

# Impaired sodium-evoked paraventricular nucleus neuronal activation and blood pressure regulation in conscious Sprague–Dawley rats lacking central $G\alpha_{i2}$ proteins

C. Y. Carmichael,<sup>1</sup> A. C. T. Carmichael,<sup>1</sup> J. T. Kuwabara,<sup>1</sup> J. T. Cunningham<sup>2</sup> and R. D. Wainford<sup>1</sup>

<sup>1</sup> Department of Pharmacology & Experimental Therapeutics and the Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, MA, USA

<sup>2</sup> Department of Integrative Physiology & Anatomy, University of North Texas Health Science Center, Fort Worth, TX, USA

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Correspondence: R. D. Wainford,  
72 E Concord St, Boston, MA  
02118, USA.  
E-mail: rwainf@bu.edu

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## Abstract

**Aim:** We determined the role of brain  $G\alpha_{i2}$  proteins in mediating the neural and humoral responses of conscious male Sprague–Dawley rats to acute peripheral sodium challenge.

**Methods:** Rats pre-treated (24-h) intracerebroventricularly with a targeted oligodeoxynucleotide (ODN) (25  $\mu$ g per 5  $\mu$ L) to downregulate brain  $G\alpha_{i2}$  protein expression or a scrambled (SCR) control ODN were challenged with an acute sodium load (intravenous bolus 3 M NaCl; 0.14 mL per 100 g), and cardiovascular parameters were monitored for 120 min. In additional groups, hypothalamic paraventricular nucleus (PVN) Fos immunoreactivity was examined at baseline, 40, and 100 min post-sodium challenge.

**Results:** In response to intravenous hypertonic saline (HS), no difference was observed in peak change in mean arterial pressure between groups. In SCR ODN pre-treated rats, arterial pressure returned to baseline by 100 min, while it remained elevated in  $G\alpha_{i2}$  ODN pre-treated rats ( $P < 0.05$ ). No difference between groups was observed in sodium-evoked increases in Fos-positive magnocellular neurons or vasopressin release.  $V_{1a}$  receptor antagonism failed to block the prolonged elevation of arterial pressure in  $G\alpha_{i2}$  ODN pre-treated rats. A significantly greater number of Fos-positive ventrolateral parvocellular, lateral parvocellular, and medial parvocellular neurons were observed in SCR vs.  $G\alpha_{i2}$  ODN pre-treated rats at 40 and 100 min post-HS challenge ( $P < 0.05$ ). In SCR, but not  $G\alpha_{i2}$  ODN pre-treated rats, HS evoked suppression of plasma norepinephrine ( $P < 0.05$ ).

**Conclusion:** This highlights  $G\alpha_{i2}$  protein signal transduction as a novel central mechanism acting to differentially influence PVN parvocellular neuronal activation, sympathetic outflow, and arterial pressure in response to acute HS, independently of actions on magnocellular neurons and vasopressin release.

**Keywords** blood pressure regulation, central nervous system,  $G\alpha$ -subunit proteins, paraventricular nucleus, sodium.

The hypothalamic paraventricular nucleus (PVN) acts as a critical site of integration of homeostatic autonomic and neuroendocrine activity to influence central sympathetic outflow in response to osmotic stimuli (Pyner 2009, Nunn *et al.* 2011). Further, there is growing support for a role of dietary salt intake in altering the excitability of the sympathetic nervous system to produce elevated arterial pressure and hypertension (Fujita & Fujita 2013, Stocker *et al.* 2013). Despite the progressive understanding of these mechanisms, the neural circuitry and neurochemical/receptor substrates within the PVN governing the immediate central nervous system (CNS) responses to acute increases in plasma sodium and osmolality in response to salt remain unclear.

The acute actions of plasma sodium on the CNS likely involve the coordinated activity of multiple brain sites, including the PVN, whose activity is driven by afferent projections from osmosensitive forebrain structures [subfornical organ, (SFO) organum vasculosum laminae terminalis (OVLT), median preoptic nucleus (MnPO)], with descending projections to brainstem [rostral ventrolateral medulla (RVLM)] and spinal cord structures [intermediolateral cell column T<sub>1</sub>–T<sub>3</sub> (IML)]. The ability of the PVN to coordinate the hormonal and neural responses to alterations in osmolality (Antunes-Rodrigues *et al.* 2004, Antunes *et al.* 2006) is mediated by two distinct cell types within this region – magnocellular and parvocellular nuclei, respectively. Within the magnocellular division are oxytocin- and vasopressin (AVP)-containing neurons – the latter a neurohypophysial hormone classically involved in body fluid homeostasis through the regulation of water retention (Swanson & Sawchenko 1980, Antunes-Rodrigues *et al.* 2004). The parvocellular division of the PVN includes sympathetic-regulatory neurons that project to the RVLM and/or the IML of the spinal cord (Swanson & Sawchenko 1980, Stocker *et al.* 2004a, Ferguson *et al.* 2008) and neuroendocrine neurons that project to the median eminence that are involved in the release of hormones into the hypophysial portal system (Swanson & Kuypers 1980, Swanson & Sawchenko 1980, Ferguson *et al.* 2008). Both acute hypertonic saline (HS) (Kantzides & Badoer 2003, Antunes *et al.* 2006) and water deprivation (Stocker *et al.* 2004a) – two stimuli that increase plasma sodium and osmolality – have been previously reported to increase PVN neuronal activation as assessed by Fos immunoreactivity.

The principal neurotransmitters of the CNS (e.g. GABA and glutamate) have been localized within the PVN, and the complex inhibitory and excitatory actions evoked by these neurotransmitters serve to produce an appropriate differential sympathetic output in the normotensive state (Pyner 2009, Nunn *et al.* 2011). Electrophysiological studies have shown

inhibition of both inhibitory and excitatory actions on PVN neurons by pre-synaptic G-protein-coupled (GPCR) GABA<sub>B</sub> receptors (Chen & Pan 2006). Cell culture studies have demonstrated that G $\alpha$ -subunit protein availability can play a critical role in determining the intracellular signalling response to GPCR activation following ligand binding (Nasman *et al.* 2001). Our laboratory has demonstrated that brain G $\alpha$ <sub>i2</sub> proteins, inhibitory intracellular signalling proteins coupled to GPCRs (e.g. GABA<sub>B</sub>) post-ligand-binding, possess a functionally selective and facilitatory role in central cardiovascular regulation. Specifically, G $\alpha$ <sub>i2</sub> proteins mediate the hypotensive response to direct central  $\alpha$ <sub>2</sub>-adrenoceptor activation, the renal sympathoinhibitory response to an acute volume expansion, and the global sympathoinhibitory response to a non-pressor sodium challenge in the normotensive, Sprague–Dawley rat – a strain which is salt-resistant (Kapusta *et al.* 2012, Wainford & Kapusta 2012, Wainford *et al.* 2013). Based on these findings, we propose that G $\alpha$ <sub>i2</sub> protein signal transduction contributes to a sympathoinhibitory GPCR-activated neuronal mechanism within the PVN. These PVN mechanisms functionally normalize sympathetic outflow and arterial pressure following an acute pressor sodium challenge in a salt-resistant rat phenotype.

In this study, we hypothesized that salt-resistant rats with compromised G $\alpha$ <sub>i2</sub> protein expression would exhibit prolonged elevated blood pressure in response to an acute HS challenge. Also, this sustained pressor response would involve altered PVN neuronal activation and impaired regulation of the release of norepinephrine (NE) and/or AVP. To address this hypothesis, we (i) used an oligodeoxynucleotide (ODN) knockdown approach to test the role of central G $\alpha$ <sub>i2</sub> proteins in mediating the blood pressure response to an acute HS challenge, (ii) examined the impact of removing central G $\alpha$ <sub>i2</sub> proteins on the sodium-evoked activation of PVN subnuclei, and (iii) examined the impact of removing central G $\alpha$ <sub>i2</sub> proteins on sodium-evoked alterations in plasma NE and AVP.

## Materials and methods

### Animals

Male Sprague–Dawley (SD) rats (Harlan Laboratories, Indianapolis, IN, USA) weighing 275–300 g were individually housed in a temperature- (range: 20–26 °C) and humidity-controlled (range: 30–70%) environment under a 12-h light/dark cycle and were randomly assigned to experimental treatment groups. Rats were allowed tap water and standard rodent diet (Test Diet, St. Louis, MO, USA) *ad libitum*. All experimental protocols were approved by the Institutional Animal

Care and Use Committee in accordance with the guidelines of Boston University School of Medicine and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

### Surgical procedures

**Intracerebroventricular cannulae implantation.** Animals were anaesthetized [ketamine, 30 mg kg<sup>-1</sup> intraperitoneally (IP) in combination with xylazine, 3 mg kg<sup>-1</sup> IP] and stereotaxically implanted with a stainless steel cannula into the right lateral cerebral ventricle 5–7 days prior to experimentation, as previously described (Wainford & Kapusta 2012).

**Acute femoral vein, artery and bladder cannulation.** On the day of the study, intracerebroventricular (ICV) control scrambled (SCR) or Gzi<sub>2</sub> ODN pre-treated rats were anaesthetized with sodium methohexital [20 mg kg<sup>-1</sup> IP, supplemented with 10 mg kg<sup>-1</sup> intravenously (IV) as required] and instrumented with catheters in the left femoral vein, left femoral artery and bladder for the administration of an isotonic saline infusion, bolus HS load or V<sub>1a</sub> receptor antagonist, measurement of heart rate (HR) and arterial blood pressure (MAP), and renal function respectively (Kapusta *et al.* 2012, Wainford & Kapusta 2012, Wainford *et al.* 2013).

