# An augmented $CO_2$ chemoreflex and overactive orexin system are linked with hypertension in young and adult spontaneously hypertensive rats

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**Key points**

- Activation of central chemoreceptors by $CO_2$ increases sympathetic nerve activity (SNA), arterial blood pressure (ABP) and breathing. These effects are exaggerated in spontaneously hypertensive rats (SHRs), resulting in an augmented $CO_2$ chemoreflex that affects both breathing and ABP.
- The augmented $CO_2$ chemoreflex and the high ABP are measureable in young SHRs (postnatal day 30–58) and become greater in adult SHRs.
- Blockade of orexin receptors can normalize the augmented $CO_2$ chemoreflex and the high ABP in young SHRs and normalize the augmented $CO_2$ chemoreflex and significantly lower the high ABP in adult SHRs.
- In the hypothalamus, SHRs have more orexin neurons, and a greater proportion of them increase their activity with $CO_2$.
- The orexin system is overactive in SHRs and contributes to the augmented $CO_2$ chemoreflex and hypertension. Modulation of the orexin system may be beneficial in the treatment of neurogenic hypertension.

**Abstract** Activation of central chemoreceptors by $CO_2$ increases arterial blood pressure (ABP), sympathetic nerve activity and breathing. In spontaneously hypertensive rats (SHRs), high ABP is associated with enhanced sympathetic nerve activity and peripheral chemoreflexes. We hypothesized that an augmented $CO_2$ chemoreflex and overactive orexin system are linked with high ABP in both young (postnatal day 30–58) and adult SHRs (4–6 months). Our main findings are as follows. (i) An augmented $CO_2$ chemoreflex and higher ABP in SHRs are measureable at a young age and increase in adulthood. In wakefulness, the ventilatory response to normoxic hypercapnia is higher in young SHRs (mean $\pm$ SEM: 179 $\pm$ 11% increase) than in age-matched normotensive Wistar–Kyoto rats (114 $\pm$ 9% increase), but lower than in adult SHRs (226 $\pm$ 10% increase; $P < 0.05$). The resting ABP is higher in young SHRs (122 $\pm$ 5 mmHg) than in age-matched Wistar–Kyoto rats (99 $\pm$ 5 mmHg), but lower than in adult SHRs (152 $\pm$ 4 mmHg; $P < 0.05$). (ii) Spontaneously hypertensive rats have more orexin neurons and more $CO_2$-activated orexin neurons in the hypothalamus. (iii) Antagonism of orexin receptors with a dual orexin receptor antagonist, almorexant, normalizes the augmented $CO_2$ chemoreflex in young and adult SHRs and the high ABP in young SHRs and significantly lowers ABP in adult SHRs. (iv) Attenuation of peripheral chemoreflexes by hyperoxia does not abolish the augmented $CO_2$ chemoreflex (breathing and ABP) in SHRs, which indicates an important role for the central chemoreflex. We suggest that an overactive orexin system may play an important role in the augmented central $CO_2$ chemoreflex and in the development of hypertension in SHRs.

## Introduction

It is well established that activation of central chemoreceptors by $CO_2/H^+$ increases breathing, sympathetic nerve activity (SNA) and blood pressure in humans and in anaesthetized and conscious animals (Hanna *et al.* 1981; Lioy & Trzebski, 1984; Somers *et al.* 1989; Nattie *et al.* 1992, 1993; Oikawa *et al.* 2005; Guyenet *et al.* 2010; Li & Nattie, 2014). Many putative central chemoreceptors sites are involved in the regulation of breathing, SNA and blood pressure. For example, the $CO_2/pH$ chemosensitive orexin neurons in the hypothalamus send projections to many brainstem putative central chemoreceptor sites, e.g. retrotrapezoid nucleus, medullary raphe and nucleus of the solitary tract, as well as to many blood pressure regulatory sites, e.g. hypothalamic paraventricular nucleus and brainstem rostral ventrolateral medulla (Peyron *et al.* 1998; Date *et al.* 1999; Marcus *et al.* 2001; Young *et al.* 2005; Nixon & Smale, 2007; Lazarenko *et al.* 2011), that participate in the regulation of respiration, SNA and blood pressure (Shahid *et al.* 2011, 2012; Li & Nattie, 2014). The neurons in the retrotrapezoid nucleus, a key putative central chemoreceptor site, are innervated by orexin neurons (Lazarenko *et al.* 2011) and in turn innervate much of the ventrolateral medulla, including the ventral respiratory column, the rostral ventrolateral medulla and the caudal ventrolateral medulla (Rosin *et al.* 2006). In conscious rats, hypercapnia induces significant increases in arterial blood pressure (ABP), renal SNA and breathing, even after denervation of carotid and aortic bodies (Oikawa *et al.* 2005).

We previously reported that blocking orexin receptor-1 ($OX_1R$; Dias *et al.* 2009, 2010) or both orexin receptors ($OX_1R$ and $OX_2R$; Dias *et al.* 2009, 2010; Li & Nattie, 2010) can significantly decrease the $CO_2$ chemoreflex in normal conscious rats. We also found that in adult spontaneously hypertensive rats (SHRs) blocking both $OX_1R$ and $OX_2R$ can significantly decrease SNA and ABP (Li *et al.* 2013a). In the present study, using SHRs at young (postnatal day 30–58) and adult ages (4–6 months), we investigated the following factors: (i) whether an enhanced $CO_2$ chemoreflex is progressively associated with the increase in ABP from young to adult age; (ii) whether the enhanced $CO_2$ chemoreflex is predominantly attributable to overactive central chemoreceptors; (iii) whether hypercapnia activates more orexin neurons in the hypothalamus; and (iv) whether blocking OXRs can normalize and/or lower (a) the augmented $CO_2$ chemoreflex in both young and adult SHRs and (b) the ABP in young SHRs. We hypothesized that the augmented central $CO_2$ chemoreflex is progressively associated with the increase in ABP from young to adult SHRs and that the overactive orexin system plays an important role in development of the augmented $CO_2$ chemoreflex and neurogenic hypertension in SHRs.

## Methods

### Ethical approval

All animal experiments and surgical protocols were within the guidelines of the National Institutes of Health for animal use and care and the American Physiological Society's *Guiding Principles in the Care and Use of Animals* and were approved by the Institutional Animal Care and Use Committee at the Geisel School of Medicine at Dartmouth. The authors have read and complied with guidelines for research in rodents outlined for *The Journal of Physiology* and *Experimental Physiology* (Grundy, 2015).

### General methods

Spontaneously hypertensive rats and their background normotensive Wistar–Kyoto (WKY) control rats were used for the experiments in this study. All the rats were housed in a temperature- and light-controlled environment set on a 12 h–12 h light–dark cycle (lights on at 00.00on h; lights off at 12.00 h). Food and water were provided *ad libitum*. The general methods are those in common use in our laboratory (Li *et al.* 2008, 2013a). At the conclusion of the experiments, the rats were killed with an overdose of sodium pentobarbital ($>75$ mg kg$^{-1}$, i.p.; Euthasol; Virbac Inc., Fort Worth, TX, USA). Two different sets of animals were used for the physiological and anatomical studies. For physiological experiments, SHR and normotensive WKY rats at two ages were used. The young SHRs (male, $n = 10$) and WKY rats (male, $n = 6$) were between postnatal days 30 and 58, while adult SHRs (male, $n = 11$) and WKY rats (male, $n = 6$) were between 4 and 6 months of age. For a $CO_2$-activated c-*fos* and orexin immunostaining study, four separate groups of young and adult SHRs and WKY rats ($n = 6$ in each) were used, and the ages and sexes of these rats were similar to those used in the physiological experiments.

