/group). *=slope is different than 0; and #=slope is different than AAV-eYFP, *P* < 0.05, linear regression analysis. See Supplementary *Tables S4–S6* for results of statistical analyses.

hormone (*CRH*) within a hypothalamic micro-dissection containing the paraventricular nucleus of the hypothalamus (*Figure 3C*). To verify that the effects of C21 on gene expression were mediated by AT2R(s) expressed on GABAergic neurons, we bred mice with a knock-in mutation of LoxP-sites flanking the *Agtr2* gene (AT2R-flox) with mice that have Cre-recombinase directed to the *VGAT* (*VGAT*-ires-cre; 016962 JAX). These AT2R-GABA-KO mice were chronically delivered aCSF or C21 as described but subsequent RT–PCR analysis found that patterns of gene expression were similar between groups (Supplementary *Figure S2*). One the one hand, this result indicates that C21 exerted effects on GABA-related gene expression via selective activation of AT2R; on the other, it indicates that AT2Rs on GABAergic cells are necessary for the decreased mRNA expression that was observed.

We next hypothesized that the down-regulation of GABAergic signalling that accompanied chronic C21 administration would alter cardiovascular function during optogenetic excitation of AT2R neurons in the NTS. To test this hypothesis, AT2R-Cre mice were injected with AAV-ChR2-eYFP into the NTS and, 3 weeks later, were implanted with osmotic minipumps and brain infusion kits to chronically deliver aCSF or C21 as above. The goal was to determine whether altered gene expression observed after chronic AT2R stimulation is predictive of blunted cardiovascular responses to light-evoked excitation of AT2R-expressing neurons in the NTS (*Figure 3D*). As above, optogenetic stimulation of AT2R neurons within the NTS elicited frequency-dependent elevations in blood pressure, heart rate, and HRV in control mice that were delivered aCSF vehicle into the brain (*Figure 3E*). Intriguingly, under these *anaesthetized* conditions, baseline blood pressure was unaltered by chronic central activation of AT2R with C21 (see Supplementary *Tables S4 and S5* for ANOVA results and absolute values for cardiovascular parameters, respectively). However, C21 significantly blunted the