### Experimental protocols

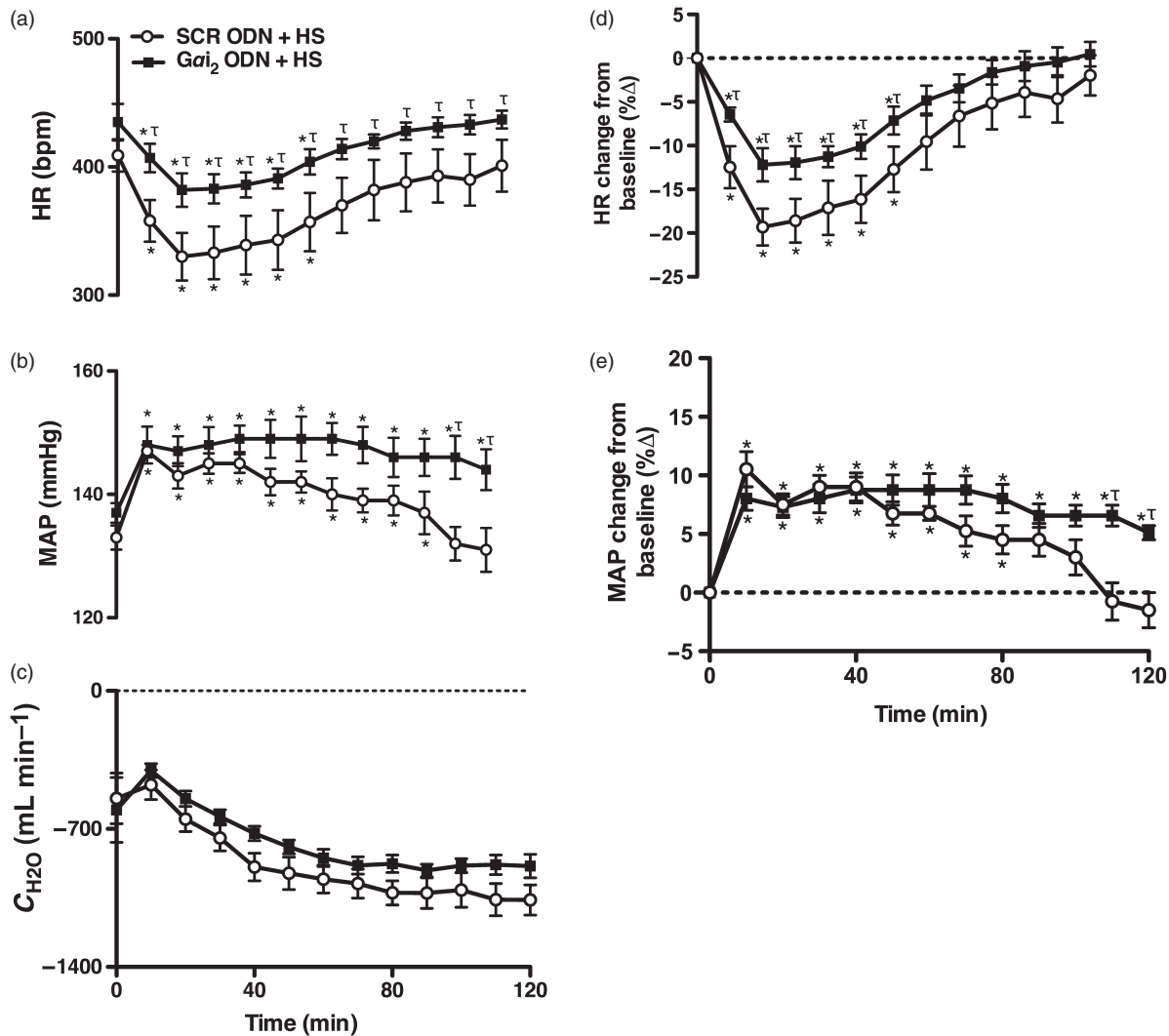
**Acute intracerebroventricular ODN administration.** Twenty-four hours prior to the day of the study, downregulation of brain Gzi<sub>2</sub> protein expression levels in rats was achieved by ICV injection (25 µg per 5 µL delivered over 60 s) of a phosphodiesterase ODN probe dissolved in isotonic saline that selectively targets Gzi<sub>2</sub> proteins (5'-CTT GTC GAT CAT CTT AGA-3') (Kapusta *et al.* 2012, Wainford & Kapusta 2012, Wainford *et al.* 2013). Control studies involved ICV injection of a SCR ODN (5'-GGG CGA AGT AGG TCT TGG-3') (The Midland Certified Reagent Company, Midland, TX, USA). An NCBI Basic Local Alignment Search Tool search of the *Rattus norvegicus* RefSeq protein database was conducted to confirm the specificity of the Gzi<sub>2</sub> ODN for the rat Gzi<sub>2</sub> protein sequence and that the SCR ODN does not match any known rat protein sequence. Multiple publications from our laboratory have confirmed effective (approx. 85%) ODN-mediated downregulation of Gzi<sub>2</sub> protein expression in the acute setting as assessed by Western blotting (Kapusta *et al.* 2012). This was not carried out in the current study due to the different methods of tissue processing required for immuno-

histochemical analysis (PFA mediated fixation) vs. Western blotting (snap-frozen) that do not allow for both methods to be performed in a single animal.

**Acute sodium challenge.** Following femoral vein, artery and bladder cannulation (as outlined above), rats were placed in a Plexiglas holder and an IV infusion of isotonic saline (20 µL min<sup>-1</sup>) was maintained for a 2-h surgical recovery period prior to experimentation to enable the animal to regain full consciousness and for cardiovascular and renal excretory functions to stabilize. HR and MAP were continuously recorded via the surgically implanted femoral artery cannula using computer-driven BIOPAC data acquisition software (MP150 and ACQKNOWLEDGE 3.8.2, BIOPAC Systems, Inc, Goleta, CA, USA) connected to an external pressure transducer (P23XL; Viggo Spectramed, San Francisco, CA, USA) (Wainford & Kapusta 2012, Wainford *et al.* 2013). Following the 2-h recovery period, HR and MAP were recorded continuously over a 20-min baseline period in conscious rats. After the measurement of baseline parameters, animals received a peripheral HS challenge: IV bolus dose of 3 M NaCl (0.14 mL per 100 g delivered over 30 s) (Antunes *et al.* 2006, Veitenheimer & Osborn 2011) and changes in HR and MAP were continuously recorded for 120 min and urine was collected in 10-min increments for the duration of the experiment (*N* = 6 per treatment group per time point).

Separate groups of animals underwent an identical HS challenge protocol, but were sacrificed at baseline, 40 and 100 min post-HS administration by either intracardiac perfusion for CNS IHC (*N* = 8 per treatment group per time point; outlined below in CNS tissue collection section) or at baseline, 10, 40 and 100 min by conscious decapitation for trunk blood collection (*N* = 5 per treatment group per time point; outlined below in Acute sodium challenge blood sample collection section). Respective time points at which PVN Fos expression was evaluated were determined based on the expression profile of Fos protein post-stimulus. An initial early time point at which sodium-evoked alterations in Fos would be detectable is represented by 40 min (Herrera & Robertson 1996) and a time point at which there was a divergence in the MAP response between groups (Fig. 1) and at which there would be robust Fos expression is represented by 100 min.

**Acute sodium challenge during V<sub>1a</sub> receptor antagonism.** An additional group of rats underwent an identical ODN pre-treatment and HS challenge protocol (outlined above in Acute intracerebroventricular ODN administration and Acute



**Figure 1** Systemic cardiovascular and free water clearance responses produced by hypertonic saline (HS) challenge (3 M, 0.14 mL per 100 g, IV) in conscious male Sprague–Dawley rats pre-treated ICV with a control scrambled (SCR) or experimental  $G\alpha_{i2}$  oligodeoxynucleotide (ODN) (25  $\mu$ g per 5  $\mu$ L) expressed as raw values (a–c) or per cent change from baseline (d, e). Data are mean  $\pm$  SEM ( $N = 8$  per group). \* $P < 0.05$ , sig. diff. vs. respective group baseline value, <sup>†</sup> $P < 0.05$ , sig. diff. vs. respective SCR ODN group value.

sodium challenge sections, respectively), but HS was injected five minutes following pre-treatment with [ $\beta$ -mercapto- $\beta$ ,  $\beta$ -cyclopentamethylene-propionyl<sup>1</sup>, O-me-Tyr<sup>2</sup>, -Arg<sup>8</sup>]-vasopressin (a  $V_{1a}$  receptor antagonist, 10  $\mu$ g  $kg^{-1}$  mL<sup>-1</sup>, IV bolus) (Veitenheimer & Osborn 2011) (Sigma-Aldrich, St. Louis, MO, USA).

**Acute sodium challenge + autonomic function.** An additional group of rats underwent an identical ODN pre-treatment and HS challenge protocol (outlined above in Acute intracerebroventricular ODN administration and Acute sodium challenge sections, respectively), but the ganglionic blocker hexamethonium (IV

30 mg  $kg^{-1}$ ) (Kapusta *et al.* 2013) was administered 110 min post-HS and peak change in MAP was recorded ( $N = 5$  per group).