## Surgery

Animals were anaesthetized with a ketamine (Putney, Inc., Portland, ME, USA) and xylazine (Lloyd Labs, Walnut, CA, USA) cocktail (90/15 mg kg$^{-1}$, I.M.). All the rats were implanted with a blood pressure telemeter, which allows us to record ABP chronically in the rats continuously and reliably, as described in our previous publications (Li *et al.* 2008, 2013*a*). In brief, the tip of the blood pressure catheter was placed into the descending aorta through the femoral artery. The adult rats were implanted with a PhysioTel C50-PXT telemetric transmitter (DSI, St Paul, MN, USA), which allowed us to measure ABP, ECG and temperature simultaneously. The positive ECG lead was secured to the xyphoid process, while the negative lead was sutured to the muscle tissue in the area of the right shoulder. The body of the transmitter was placed inside the abdominal cavity and secured to the abdominal wall. The young rats were implanted with a PA-C10 blood pressure telemetric transmitter (DSI), and the body of the transmitter was placed in the right flank region subcutaneously. All the rats were implanted with EEG and neck EMG electrodes for sleep–wake study as previously described (Li *et al.* 2008, 2013*a*; Li & Nattie, 2010). In brief, three EEG electrodes were screwed onto the skull and two EMG electrodes were sutured onto the dorsal neck muscles. All the electrodes were connected to a six-prong plastic pedestal. The rats were then allowed at least 7 days of recovery before conducting any experiment. All the experiments were conducted in a Plexiglas chamber with the lights controlled as appropriate for the phase of the rat's diurnal cycle under study.

## Ventilatory and ABP measurements and data collection

The methods used to measure the ventilation ($\dot{V}_E$), ABP, ECG, body temperature, EEG and EMG were those in common use in our laboratory (Nattie & Li, 2000; Taylor *et al.* 2005; Li *et al.* 2008, 2013*a*; Li & Nattie, 2010). In brief, whole-body plethysmography was used to measure breathing, with the analog output signal from the pressure transducer sampled at 150 Hz and converted into a digital signal by a computer using LabChart (ADInstruments, Colorado Springs, CO, USA). To calibrate the plethysmograph before each experiment, we obtained pressure data from five 0.3 ml air injections made with a 1 ml syringe. The rates of inflow and outflow to the plethysmograph were balanced with a flowmeter so that the plethysmograph remained at atmospheric pressure. The $CO_2$ and $O_2$ fractions were sampled from the outflow line at ~100 ml min$^{-1}$ by a $CO_2$ and $O_2$ gas analyser (Applied Electrochemistry, Pittsburgh, PA, USA). Oxygen consumption was calculated by application of the Fick principle, using the difference

in $O_2$ content between inflow gas and outflow gas and the flow rate, as described previously (Nattie & Li, 2000; Taylor *et al.* 2005; Li & Nattie, 2010). Core body temperature was measured continuously via the signal from the telemetric temperature probe within the abdomen, and the temperature of the experimental chamber was measured using a thermometer within the plethysmograph.

To measure ABP, the signals of blood pressure or the blood pressure–ECG–body temperature combination telemeter were converted into an analog signal using a calibrated pressure output adapter and recorded continuously using LabChart (ADinstruments). For adult animals with a PhysioTel C50-PXT telemeter, ABP and ECG (heart rate) were sampled at 1000 Hz, whereas body temperature was sampled at 150 Hz. For the young animals with PA-C10 blood pressure only telemeters, ABP was sampled at 1000 Hz, heart rate was derived from pulse pressure of the ABP using LabChart, and the rectal body temperature was measured before and after the experiment. Raw EEG and EMG outputs from the skull and neck skeletal muscle electrodes were filtered at 0.3–70 and 0.1–100 Hz, respectively, using a Grass Physiodata Amplifier System (Natus Neurology Inc., Grass Products, Middleton, WI, USA) and sampled at 150 Hz via LabChart.

## Inspired gases and drugs

To test the hypercapnic chemoreflex, the following three inspired gases were used: (i) room air (21% $O_2$ balanced with nitrogen) to measure the resting control conditions; (ii) 7% $CO_2$–21% $O_2$ balanced with nitrogen to measure the responses to normoxic hypercapnia; and (iii) 7% $CO_2$ –93% $O_2$ to measure the responses to the hyperoxic hypercapnia when the peripheral chemoreceptors were attenuated.

Almorexant (Almxt; (2*R*)-2-{(1*S*)-6,7-dimethoxy-1-[2-(4-trifluoromethylphenyl)-ethyl]-3,4-dihydro-1H-isoquinolin-2-yl}-*N*-methyl-2-phenyl-acetamide) is a selective potent dual orexin receptor antagonist that can be administrated orally, rapidly crosses the blood–brain barrier, and blocks both $OX_1R$ and $OX_2R$ in the central nervous system (Brisbare-Roch *et al.* 2007). Adult SHRs received Almxt gavage (200 mg kg$^{-1}$) made of Almxt dissolved in 0.25% methylcellulose solution as previously described (Brisbare-Roch *et al.* 2007; Li & Nattie, 2010; Li *et al.* 2013*a*). Young rats received injection of Almxt (32 mg kg$^{-1}$, S.C.). The doses used for gavage and subcutaneous injection are comparable based on previous studies (Brisbare-Roch *et al.* 2007; Dugovic *et al.* 2009), in which the authos examined the selectivity and pharmacodynamics of the drug and the brain and systemic concentrations after treatment. Almorexant was generously supplied by Actelion Pharm, Ltd.

## Experimental design and protocol

The rats were divided into three testing groups. In group 1, SHRs, young ($n = 5$) and adult ($n = 9$), were tested with normoxic hypercapnia (7% $CO_2$–21% $O_2$) before and after Almxt treatment. In group 2, SHRs, young ($n = 5$) and adult ($n = 6$), were tested with normoxic hypercapnia and hyperoxic hypercapnia (7% $CO_2$–93% $O_2$). In group 3, normotensive WKY control rats, young ($n = 6$) and adult ($n = 6$), were tested with normoxic hypercapnia.

    All the animals were studied in the dark period of the diurnal cycle, when the orexin neurons are more active. On the day of the experiment, the rat was placed into the experimental chamber and allowed to acclimate for at least 1 h until the rat started to have normal wake–sleep cycles. Then 1.5–2 h of air-breathing baseline data were collected before switching the inspired gas to 7% $CO_2$–21% $O_2$ or 7% $CO_2$–93% $O_2$ for 1–1.5 h to ensure sufficient data in wakefulness, non-rapid eye movement (NREM) and rapid eye movement (REM) sleep. For Almxt treatment, the rats received Almxt after baseline data collection and were then returned to the recording chamber; the treatment data were collected from 30 min post-treatment.