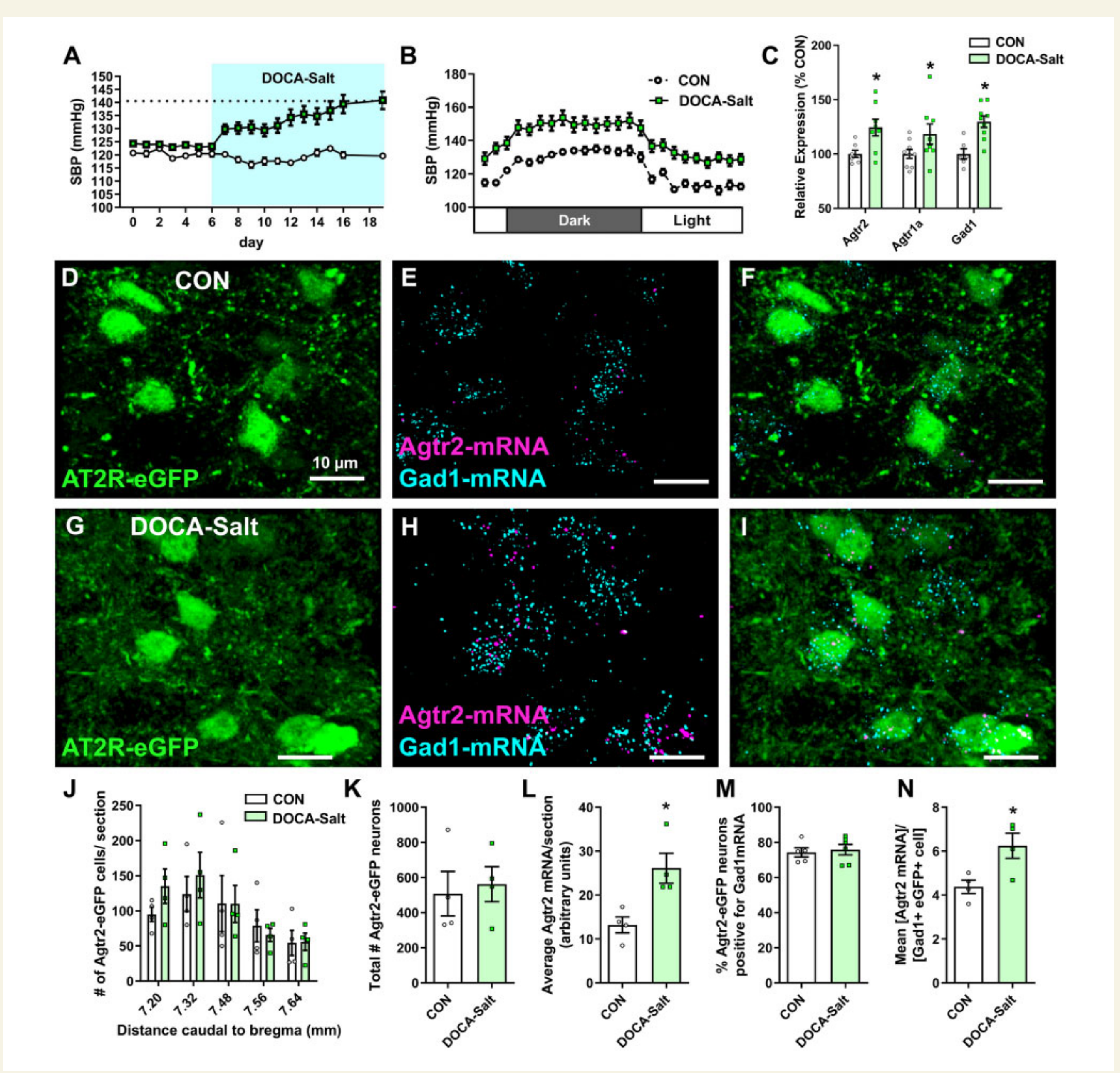


Figure 4 Experimentally induced hypertension augments the co-expression of Gad1 and *Agtr2* mRNAs within the NTS. (*A*) Daily SBP during the establishment of DOCA-Salt hypertension. (*B*) A 24 h SBP trace of DOCA-Salt hypertensive vs. normotensive control (CON) mice. (*C*) Relative gene expression of *Agtr2, Agtr1a*, and *Gad1* within the DVC of DOCA-Salt mice vs. CON as determined via RT–PCR (*n*=8/group). Representative projection images through the NTS of (*D–F*) CON or (*G–I*) DOCA-Salt AT2R-eGFP mice depicting (*D, G*) eGFP, (*E, H*) *Agtr2*, and *Gad1* mRNAs in magenta and cyan, respectively, and (*F, I*) the merged images. Distribution of AT2R-eGFP cells (*J*) across various distances caudal to bregma and (*K*) throughout the entire NTS. (*L*) Total *Agtr2* mRNA levels, (*M*) percentage of AT2R-eGFP neurons containing *Gad1* mRNA, and (*N*) level of *Agtr2* mRNAs per *Gad1*+ AT2R-eGFP+ cell throughout the NTS of DOCA-Salt mice vs. CON. *n*=4/group. *=P<0.05, *t*-test. Bars=SEM.

increased blood pressure and heart rate responses to blue light stimulation and these effects persisted across all patterns of stimulation (*Figure 3E and F*; see Supplementary *Table S4* for ANOVA results revealing significant effects of time, treatment, and time–treatment interactions). Furthermore, linear regression analyses revealed that the impact of optical stimulation on SBP and MAP was frequency-dependent for both groups; however, this effect was significantly blunted in the mice that received C21. Notably, the increase in HRV produced by blue light stimulation was abrogated by C21, at all stimulation frequencies (*Figure 3E and F*). Collectively, these results suggest that chronic icv administration of C21, disrupts indices of GABAergic signalling within the NTS to uncouple blood pressure and heart rate from the firing of