**Acute sodium challenge blood sample collection.** Conscious decapitation was performed either at baseline, 10, 40 or 100 min time points post-HS administration, and trunk blood was collected in a heparinized tube. Following plasma extraction, samples were frozen at  $-80$  °C until later analysis of sodium, osmolality, NE and AVP.

**Cardiac baroreflex curve.** Baroreflex regulation of HR was assessed in a separate group of 24-h ICV SCR or

Gxi<sub>2</sub> ODN pre-treated conscious rats instrumented with femoral vein and artery cannulae, as outlined above ( $N = 6$  per treatment group). Doses of phenylephrine and sodium nitroprusside ( $10 \mu\text{g}$  in  $0.2 \text{ mL}$  saline for each) were infused (IV;  $0.05 \text{ mL}$  every  $10 \text{ s}$ ) over  $40 \text{ s}$  to increase and decrease blood pressure by  $50\text{--}60 \text{ mmHg}$ , respectively, to generate a dynamic baroreflex gain curve for HR. Changes in HR and MAP were continuously recorded via the surgically implanted femoral artery cannula using computer-driven BIOPAC data acquisition software connected to an external pressure transducer. A four-parameter logistic equation was used to generate a baroreflex gain curve in which average values of HR were calculated for each  $5 \text{ mmHg}$  change in MAP using the 1-s values stored on the hard drive. This allowed for the calculation of the range over which the curve operated ( $A_1$ ), the sensitivity or curvature coefficient of the relationship ( $A_2$ ), the mid-point mean blood pressure of the curve ( $A_3$ ) and the lowest point to which the HR could be driven ( $A_4$ ) (Huang *et al.* 2006).

#### CNS tissue collection

Following completion of the HS challenge protocol, rats were deeply anaesthetized with sodium methohexital ( $10 \text{ mg kg}^{-1}$  IV) and immediately perfused transcardially with  $0.2\text{--}0.3 \text{ L}$  of  $0.1 \text{ M}$  phosphate-buffered saline (PBS) followed by  $4\%$  paraformaldehyde (PFA) in  $0.1 \text{ M}$  PBS ( $4 \text{ }^\circ\text{C}$ ,  $0.3\text{--}0.5 \text{ L}$ ). The brains were removed and placed in a vial containing  $4\%$  PFA in PBS overnight and then switched to a  $30\%$  w/v sucrose solution for 2 days (Randolph *et al.* 1998). The PVN was sectioned into three separate sets of serial  $40\text{-}\mu\text{m}$  coronal sections that were collected into a cryoprotectant ( $30\%$  sucrose +  $30\%$  ethylene glycol +  $1\%$  polyvinyl-pyrrolidone in  $0.1 \text{ M}$  PBS) and stored at  $-20 \text{ }^\circ\text{C}$  until they were processed for immunohistochemistry.

#### Fos immunoreactivity and histological analysis

Free-floating sections from each brain were processed for Fos immunohistochemistry using a rabbit polyclonal anti-Fos antibody (Calbiochem, San Diego, CA, USA) as previously described (Randolph *et al.* 1998). Briefly, sections were brought to room temperature and rinsed twice for  $30 \text{ min}$  in  $0.1 \text{ M}$  PBS to remove cryoprotectant. Sections were incubated in  $0.3\%$  hydrogen peroxide in distilled water for  $30 \text{ min}$  at room temperature and then rinsed for  $30 \text{ min}$  in  $0.1 \text{ M}$  PBS. Sections were then incubated for  $2 \text{ h}$  at room temperature in PBS diluent ( $3\%$  normal horse serum in  $0.1 \text{ M}$  PBS containing  $0.25\%$  Triton-100; Sigma-Aldrich). The rabbit polyclonal anti-Fos antibody was

diluted to  $1 : 30\,000$  in PBS diluent, and the sections were incubated in the primary antibody for  $48 \text{ h}$  at  $4 \text{ }^\circ\text{C}$ . After two  $30\text{-min}$  rinses in  $0.1 \text{ M}$  PBS, sections were incubated in a biotinylated horse anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) diluted to  $1 : 200$  in PBS diluent for  $2 \text{ h}$  at room temperature. The tissue was reacted with an avidin–peroxidase conjugate (ABC-Vectastain kit; Vector Laboratories) and PBS containing  $0.04\%$  3,3'-diaminobenzidine hydrochloride and  $0.04\%$  nickel ammonium sulphate (Sigma-Aldrich). Sections were mounted on gel-coated slides, processed through a series of dehydrating alcohols followed by xylenes, and placed under a coverslip with Permount mounting medium.

Tissue sections were analysed using an Olympus microscope (BX41) equipped for epifluorescence and an Olympus DP70 digital camera with DP MANAGER software (v 2.2.1) (Olympus, Center Valley, PA, USA). The PVN was identified using the stereotaxic atlas of Paxinos & Watson (1998) and sampled at three rostral–caudal levels, as previously described (Stocker *et al.* 2004a). Care was taken to ensure that the sections used were from the same rostral–caudal plane in each brain. Analysis was performed on two sets of tissue for each animal. The number of Fos-positive cells was visually quantified by participants blind to the experimental conditions using National Institutes of Health IMAGEJ software (NIH, Bethesda, MD, USA), and the counts for each PVN subnucleus was averaged for each animal.

#### Analytical techniques

**Analysis of urine.** Urine volume was determined gravimetrically assuming  $1 \text{ g} = 1 \text{ mL}$ . Urinary osmolality was measured using a Vapor Pressure Osmometer (model 5600; Wescor, South Logan, UT, USA). Free water clearance ( $C_{\text{H}_2\text{O}}$ ) was calculated as a difference between the rate of urine volume ( $\mu\text{L min}^{-1}$ ) and the osmolar clearance (Kapusta *et al.* 2013).

**Analysis of plasma samples.** Plasma sodium was determined by flame photometry (model 943; Instrumentation Laboratory, Bedford, MA, USA), and plasma osmolality was measured using a Vapor Pressure Osmometer (model 5600; Wescor) (Kapusta *et al.* 2013, Wainford *et al.* 2013, 2015). Plasma NE content was determined using a NE ELISA kit as per manufacturers' instruction (Immuno-Biological Laboratories, Minneapolis, MN, USA). For AVP analysis, equal volumes of  $0.1\%$  (v/v) trifluoroacetic acid (TFA) and distilled water were added to plasma samples that were centrifuged for  $15 \text{ min}$  ( $17\,000 \text{ g}$  at  $4 \text{ }^\circ\text{C}$ ). Sep-Pak C<sub>18</sub> columns (EMD Millipore, Billerica, MA, USA) were preconditioned with  $1 \text{ mL}$  acetonitrile fol-

lowed by 10–25 mL 0.1% TFA, and the supernatant was added. The columns were washed with 10–25 mL of 0.1% TFA, and the peptides were eluted by 3 mL of 60 : 40 acetonitrile and 0.1% TFA and 1 mL fractions were collected. All samples were evaporated to dryness on a Savant Speed Vac and reconstituted in 250  $\mu$ L of assay buffer. Following extraction, plasma AVP concentration was determined using an AVP ELISA kit as per manufacturers' instruction (Enzo Life Sciences, Farmingdale, NY, USA; Wainford & Kapusta 2010).

**Statistical analysis.** Results are expressed as mean  $\pm$  SEM. The magnitude of change in cardiovascular and free water clearance parameters at different time points after HS or  $V_{1a}$  receptor antagonism + HS were compared with respective group control values by a one-way repeated-measures (RM) ANOVA with a subsequent Dunnett's multiple comparisons test (GRAPHPAD PRISM v. 6.00 for Mac OS X; GraphPad Software, San Diego, CA, USA). Differences occurring between treatment groups were assessed by a two-way RM (mixed model) ANOVA (treatment  $\times$  time) with a subsequent Sidak's multiple comparisons test. Baroreflex gain curves were generated using a nonlinear least squares fit approach and analysed by a two-way RM ANOVA with a subsequent Sidak's multiple comparisons test. Baroreflex parameters ( $A_1$ – $A_4$ ) were analysed by independent  $t$ -tests. Plasma sodium, osmolality, NE content and AVP content were compared with respective group control values by a one-way ANOVA with a subsequent Sidak's multiple comparisons test. Differences occurring between treatment groups were assessed by a two-way ANOVA with a subsequent Sidak's multiple comparisons test. The number of Fos-positive cells in each subregion of interest was compared with respective group control values by a one-way ANOVA and between groups by a two-way ANOVA with a subsequent Dunnett's and Sidak's multiple comparisons test respectively. In each case, statistical significance was defined as  $P < 0.05$ .

## Results

### Effect of HS on cardiovascular and renal excretory parameters

Table 1 presents HR and MAP values in *all* conscious SD rats pre-treated ICV with a SCR or  $G\alpha_{i2}$  ODN used through the course of these experiments to illustrate there was no difference in baseline hemodynamic parameters prior to HS (3 M, IV) stimulus (i.e. no ODN-specific effect). Following HS in male SD rats pre-treated (24-h) with a control SCR or  $G\alpha_{i2}$  ODN,

peak bradycardia was significantly greater in SCR as compared to  $G\alpha_{i2}$  ODN pre-treated groups [HS  $\Delta$ HR (beats  $\text{min}^{-1}$ ); SCR ODN:  $-79 \pm 15$  vs.  $G\alpha_{i2}$  ODN:  $-59 \pm 12$ ,  $P < 0.05$ ]. No difference was observed in the peak change in MAP between ODN pre-treatment groups [HS  $\Delta$ MAP (mmHg) SCR ODN:  $+17 \pm 4$  mmHg vs.  $G\alpha_{i2}$  ODN:  $+13 \pm 3$ ,  $P > 0.05$ ]. In SCR ODN pre-treated rats, MAP returned to baseline by 100 min, whereas in  $G\alpha_{i2}$  ODN pre-treated rats, MAP remained significantly elevated [MAP 100 min (mmHg) SCR ODN:  $134 \pm 2$  vs.  $G\alpha_{i2}$  ODN:  $146 \pm 3$ ,  $P < 0.05$ ]. No differences in free water clearance were observed between ODN pre-treatment groups at any time point (Fig. 1).