## Carbon dioxide-activated c-*fos* expression experiments

To test whether $CO_2$ activated more orexin neurons in the hypothalamus, we evaluated $CO_2$-induced c-*fos* in orexin neurons in SHRs and WKY rats at young and adult ages ($n = 6$ in each group). After exposure to either 7% $CO_2$–21% $O_2$ or air for 1.5 h during the dark period, the rats were overdosed with the ketamine and xylazine cocktail, then transcardially perfused with saline followed by 4% paraformaldehyde. The brains were collected and postfixed overnight in 4% paraformaldehyde at 4°C, after which they were cryoprotected in 30% sucrose for 48 h. The general methods were similar to those described previously (Li & Nattie, 2006). In short, the brains were cut into 40-$\mu$m-thick coronal sections, with every other section processed for double immunohistochemical staining of c-*fos* and orexin A, using the diaminobenzidine reaction for visualization. The free-floating sections were washed using 0.1% Triton X-100 phosphate-buffered saline (PBST), incubated in a rabbit polyclonal anti-c-*fos* antibody (1:10,000 dilution; Ab-5, catalogue no. PC38; Calbiochem, Billerica, MA, USA) for 48 h, followed by a biotinylated goat anti-rabbit IgG secondary antibody (1:1000 dilution; Vector Lab, Burlingame, CA, USA) overnight at 4°C or for 2 h at room temperature. The peroxidase and diaminobenzidine with nickel were used to visualize the c-*fos* expression (black). After finishing c-*fos* staining, all the sections were washed with PBST–azide and PBST, then incubated in a goat polyclonal anti-orexin A (OXA) antibody (1:10,000 dilution; SC-8070; Santa Cruz, Dallas,

TX, USA) for 48 h, a biotinylated horse anti-goat IgG secondary antibody (1:100 dilution; Vector Lab) overnight at 4°C, and then the peroxidase and diaminobenzidine with no nickel to visualize the OXA expression (brown). All sections were mounted and dehydrated with graded alcohol (from 25 to 100% EtOH), cleared with xylene and coverslipped. The OXA-immunoreactive (OXA-ir) and c-*fos*-expressing orexin (*fos*–OXA-ir) neurons were counted using the Stereo Investigator System (MBF Bioscience, Williston, VT, USA).

    For the cell counts, we divided the hypothalamus into three zones, the dorsomedial hypothalamus (DMH), the perifornical hypothalamus (PeF) and the lateral hypothalamic area (LHA), by drawing four vertical lines in the hypothalamus (see Fig. 5), modified from Clifford *et al.* (2015). The five vertical placement lines were positioned as follows (see Fig. 5*A*): line 1 at the centre of the fornix; line 2 at the edge of the third ventricle; line 3 two-thirds of the distance from the second line (edge of the third ventricle); line 4 at the same distance between lines 1 and 3 on the lateral side of the fornix; and line 5 at the edge of the LHA. The DMH zone was between the second and third vertical lines, the PeF zone between the third and fourth vertical lines, and the LHA zone between the fourth and and fifth lines (Fig. 5*A*). We identified *fos*–OXA-ir cells as brown with black nuclei double stain (Fig. 5*B*, red arrows), non-*fos* OXA-ir cells as brown cells without black nuclei (Fig. 5*B*, blue arrows), and non-OXA *fos*-ir as black nucleic stain only (Fig. 5*B*, green arrow). All OX-ir and *fos*–OXA-ir positive cells within these three zones of both sides of the sections were counted and averaged based on their location, and grouped as young WKY rat air control and 7% $CO_2$, young SHR air and 7% $CO_2$, adult WKY rat air and 7% $CO_2$, and adult SHR air and 7% $CO_2$.

## Data and statistical analysis

The methods for sleep analysis were as described previously (Nattie & Li, 2001; Li & Nattie, 2010). In brief, a fast Fourier transform was performed on the EEG signal at 4.0-s-long epochs using SleepSign software (Kissei Comtec Co. Ltd, Matsumoto, Nagano, Japan). The vigilance states were categorized as wakefulness, NREM and REM sleep. The $\dot{V}_E$ and mean ABP were averaged based on the vigilance state in different inspired gases in each rat, and the data are presented as means ± SEM (Figs 1–4).

    Breath-by-breath analysis was performed using the pressure deflections and the respiratory cycle time for each breath as described previously (Nattie & Li, 2000; Taylor *et al.* 2005; Li *et al.* 2008; Li & Nattie, 2010). Any breaths occurring during activity (sniffing, grooming and moving) were excluded. Ventilation was the product of tidal volume and breathing frequency ($f_R$). Tidal volume and $\dot{V}_E$ were calculated for each breath, with rat body temperature, plethysmograph chamber temperature and

the barometric pressure of the day the experiment was performed, and normalized with body weight (Bartlett & Tenney, 1970). Ventilatory parameters are expressed as mean values ± SEM for quiet wakefulness, NREM and REM sleep during room air or 7% $CO_2$ conditions. To evaluate the central chemoreflex, $\dot{V}_E$ and mean ABP responses to 7% $CO_2$–21% $O_2$ with and without Almxt treatment and to 7% $CO_2$–93% $O_2$, we first categorized the vigilance states into wakefulness, NREM and REM sleep in each group, and then compared the changes in 7% $CO_2$–21% $O_2$ with and without Almxt treatment and 7% $CO_2$–93% $O_2$ using a one-way ANOVA separately for each vigilance state, applying the Holm–Sidak or Student–Newman–Keuls *post hoc* test when appropriate in each age group.

To compare the changes of $CO_2$ chemoreflex ($\dot{V}_E$ and ABP; the value in hypercapnia minus the value in resting air) in SHRs and WKY rats at young and adult ages, a two-way ANOVA was used separately for each vigilance state. The two factors were age (young and adult) and genotype (SHRs and WKY rats), applying the Holm–Sidak *post hoc* test when appropriate.

To evaluate whether SHRs have more orexin neurons and whether hypercapnia activated more orexin neurons in SHRs, the differences in the total number of OXA-ir and *fos*–OXA-ir neurons in each of the three zones and in the hypothalamus (all three zones combined) between SHRs and WKY rats at young and adult age were compared using a one-way ANOVA and applying the Holm–Sidak or Student–Newman–Keuls *post hoc* test when appropriate. To evaluate whether there was an age difference in the total number of OXA-ir or *fos*–OXA-ir neurons between young and adult SHRs or WKY rats, a one-way ANOVA was used with *post hoc* analysis using the Holm–Sidak method when appropriate.

## Results

### Ventilatory $CO_2$ chemoreflex in young and adult SHRs

**Room-air breathing.** There was no significant difference in air breathing between young and adult SHRs and between age-matched SHRs and WKY rats in wakefulness, NREM and REM sleep ($P \geq 0.05$, one-way ANOVA; Fig. 1).

**Ventilatory response to normoxic and hyperoxic hypercapnia.** At each age, young and adult, the SHRs had a significantly higher ventilatory ($\dot{V}_E$) response to normoxic hypercapnia and to hyperoxic hypercapnia than age-matched normotensive WKY rats in wakefulness and NREM and REM sleep ($P < 0.05$, one-way ANOVA; Figs 1 and 2). In SHRs during wakefulness, the $\dot{V}_E$ response to normoxic hypercapnia and to hyperoxic hypercapnia in young ($179 \pm 11$ and $162 \pm 18\%$ increase, respectively) and adult animals ($226 \pm 10$ and $218 \pm 9\%$ increase, respectively) was higher than that of age-matched normotensive young ($114 \pm 9$ and $113 \pm 6\%$ increase, respectively) and adult WKY rats ($137 \pm 10$ and $126 \pm 28\%$ increase, respectively; $P < 0.05$, one-way ANOVA; Figs 1 and 2). In SHRs, there was no significant difference between the $\dot{V}_E$ response to normoxic hypercapnia and to hyperoxic hypercapnia, when peripheral chemoreceptors are attenuated, at either young or adult age in wakefulness or NREM or REM sleep ($P \geq 0.05$, one-way ANOVA; Fig. 1).

When comparing age and genotype, a two-way ANOVA with Holm–Sidak *post hoc* test showed that there was a significant difference in the $\dot{V}_E$ response to $CO_2$ (expressed as a percentage change) between the genotypes (SHR and WKY rat, $P < 0.001$) and ages (young and adult, $P = 0.009$), with significant interaction ($P \leq 0.001$). The Holm–Sidak *post hoc* test showed that SHRs had a significantly higher $CO_2$ chemoreflex than age-matched normotensive WKY rats at both young and adult ages ($P = 0.002$ and $P < 0.001$, respectively), and adult SHRs had significantly higher $CO_2$ chemoreflex than young SHRs ($P = 0.01$; Fig. 2). In WKY rats, there was no significant difference in the $\dot{V}_E$ response to $CO_2$ between young and adult age ($P = 0.2$; Figs 1 and 2).