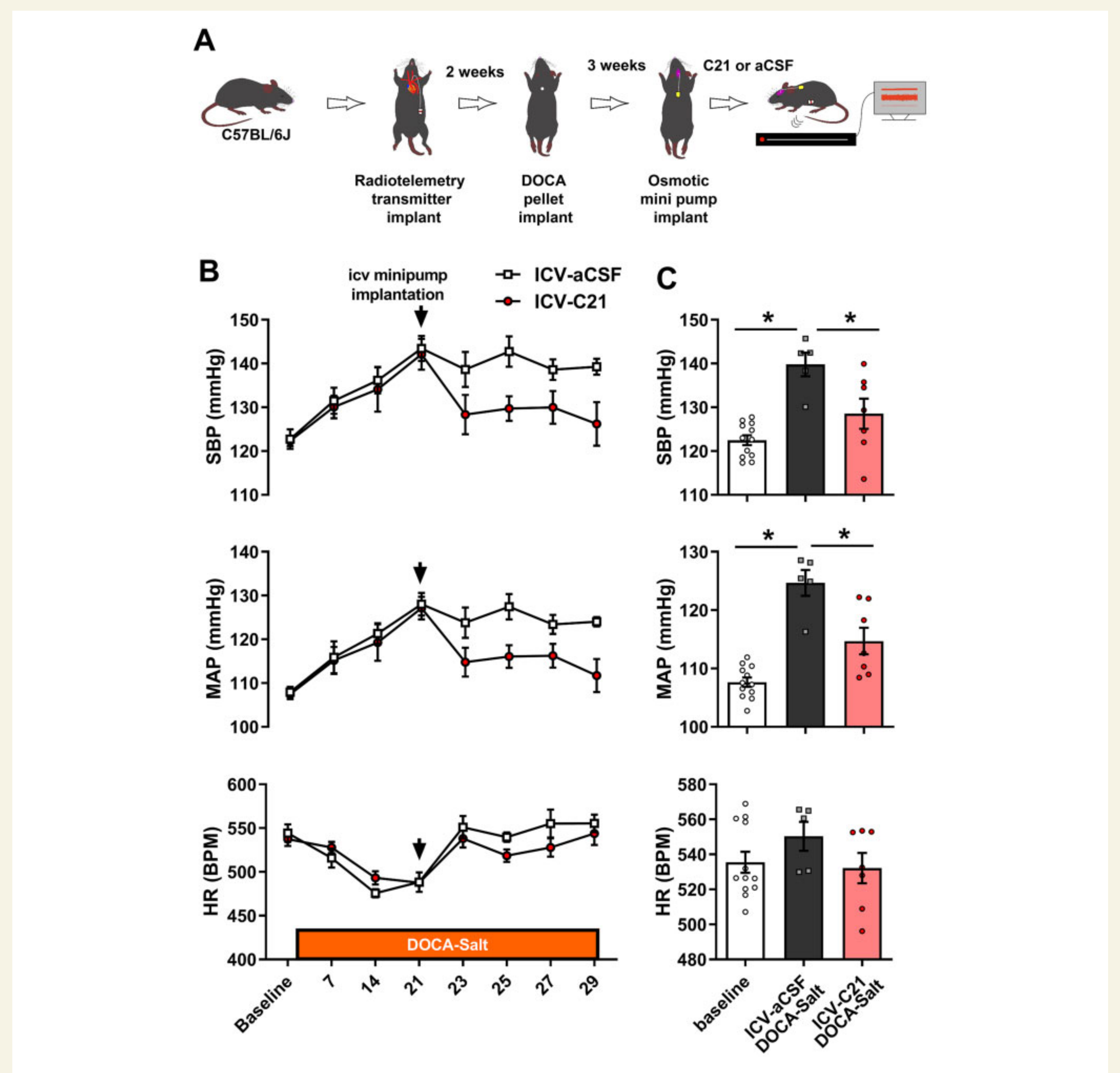


Figure 5 DOCA-Salt induced elevations in blood pressure are reversed by chronic icv C21. (*A*) Schematic of experimental design used to evaluate the ability of C21 (7.5 ng/kg/h; 8 days) to reduce blood pressure in mice rendered DOCA-Salt hypertensive. (*B*) Daily SBP (top), MAP (middle), and HR (bottom) throughout the study for the CON and C21 groups. Two-way repeated measures ANOVA revealed significant main effects of time for both MAP and SBP, and of treatment for MAP, $P<0.05$. (*C*) Average SBP (top), MAP (middle), and HR (bottom) of normotensive controls ($n=12$) and of the DOCA-Salt mice given C21 ($n=7$) or vehicle ($n=5$) during the period post icv minipump implantation, *$P<0.05$, one-way ANOVA. Bars=SEM. See Supplementary *Table S7* for results of statistical analyses.

AT2R-expressing neurons. Based on the HRV data, these effects of C21 appear to involve suppression of sympathetic activity.

3.4 Experimentally induced hypertension augments the co-expression of *Gad1* and *Agtr2* mRNAs within the NTS

Experimentally induced hypertension is associated with enhanced GABAergic signalling within the NTS.[20] Given that AT2R-expressing neurons in the NTS are largely GABAergic and that their excitation is coupled to elevated blood pressure, we hypothesized that hypertension would modulate the expression of AT2R within the NTS. The prediction is that this plasticity may then be exploited to treat the disease. To probe for such alterations in AT2R expression, we first used wild-type mice that were implanted with telemetry devices. After surgical recovery and baseline recordings, mice were implanted with pellets containing DOCA (100 mg, sc; DOCA-Salt) or sham (CON) and given *ad libitum* access to isotonic saline. We selected the DOCA-Salt model of experimentally

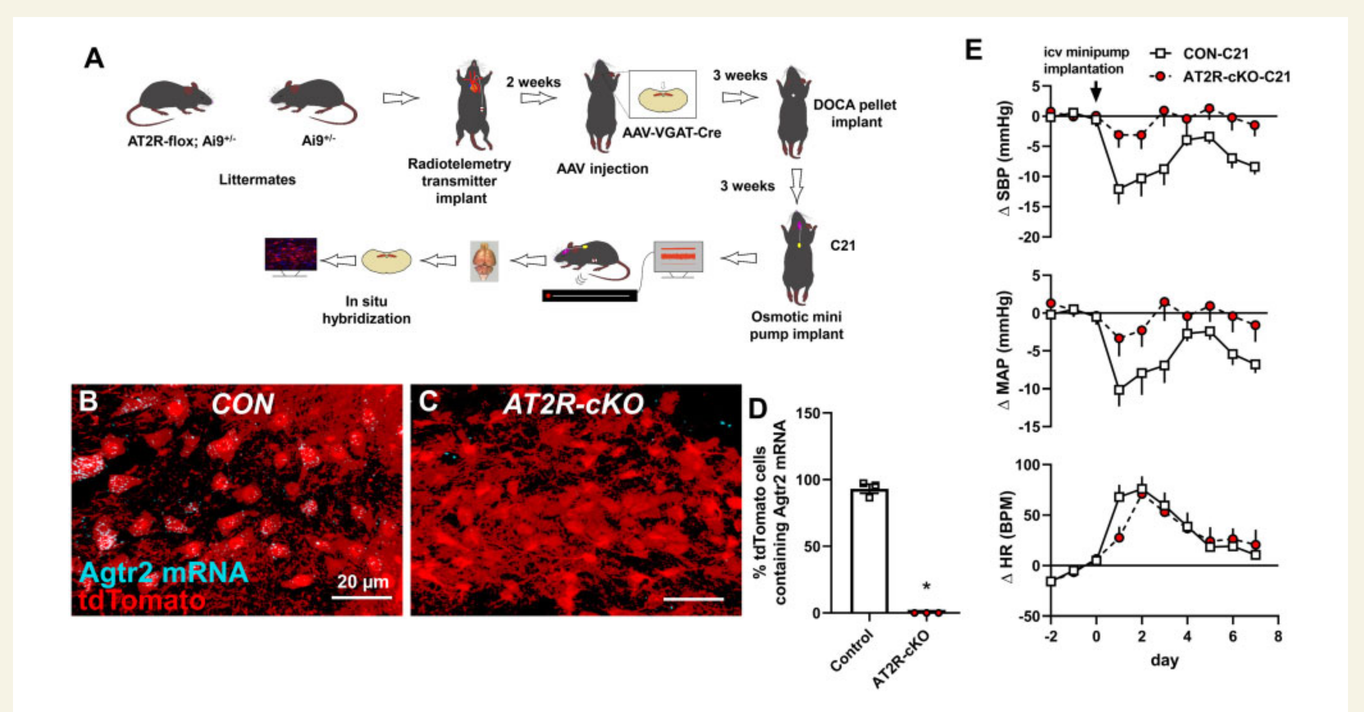


Figure 6 Deletion of AT2R from GABAergic neurons in the NTS prevents the ability of C21 to reduce blood pressure in mice previously rendered hypertensive. (*A*) Schematic of experimental design. (*B–D*) Validation of the specific deletion of *Agtr2* from tdTomato neurons in the AT2R-cKO mice relative to Ai9 controls. Images through the NTS of (*B*) a control mouse and (*C*) an AT2R-cKO mouse depicting mRNAs for *Agtr2* (Cyan) and thereby highlighting the lack of *Agtr2* mRNA within these neurons. (*D*) Quantification of the number of tdTomato neurons containing *Agtr2* mRNA in CON (*n*=3 mice; 350 of 374 tdTomato^{+} cells contain Agtr2 mRNAs) vs. AT2R-cKO mice (*n*=3 mice; 0 of 489 tdTomato^{+} cells contain Agtr2 mRNAs). **P*<0.05, *t*-test. (*E*) Change in SBP (top), MAP (middle), and HR (bottom) subsequent to the initiation of icv C21 administration to AT2R-cKO mice and CON (relative to the baseline DOCA-Salt hypertensive levels). Two-way repeated measures ANOVA revealed significant main effects of time on all cardiovascular parameters assessed, main effects of genotype on MAP and SBP, and a significant time by genotype interaction for SBP, $P < 0.05$. *n*=7/group. Bars=SEM. See Supplementary *Tables S8 and S9* for results of statistical analyses.