### Effect of central $G\alpha_{i2}$ protein downregulation on the cardiac baroreflex

The impact of central  $G\alpha_{i2}$  protein downregulation on the ability of the arterial baroreceptors to reflexively regulate HR was investigated in separate groups of ODN pre-treated animals (Fig. 2). Table 2 presents the four parameters of the baroreflex gain curves for HR in animals pre-treated centrally with a SCR or  $G\alpha_{i2}$  ODN. No differences were observed between ODN pre-treatment groups in the range of the curves ( $A_1$ ), the sensitivity or curvature coefficient of the relationship ( $A_2$ ), or the mid-point mean blood pressure ( $A_3$ ). Central  $G\alpha_{i2}$  protein downregulation significantly altered the lowest point to which HR could be driven ( $A_4$ ) in an acute setting [lowest HR (beats  $\text{min}^{-1}$ ) SCR ODN:  $258.8 \pm 9.9$  vs.  $G\alpha_{i2}$  ODN:  $288.2 \pm 13.7$ ,  $P < 0.05$ ]. Separate analysis of the high-pressure baroreflex revealed a significant difference between pre-treatment groups in baroreflex sensitivity ( $A_2$ ) [(beats  $\text{min}^{-1}$  mmHg $^{-1}$ ) SCR ODN:  $0.044 \pm 0.004$  vs.  $G\alpha_{i2}$  ODN:  $0.083 \pm 0.004$ ,  $P < 0.05$ ].

**Table 1** HR (beats  $\text{min}^{-1}$ ) and MAP (mmHg) in all conscious Sprague–Dawley rats pre-treated ICV with a control SCR or experimental  $G\alpha_{i2}$  ODN (25  $\mu$ g per 5  $\mu$ L) to illustrate no difference in baseline hemodynamic parameters prior to HS stimulus

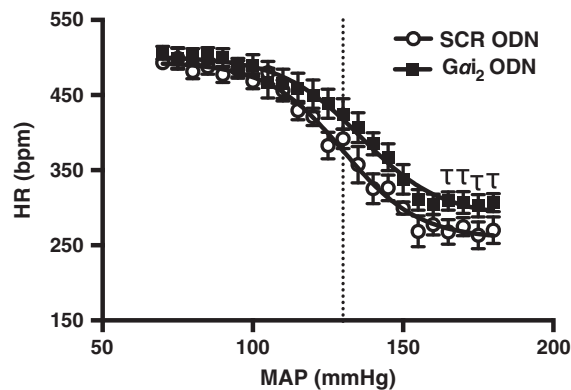
	Baseline ( $N = 57$ per group)	
	SCR ODN	$G\alpha_{i2}$ ODN
HR (beats $\text{min}^{-1}$ )	$427 \pm 8$	$436 \pm 9$
MAP (mmHg)	$126 \pm 2$	$128 \pm 2$

HR, heart rate; MAP, mean arterial pressure; ICV, intracerebroventricular; ODN, oligodeoxynucleotide; SCR, scrambled; HS, hypertonic saline.

Data are mean  $\pm$  SEM ( $N = 57$  per group).

### Effect of HS on Fos immunoreactivity in PVN parvocellular neurons

Paraventricular nucleus Fos immunoreactivity was analysed by subnuclei whose cytoarchitectonic boundaries were determined based on the rostral–caudal level of each section (Stocker *et al.* 2004a). Table S1 presents the mean HR and MAP group values in response to an IV 3 M NaCl bolus from ODN pre-treated animals in which cFos IHC was conducted at baseline, 40 and 100 min. These data demonstrate that the cardiovascular responses were not different as those observed in animals from which the initial physiological data were derived (Fig. 1), confirming the reproducibility of our experimental paradigm. PVN parvocellular neurons were analysed as dorsal (DP), medial (MP), ventrolateral (VLP) and lateral (LP) subregions based on the respective rostral–caudal level of the PVN. No differences between the ODN pre-treatment groups in the number of Fos-positive nuclei were observed in any parvocellular subregion prior to HS. At 40 min, HS significantly increased Fos immunore-



**Figure 2** Cardiac baroreflex relationship produced by IV infusion of phenylephrine and sodium nitroprusside in conscious male Sprague–Dawley rats pre-treated ICV with a control scrambled (SCR) or experimental G $\alpha$ i<sub>2</sub> oligodeoxynucleotide (ODN) (25  $\mu$ g per 5  $\mu$ L). Mid-point mean arterial pressure (MAP) (mmHg) indicated by dashed line. Data are mean  $\pm$  SEM ( $N = 6$  per group).  $^{\dagger}P < 0.05$ , sig. diff. vs. respective SCR ODN group value.

**Table 2** Baroreflex parameters for heart rate (HR) obtained in conscious Sprague–Dawley rats pre-treated ICV with a control SCR or experimental G $\alpha$ i<sub>2</sub> ODN (25  $\mu$ g per 5  $\mu$ L)

	A <sub>1</sub> (beats min <sup>-1</sup> )	A <sub>2</sub> (beats min <sup>-1</sup> mmHg <sup>-1</sup> )	A <sub>3</sub> (mmHg)	A <sub>4</sub> (beats min <sup>-1</sup> )
SCR ODN	235.1 $\pm$ 14.7	0.034 $\pm$ 0.005	129.9 $\pm$ 2.0	258.8 $\pm$ 9.9
G $\alpha$ i <sub>2</sub> ODN	213.0 $\pm$ 18.4	0.033 $\pm$ 0.006	135.8 $\pm$ 2.7	288.2 $\pm$ 13.7*

ICV, intracerebroventricular; ODN, oligodeoxynucleotide; SCR, scrambled. A<sub>1</sub>: range of the curve; A<sub>2</sub>: sensitivity of the curve; A<sub>3</sub>: mid-point mean of the blood pressure; A<sub>4</sub>: lowest point to which HR could be driven.

Data are mean  $\pm$  SEM ( $N = 6$  per group).

\* $P < 0.05$ , sig. diff. vs. respective SCR ODN group value.

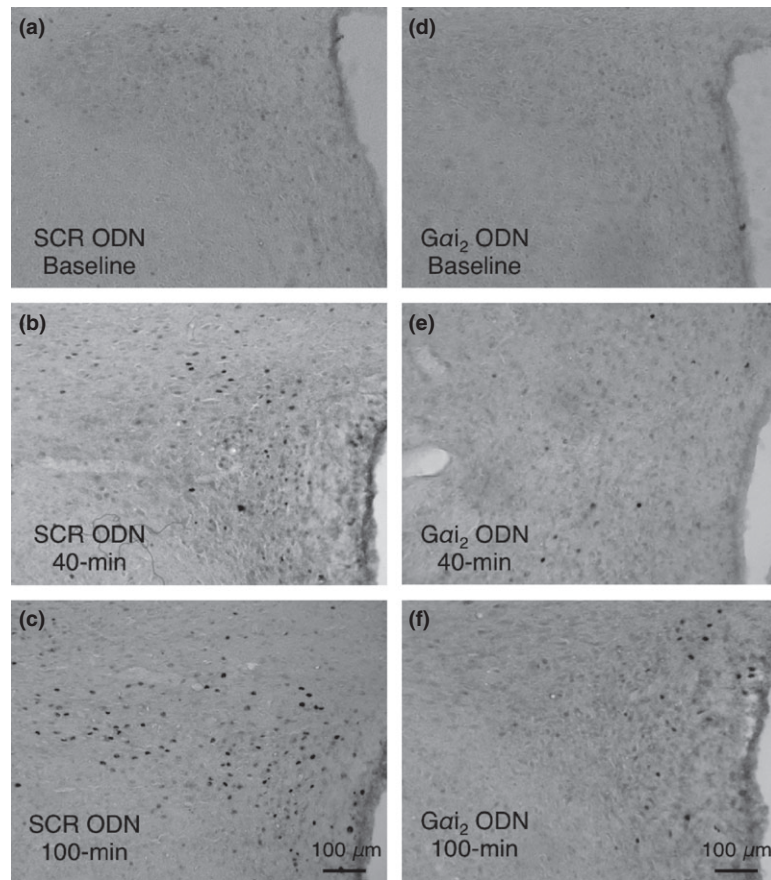
activity in the MP region in the SCR ODN pre-treatment group [(MP Fos<sup>+</sup> cells) SCR ODN baseline: 8  $\pm$  1 vs. 40 min: 29  $\pm$  4,  $P < 0.05$ ], while no differences in any region were observed in the G $\alpha$ i<sub>2</sub> ODN group as compared to respective baseline levels. At 100 min, SCR ODN pre-treated rats exhibited significant increases in DP, MP, VLP and LP regions as compared to baseline. As compared to SCR ODN pre-treated rats, G $\alpha$ i<sub>2</sub> ODN pre-treated rats exhibited significantly less Fos-positive cells in MP, VLP and LP regions at the 100 min post-HS challenge time point. No significant differences were observed between ODN pre-treatment groups in the DP region at any time point (Figs 3 and 4).