There was no significant difference between the percentage change of the $\dot{V}_E$ response to normoxic and hyperoxic hypercapnia in SHRs ($P > 0.05$, one-way ANOVA); therefore, the data are not shown here.

In both young and adult SHRs, Almxt treatment significantly lowered the augmented respiratory $CO_2$ chemoreflex to a level that was no longer significantly different from that of age-matched normotensive WKY rats in wakefulness, NREM and REM sleep ($P > 0.05$, one-way ANOVA; Figs 1 and 2). In adult WKY rats ($n = 5$), Almxt treatment decreased the respiratory response to hypercapnia by ∼20% within the range we previously reported in Sprague–Dawley rats (Li & Nattie, 2010; data not shown).

### Arterial blood pressure in young and adult SHRs

**Resting ABP.** There was a significant difference in resting mean ABP between the genotypes (SHR and WKY rat, $P < 0.001$) and ages (young and adult, $P < 0.001$), whereby SHRs had significantly higher mean ABP than that of age-matched normotensive WKY rats at both young and adult ages ($P < 0.001$) and an age-dependent increase in mean ABP ($P < 0.001$; two-way ANOVA with Holm–Sidak *post hoc* test; Fig. 3). The resting mean ABP in SHRs at postnatal day 30–58 was already higher than that of age-matched normotensive WKY rats in wakefulness, NREM and REM sleep (e.g. in wakefulness $122 \pm 5$ and $99 \pm 5$ mmHg, respectively, $P < 0.05$), but lower than that of adult SHRs ($152 \pm 4$ mmHg in wakefulness, $P < 0.05$, one-way ANOVA; Fig. 3). A complete study on
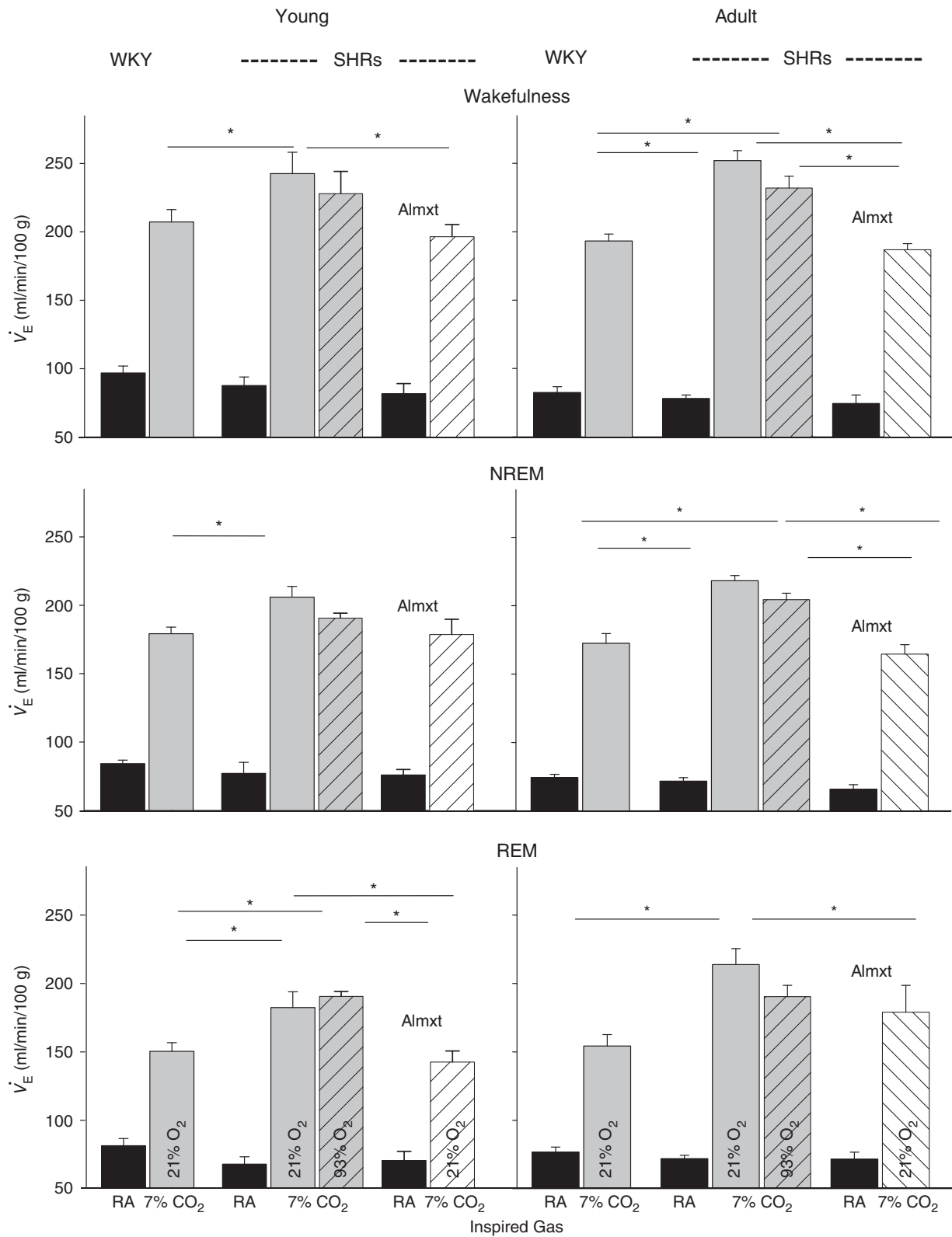
**Figure 1. Comparisons of ventilation ($\dot{V}_E$) between spontaneously hypertensive rats (SHRs) and age-matched Wistar–Kyoto (WKY) rats at young and adult ages in wakefulness, non-rapid eye movement (NREM) and rapid eye movement (REM) sleep**

Data are shown as absolute values (means + SEM). Abbreviations: Almxt, almorexant; and RA, room air. *Values are significantly different in hypercapnia with Holm–Sidak or Student–Newman–Keuls *post hoc* test ($P < 0.05$).

the effect of Almxt treatment on ABP in adult SHRs has been reported previously (Li *et al.* 2013*a,b*), and here we provide further comparison of the effect of Almxt on ABP in both young and adult SHRs in resting, air-breathing conditions. In young SHRs, Almxt significantly lowered ABP from 121 to 103 mmHg, a level similar to that of age-matched WKY rats (99 mmHg), while in adult SHRs, Almxt significantly lowered ABP from 152 to 130 mmHg, a level that was still significantly higher than that of age-matched normotensive WKY rats (103 mmHg; Fig. 3).

**Arterial blood pressure response to hypercapnia.** Hypercapnic stimulation induced an increase in ABP in both hypertensive and normotensive rats (Fig. 4). In SHRs, however, the increase in ABP was greater than in age-matched normotensive WKY rats, a difference that increased with age (Fig. 4). In wakefulness in young SHRs and WKY rats, hypercapnia increased ABP by 6.6 and 4.5 mmHg, respectively, and the difference between young SHRs and WKY rats was not significant ($P \geq 0.05$, one-way ANOVA; Fig. 4). In adult SHRs and WKY rats, however, hypercapnia increased ABP by 16.8 and 6.8 mmHg, respectively, and the difference between adult SHRs and WKY rats was highly significant ($P < 0.001$, one-way ANOVA; Fig. 4). In adult SHRs, Almxt treatment significantly lowered the $CO_2$-augmented ABP response to a level similar to that of normotensive WKY rats in wakefulness and sleep (Fig. 4). It was also noted that in SHRs the augmented ABP response to hypercapnia was progressively increased with age, and the difference between young (6.6 mmHg) and adult SHRs (16.8 mmHg) was significant ($P < 0.001$, one-way ANOVA; Fig. 4).