induced hypertension since it results in consistent increases in blood pressure in mice and has a strong neurogenic component.[21] Cardiovascular parameters were recorded for 3 weeks, after which microdissections of the DVC were used to assess *Agtr2, Agtr1a*, and *Gad1* mRNAs via RT–PCR. As expected, DOCA-Salt significantly increased daily SBP (*Figure 4A*) and this effect persisted across the light-dark cycle as determined by mean hourly SBP (*Figure 4B*). Within the DVC, DOCA-Salt hypertension significantly increased expression of *Agtr2, Agtr1a*, and *Gad1* mRNAs relative to normotensive controls (*Figure 4C*). We have previously determined that DOCA-Salt does not lead to a redistribution of AT2R from neurons to glia, indicating that the rise is Agtr2 mRNAs are not likely due to increased expression on microglia or astrocytes.[14] Here, follow-up experiments evaluated whether (i) DOCA-Salt hypertension altered the number or distribution of neurons in the NTS that express *Agtr2* and (ii) if augmented expression of *Agtr2* occurred in NTS neurons that also express *Gad1*.

Mice genetically engineered to have the expression of eGFP driven by an *Agtr2* bacterial artificial clone gene (AT2R-eGFP)[10] underwent DOCA-pellet implantation or sham surgeries and were provided access to isotonic saline as described. Afterwards, sections through the NTS were processed for immunohistochemistry for eGFP and dual RNAscope *ISH* for *Agtr2* and *Gad1* mRNAs (*Figure 4D–I*). Cell counts throughout the NTS found no effect of DOCA-Salt on the overall number or distribution of eGFP-labelled neurons (*Figure 4J and K*). Rather, *ISH* revealed that DOCA-Salt resulted in elevated *Agtr2* mRNA expression in the NTS (*Figure 4L*). Further quantification revealed that DOCA-Salt hypertension is not associated with a shift in the percentage of AT2R neurons that are GABAergic (*Figure 4M*), indicating that DOCA-Salt does not shift the phenotype of AT2R neurons in the NTS. Instead, DOCA-Salt increased the number of *Agtr2* mRNA transcripts specifically within NTS neurons that contain *Gad1* mRNA and report *Agtr2* gene transcription with the expression of eGFP (*Figure 4N*), an effect that we hypothesize may represent an endogenous depressor mechanism that can be engaged to reverse augmented GABA synthesis and the development of hypertension. Taken together, these results suggest that chronic elevations in blood pressure increase the synthesis of AT2R in NTS neurons that express GABA.

3.5 Central delivery of C21 reduces experimental hypertension and this effect is abrogated by the selective deletion of AT2R from GABAergic neurons in the NTS

Based on the localization and plasticity of AT2R in hypertension, we hypothesized that activation of AT2R in the NTS would suppress increased GABA production and thereby represent a depressor mechanism that can be engaged to reverse hypertension. Consistent with this notion, elevations in blood pressure that followed DOCA-Salt treatment in wild-type mice were abolished with chronic central delivery of C21 using the same infusion protocol as above, an effect that was absent in controls given the aCSF

vehicle (*Figure 5A–C*). Thus, a final series of experiments tested the hypothesis that AT2Rs expressed on GABAergic neurons in the NTS are necessary for the antihypertensive effects of centrally delivered C21.

An experimental timeline is depicted in *Figure 6A*. In order to visualize and quantify cells undergoing Cre-recombination, we bred female mice heterozygous for AT2R-flox with homozygous Ai9 reporter male mice. This produced male offspring carrying: (i) the AT2R-flox gene and ROSA driven expression of the tdTomato gene preceded by a stop codon flanked by loxP-sites (AT2R-flox mice) or (ii) littermate Ai9 control mice carrying only the tdTomato stop-flox manipulation. Mice were implanted with telemetry devices to measure cardiovascular parameters. To target Cre-recombination specifically to GABA-synthesizing cells in the NTS, mice were injected with an AAV that expresses Cre-recombinase under the control of the *VGAT* promoter (AAV8-*VGAT*-Cre). Mice were then rendered hypertensive using the DOCA-Salt paradigm, afterwhich, they were chronically delivered C21 icv using osmotic minipumps. *Figure 6B and C* highlight the co-localization of tdTomato and *Agtr2* mRNA in control and AT2R-flox mice administered AAV-*VGAT*-Cre into the NTS. In control mice (n=3) ≈93% of tdTomato expressing cells (n=374) were also found to express *Agtr2* mRNA. In contrast, AT2R-flox mice (n=3) given AAV-*VGAT*-Cre had no co-localization of tdTomato and *Agtr2* mRNA in the cells examined (n=489). Importantly, *Agtr2* mRNA was detected in cells devoid of tdTomato that were presumably non-GABAergic. Collectively, these results confirm that delivery of AAV8-*VGAT*-Cre into the NTS has no effect on *Agtr2* mRNA expression in control mice but deletes AT2R from GABAergic neurons within the NTS of AT2R-flox mice. As expected, due to the overwhelming actions of endogenous Ang-II at AT1aR during hypertension, the expression of which we found also to be elevated in the DVC during DOCA-Salt (*Figure 4C*), the deletion of AT2Rs specifically from GABAergic neurons in the NTS had no effect on blood pressure during establishment of hypertension (Supplementary *Table S7*). Rather, this deletion abolished the antihypertensive effects of C21 (*Figure 6E*). That is, two-way ANOVA analyses revealed a significant genotype–time interaction on SBP (Supplementary *Table S8*). Main effects of genotype and time were also revealed for MAP and SBP. Furthermore, analyses of mean SBP, MAP, and HR similarly highlighted a blunted antihypertensive effect of C21, without an impact on HR. Taken together, these results suggest that AT2Rs expressed on GABAergic neurons residing within the NTS are required for the reductions in blood pressure that follow central administration of C21.