### Effect of HS on Fos immunoreactivity in PVN magnocellular neurons

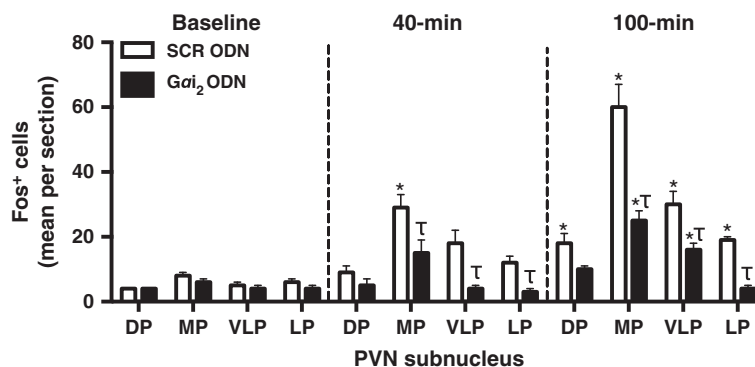
The total number of Fos-positive magnocellular neurons [i.e. posterior magnocellular (PM)] was significantly greater at 100 min as compared to baseline in both SCR and G $\alpha$ i<sub>2</sub> ODN pre-treatment groups [(PM Fos<sup>+</sup> cells) SCR ODN baseline: 3  $\pm$  1 vs. 100 min: 31  $\pm$  5; G $\alpha$ i<sub>2</sub> ODN baseline: 2  $\pm$  0 vs. 100 min: 26  $\pm$  4,  $P < 0.05$ ]. There were no significant differences in Fos-positive magnocellular neurons between SCR and G $\alpha$ i<sub>2</sub> ODN pre-treatment groups at any time point (Figs 5 and 6). Analysis of free water clearance revealed a decrease in free water clearance in both ODN pre-treatment groups, but there were no significant differences in this parameter between the groups at any time point (Table S1).

### Effect of HS on plasma sodium, osmolality and circulating NE and AVP

An IV HS challenge produced a significant increase in plasma sodium at 10 min in both SCR [plasma Na<sup>+</sup> ( $\mu$ eq) baseline: 138.5  $\pm$  1.7 vs. 10 min: 150.1  $\pm$  0.9,  $P < 0.05$ ] and G $\alpha$ i<sub>2</sub> [plasma Na<sup>+</sup> ( $\mu$ eq) baseline: 138.7  $\pm$  1.5 vs. 10 min: 149.2  $\pm$  0.9,  $P < 0.05$ ] ODN pre-treatment groups (Table 3). IV HS challenge also produced a significant increase in plasma osmolality at



**Figure 3** Representative images of total paraventricular nucleus (PVN) Fos-positive nuclei at level 3 in male Sprague–Dawley rats pre-treated ICV with a control scrambled (SCR) (a–c) or experimental *Gαi<sub>2</sub>* (d–f) oligodeoxynucleotide (ODN) (25 μg per 5 μL) at baseline, 40 and 100 min post hypertonic saline (HS) challenge. Counts are summarized in Figs. 4 and 6.

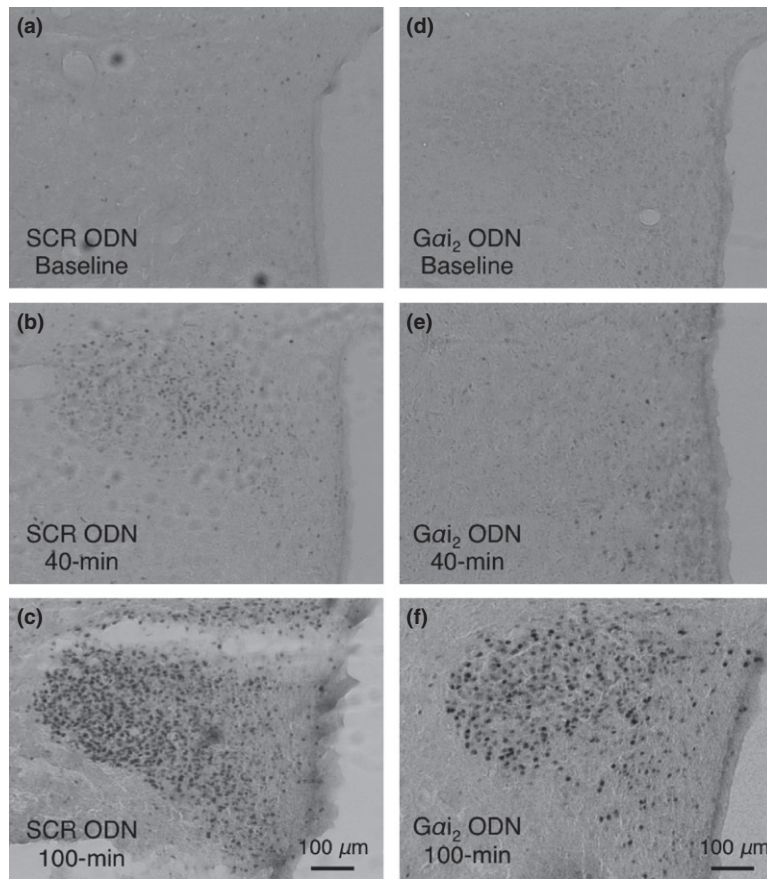


**Figure 4** Mean number (per section) of Fos-positive parvocellular nuclei averaged across all rostral–caudal levels in male Sprague–Dawley rats pre-treated ICV with a control scrambled (SCR) or experimental *Gαi<sub>2</sub>* oligodeoxynucleotide (ODN) (25 μg per 5 μL) at baseline, 40 and 100 min post-hypertonic saline (HS) challenge. Data are mean ± SEM ( $N = 8$  per group per time point). \* $P < 0.05$ , sig. diff. vs. respective group baseline value, <sup>†</sup> $P < 0.05$ , sig. diff. vs. respective SCR ODN group value.

10 min in both SCR [plasma osmolality ( $\text{mmol kg}^{-1}$ ) baseline:  $298 \pm 2$  vs. 10 min:  $319 \pm 1$ ,  $P < 0.05$ ] and *Gαi<sub>2</sub>* [plasma osmolality ( $\text{mmol kg}^{-1}$ ) baseline:  $296 \pm 3$  vs. 10 min:  $317 \pm 1$ ,  $P < 0.05$ ] ODN pre-treatment groups. Control values and the resultant changes in plasma sodium and osmolality were similar between ODN pre-treatment groups at all time points ( $P > 0.05$ ) and returned to baseline levels at 40 min

(Table 3). No differences in plasma NE or AVP content were observed between ODN pre-treatment groups at baseline time points prior to HS ( $P > 0.05$ ) (Fig. 7). Following HS challenge, plasma NE content significantly decreased at 10 min in SCR ODN pre-treated rats as compared to baseline [plasma NE ( $\text{nmol L}^{-1}$ ) baseline:  $44.1 \pm 4.9$  vs. 10 min:  $17.4 \pm 3.9$ ,  $P < 0.05$ ], while no change was observed in *Gαi<sub>2</sub>*





**Figure 5** Representative images of total paraventricular nucleus (PVN) Fos-positive nuclei at level 2 in male Sprague–Dawley rats pre-treated ICV with a control scrambled (SCR) (a–c) or experimental G $\alpha$ <sub>i2</sub> (d–f) oligodeoxynucleotide (ODN) (25  $\mu$ g per 5  $\mu$ L) at baseline, 40 and 100 min post-hypertonic saline (HS) challenge. Counts are summarized in Figs. 4 and 6.

ODN pre-treated rats ( $P > 0.05$ ). No differences in plasma NE content were observed at 40 and 100 min compared to baseline or between ODN pre-treatment groups (Fig. 7a). Significant increases in plasma AVP content were observed in both ODN pre-treatment groups at 10 min [plasma AVP ( $\text{pg mL}^{-1}$ ) SCR ODN baseline:  $12.2 \pm 1.6$  vs. 10 min:  $62.8 \pm 6.9$ , G $\alpha$ <sub>i2</sub> ODN baseline:  $12.1 \pm 1.5$  vs. 10 min:  $67.7 \pm 7.7$ ,  $P < 0.05$ ] (Fig. 7b). At 40 and 100 min, plasma AVP content was not significantly different than baseline in either ODN pre-treatment group ( $P > 0.05$ ). Table S2 presents the mean HR and MAP group values in response to an IV 3 M NaCl bolus from ODN pre-treated animals in which plasma sodium, osmolality, NE and AVP were conducted at baseline, 10, 40 and 100 min. These data demonstrate that the cardiovascular responses were the same as those observed in animals from which the initial physiological data were derived (Fig. 1).