## Other physiological parameters

The changes of body temperature and metabolic rate before and after Almxt were not statistically significant
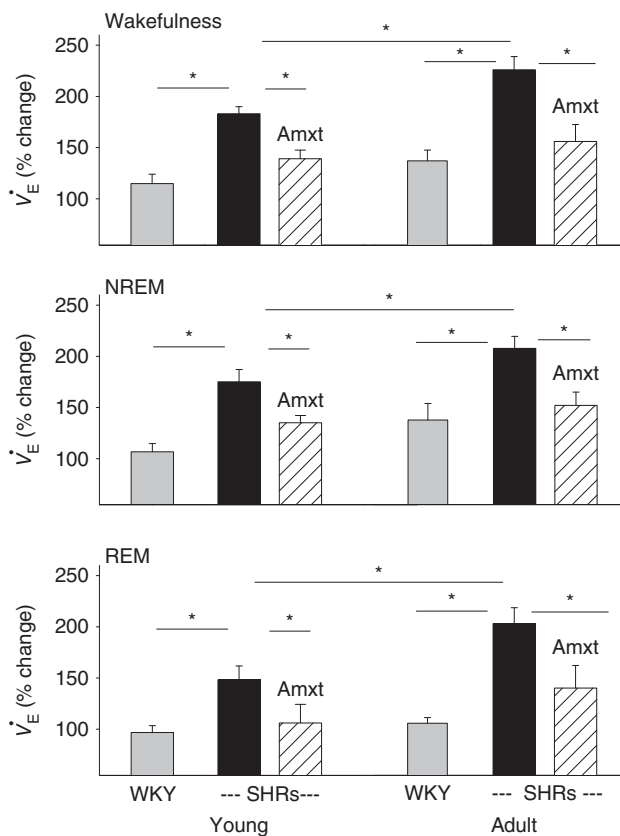


**Figure 2. Ventilatory response ($\dot{V}_E$; expressed as the percentage change) to hypercapnia**
The percentage change of $\dot{V}_E$ in normoxic hypercapnia with almorexant (Amxt) treatment (hatched bars) and without (black bars) in young and adult SHRs *vs.* that in normotensive young and adult WKY rats (grey bars). Value are shown as means + SEM. *Values are significantly different with Holm–Sidak or Student–Newman–Keuls *post hoc* test ($P = 0.05$–$0.001$).
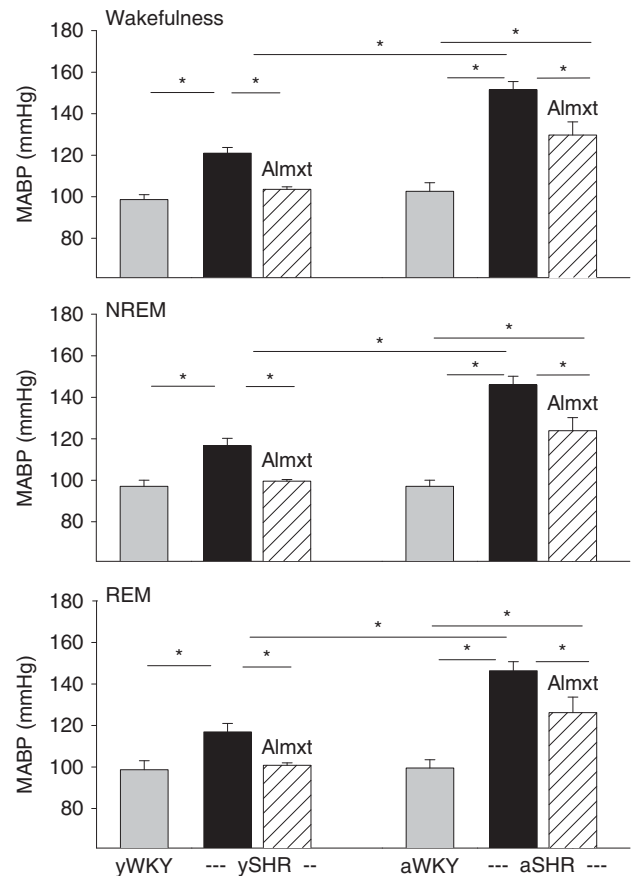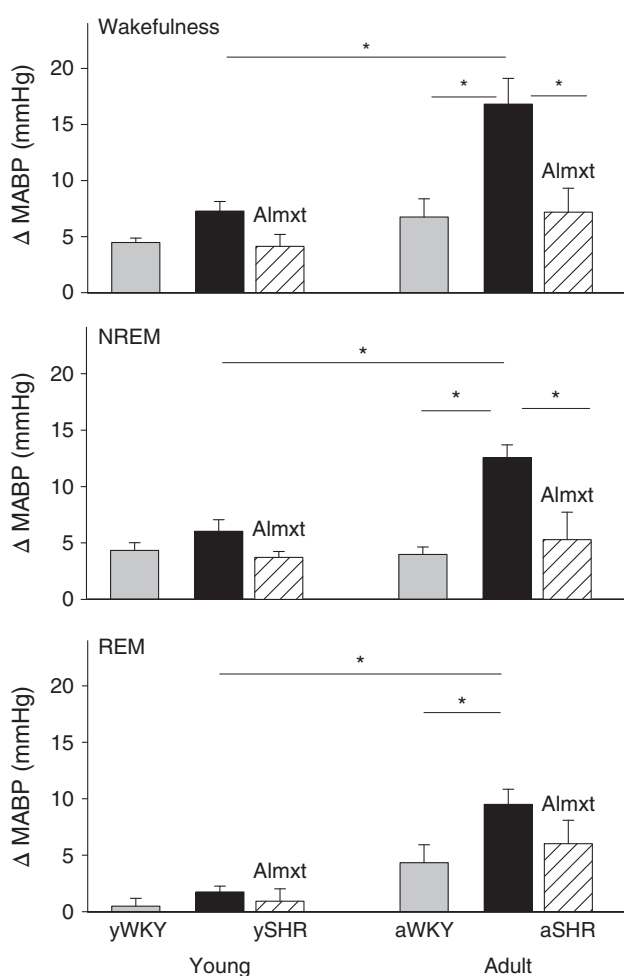
**Figure 3. Resting mean arterial blood pressure (MABP) with almorexant (Amxt) treatment (hatched bars) and without (black bars) in young and adult SHRs *vs.* that in age-matched WKY rats (grey bars)**
Data are shown as absolute values (means + SEM). *Values are significantly different with Student–Newman–Keuls *post hoc* tests ($P < 0.001$).

in wakefulness, NREM and REM sleep in SHRs and WKY rats, or between SHRs and WKY rats, possibly because of the lower dose of Almxt used in this set of experiments relative to our previous experiment (Li & Nattie, 2010; data not shown). The effects of Almxt have been reported in detail previously (Brisbare-Roch *et al.* 2007), and in the present experiment we focused on the cardiorespiratory changes in different vigilance states, namely wakefulness, NREM and REM sleep.

