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RESEARCH PAPER

Orexin A in rat rostral ventrolateral medulla is pressor, sympatho-excitatory, increases barosensitivity and attenuates the somato-sympathetic reflex

Israt Z Shahid[1,2,*], Ahmed A Rahman[1,2,*] and Paul M Pilowsky[1]

[1]*Australian School of Advanced Medicine, Macquarie University, Sydney, Australia, and*
[2]*Pharmacy Discipline, Life Science School, Khulna University, Khulna, Bangladesh*

Correspondence
Paul M. Pilowsky, Professor of Medical Physiology, The Australian School of Advanced Medicine, F10A, Macquarie University, Sydney, NSW 2109, Australia. E-mail: paul.pilowsky@mq.edu.au

*I.Z.S. and A.A.R. contributed equally to this work.

Keywords
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BACKGROUND AND PURPOSE
The rostral ventrolateral medulla (RVLM) maintains sympathetic nerve activity (SNA), and integrates adaptive reflexes. Orexin A-immunoreactive neurones in the lateral hypothalamus project to the RVLM. Microinjection of orexin A into RVLM increases blood pressure and heart rate. However, the expression of orexin receptors, and effects of orexin A in the RVLM on splanchnic SNA (sSNA), respiration and adaptive reflexes are unknown.

EXPERIMENTAL APPROACH
The effect of orexin A on baseline cardio-respiratory variables as well as the somato-sympathetic, baroreceptor and chemoreceptor reflexes in RVLM were investigated in urethane-anaesthetized, vagotomized and artificially ventilated male Sprague-Dawley rats (n = 50). orexin A and its receptors were detected with fluorescence immunohistochemistry.

KEY RESULTS
Tyrosine hydroxylase-immunoreactive neurones in the RVLM were frequently co-localized with orexin 1 (OX_1) and orexin 2 (OX_2) receptors and closely apposed to orexin A-immunoreactive terminals. Orexin A injected into the RVLM was pressor and sympatho-excitatory. Peak effects were observed at 50 pmol with increased mean arterial pressure (42 mmHg) and SNA (45%). Responses to orexin A (50 pmol) were attenuated by the OX_1 receptor antagonist, SB334867, and reproduced by the OX_2 receptor agonist, [Ala^{11}, D-Leu^{15}]orexin B. Orexin A attenuated the somato-sympathetic reflex but increased baroreflex sensitivity. Orexin A increased or reduced sympatho-excitation following hypoxia or hypercapnia respectively.

CONCLUSIONS AND IMPLICATIONS
Although central cardio-respiratory control mechanisms at rest do not rely on orexin, responses to adaptive stimuli are dramatically affected by the functional state of orexin receptors.

Abbreviations
AUC, area under the curve; HR, heart rate; IHC, immunohistochemistry; LTF, long term facilitation; MAP, mean arterial pressure; NTS, nucleus tractus solitarius; OX_1, orexin receptor 1; OX_2, orexin receptor 2; PNA, phrenic nerve activity; PNamp, phrenic nerve amplitude; PNf, phrenic nerve frequency; RTN, retrotrapezoid nucleus; RVLM, rostral ventrolateral medulla; RVMM, rostral ventromedial medulla; SB334867, (*N*-(2-methyl-6-benzoxazolyl)-*N*′-1,5-naphthyridin-4-yl-urea; SNP, sodium nitroprusside; SPN, sympathetic preganglionic neurones; sSNA, splanchnic sympathetic nerve activity; TH, tyrosine hydroxylase

Introduction

Tonically active sympathetic premotor neurones in the rostral ventrolateral medulla (RVLM) project to the intermediolateral cell column of the spinal cord and provide an excitatory input to sympathetic preganglionic neurones so as to maintain the resting level of sympathetic vasomotor tone (Pilowsky and Goodchild, 2002; Pilowsky *et al.*, 2009). The RVLM is the key site for cardiovascular homoeostasis. Neurones in the RVLM integrate information from the centre and periphery, including: respiration, and baro-, chemo- and somato-sympathetic reflex afferent neurones. Although synaptic input modulates the excitability of RVLM neurones (Pilowsky *et al.*, 2009), the sources of this input and the transmitters involved are not thoroughly characterized. Glutamate and GABA are the key contributors for the short-term control of RVLM neurones. Regulation of RVLM neurones by metabotropic transmitters, including neuropeptides that have long-term effects on cell function, may affect adaptive reflexes, and encode distinct patterns of autonomic output (Pilowsky *et al.*, 2009).