4. Discussion

These experiments reveal an unique population of GABAergic neurons residing within the NTS whose firing is coupled to changes in blood pressure. These neurons express AT2Rs and form dense inhibitory synapses onto other neurons residing in the NTS and DMNX. Experimentally induced hypertension up-regulates *Agtr2* transcription within these GABAergic NTS neurons; and chronic central activation of these elevated AT2R with the selective agonist, C21, reverses hypertension. Moreover, AT2Rs on GABAergic neurons in the NTS appear to be required for these actions because their selective deletion prevents the lowered blood pressure that follows central delivery of C21. The collective implication is that neurons in the NTS that express AT2R may serve as an access point for reversing hypertension. Whether or not activation of these NTS AT2R also reverses the hypertension-induced damage to target organs, such as the kidney and heart, remains to be investigated.

The expression of AT2R on discrete neurons in the NTS that are intimately linked to blood pressure regulation is a discovery that is both novel and intriguing. Prior neuroanatomical studies determined that AT2R are localized to the NTS,[18] and over a decade later, the advent of genetic reporting revealed that they are mostly distributed on GABAergic neurons.[10] The present results functionally link the activity of these neurons to changes in blood pressure. Specifically, optogenetic excitation of neurons in the NTS that express AT2Rs increases blood pressure in a frequency-dependent manner and these elevations persist for minutes after blue light illumination ceases. The mechanisms by which these neurons regulate blood pressure are unknown; however, insight can be gained from their connectivity and responsiveness. The brain monitors arterial pressure, in part, via baroreceptor afferents that make excitatory synapses onto second order neurons in the NTS. Canonically, second order neurons are depicted as glutamatergic neurons whose excitatory projections to the caudal ventrolateral medulla form a portion of the efferent limb of the baroreflex arc. However, electrophysiological recordings from brain slices obtained from GABA reporter mice indicate that ≈70% of GABAergic neurons in the NTS receive visceral sensory afferents,[22] suggesting that some portion of second order neurons are GABAergic. Therefore, it is possible that AT2R-expressing neurons in the NTS, which are also GABAergic, sense arterial pressure via direct connections from primary baroreceptor afferents. While our studies do not probe for conditions that acutely activate or inhibit these neurons, they do reveal that chronic elevations in blood pressure, lead to plasticity in the expression of the AT2R, implying that elevations in blood pressure may change their functionality. Furthermore, our findings clearly indicate that once excited, these neurons prompt a robust rise in arterial pressure, suggestive of a prominent role in the neural circuits that regulate blood pressure.

When considering the ubiquitous actions of GABA within the NTS, there is clear evidence for a pressor action of the inhibitory neurotransmitter. For example, microinjections of GABA receptor agonists into the NTS increase blood pressure,[7] an effect mediated by augmented sympathetic outflow.[23] The current thinking is that application of GABA to the NTS inhibits second order neurons, which elevates blood pressure by liberating sympathetic outflow from tonic inhibition, thus dampening the impact of baroreflex stimulation. Despite the general acceptance that GABA exerts pressor actions within the area, the specific circuitry underlying these pressor actions has not been defined. The NTS contains an abundance of GABAergic interneurons that may mediate these effects; however, the transmitter also arises from projections from forebrain areas like the central amygdala.[24] Our *in vitro* optogenetic experiments determined that AT2R-expressing neurons within the NTS form dense inhibitory synapses onto neighbouring neurons in the NTS and DMNX. Thus, it is likely that excitation of AT2R-expressing neurons increases blood pressure by inhibiting second order neurons to augment sympathetic outflow. Indeed, this notion is supported by our HRV data in *Figures 2 and 3*, which suggest that optogenetic activation of AT2R-expressing neurons increases sympathetic activity. Alternatively, or in addition, AT2R-expressing neurons in the NTS may increase blood pressure via connections to the forebrain,[25] that regulate release of hormones like *AVP* into the systemic vasculature.[7] Ultimately, cardiovascular disorders, like hypertension, result from autonomic and humoral dysregulation that chronically increases blood pressure.[26] Given that AT2R-expressing neurons in the NTS appear to play a role in the aetiology of hypertension, it is likely that such neurons also engage autonomic and humoral nodes to couple their activity to hemodynamic status.