### The number of orexin neurons and $CO_2$-activated *c-fos*-expressing orexin neurons in SHRs

The numbers of OXA-ir neurons in the hypothalamus (Hyp; three zones combined) and the three individual



**Figure 4. The change of mean arterial blood pressure (ΔMABP) in response to normoxic hypercapnia with almorexant (Amxt) treatment (hatched bars) and without (black bars) in young and adult SHRs *vs.* that in age-matched WKY rats (grey bars)**
Data are shown as absolute values (means + SEM). *Values are significantly different with Student–Newman–Keuls *post hoc* tests ($P < 0.001$).

zones (DMH, PeF and LHA) are summarized and shown in Fig. 5C. The total number of OXA-ir neurons in the hypothalamus was significantly higher in both young (2791 ± 184) and adult SHRs (2984 ± 86) than that in age-matched normotensive WKY rats (2160 ± 104 and 2155 ± 128, respectively, $P = 0.01$ and $P = 0.02$, one-way ANOVA applied to SHRs and WKY rats; Fig. 5C). The significance was solely attributable to the difference in the PeF zone, [young SHRs (1718 ± 93) *vs.* young WKY rats (1228 ± 67), $P < 0.001$; adult SHRs (1783 ± 86) *vs.* adult WKY rats (1118 ± 64), $P < 0.001$; one-way ANOVA with Holm–Sidak *post hoc* test]; there was no significant difference in the DMH and LHA zones between SHRs and WKY rats at either age ($P > 0.05$, one-way ANOVA; Fig. 5C).

The numbers of *fos*–OXA-ir double stained neurons in the hypothalamus (Hyp; three zones combined) and the three individual zones (DMH, PeF and LHA) are summarized and shown in Fig. 5D. In room-air control conditions, fewer *fos*–OXA-ir positive neurons were found in both SHRs and WKY rats; however, both young and adult SHRs (143 ± 17 and 87 ± 8, respectively) had more *fos*–OXA-ir neurons than age-matched WKY rats (75 ± 20 and 35 ± 5, respectively) in Hyp ($P ≤ 0.011$, one-way ANOVA; Fig. 5D, grey bars). The difference was mainly contributed by DMH and PeF regions, and the only individual area that showed a significant difference was the PeF region between adult SHRs and WKY rats ($P ≤ 0.001$, one-way ANOVA; Fig. 5). Hypercapnia (7% $CO_2$) activated more orexin neurons (*fos*–OXA-ir) in the hypothalamus in both young (255 ± 29) and adult SHRs (206 ± 4) than in age-matched WKY rats (92 ± 35 and 44 ± 13, respectively, $P = 0.002$, one-way ANOVA with Student–Newman–Keuls *post hoc* test; Fig. 5D). In this situation, both the PeF and DMH zones contributed to the effect. In the Pef zone, both young and adult SHRs had significantly more *fos*–OXA-ir neurons than age-matched WKY control rats ($P < 0.001$, one-way ANOVA with Student–Newman–Keuls *post hoc* test; Fig. 5D). In the DMH zone, both young and adult SHRs had more *fos*–OXA-ir neurons than age-matched WKY rats, but only the difference between the adult SHRs and WKY rats reached significance ($P = 0.007$, one-way ANOVA with Student–Newman–Keuls *post hoc* test). In the LHA, very few orexin neurons were activated by the hypercapnia (7% $CO_2$), and there was no significant difference between SHRs and WKY rats at either age.

When comparing the difference in numbers of *fos*–OXA-ir neurons between room air and 7% $CO_2$ in the three individual zones and combined Hyp between SHRs and WKY rats at young or adult ages, we found that the significant differences were only in the PeF zone and Hyp in young SHRs ($P ≤ 0.001$, one-way ANOVA) and in the PeF and DMH zones and Hyp in adult SHRs ($P ≤ 0.002$, one-way ANOVA; Fig. 5D).
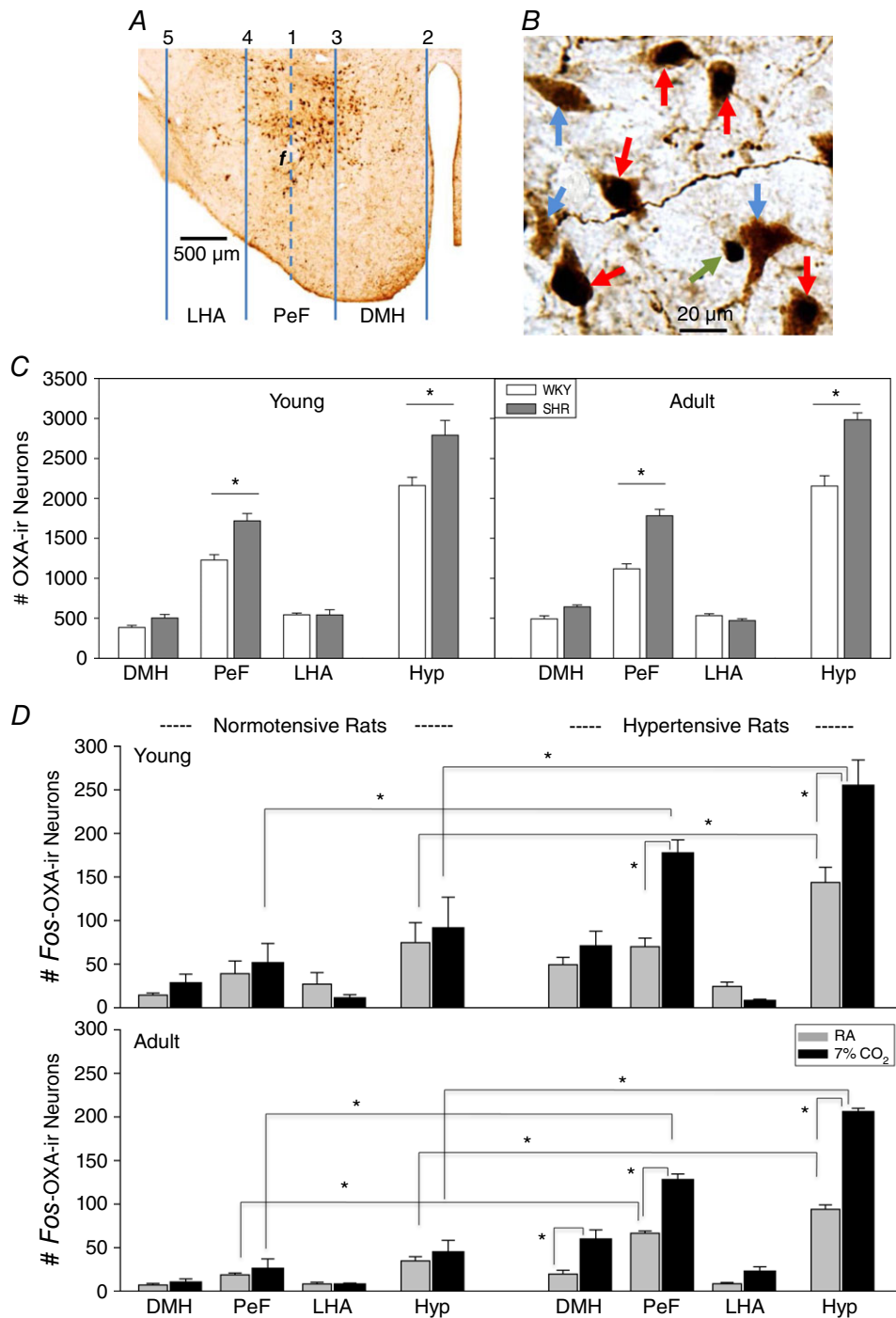
**Figure 5. Distribution of orexin A-immunoreactive (OXA-ir) and *c-fos*-expressing orexin (*fos*–OXA-ir) neurons in the hypothalamus**
Representative sections show the distribution of OX-ir and *fos*–OXA-ir neurons and the three subdivided zones in the hypothalamus (*A*) and an example of OXA-ir (blue arrows; brown cells with no staining of nuclei), *fos*–OXA-ir (red arrows; brown cells with black nuclei) and non-OXA *fos*-ir neurons (green arrow; black nucleus only; *B*). *C* shows the distributions of OXA-ir in the three zones and Hyp in young and adult SHRs and WKY rats. *D* shows the distributions of *fos*–OXA-ir neurons in room air and 7% $CO_2$ in the three zones and Hyp (all three zones) in young and adult SHRs and WKY rats. Values are shown as means + SEM. *Values are significantly different with Student–Newman–Keuls *post hoc* tests ($P \leq 0.03$). Abbreviations: DMH, dorsomedial hypothalamus; *f*, fornix; Hyp, hypothalamus (all three zones); LHA, lateral hypothalamic area; PeF, perifornical hypothalamus; and RA, room air.