Orexin A and orexin B (also known as hypocretin 1 and hypocretin 2) have well-established roles in feeding and sleep-wakefulness. Recent evidence suggests a role for orexins as neuromodulators in central cardio-respiratory regulation. Orexin A increases BP and heart rate (HR) following intracisternal injection, or microinjection into the RVLM, in conscious or anaesthetized rats (Chen *et al.*, 2000; Machado *et al.*, 2002). However, the effects of activating orexin receptors in the RVLM on sympathetic nerve activity, or autonomic reflexes, are unknown.

Although orexin-containing cell bodies are restricted to the lateral hypothalamus, perifornical area and dorsomedial hypothalamus, orexin-immunoreactive fibres are widely distributed in the brain, including many brainstem areas that influence cardio-respiratory function, including the nucleus tractus solitarius (NTS), RVLM, rostral ventromedial medulla (RVMM), pre-Bötzinger complex, retrotrapezoid nucleus (RTN), dorsal motor nucleus of the vagus and the raphe (de Lecea *et al.*, 1998; Peyron *et al.*, 1998; Date *et al.*, 1999; Nambu *et al.*, 1999; Machado *et al.*, 2002; Ciriello *et al.*, 2003; Young *et al.*, 2005). Orexin binds to two GPCRs (coupled to $G\alpha_{q/11}$ and/or $G\alpha_i$), orexin 1 (OX_1) and orexin 2 (OX_2) receptors (nomenclature follows Alexander *et al.*, 2011). OX_1 receptors have preferential affinity for orexin A, whereas OX_2 receptors exhibit similar affinities for orexin A and orexin B *in vitro* (Sakurai *et al.*, 1998). Orexin receptors are also widespread throughout the brain including thalamus, hypothalamus, brainstem areas like the nucleus ambiguus, RVMM, pre-Bötzinger complex, NTS and spinal cord (Trivedi *et al.*, 1998; Marcus *et al.*, 2001; Sunter *et al.*, 2001; Cluderay *et al.*, 2002; Ciriello and de Oliveira, 2003). The distribution of OX_1 and OX_2 receptors in relation to putative sympatho-excitatory neurones in the RVLM is unknown.

The aims of this study were (i) to determine whether OX_1 and OX_2 receptors were expressed within neurones in the RVLM, and to determine if they were co-localized with putative sympatho-excitatory catecholamine-containing neurones of the rostral C1 cell group; (ii) to evaluate the actions of orexin A in the RVLM on splanchnic sympathetic (sSNA) and phrenic nerve activity (PNA), and on the baro-, chemo- and somato-sympathetic reflexes; and (iii) to determine which type of orexin receptor mediated the central effects of orexin.

Our principal findings are that OX_1 and OX_2 receptors were expressed abundantly in the RVLM neurones. Bilateral injection of orexin A in the RVLM increased mean arterial pressure (MAP), HR, sSNA and phrenic nerve amplitude (PNamp). Orexin A in the RVLM attenuated the somato-sympathetic reflex but increased barosensitivity. Following orexin A injection into the RVLM, sympatho-excitatory responses to hypoxia or hypercapnia were increased or reduced respectively. The findings demonstrate a role for orexin A in the RVLM in both the tonic and reflex regulation of central cardio-respiratory control.

Methods

All animal care and experimental procedures complied with the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (http://www.nhmrc.gov.au/guidelines/publications/ea16) and were approved by the Animal Ethics Committee of Macquarie University, Sydney, Australia. We used male Sprague-Dawley rats (n = 50, 350–500 g), housed under controlled conditions with a standard 12 h light/dark cycle. Standard laboratory rat chow and tap water were available *ad libitum*. They were allowed to acclimatize for at least 7 days before experimental manipulations. Food and water were withdrawn 30 min before anaesthesia.