Collectively, our optogenetic studies reveal a specific population of GABAergic neurons that are involved in the central control of blood pressure and suggest that AT2R serve as a phenotypic marker for targeting this specific neural circuit. To determine the role of AT2R, *per se*, in the central regulation of blood pressure, we combined the use of the AT2R agonist, C21, with optogenetics and with the Cre-loxP-mediated deletion of AT2Rs. Our initial experiments indicated that central infusion of C21 attenuated the increase in blood pressure elicited by optogenetic stimulation of AT2R-expressing neurons in the NTS (*Figure 3*), and HRV analyses of the data revealed that this C21 effect involved abrogation of the sympathetic component of the optical stimulation. While AT2R has been considered a potential therapeutic for cardiovascular disease for decades,[27] the central site of action and mechanism(s) underlying its protective effects have remained an enigma. Whole body deletion of AT2Rs in mice results in heightened pressor responses to systemically delivered angiotensin II.[28,29] Follow-up studies implicated the CNS as a potential site of action by demonstrating that central administration of angiotensin II to AT2R KO mice also produces augmented pressor responses[30] and virally mediated overexpression of AT2Rs within the baroreflex circuit reduces blood pressure and improves baroreflex sensitivity.[31] The development of C21 as a selective agonist[13] provides a pharmacological tool to probe the effects of AT2R activation. Indeed, consistent with our results, central administration of C21 lowers blood pressure during experimentally induced hypertension.[11,12] However, recent studies using microdialysis revealed that acute application of C21 to the NTS has no effect on levels of GABA sampled from the same nucleus, which casts uncertainty on AT2R and GABA interactions that affect blood pressure.[32] This contrasts with our results showing that *chronic* administration of C21 elicits robust and consistent decreases in mRNAs for GABA synthetic enzymes and blood pressure that counteract the development of hypertension; these effects of chronically administered C21 require the presence of the AT2R on GABAergic neurons in the NTS. An explanation for the differences between our current findings and those of Legat *et al.*[32] may reside in the time course of C21 administration. Our results indicate that the blood pressure-lowering action of chronic AT2R activation is associated with reduced levels of GABA synthetic enzyme mRNAs. If the antihypertensive action of C21 requires a decrease in GABA synthesis in AT2R neurons, reductions in GABA levels would not be achieved by an acute perfusion protocol as employed by Legat *et al.*[32] Regardless of the mechanism, our collective results reveal novel pathophysiology that can be reversed with chronic stimulation of brain AT2Rs and this discovery may be exploited to develop and improve therapeutics aimed at relieving resistant hypertension. While our data strongly suggest that the locus of this AT2R-mediated antihypertensive effect is the NTS (*Figure 6*), at this point, we are unable to completely rule out an involvement of other AT2R-containing brain regions. In addition, we cannot rule out that C21 influences neurohormonal processes, which may decrease blood pressure. For example, even though our data indicate that icv infusion of C21 does not alter hypothalamic AVP mRNA levels (*Figure 3C*), our previous studies demonstrated that icv infusion of C21 lowers circulating AVP.[33]

An interesting facet of our data is the differing effects of C21 on blood pressure and heart rate. For example, central C21 infusion attenuated the increase in blood pressure produced by optogenetic stimulation of AT2R-expressing neurons in the NTS, but at the same time completely blocked the tachycardia induced by optical stimulation, and even lowered heart rate. An explanation may reside in the phenotype(s) of cells in the NTS that contain AT2R. As discussed above, the majority of the AT2R-containing neurons in the NTS are GABAergic, and we believe that C21 lowers blood pressure through reducing GABA synthesis. A reduction in GABA synthesis may lead to reduce optogenetic-stimulation induced GABA release onto DMNX neurons that provide parasympathetic input to the heart, thereby contributing to reduced/abrogated blood pressure/heart rate responses. Another layer of complexity is that in addition to being expressed on GABA neurons, there is also a subset of AT2R neurons in the DMNX itself that are cholinergic,[10] and optogenetic stimulation of DMNX cholinergic neurons, in general, is known to reduce heart rate.[34] While the particular cholinergic neurons that express AT2R were not directly adjacent to the fibre optic post in our subjects, the close proximity of the area to the NTS, combined with the known contribution of the area to the parasympathetic innervation of the heart, as well as the observed bradycardic response to C21 administration (*Figure 3*), bring up the possibility that the removal the GABAergic influence of AT2R neurons in the NTS by way of chronic C21 administration, effectively 'unmasks' a direct effect of the DMNX neurons to reduce heart rate.

Another thing that is apparent from our data is that deletion of AT2R from NTS GABAergic neurons does not alter baseline blood pressure or the development of DOCA-Salt hypertension. These findings were not unexpected, for a number of reasons. Hypertension is a multi-factorial disease and it is doubtful that removing one protective factor, such as the AT2R would provide a significant influence over baseline blood pressure. This is especially true as the levels of AT2R in the NTS are low to begin with,[18] and even though their levels increase in hypertension (*Figure 4*), their endogenous activity is likely overridden by the pro-hypertensive AT1R, which are expressed at higher levels in the NTS and also increase in hypertension (*Figure 4*). A lack of changes in baseline blood pressure after deletion of brain angiotensin receptors has also been demonstrated in other studies. Namely, while deletion of AT1R from catecholaminergic neurons in the rostral ventrolateral medulla attenuated angiotensin II-induced hypertension, it did not alter baseline blood pressure.[35] The key points are that the endogenous AT2R-depressor mechanism that we have discovered to exist in the NTS is likely to be a failed compensatory mechanism during high blood pressure; however, it can be taken advantage of by using a selective AT2R-agonist to decrease BP in hypertension, something that is clearly demonstrated by our data. Furthermore, combining an AT2R agonist with an AT1R blocker may result in an enhanced antihypertensive strategy.

Increasing evidence implicates the nervous system in the development or reversal of resistant hypertension. Accordingly, emerging therapies are using medications,[36] devices,[37] or surgical approaches[38] that alter neural and humoral control of circulation to lower blood pressure. For example, firibastat, a new class of drug that targets the brain renin-angiotensin system by suppressing production of angiotensin III and thus decreasing its interaction with AT1Rs, was recently found to be efficacious at lowering blood pressure in patient populations that have historically suffered from resistant hypertension.[36,39] Here, we propose that the antihypertensive effects of centrally acting therapies, especially those targeting the brain renin-angiotensin-system, likely engage subsets of GABAergic neurons in NTS, possibly via activation of AT2Rs, to reverse the neuronal plasticity underlying the autonomic and neuroendocrine dysfunction that promotes the development of resistant hypertension. This might be taken advantage of in the clinical setting by developing means (including drugs) that would produce selective- and long-lasting activation of the AT2R on GABAergic neurons in the NTS. This is a reasonable suggestion, as it has been demonstrated that certain blood vessels in the dorsomedial NTS lack a blood-brain barrier (BBB),[40] and that during hypertension the BBB in the NTS becomes leaky.[41] Alternatively,

as these NTS GABAergic neurons appear to be an important component of the mechanisms that contribute to resistant hypertension, strategies that dampen their activity, with or without AT2R agonists, might prove useful in this disease.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Authors' contributions

A.D.d.K., E.G.K., and C.S. conceived, designed, supervised, and coordinated the study; M.M., D.N.J., L.A.W., S.W.H., W.S., E.A.S., K.A.S., and A.D.d.K. conducted experiments and acquired data; M.M., D.N.J., L.A.W., S.W.H., K.E., C.J.F., and A.D.d.K. analysed data; U.M.S., M.B., and A.D.d.K. generated mice; A.D.d.K., E.G.K., C.S., M.M., K.E., C.J.F., M.B., and U.M.S. wrote, edited, and revised the article.