#### Effect of ganglionic blockade on blood pressure following acute sodium challenge

Hexamethonium-mediated ganglionic blockade at 110 min post-HS challenge resulted in a significantly greater reduction in MAP in hypertensive G $\alpha$ <sub>i2</sub> ODN

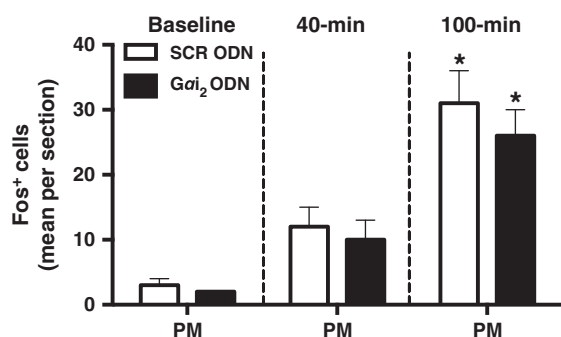
pre-treated rats compared with SCR ODN pre-treated rats [ $\Delta$ MAP (mmHg) SCR ODN:  $-26 \pm 3$  vs. G $\alpha$ <sub>i2</sub> ODN:  $-38 \pm 2$ ,  $P < 0.05$ ]. Ganglionic blockade reduced baseline blood pressure to a similar level in both pre-treatment groups (Table 4).

#### Effect of acute sodium challenge during V<sub>1a</sub> receptor antagonism on cardiovascular and renal excretory parameters

Pre-treatment with a peripheral V<sub>1a</sub> receptor antagonist abolished the rapid (within 10 min) elevation in MAP and the bradycardiac response to HS observed in both SCR and G $\alpha$ <sub>i2</sub> ODN pre-treatment groups. V<sub>1a</sub> receptor blockade also prevented any significant HS-evoked increases in MAP in SCR ODN pre-treated rats. In contrast, G $\alpha$ <sub>i2</sub> ODN pre-treated rats showed a delayed peak MAP but remained significantly elevated [MAP 100 min (mmHg) SCR ODN:  $125 \pm 2$  vs. G $\alpha$ <sub>i2</sub> ODN:  $135 \pm 0$ ,  $P < 0.05$ ] (Fig. 8).

#### Discussion

The current studies were designed to determine whether central G $\alpha$ <sub>i2</sub> subunit proteins are critical to physiological blood pressure regulation following an



**Figure 6** Mean number (per section) of magnocellular Fos-positive nuclei averaged across all rostral–caudal levels in male Sprague–Dawley rats pre-treated ICV with a control scrambled (SCR) or experimental  $G\alpha_{i2}$  oligodeoxynucleotide (ODN) (25  $\mu\text{g}$  per 5  $\mu\text{L}$ ) at baseline, 40 and 100 min post-hypertonic saline (HS) challenge. Data are mean  $\pm$  SEM ( $N = 8$  per group per time point). \* $P < 0.05$ , sig. diff. vs. respective group baseline value.

acute HS challenge. Both control SCR and experimental  $G\alpha_{i2}$  ODN pre-treatment groups exhibited characteristic rapid increases in plasma sodium, osmolality and MAP in response to IV HS, as has been previously reported in conscious rats (Garcia-Estan *et al.* 1989, Bealer 2000, Antunes *et al.* 2006). MAP returned to baseline by 100 min in control SCR ODN pre-treated rats, while  $G\alpha_{i2}$  ODN pre-treated rats exhibited a significant persistent elevation in MAP during the course of the 120-min experimental protocol. Reflexive bradycardia was observed in both groups following IV HS administration, but peak changes in HR were significantly greater in control SCR ODN pre-treated rats. Downregulation of brain  $G\alpha_{i2}$  proteins was not directly measured in these studies due to the different methods of tissue processing for IHC vs. Western blotting. Nonetheless,

based on our extensive prior studies, we are confident that ICV  $G\alpha_{i2}$  ODN administration resulted in significant and widespread downregulation of brain and PVN  $G\alpha_{i2}$  proteins (Kapusta *et al.* 2012, 2013, Wainford *et al.* 2013, 2015). Collectively, these data demonstrate that central  $G\alpha_{i2}$  proteins contribute to the normalization of MAP in response to an acute hyperosmotic stimulus, while the absence of these inhibitory proteins produces a prolonged increase in MAP that did not return to baseline during the 120-min protocol. Thus, impaired  $G\alpha_{i2}$  function resulted in blood pressure dysregulation following an acute HS challenge.

Because the bradycardia observed following acute HS administration was significantly less in animals lacking  $G\alpha_{i2}$  proteins at all time points in our experiment, we examined potential impairments in the cardiac baroreflex in a separate group of animals as a possible mechanism contributing to the observed pathophysiological hypertensive pressor response. Cardiac baroreflex sensitivity is known to be reduced in hypertensive animals, and altered baroreflex responses are often implicated as a mechanism whereby blood pressure remains elevated in the experimental setting (Gordon *et al.* 1981, Bunag & Miyajima 1984). In our cardiac baroreflex studies,  $G\alpha_{i2}$  ODN pre-treated rats showed a significant increase in the lowest point to which HR could be driven ( $A_4$ ) (i.e. these rats did not lower their HR to the same level as rats with intact  $G\alpha_{i2}$  proteins at higher blood pressures). Further, a decrease in baroreflex sensitivity was evident in  $G\alpha_{i2}$  ODN pre-treated rats when the high-pressure baroreflex was analysed independent of the low-pressure baroreflex curve. Alterations in PVN projections to parasympathetic cardiac vagal neurons (CVN) in the dorsal motor nucleus of the vagus may also contribute to dysregulation of central autonomic func-

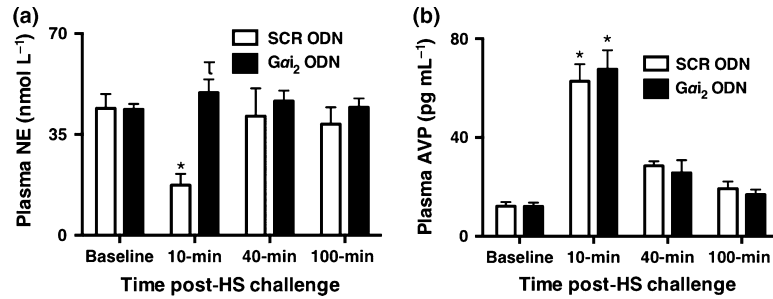
**Table 3** Plasma sodium ( $\mu\text{eq}$ ) and osmolality ( $\text{mmol kg}^{-1}$ ) during baseline, 10, 40 and 100 min post-hypertonic saline challenge in conscious Sprague–Dawley rats pre-treated ICV with a SCR or  $G\alpha_{i2}$  ODN (25  $\mu\text{g}$  per 5  $\mu\text{L}$ )

	Baseline ( $N = 5$ per group)		10 min ( $N = 5$ per group)		40 min ( $N = 5$ per group)		100 min ( $N = 5$ per group)	
	SCR ODN	$G\alpha_{i2}$ ODN	SCR ODN	$G\alpha_{i2}$ ODN	SCR ODN	$G\alpha_{i2}$ ODN	SCR ODN	$G\alpha_{i2}$ ODN
Plasma $\text{Na}^+$ ( $\mu\text{eq}$ )	138.5 $\pm$ 1.7	138.7 $\pm$ 1.5	150.1 $\pm$ 0.9*	149.2 $\pm$ 0.9*	139.0 $\pm$ 1.5	139.9 $\pm$ 1.2	137.2 $\pm$ 3.1	139.2 $\pm$ 2.5
Plasma Osm ( $\text{mmol kg}^{-1}$ )	298 $\pm$ 2	296 $\pm$ 3	319 $\pm$ 1*	317 $\pm$ 1*	303 $\pm$ 2	304 $\pm$ 2	299 $\pm$ 1	300 $\pm$ 2

$\text{Na}^+$ , sodium; Osm, osmolality; ICV, intracerebroventricular; SCR, scrambled; ODN, oligodeoxynucleotide.

Data are mean  $\pm$  SEM ( $N = 5$  per group).

\* $P < 0.05$ , sig. diff. vs. respective baseline group value.



**Figure 7** (a) Plasma norepinephrine (NE) and (b) plasma vasopressin (AVP) content in male Sprague–Dawley rats pre-treated ICV with a control scrambled (SCR) or experimental G $\alpha$ <sub>2</sub> oligodeoxynucleotide (ODN) (25  $\mu$ g per 5  $\mu$ L) at baseline, 10, 40 and 100 min post-hypertensive saline (HS) challenge. Data are mean  $\pm$  SEM ( $N = 5$  per group per time point). \* $P < 0.05$ , sig. diff. vs. respective group baseline value,  $^{\dagger}P < 0.05$ , sig. diff. vs. respective SCR ODN group value.

**Table 4** MAP (mmHg) during baseline, 110 min post-hypertensive saline challenge, the change in MAP following IV hexamethonium and 120 min (endpoint) MAP in conscious Sprague–Dawley rats pre-treated ICV with a SCR or G $\alpha$ <sub>2</sub> ODN (25  $\mu$ g per 5  $\mu$ L) and given a ganglionic blocker at 110 min

	SCR ODN	G $\alpha$ <sub>2</sub> ODN
Baseline MAP (mmHg)	136 $\pm$ 2	137 $\pm$ 3
110-min MAP (mmHg)	135 $\pm$ 4	146 $\pm$ 3 <sup>*,†</sup>
$\Delta$ MAP (mmHg) post-hex	-26 $\pm$ 3	-38 $\pm$ 3 <sup>†</sup>
Endpoint MAP (mmHg)	109 $\pm$ 5*	108 $\pm$ 5*

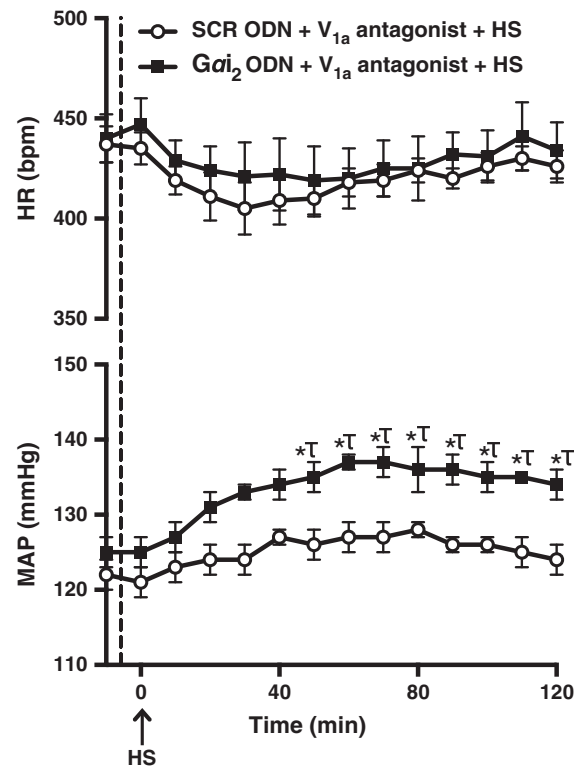
MAP, mean arterial pressure; ICV, intracerebroventricular; IV, intravenous; SCR, scrambled; ODN, oligodeoxynucleotide.