When comparing young and adult animals within each strain, we found no significant difference in the total number of OXA-ir or *fos*–OXA-ir neurons in the hypothalamus or in any of the three zones between young and adult SHRs or WKY rats ($P > 0.05$, one-way ANOVA).

## Discussion

The main findings of this study are as follows: (i) SHRs have an augmented $CO_2$ chemoreflex compared with age-matched normotensive rats; (ii) both the augmented $CO_2$ chemoreflex and the higher ABP are measureable in young rats (postnatal day 30–58) and become greater in adult SHRs; (iii) attenuation of the peripheral chemoreflexes by hyperoxia does not diminish the augmented $CO_2$ chemoreflex in SHRs; (iv) SHRs have more orexin neurons in the hypothalamic PeF zone, and hypercapnia activates significantly more orexin neurons (PeF zone in young rats; PeF and DMH zones in adult rats); and (v) blocking orexin receptors with a dual orexin receptor antagonist, Almxt, in SHRs normalizes the augmented $CO_2$ chemoreflex in both young and adult animals, normalizes the higher ABP in the young rats, and significantly lowers the higher ABP in the adults.

### Animal models

Spontaneously hypertensive rats are the most commonly used animal model to study neurogenic hypertension. The SHR strain was obtained from selective breading of the Wistar rats at Kyoto University with high blood pressure during 1960s by Dr Okamoto and his colleagues (Okamoto & Aoki, 1963), and the inbred WKY rats were used as the background normotensive control animals for the SHR (Okamoto & Aoki, 1963). There is a mild elevation in ABP in normotensive rats between the fourth and 10th week of age, but it is more severe in SHRs, in which ABP increases by up to 30% above that of WKY rats (Zicha & Kunes, 1999). Spontaneously hypertensive rats develop an established hypertension between 8 and 10 weeks of age without physiological, pharmacological, environmental or surgical intervention (Dickhout & Lee, 1998; Zicha & Kunes, 1999; Dornas & Silva, 2011). In the present study, we focused on two ages, postnatal day 30–58, when hypertension is actively developing, and 4–6 months, when the hypertension is completely established. Our data show that a higher $CO_2$ chemoreflex and ABP are already measureable at the age of postnatal day 30–58 in SHRs and progressively increase with age relative to the age-matched normotensive WKY control rats, at least at the ages we studied.

## More OXA-ir and $CO_2$-activated *fos*–OXA-ir neurons in hypothalamus in SHRs

Overall, SHRs have more orexin neurons in the hypothalamus than normotensive WKY control rats at both young and adult ages, and our data are consistent with two recent publications (Clifford *et al.* 2015; Lee *et al.* 2015) and a recent report on the genetically hypertensive BPH/2J mice (Jackson *et al.* 2016). Moreover, hypercapnia (7% $CO_2$) activated more orexin neurons in the hypothalamus in both young and adult SHRs than in age-matched normotensive rats. Both the DMH and PeF zones contribute to the significant changes in SHRs. It is known that orexin neurons in the hypothalamus, particularly in the PeF and DMH areas, are involved in cardiorespiratory function (Young *et al.* 2005; Furlong & Carrive, 2007; Dampney *et al.* 2008) and responses to stress (Johnson *et al.* 2010; Nollet *et al.* 2011; Iigaya *et al.* 2012).

It is worth noting that in room-air control conditions, fewer *fos*–OXA-ir neurons were found in the hypothalamus in both SHRs and WKY rats, but the difference in total numbers between SHRs and age-matched WKY rats was significant. When comparing the change in numbers of *fos*–OXA-ir neurons in the hypothalamus between room-air control conditions and hypercapnia in SHRs and WKY rats, we found that this change is much more profound in SHRs than in age-matched WKY rats. For example, in Hyp (all three hypothalamic zones combined) relative to room-air control conditions, hypercapnia (7% $CO_2$) increased the number of *fos*–OXA-ir neurons by 77 and 122% in young and adult SHRs, respectively, and by only 24 and 33%, respectively, in age-matched WKY rats (Fig. 5*D*). From these data we suggest that orexin neurons in SHRs are hyperactive in room-air conditions and hyper-responsive to hypercapnia relative to normotensive WKY control rats. We speculate that orexin signalling is higher in SHRs from a very young age and contributes significantly to the development of an augmented $CO_2$ chemoreflex and hypertension. More studies may be needed to draw a definitive conclusion on this issue.

It has been demonstrated that $CO_2$ can activate orexin neurons *in vitro* and *in vivo* in normal animals (Williams *et al.* 2007; Sunanaga *et al.* 2009). In the present study, three indices [(i) numbers of $CO_2$-activated *fos*–OXA-ir neurons; (ii) magnitude of changes in ventilation; and (iii) magnitude of changes in ABP] were used to evaluate the $CO_2$ chemoreflex, and we showed that all three indices were significantly higher in SHRs than in age-matched normotensive WKY control rats (Figs 1–5). It is very interesting to note that Almxt significantly decreased the $\dot{V}_E$ response to hypercapnia in both SHRs and WKY rats; however, the magnitude of change of $\dot{V}_E$ response to $CO_2$ with Almxt treatment was much larger in SHRs than in

WKY rats. For example, the $\dot{V}_E$ response to hypercapnia with Almxt was ~20 and ~71% lower than no-treatment control in adult WKY rats and SHRs respectively, and in SHRs the Almxt treatment decreased the augmented $CO_2$ chemoreflex to a level that was no longer significantly higher than that of the normotensive control rats. The results suggest that Almxt may be more potent when the orexin system is highly activated. A similar phenomenon has been observed previously, e.g. an orexin receptor antagonist has little effect on resting cardiorespiratory parameters, but it can block the cardiorespiratory response to exogenous orexin, fear and anxiety panic (Shirasaka *et al.* 2002; Johnson *et al.* 2010; Iigaya *et al.* 2012).

## Carbon dioxide chemoreception in SNA, ABP and hypertension

The sympathetic nervous system plays a crucial role in the regulation of ABP, and increased central sympathetic drive is one of the hallmarks of essential hypertension in humans and in SHRs. Many factors, including chemoreflexes, are known to affect sympathetic tone, and both hypoxia and hypercapnia are capable of inducing increased ventilation, sympathoexcitation and increased ABP (Hanna *et al.* 1981; Trzebski & Kubin, 1981; Lioy & Trzebski, 1984; Somers *et al.* 1989; Nattie *et al.* 1992, 1993; Oikawa *et al.* 2005; Guyenet *et al.* 2010; Li & Nattie, 2014). The role of peripheral chemoreceptors in neurogenic hypertension and in heart failure has generated attention recently (Paton *et al.* 2013*a*; Schultz *et al.* 2015). Studies have shown that an enhanced peripheral chemoreflex may contribute to the development of hypertension by altering respiratory–sympathetic coupling and increasing the sympathetic vascular tone (Trzebski & Kubin, 1981; Trzebski *et al.* 1982; Somers *et al.* 1988; Zoccal *et al.* 2009; Sinski *et al.* 2012) and that denervation of the carotid body can significantly lower ABP in patients with persistent hypertension and in SHRs (Abdala *et al.* 2012; Paton *et al.* 2013*b*). However, few studies have focused on the role of central chemoreceptors or the $CO_2$ chemoreflex in pathological conditions, such as neurogenic hypertension, even though it is known that in conscious normal rats hypercapnia induces significant increases in renal SNA, mean ABP and respiratory rate in rats with and without peripheral carotid and/or aortic chemoreceptors (Oikawa *et al.* 2005).