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

References

1. Heron M. Deaths: leading Causes for 2016. *Natl Vital Stat Rep* 2018;**67**:1–77.
2. Achelrod D, Wenzel U, Frey S. Systematic review and meta-analysis of the prevalence of resistant hypertension in treated hypertensive populations. *Am J Hypertens* 2015;**28**:355–361.
3. Osborn JW. Hypothesis: set-points and long-term control of arterial pressure. A theoretical argument for a long-term arterial pressure control system in the brain rather than the kidney. *Clin Exp Pharmacol Physiol* 2005;**32**:384–393.
4. Andresen MC, Kunze DL. Nucleus tractus solitarius–gateway to neural circulatory control. *Annu Rev Physiol* 1994;**56**:93–116.
5. Dampney RAL. Resetting of the baroreflex control of sympathetic vasomotor activity during natural behaviors: description and conceptual model of central mechanisms. *Front Neurosci* 2017;**11**:461.
6. Lohmeier TE, Iliescu R. The baroreflex as a long-term controller of arterial pressure. *Physiology (Bethesda)* 2015;**30**:148–158.
7. Catelli JM, Giakas WJ, Sved AF. GABAergic mechanisms in nucleus tractus solitarius alter blood pressure and vasopressin release. *Brain Res* 1987;**403**:279–289.
8. Zhang W, Mifflin S. Plasticity of GABAergic mechanisms within the nucleus of the solitary tract in hypertension. *Hypertension* 2010;**55**:201–206.
9. Landulpho CD, Dias AC, Colombari E. Cardiovascular mechanisms activated by microinjection of baclofen into NTS of conscious rats. *Am J Physiol Heart Circ Physiol* 2003;**284**:H987–H993.
10. de Kloet AD, Wang L, Ludin JA, Smith JA, Pioquinto DJ, Hiller H, Steckelings UM, Scheuer DA, Sumners C, Krause EG. Reporter mouse strain provides a novel look at angiotensin type-2 receptor distribution in the central nervous system. *Brain Struct Funct* 2016;**221**:891–912.
11. Dai SY, Zhang YP, Peng W, Shen Y, He JJ. Central infusion of angiotensin II type 2 receptor agonist compound 21 attenuates DOCA/NaCl-induced hypertension in female rats. *Oxid Med Cell Longev* 2016;**2016**:3981790.
12. Brouwers S, Smolders I, Wainford RD, Dupont AG. Hypotensive and sympathoinhibitory responses to selective central AT2 receptor stimulation in spontaneously hypertensive rats. *Clin Sci (Lond)* 2015;**129**:81–92.
13. Steckelings UM, Larhed M, Hallberg A, Widdop RE, Jones ES, Wallinder C, Namsolleck P, Dahlof B, Unger T. Non-peptide AT2-receptor agonists. *Curr Opin Pharmacol* 2011;**11**:187–192.
14. Sumners C, Alleyne A, Rodriguez V, Pioquinto DJ, Ludin JA, Kar S, Winder Z, Ortiz Y, Liu M, Krause EG, de Kloet AD. Brain angiotensin type-1 and type-2 receptors: cellular locations under normal and hypertensive conditions. *Hypertens Res* 2020;**43**:281–295.
15. Danyel LA, Schmerler P, Paulis L, Unger T, Steckelings UM. Impact of AT2-receptor stimulation on vascular biology, kidney function, and blood pressure. *Integr Blood Press Control* 2013;**6**:153–161.
16. Joseph JP, Mecca AP, Regenhardt RW, Bennion DM, Rodriguez V, Desland F, Patel NA, Pioquinto DJ, Unger T, Katovich MJ, Steckelings UM, Sumners C. The angiotensin type 2 receptor agonist Compound 21 elicits cerebroprotection in endothelin-1 induced ischemic stroke. *Neuropharmacology* 2014;**81**:134–141.
17. Franklin KBJ, Paxinos G, *The Mouse Brain: In Stereotaxic Coordinates*. 3rd ed. New York, NY: Elsevier; 2008.
18. Lenkei Z, Palkovits M, Corvol P, Llorens-Cortes C. Distribution of angiotensin II type-2 receptor (AT2) mRNA expression in the adult rat brain. *J Comp Neurol* 1996; **373**:322–339.
19. de Kloet AD, Wang L, Pitra S, Hiller H, Smith JA, Tan Y, Nguyen D, Cahill KM, Sumners C, Stern JE, Krause EG. A unique "Angiotensin-Sensitive" neuronal population coordinates neuroendocrine, cardiovascular, and behavioral responses to stress. *J Neurosci* 2017;**37**:3478–3490.
20. Tolstykh G, Belugin S, Tolstykh O, Mifflin S. Responses to GABA(A) receptor activation are altered in NTS neurons isolated from renal-wrap hypertensive rats. *Hypertension* 2003;**42**:732–736.
21. Basting T, Lazartigues E. DOCA-salt hypertension: an update. *Curr Hypertens Rep* 2017;**19**:32.
22. Bailey TW, Appleyard SM, Jin YH, Andresen MC. Organization and properties of GABAergic neurons in solitary tract nucleus (NTS). *J Neurophysiol* 2008;**99**:1712–1722.
23. Moreira TS, Takakura AC, Colombari E. Important GABAergic mechanism within the NTS and the control of sympathetic baroreflex in SHR. *Auton Neurosci* 2011;**159**:62–70.
24. Saha S, Batten TF, Henderson Z. A GABAergic projection from the central nucleus of the amygdala to the nucleus of the solitary tract: a combined anterograde tracing and electron microscopic immunohistochemical study. *Neuroscience* 2000;**99**:613–626.
25. Sawchenko PE, Swanson LW. The organization of noradrenergic pathways from the brainstem to the paraventricular and supraoptic nuclei in the rat. *Brain Res* 1982;**257**:275–325.
26. Lozic M, Sarenac O, Murphy D, Japundzic ZN. Vasopressin, central autonomic control and blood pressure regulation. *Curr Hypertens Rep* 2018;**20**:11.
27. Sumners C, de Kloet AD, Krause EG, Unger T, Steckelings UM. Angiotensin type 2 receptors: blood pressure regulation and end organ damage. *Curr Opin Pharmacol* 2015;**21**:115–121.
28. Hein L, Barsh GS, Pratt RE, Dzau VJ, Kobilka BK. Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor in mice. *Nature* 1995;**377**:744–747.
29. Ichiki T, Labosky PA, Shiota C, Okuyama S, Imagawa Y, Fogo A, Niimura F, Ichikawa I, Hogan BL, Inagami T. Effects on blood pressure and exploratory behaviour of mice lacking angiotensin II type-2 receptor. *Nature* 1995;**377**:748–750.
30. Li Z, Iwai M, Wu L, Shiuchi T, Jinno T, Cui TX, Horiuchi M. Role of AT2 receptor in the brain in regulation of blood pressure and water intake. *Am J Physiol Heart Circ Physiol* 2003;**284**:H116–H121.
31. Blanch GT, Freiria-Oliveira AH, Speretta GF, Carrera EJ, Li H, Speth RC, Colombari E, Sumners C, Colombari DS. Increased expression of angiotensin II type 2 receptors in the solitary-vagal complex blunts renovascular hypertension. *Hypertension* 2014;**64**:777–783.
32. Legat L, Smolders IJ, Dupont AG. Investigation of the role of AT2 receptors in the nucleus tractus solitarii of normotensive rats in blood pressure control. *Front Neurosci* 2019;**13**:589.
33. de Kloet AD, Pitra S, Wang L, Hiller H, Pioquinto DJ, Smith JA, Sumners C, Stern JE, Krause EG. Angiotensin type-2 receptors influence the activity of vasopressin neurons in the paraventricular nucleus of the hypothalamus in male mice. *Endocrinology* 2016;**157**:3167–3180.
34. Machhada A, Hosford PS, Dyson A, Ackland GL, Mastitskaya S, Gourine AV. Optogenetic stimulation of vagal efferent activity preserves left ventricular function in experimental heart failure. *JACC Basic Transl Sci* 2020;**5**:799–810.
35. Jancovski N, Carter DA, Connelly AA, Stevens E, Bassi JK, Menuet C, Allen AM. Angiotensin type 1A receptor expression in C1 neurons of the rostral ventrolateral medulla contributes to the development of angiotensin-dependent hypertension. *Exp Physiol* 2014;**99**:1597–1610.
36. Ferdinand KC, Balavoine F, Besse B, Black HR, Desbrandes S, Dittrich HC, Nesbitt SD, On behalf of the NEW HOPE Investigators. Efficacy and safety of firibastat, a first-in-class brain aminopeptidase A inhibitor, in hypertensive overweight patients of multiple ethnic origins. *Circulation* 2019;**140**:138–146.
37. Spiering W, Williams B, Van der Heyden J, van Kleef M, Lo R, Versmissen J, Moelker A, Kroon A, Reuter H, Ansel G, Stone GW, Bates M, Spiering W, Williams B, Stone GW, Bates M. Endovascular baroreflex amplification for resistant hypertension: a safety and proof-of-principle clinical study. *Lancet* 2017;**390**:2655–2661.
38. Azizi M, Pereira H, Hamdidouche I, Gosse P, Monge M, Bobrie G, Delsart P, Mounier-Vehier C, Courand PY, Lantelme P, Denolle T, Dourmap-Collas C, Girerd X, Michel Halimi J, Zannad F, Ormezzano O, Vaisse B, Herpin D, Ribstein J,