Immunohistochemical experiments

Rats (n = 3) were anaesthetized (pentobarbitone sodium, 70 $mg \cdot kg^{-1}$, i.p.), transcardially perfused and brainstems were processed for fluorescence immunohistochemistry (IHC) as described previously (Li *et al.*, 2005; Padley *et al.*, 2007). In brief, 40 µm sections were incubated with species-specific primary antibodies to detect tyrosine hydroxylase (TH; mouse, 1:2500), orexin A (rabbit, 1:2000), OX_1 receptors (rabbit, 1:50) and OX_2 receptors (goat, 1:50). Sections were then washed (3 × 30 min) in TPBS buffer (10 mM Tris HCl, 10 mM sodium phosphate buffer, 0.9% NaCl, pH 7.4) and incubated with fluorophore-conjugated secondary antibodies (Alexa Fluor 488 donkey anti-mouse IgG, and Cy3 donkey anti-rabbit IgG or Cy3 donkey anti-sheep IgG; 1:500) to reveal TH, orexin A, OX_1 and OX_2 receptors respectively. Finally, sections were mounted on glass slides, coverslipped with Vectashield (Vector Laboratories, QLD, Australia) and sealed with nail polish.

Brainstem sections, including the RVLM region, extending from 11.6 to 12.8 mm caudal to Bregma, were examined using an epifluorescence microscope (Axio-Imager Z1; Zeiss, Göttingen, Lower Saxony, Germany). The RVLM was defined as a triangular area ventral to the nucleus ambiguus, medial to the spinal trigeminal tract, and lateral to the pyramidal tract or the inferior olive. Labelled neurones in the RVLM were counted from six to seven sections, 200 µm apart. Counts were made for OX_1, OX_2 receptors and TH-immunoreactive neurones, as well as all double-labelling combinations. Results were expressed as the mean ± SEM at 200 µm intervals. Images were captured in grey scale with an Axiocam MR3 digital camera

(Göttingen, Lower Saxony, Germany) and pseudocolouring was applied. Staining for all antigens was observed throughout the depth of the section indicating adequate antibody penetration.

Electrophysiological experiments

Experimental design. Electrophysiological experiments were conducted as described previously (Shahid *et al.*, 2011). Briefly, rats (n = 47) were anaesthetized with urethane (1.2–1.4 g·kg^{-1}, i.p.). Supplemental doses of urethane (30–40 mg, i.v.) were given when necessary if nociceptive stimuli (tested every half an hour) caused a change in MAP of more than 10 mmHg. Rectal temperature was maintained at ~36.5°C with a thermostatically controlled heating pad (Harvard Apparatus, Holliston, MA, USA) and infrared heat lamp.

The left jugular vein and right carotid artery were cannulated with polyethylene tubing (internal diameter = 0.58 mm; outer diameter = 0.96 mm) for administration of drugs and fluids and for the measurement of BP. In some experiments, both femoral veins were cannulated to enable administration of sodium nitroprusside (SNP) or phenylephrine hydrochloride. The trachea was cannulated to enable artificial ventilation and a 3-lead electrocardiogram was fitted. HR was derived from the BP. The left greater splanchnic nerve and phrenic nerve were isolated, tied with silk thread, and cut distally to permit recording of efferent sSNA and PNA respectively. In an additional subset of animals, the sciatic nerve was isolated, tied and cut distally for electrical stimulation to allow activation of somato-sympathetic reflexes. Rats were secured in a stereotaxic frame, vagotomized, paralyzed (pancuronium bromide; 0.8 mg initially, then 0.4 mg·h^{-1}) and artificially ventilated with oxygen-enriched room air. End-tidal CO_2 was monitored and maintained between 4.0% and 4.5% (Capstar 100, CWE Inc., Ardmore, PA, USA). Arterial blood gas was analysed to maintain pH at 7.35–7.45 (pH 7.4 ± 0.03; $PaCO_2$ 40.4 ± 0.9) by electrolyte and blood gas analyzer (IDEXX Vetstat, West brook, ME, USA). Animals were infused with 5% glucose in water (1.0–2.0 mL·h^{-1}) to ensure hydration. Nerve recordings were made with bipolar silver wire electrodes. The neurograms were amplified (×10 000, CWE Inc.), bandpass filtered (0.1–2 kHz), sampled at 3 kHz (1401 plus, CED Ltd., Cambridge, UK) and recorded on computer using Spike2 software (v7, CED Ltd.).