Data are mean  $\pm$  SEM.

\* $P < 0.05$ , sig. diff. vs. respective baseline group value,

<sup>†</sup> $P < 0.05$ , sig. diff. vs. respective SCR ODN group value.

tion and baroreflex control of HR (Dergacheva *et al.* 2014). Dergacheva *et al.* (2014) have demonstrated that impaired glutamatergic PVN pathways to brain stem CVNs attenuate cardiac parasympathetic activity that may underlie blunted baroreflex control associated with cardiovascular diseases. Although not directly tested in the current studies, we speculate that our data demonstrating altered PVN neuronal activation in rats lacking brain G $\alpha$ <sub>2</sub> proteins during HS challenge may be affecting the PVN projections to CVNs mediating the observed impairment in the high-pressure baroreflex activity during HS challenge and acute pharmacological baroreflex activation. On the basis of our prior studies in which chronic downregulation of central G $\alpha$ <sub>2</sub> proteins resulted in the development of salt-sensitive hypertension in multiple salt-resistant rat phenotypes (Kapusta *et al.* 2013, Wainford *et al.* 2015), we speculate that brain G $\alpha$ <sub>2</sub> protein-mediated attenuation of the high-pressure



**Figure 8** Systemic cardiovascular responses produced by V<sub>1a</sub> receptor antagonism (10  $\mu$ g kg<sup>-1</sup> mL<sup>-1</sup>, IV) followed by hypertensive saline (HS) challenge (3 M, 0.14 mL per 100 g, IV) in conscious male Sprague–Dawley rats pre-treated ICV with a scrambled (SCR) or G $\alpha$ <sub>2</sub> oligodeoxynucleotide (ODN) (25  $\mu$ g per 5  $\mu$ L). Antagonist pre-treatment occurred 5 min prior to HS, indicated by the dashed line. Data are mean  $\pm$  SEM ( $N = 6$  per group). \* $P < 0.05$ , sig. diff. vs. respective group baseline value,  $^{\dagger}P < 0.05$ , sig. diff. vs. respective SCR ODN group value.

baroreflex is a factor contributing to the development of sodium-dependent hypertension.

The PVN is a critical brain region involved in CNS responses to osmotic stimuli (Scrogin *et al.*

1999) that functions in concert with peripheral osmoreceptors to regulate the sympathetic responses to sodium challenge (Morita *et al.* 1997). Our laboratory has previously reported that endogenous upregulation of PVN G $\alpha$ <sub>i2</sub> proteins is required to maintain physiological blood pressure regulation following chronic dietary sodium challenge in salt-resistant rat phenotypes (Kapusta *et al.* 2012, 2013, Wainford *et al.* 2015). However, the effect of downregulation of central G $\alpha$ <sub>i2</sub> proteins on PVN neuronal activation in response to an acute HS challenge is not known. Increased expression of the protein product Fos results from a substance binding to cell surface receptors to induce the immediate early gene *c-fos* (Dragunow & Faull 1989, Morgan & Curran 1991, Herrera & Robertson 1996) – therefore, the presence of Fos (i.e. activation of that cell) does not necessarily imply neuronal excitation but merely that an extrinsic signal (e.g. neurotransmitter) modified the cell's function via receptor binding (Herrera & Robertson 1996). The current study analysed Fos protein expression in PVN neurons as a surrogate marker of neuronal activation to determine whether impaired activation in this critical site of autonomic integration underlies the pathophysiological prolonged elevation in arterial pressure observed in the acute HS paradigm following downregulation of brain G $\alpha$ <sub>i2</sub> proteins. No differences in the number of Fos-positive nuclei were observed between the ODN pre-treatment groups (i.e. control SCR vs. experimental G $\alpha$ <sub>i2</sub>) in any PVN subregions under baseline conditions 24 h post-ODN administration. These data suggest that during basal conditions in which plasma sodium/osmolality is stable, loss of brain G $\alpha$ <sub>i2</sub> protein expression does not impact tonic activation of neurons within the PVN. This is in line with electrophysiological data showing that pharmacological blockade of inhibitory-mediated receptor systems has little effect on firing activity of PVN neurons in the normotensive state (Li & Pan 2006).

In our studies, acute HS-evoked increases in total PVN Fos-positive nuclei were observed in SCR ODN pre-treated rats at both 40 and 100 min post-HS challenge – a finding in agreement with the notion that elevations in plasma osmolality induce increases in Fos immunoreactivity (Randolph *et al.* 1998, Kantzides & Badoer 2003, Stocker *et al.* 2004a). Varied evidence exists for the roles of PVN magnocellular neurosecretory, parvocellular neuroendocrine and parvocellular sympathetic neurons with regard to alterations in body fluid homeostasis that influence the sympathetic nervous system to regulate blood pressure (Randolph *et al.* 1998, Kantzides & Badoer 2003, Stocker *et al.* 2004a, Antunes *et al.* 2006). The PVN was analysed by subnuclei dependent on respective

rostral–caudal level to account for the heterogeneous structure and accompanying function of the PVN.

Multiple studies have established that alterations in sympathetic nerve discharge are elicited by a hyperosmotic challenge, with PVN parvocellular neurons acting as a key mediator of sympathetic outflow in this setting (Swanson & Kuypers 1980, Kannan *et al.* 1989, Badoer 1996, Toney *et al.* 2003). G $\alpha$ <sub>i2</sub> ODN pre-treatment significantly attenuated HS-induced Fos staining in the MP, VLP and LP subnuclei of PVN demonstrating an impairment in parvocellular neuronal activation following central G $\alpha$ <sub>i2</sub> protein downregulation. SCR ODN pre-treated rats significantly suppressed circulating levels of plasma NE at 10 min post-HS. There was no HS-evoked reduction in plasma NE content in G $\alpha$ <sub>i2</sub> ODN pre-treated rats. The levels of plasma NE are similar to previous findings from our laboratory, and these data support our prior report that central G $\alpha$ <sub>i2</sub> proteins are required to suppress the release of NE during an acute sodium challenge (Kapusta *et al.* 2013). Taken together with our Fos data, these results are consistent with the hypothesis that HS-evoked PVN parvocellular neuronal activation represents, in part, activation of sympathoinhibitory pathways in control SCR ODN pre-treated rats that may involve the MP, VLP and LP portions of PVN. Our hypothesis of an inhibitory role of the PVN on sympathetic outflow to reduce blood pressure post-acute HS challenge is supported by prior reports of inhibitory PVN-mediated modulation of renal sympathetic nerve activity to attenuate elevations in arterial pressure (Akine *et al.* 2003, Stocker *et al.* 2004b). Directly relevant to our finding of sympathoinhibition in response to acute HS challenge are data generated in conscious sheep demonstrating the existence of central, likely PVN-centric, sodium-sensitive pathways that selectively suppress renal sympathetic nerve activity in response to elevated sodium content, independently of the high-pressure baroreflex (May & McAllen 1997, Frithiof *et al.* 2009, 2014). Further, studies utilizing selective destruction of PVN parvocellular neurons – while leaving magnocellular neurons intact – have previously demonstrated that parvocellular neurons are a critical synaptic relay site in the activated reflex arc to reduce renal nerve discharge during an osmotic challenge (Haselton *et al.* 1994).

Hypertonic saline-evoked increases in Fos-positive magnocellular nuclei were observed at 100 min in both SCR and G $\alpha$ <sub>i2</sub> ODN pre-treated rats. No differences were observed between the two ODN pre-treatment groups at any time point, suggesting that the downregulation of brain G $\alpha$ <sub>i2</sub> proteins does not impair the activity of PVN magnocellular neurons in response to acute HS. Consistent with these results, we observed that G $\alpha$ <sub>i2</sub> ODN pre-treatment had no effect

on the observed reduction in free water clearance by the kidneys seen after acute HS challenge in the same animals used for Fos staining. To address the direct impact of Gzi<sub>2</sub> proteins on AVP release to an acute HS challenge, circulating plasma AVP was directly measured in separate groups of ODN pre-treated animals because the volume of blood required for the assay was not compatible with repeated sampling. A significant increase in AVP levels was detected 10 min post-HS challenge in both ODN pre-treatment groups, while no differences were observed between the groups at any time point, and these levels are similar to prior data from our laboratory (Wainford & Kapusta 2010). The similar levels of AVP release in both SCR and Gzi<sub>2</sub> ODN pre-treated groups are concurrent with the observed pattern of increased Fos-positive nuclei in magnocellular neurons and suggest that brain Gzi<sub>2</sub> proteins are not involved in the neural regulation of AVP release following acute HS challenge. Previous work has established that a rise in plasma sodium concentration promotes neurohypophyseal release of AVP (Bourque 2008) and elevated circulating levels of vasopressin following peripheral administration of HS (Russ *et al.* 1992, Antunes *et al.* 2006), a process largely determined by the activity of magnocellular neurons (Badoer 2010). Collectively, these data indicate that both neuroendocrine and sympathetic-regulatory parvocellular neurons are activated in response to acute HS and brain Gzi<sub>2</sub> signal transduction is required to mediate sodium-evoked parvocellular autonomic, but not magnocellular vasopressinergic, responses to maintain physiologically appropriate blood pressure regulation.