Chamontin B, Mourad JJ, Ferrari E, Plouin PF, Jullien V, Sapoval M, Chatellier G, Investigators D, DENERHTN Investigators. Adherence to antihypertensive treatment and the blood pressure-lowering effects of renal denervation in the renal denervation for hypertension (DENERHTN) trial. *Circulation* 2016;**134**:847–857.
39. Llorens-Cortes C, Touyz RM. Evolution of a new class of antihypertensive drugs: targeting the brain renin-angiotensin system. *Hypertension* 2020;**75**:6–15.
40. Maolood N, Meister B. Protein components of the blood-brain barrier (BBB) in the brainstem area postrema-nucleus tractus solitarius region. *J Chem Neuroanat* 2009;**37**:182–195.
41. Biancardi VC, Son SJ, Ahmadi S, Filosa JA, Stern JE. Circulating angiotensin II gains access to the hypothalamus and brain stem during hypertension via breakdown of the blood-brain barrier. *Hypertension* 2014;**63**:572–579.

Translational perspective

Hypertension is a widespread health problem and risk factor for cardiovascular disease and stroke. Although treatment options exist, many patients suffer from resistant hypertension, which is associated with enhanced sympathetic drive. Thus, many available therapeutics focus on dampening pressor mechanisms. The present studies take the alternative approach of treating hypertension by exploiting an endogenous depressor mechanism.