The dorsal medulla was exposed after an occipital craniotomy. Bilateral microinjections of L-glutamate (100 mM, 5 nmol in 50 nL), orexin A (250 μM, 500 μM, 1 mM, 2 mM equivalent to 12.5, 25, 50 and 100 pmol; 50 nL per side) and vehicle (PBS) into the RVLM were made, using single- or multi-barrel glass pipettes, as described previously (Miyawaki *et al.*, 2001; Rahman *et al.*, 2011a). The RVLM was functionally identified by an increase of >30 mmHg in MAP following microinjection of L-glutamate. All variables were allowed to return to baseline (>30 min) before microinjection of PBS. After a further 30 min, orexin A was microinjected into the same site and observation was continued for a further 60 min. For all experiments, microinjections (50 nL) were performed over 5 s. Injection sites were marked with 2% rhodamine beads added to the PBS (50 nL) which was also microinjected as a volume and vehicle control.

Cardiorespiratory reflexes were evoked as described previously (Abbott and Pilowsky, 2009; Shahid *et al.*, 2011; Rahman *et al.*, 2011b), with sequential i.v. injection of SNP and phenylephrine (10 μg·kg^{-1}) or electrical stimulation (10–25 V, 50, 0.2 ms pulses at 1 Hz) of the sciatic nerve. Chemoreceptors were activated by ventilating animals with either 100% N_2 (12–14 s, brief hypoxia; peripheral; BOC gas and gear, NSW, Australia) or 5% CO_2:95%O_2 (3 min, hypercapnia; central; BOC gas and gear). Reflexes were activated before and after bilateral microinjection of PBS or orexin A (50 pmol, per side, 50 nL). Following death (3 M KCl, 0.5 mL, i.v.), brainstems were removed and injection sites were verified. Preliminary experiments revealed that tachyphylaxis occurs following orexin A injection; therefore, each animal received only one injection of orexin A. Different reflexes and antagonist studies were conducted on different sets of animals. Cardio-respiratory coupling was analysed from the same group used to determine the cardio-respiratory responses.

Data acquisition

Neurograms were rectified and smoothed (sSNA, 1 s time constant; PNA, 50 ms). Minimum background activity after death was taken as zero sSNA, and this value was subtracted from sSNA before analysis with off-line software (Spike 2 version 7). Baseline values were obtained by averaging 60 s of data 5 min prior to orexin A or PBS injection and maximum changes were expressed as absolute or percentage changes from baseline values. Phrenic-triggered ensemble averages of sSNA were generated from 60 s portions of data to evaluate cardio-respiratory coupling. The area under the curve (AUC), less baseline, of sSNA activity during the inspiratory and post-inspiratory phases was determined. sSNA was rectified and smoothed at 1 s and 5 ms time constants to analyse baroreceptor reflex and somato-sympathetic reflex respectively. To analyse reflexes, sSNA was normalized between the activity of sSNA before PBS injection (100%) and the sSNA after death (0%). The sSNA response to sciatic nerve stimulation was analysed using peristimulus waveform averaging. The AUC of the sympatho-excitatory peaks was analysed. The response to hypoxia (100% N_2 inhalation for 12–14 s) was quantified by comparing the average maximum sSNA during hypoxia compared with a control period during normal hyperoxic ventilation. The maximum response to stimulation was then expressed as a percentage change from the baseline (control) considering maximum reflex response in control as 100%.

Data analysis

Grouped data are expressed as mean ± SEM. Statistical analysis was conducted with GraphPad Prism (version 5.0) (GraphPad, La Jolla, CA, USA).. A two-way, repeated measures, ANOVA with Bonferroni's correction was used to compare values after orexin A (50 pmol) administration on sSNA and PNA with the PBS value. Paired t-test was used to analyse peak effects and reflexes. $P < 0.05$ was considered significant.