We postulate that the differences in PVN Fos staining following HS between the SCR and Gzi<sub>2</sub> pre-treated rats could reflect, in part, activation of neuronal pathways mediating sympathoinhibition necessary for physiological blood pressure control. Evidence exists for a PVN-mediated inhibition of renal sympathetic nerve activity associated with increases in arterial pressure (Akine *et al.* 2003, Stocker *et al.* 2004b). As such, we speculate that some PVN neurons activated by HS in SCR ODN pre-treated rats promote inhibitory-mediated signalling required to reduce sympathetic nerve discharge and return MAP to basal levels. In contrast, in Gzi<sub>2</sub> ODN pre-treated rats, failure to activate PVN sympathoinhibitory neurons represents a potential mechanism by which impairment of Gzi<sub>2</sub> signal transduction may contribute to the prolonged elevation in MAP.

Alterations of inhibitory mechanisms within the PVN, namely mediated by GABA and NO, underlie the neural component of cardiovascular reflexes by influencing renal sympathetic nerve discharge (Li & Patel 2003). The presence of GABA neurons and NOS

immunoreactive neurons in the PVN has been well characterized (Decavel & Van den Pol 1990) and pharmacological blockade of these inhibitory systems elicit significant increases in efferent renal sympathetic nerve traffic and MAP (Li & Patel 2003). This supports the present findings that implicate activation of PVN parvocellular neurons as a critical component in the central neural circuitry contributing to physiological blood pressure regulation. In congruence with this, GABAergic inhibition in the modulation of sympathetic outflow may be impaired in hypertension as a consequence of a functional change in pre- and post-synaptic GABA receptor activity, specifically in the firing activity of PVN-RVLM projecting neurons (Chen & Pan 2006, Li & Pan 2006, 2007). It has been previously reported that GABA<sub>B</sub> receptors play a significant role in tonic GABAergic inhibition of the excitability of the PVN output neurons in hypertension. This increased excitability of PVN-RVLM neurons is due to inadequate GABAergic inhibition (disinhibition) and may play a role in the heightened sympathetic vasomotor tone in adult hypertensive rats (Li & Pan 2006). Owing to the fact that GABA<sub>B</sub> receptors are coupled to intracellular Gzi<sub>2</sub> proteins, we speculate that the observed impact of Gzi<sub>2</sub> down-regulation on cardiovascular hemodynamics is reflective of altered GABAergic signalling.

Several studies utilizing a similar hyperosmotic challenge have reported variable effects of acute HS on the neuronal circuits mediating elevations in MAP. Antunes *et al.* (2006) postulated a V<sub>1a</sub> receptor-sensitive mechanism to be solely mediating prolonged elevations of MAP, while Veitenheimer & Osborn (2011) found that spinal V<sub>1a</sub> receptors are not required for elevations of MAP in conscious rats under acute or chronic osmotic stress conditions. In the current study, it is acknowledged that AVP contributes to initial HS-evoked increases in MAP – as the rapid peak pressor response was abolished in both ODN pre-treatment groups during V<sub>1a</sub> receptor antagonism. However, pharmacological antagonism of V<sub>1a</sub> receptors did not attenuate the prolonged elevation in MAP in Gzi<sub>2</sub> ODN pre-treated rats following HS challenge. This suggests that V<sub>1a</sub> receptors are not solely responsible for regulating MAP in conditions of acute hyperosmolality. Further, it demonstrates that a significant component of blood pressure control in this setting is regulated by the sympathetic nervous system – as supported by our observation of attenuated activation of PVN parvocellular neurons and a failure to suppress circulating levels of NE in Gzi<sub>2</sub> ODN pre-treated rats.

Despite the observed differential levels of Fos-positive PVN parvocellular neurons between control SCR and Gzi<sub>2</sub> ODN pre-treatment groups – which we

hypothesize underlie the observed prolonged elevation in MAP – Fos immunoreactivity possesses limitations (Dragunow & Faull 1989, Morgan & Curran 1991). It cannot be assumed that an increase in Fos expression results in changes in neuronal firing, and thus, it is difficult to conclude altered intracellular signal transduction as a consequence of brain Gzi<sub>2</sub> protein downregulation. Further, inhibitory neurons also express Fos (i.e. activation does not equate to excitation). Therefore, this technique does not allow one to directly understand the precise downstream activity of synaptically coupled neurons (i.e. whether this resulted in excitatory or inhibitory post-synaptic potentials). Although not feasible in the current study, whole-cell patch-clamp recordings performed on retrogradely labelled PVN neurons projecting to the RVLM in brain slices have revealed reduced frequency and amplitude of GABAergic inhibitory post-synaptic currents in the spontaneously hypertensive rat (Li & Pan 2006). Taken together, modulation of PVN parvocellular neurons by inhibitory neurotransmitter receptor systems is critical in the regulation of sympathetic outflow and we speculate decreased activation of these inhibitory neurons contributes to the observed HS-evoked elevations in arterial pressure in rats lacking brain Gzi<sub>2</sub> proteins.

Multiple lines of evidence support increased PVN Fos immunoreactivity following osmotic challenge [i.e. hypertonic sodium injection (Antunes *et al.* 2006), water deprivation (Stocker *et al.* 2004a) and sodium infusion (Kantzides & Badoer 2003)], although the subset of activated PVN neurons vary with stimuli. Although the projections of activated neurons were not investigated in the current study, studies by Stocker *et al.* (2004a) reported that water deprivation increased Fos immunoreactivity in RVLM- and spinally projecting PVN neurons, demonstrating a role for elevated plasma osmolality in increased activation of PVN autonomic neurons. Kantzides & Badoer (2003) observed an increase in total PVN Fos production in response to HS infusion in conscious SD rats, but this increase was not localized to PVN neurons with projections to the RVLM and IML. Observed differences between the subpopulations of activated PVN nuclei could exist due to the change in MAP produced by a bolus sodium challenge in the current study that was not seen in response to HS infusion. This is plausible as parvocellular neurons regulate cardiovascular function through their afferent projections to medullary and spinal autonomic control centres (Ferguson *et al.* 2008), thus showing increased levels of activation in settings of elevated MAP.

The current data highlight Gzi<sub>2</sub> protein signal transduction as a novel CNS mechanism acting to influence PVN neuronal activation in response to an acute HS

challenge in a salt-resistant phenotype. Increased understanding of the acute CNS responses to elevations in plasma osmolality has valuable implications for pathophysiological changes that may contribute to the development of hypertension, and particularly salt-sensitive hypertension. These data extend our understanding of the CNS regulatory mechanisms influencing blood pressure regulation and contributes novel insight to the ongoing pursuit to define the central neural circuitry that is activated in response to increased plasma sodium and osmolality. We speculate that the acute activation of PVN neurons in response to elevated plasma sodium/osmolality reflects a mechanism mediating sympathoinhibition to facilitate the maintenance of a normotensive state. Under conditions in which CNS Gzi<sub>2</sub> protein signal transduction is impaired (e.g. the Dahl salt-sensitive rat phenotype) (Wainford *et al.* 2015), alterations in PVN neuronal activity may play a role in driving sympathetically mediated sodium retention and the pathophysiology of salt-sensitive hypertension. Following the discovery of a correlation between single nucleotide polymorphisms in the human *GNAI2* gene and increased hypertension risk (Menzaghi *et al.* 2006), potential translational implications of our findings exist with regard to the pathogenesis of human hypertension and for the development of novel anti-hypertensive therapeutics designed to target Gzi<sub>2</sub> protein-gated signal transduction pathways.

### Conflict of interest

The authors have no conflict of interest to report.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** HR (beats  $\text{min}^{-1}$ ), MAP (mmHg), and  $\text{C}_{\text{H}_2\text{O}}$  ( $\text{mL min}^{-1}$ ) during baseline, 10-, 40-, and 100-min post-sodium challenge in conscious Sprague–Dawley rats pretreated ICV with a SCR or  $G\alpha_{i2}$  ODN (25  $\mu\text{g}$  per 5  $\mu\text{L}$ ) and sacrificed at baseline, 40-, or 100-min for cFos immunohistochemistry.

**Table S2.** HR (beats  $\text{min}^{-1}$ ) and MAP (mmHg) during baseline, 10-, 40-, and 100-min post-sodium challenge in conscious Sprague–Dawley rats pretreated ICV with a SCR or  $G\alpha_{i2}$  ODN (25  $\mu\text{g}$  per 5  $\mu\text{L}$ ) and sacrificed for plasma sodium, osmolality, norepinephrine, and vasopressin measurement.