How central and peripheral chemoreceptors interact in regulating the $CO_2$ chemoreflex remains a controversial topic (Duffin & Mateika, 2013; Dempsey *et al.* 2014). Studies in humans and dogs suggest that central and peripheral chemoreceptors are not functionally separate but rather that they are interdependent in responding to the change in arterial $CO_2$/pH (Duffin & Mateika, 2013; Dempsey *et al.* 2014). It is unknown what the relationship between central and peripheral chemo-

receptors is in pathological conditions, i.e. neurogenic hypertension. In the present study, we showed that SHRs have an augmented $CO_2$ chemoreflex that is progressively associated with the increase in ABP from postnatal day 30–58 to adulthood, and the enhanced $CO_2$ chemoreflex may be predominantly attributable to overactive central chemoreceptors, because it persisted while the activity of the peripheral chemoreceptors was attenuated by hyperoxia. We speculate that both central and peripheral chemoreceptors are overactive in SHRs and contribute to the enhanced SNA and respiratory–sympathetic coupling in SHRs.

## Orexin in enhanced $CO_2$ chemoreflex and high ABP in SHRs

The finding that hypothalamic orexin participates in the regulation of the $CO_2$ chemoreflex (Williams *et al.* 2007; Dias *et al.* 2009, 2010; Sunanaga *et al.* 2009; Kuwaki *et al.* 2010; Li & Nattie, 2010; Lazarenko *et al.* 2011), SNA and blood pressure (Chen *et al.* 2000; Matsumura *et al.* 2001; Shirasaka *et al.* 2002; Shahid *et al.* 2012) has been well documented. In normal rats, antagonism of orexin receptors can significantly decrease the $CO_2$ chemoreflex (Dias *et al.* 2009, 2010; Li & Nattie, 2010), and central injection of orexin-A can significantly increase breathing, SNA and ABP (Chen *et al.* 2000; Matsumura *et al.* 2001; Shirasaka *et al.* 2002; Shahid *et al.* 2012). In adult SHRs, we have shown that blocking both orexin receptors via an oral administration of Almxt can significantly lower SNA and ABP (by up to 30 mmHg) in both wakefulness and sleep, an effect accompanied by decreased levels of noradrenaline in cerebrospinal fluid and blood (Li *et al.* 2013*a*). In the present study, we provide further evidence supporting our hypothesis that SHRs have an overactive orexin system, as follows. (i) Blocking orexin receptors with Almxt can not only significantly lower ABP but also normalize the augmented $CO_2$ chemoreflex in SHRs. (ii) Spontaneously hypertensive rats have more orexin neurons, and a greater proportion of orexin neurons are activated by hypercapnia (7% $CO_2$) than in age-matched normotensive WKY rats. This suggests that SHRs not only have more orexin neurons but they also have more hyperactive or hypersensitive orexin neurons in the hypo-thalamus, at least in response to $CO_2$. It is likely that these hyperactive orexin neurons are responsible for the augmented $CO_2$ chemoreflex and possibly the overactive SNA and the development of hypertension in SHRs.

In SHRs, the increased ABP and $CO_2$ chemoreflex are measureable from a young age (postnatal day 30–58) and become progressively greater with age. Almorexant treatment in young SHRs significantly lowered ABP to a level that was no longer significantly different from that of age-matched normotensive WKY rats, whereas in adult SHRs the ABP post-treatment was still significantly higher

than that of age-matched WKY rats. We speculate that in SHRs the upregulated orexin system produces a long-term excitatory drive to many cardiorespiratory-related nuclei in the brainstem and spinal cord projection sites and results in a long-term increase in the $CO_2$ chemoreflex, SNA and blood pressure. The sustained increase in SNA may also induce partly irreversible changes in vascular smooth muscle of resistance vessels. Downregulation of the orexin system, e.g. by blocking orexin receptors, can, at least in part, remove the elevated orexin excitatory input on these cardiovascular nuclei and vascular smooth muscle cells, resulting in a depressor effect, that lowers SNA, ABP and the $CO_2$ chemoreflex. The overactive orexin system could also overexcite hypothalamic–pituitary–adrenal axis functions, which could also lead to altered cardio-respiratory changes in SHRs.

## Perspectives

It is not clear at present why there is an increased number of orexin neurons in SHRs and whether the increased number of orexin neurons is the cause of hypertension in SHRs. Mounting evidence in the past decade has shown that the orexin system is linked with hyperarousal, anxiety/stress and autonomic functions (Kayaba *et al.* 2003; Furlong *et al.* 2009; Huang *et al.* 2010; Johnson *et al.* 2010; Li & Nattie, 2010; de Lecea, 2012; Shahid *et al.* 2012; Li *et al.* 2013*a*; Bonnavion *et al.* 2015; Clifford *et al.* 2015; de Lecea, 2015), and it has been proposed that the orexin system may be the link between auto-nomic function and stress (Kuwaki *et al.* 2008, 2010). Some reports suggest that stress can lead to an upregulated orexin system. For example, exposing normal rats to an acute episode of footshock led to an increase in prepro-orexin mRNA for 6 and 14 days (Chen *et al.* 2014), whereas daily footshock for 2 weeks induced hypertension and doubled the number of orexin neurons (Xiao *et al.* 2013). The number of orexin neurons increased by 20% when rats were REM sleep deprived using the technique of multiple small platforms over water for a week (Allard *et al.* 2007). These data suggest that stress or extreme conditions can alter the orexin system in the healthy normotensive individual. In SHRs, in contrast, a genetic trait may play an important role in development of hypertension, because SHRs will develop hypertension without any physiological, pharmacological, environmental or surgical intervention. More studies are needed to gain a better understanding of why SHRs have an augmented $CO_2$ chemoreflex and upregulated orexin system, and their role in the development of neurogenic hypertension.

## Summary and conclusions

The augmented $CO_2$ chemoreflex and overactive orexin system are associated with high ABP in neurogenic hypertension in SHRs. This link between the overactive orexin system and the augmented $CO_2$ chemoreflex and high ABP is already present at an age of post-natal day 30–58 in SHRs, and downregulation of the overactive orexin system has significant antihypertensive effects. We hypothesize that this overactive orexin system may overstimulate many key central chemoreceptor and cardiovascular sites as well as vascular smooth muscle of resistance vessels, causing or facilitating the increase of SNA, $CO_2$ chemoreflex and blood pressure in the neuro-genic hypertension of SHRs. Modulation of the orexin system and central $CO_2$ chemoreflex may be beneficial in treatment of neurogenic hypertension.

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## Additional information

### Competing interests

None declared.

### Author contributions

All experiments were conducted at Geisel School of Medicine at Dartmouth. A.L.: designed the experiments, performed physiology experiments and data analysis, and wrote the manuscript. S.H.R.: performed cell counts and staining. A.L. & E.E.N.: contributed equally to conceive the hypothesis and conceptualize the findings, revised and finalized the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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