Materials

Orexin A (MW = 3561.16; Cat. No: H-4172; Lot No: 1013059) was obtained from, Bachem AG (Bubendorf, Switzerland); the OX_1 receptor antagonist, SB334867 (*N*-(2-methyl-6-benzoxazolyl)-*N*′-1,5-naphthyridin-4-yl urea) (Cat. No: 1960; Batch No: 5) and the OX_2 receptor agonist, [Ala^{11},

D-Leu[15]]orexin B (Cat. No: 2142; Batch No: 10), were from Tocris Bioscience (Bristol, UK). Urethane, L-glutamate, glucose, phenylephrine and SNP were purchased from Sigma-Aldrich (Sydney, Australia); pancuronium bromide was from AstraZeneca Pty Ltd (Sydney, Australia); pentobarbitone sodium was from Merial Australia Pty Ltd (NSW, Australia); rhodamine microbeads were from Molecular Probes (Sydney, Australia); and PBS (10 mM in 0.9% NaCl) tablets were from AMRESCO Inc. (Solon, OH, USA). Primary antibodies for orexin A (Cat No: PC362), OX_1 receptors (Cat. No: AB3092; Lot No: LV1387169), OX_2 receptors (Cat. No: sc-8074) and TH were from Merck (Darmstadt, Germany), Millipore (Billerica, MA, USA), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Sigma-Aldrich respectively. Fluorophore-conjugated secondary antibodies such as Alexa Fluor 488 donkey anti-mouse IgG (Cat. No: A21202; Lot No: 811493), Cy3 donkey anti-rabbit IgG (Cat. No: 711–166-152; Lot No: 92082) or Cy3 donkey anti-sheep IgG (Cat. No: 713–166-147; Lot No: 89763) were from Invitrogen (Victoria, Australia) and Jackson ImmunoResearch Laboratories (West Grove, PA, USA), and vectashield was from Vector Laboratories. orexin A, [Ala[11], D-Leu[15]]orexin B and rhodamine (2% v/v) were dissolved and further diluted in PBS (10 mM; pH 7.4). SB334867 was dissolved and further diluted in 10% dimethyl sulphoxide. PBS, phenylephrine and SNP were prepared in de-ionized water. Urethane was dissolved in 0.9% NaCl and L-glutamate in PBS.

Results

Expression of orexin A terminals and its receptors in the RVLM

The expression of orexin A, OX_1 and OX_2 receptors in the RVLM was examined using fluorescence IHC. Orexin A-immunoreactive fibres and terminals were found commonly throughout the RVLM (Figure 1A). Orexin A-immunoreactive terminals were also closely apposed to TH-immunoreactive cell bodies as well as dendrites (n = 3, Figure 1A).

Both OX_1 and OX_2 receptors were expressed in the RVLM. Intense immunoreactivity for both receptors was observed on cell bodies as well as on dendrites, fibres and terminals throughout the RVLM (Figure 1B,C). Immunoreactivity for OX_1 receptors and TH were frequently co-localized in the RVLM neurones (Figure 1B). OX_1 receptor immunoreactivity was found in 78 ± 2% (561 of 727 neurones across the region counted from –11.6 to –12.8; n = 3) of TH-immunoreactive neurones in the RVLM (Figure 1B); OX_2 receptor immunoreactivity was found in 77 ± 3% (475 of 611 neurones) of TH-immunoreactive neurones in the RVLM (Figure 1C). Within the RVLM, about 51% of OX_1 receptors (590 of 1151 neurones across the entire region counted from –11.6 to –12.8, n = 3) and 56% of OX_2 receptors (615 of 1090 neurones across the entire region counted from –11.6 to –12.6, n = 3) were expressed in non-TH-immunoreactive or non-C1 neurones, suggesting that both C1 and non-C1 neurones in the RVLM contain both orexin receptors (Figure 1B,C). Immunoreactivities to OX_1 and OX_2 receptors and to TH showed similar patterns of distribution in the RVLM, being expressed most frequently in the rostral pole (Figure 1B,C).

Effects of orexin A in the RVLM on cardio-respiratory parameters

Bilateral microinjection of orexin A at four different doses (12.5, 25, 50 and 100 pmol, per side) evoked a pressor response, tachycardia and sympatho-excitation in a dose-dependent manner (Figures 2A, 2B) with maximum effects attained following injection of 50 pmol orexin A (Figure 2B). Bilateral injection of PBS (vehicle) in the RVLM was without effect on MAP, HR or sSNA (Figure 2B).

Orexin A injected bilaterally (12.5, 25, 50 and 100 pmol, per side) in the RVLM evoked a significant increase in PNamp without any effect on phrenic nerve frequency (PNf) (Figure 2A,B). The maximum increase in PNamp (n = 6, P < 0.001) was elicited by 50 pmol orexin A (Figure 2B). No significant change in PNamp or PNf was observed after injection of PBS (vehicle) (Figure 2A,B).

In some experiments (n = 5), bilateral injection of orexin A (50 or 100 pmol, per side) in the RVLM evoked a long-lasting increase in sSNA and PNA (Figure 2A,C) that mimicked the pattern of long term facilitation (LTF) of sSNA and PNA induced by acute intermittent hypoxia (Baker-Herman and Mitchell, 2002; Dick *et al.*, 2007) or by intrathecal injection of orexin A (Shahid *et al.*, 2011). All injection sites were centred in the RVLM (Figure 2D).

Effects of orexin receptor antagonism or agonism on cardio-respiratory responses mediated by orexin A

In the absence of orexin A, SB334867 (1 nmol, per side) did not affect MAP, HR, sSNA, PNamp or PNf (n = 5) (data not shown). On the other hand, the cardio-respiratory effects of orexin A (50 pmol per side) in the RVLM were significantly reduced by prior bilateral injection of SB334867 (P < 0.01,; n = 5, Figure 3).

Bilateral microinjection of the OX_2 receptor agonist, [Ala[11], D-Leu[15]]orexin B (0.75 pmol, per side; n = 6), into the RVLM significantly increased MAP, HR, sSNA and PNamp to an extent that was similar or slightly higher in magnitude with the responses to orexin A (50 pmol) (Figure 3). The only exception was a decrease in PNf as compared with PBS (Figure 3).

Effects of orexin A in the RVLM on cardio-respiratory coupling

Peri-phrenic averaging of the sympathetic nerve activity revealed an inspiratory and post-inspiratory peak of sSNA (Figure 4A). The AUC of both inspiratory and post-inspiratory peaks of sSNA was significantly increased by bilateral injection of 50 pmol orexin A (P < 0.05; n = 4, Figure 4B) compared with injection of PBS.

Effects of orexin A in the RVLM on the somato-sympathetic reflex

Intermittent stimulation of the sciatic nerve resulted in two characteristic excitatory peaks in sSNA with latencies of 84 ± 6 ms and 186 ± 7 ms, before microinjection (n = 5,

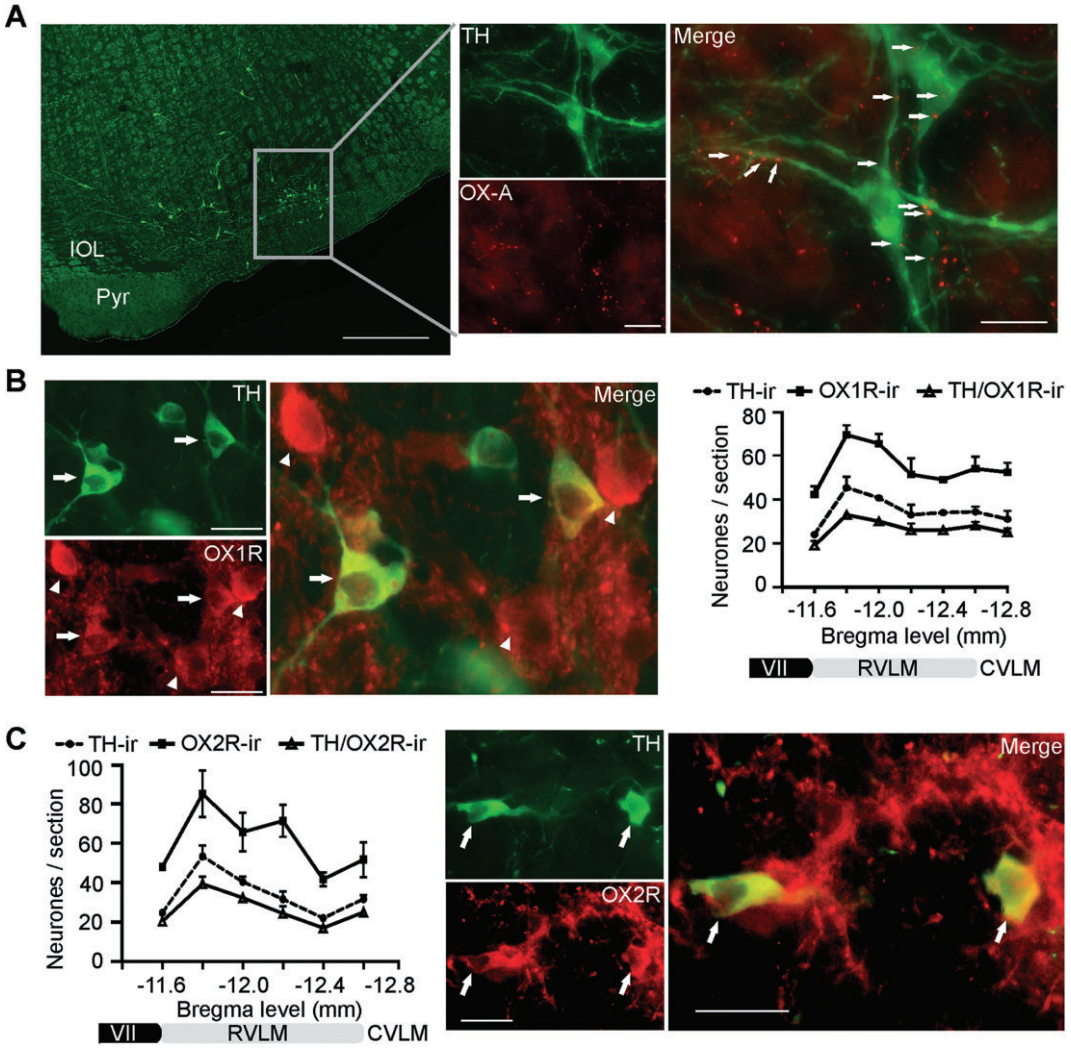

Figure 1

Immunohistochemical location of orexin A (OX-A) terminals, OX_1 and OX_2 receptors in the RVLM. (A) TH-positive (TH-ir) C1 neurones (green) in the RVLM are surrounded by orexin A-immunoreactive (OX-A-ir) varicosities (red) that form close appositions with TH-positive cell bodies and dendrites (arrows). (B) Immunostaining for OX_1 receptors in the RVLM. OX_1 receptors (OX1R-ir; red) were expressed in TH-immunoreactive neurones (green) (arrows) as well as in some non-TH neurones (arrowheads). XY plot represents rostrocaudal distribution of neurones staining for TH (TH-ir), those staining for OX_1 receptors (OX1R-ir) and those staining for both TH and OX_1- receptors(TH/OX1R-ir). (C) XY plot represents rostrocaudal distribution of neurones staining for TH (TH-ir), those staining for OX_2 receptors (OX2R-ir)and those staining for both TH and OX_2-receptors (TH/OX2-ir). OX_2 receptors (red) are co-localized with TH-immunoreactive neurones (green; arrows). VII, facial nucleus; CVLM, caudal ventrolateral medulla. Scale bars: 500 μm (A, left); 20 μm (A, right); 25 μm (B and C).

Figure 5A). The latencies of the peaks were not significantly altered by PBS [89 ± 5 ms and 190 ± 7 ms, n = 5, non-significant (ns)], or by orexin A (88 ± 5 ms and 189 ± 8 ms, n = 5, ns), injection. Bilateral orexin A injection (50 pmol per side) in the RVLM markedly attenuated the AUC of both excitatory peaks by 32% of baseline ($P < 0.01$; n = 5, Figure 5B).

Effects of orexin A in the RVLM on baroreflex

In five animals, the changes in sSNA were plotted against the changes in MAP evoked by i.v. injection of SNP and phenylephrine. Bilateral RVLM microinjection of orexin A (50 pmol per side) significantly enhanced the reflex sympathoinhibitory responses evoked by phenylephrine (Figure 6A). Orexin A significantly increased the upper plateau, range of sSNA, operating range and maximum gain of the sSNA without significantly altering the lower plateau, the threshold level, midpoint and the saturation levels of MAP as compared with control (Figure 6B and Table 1).

Effects of orexin A in the RVLM on chemoreflex

Activation of peripheral chemoreceptors with brief hypoxia evoked an increase in MAP, sSNA, HR, PNamp and PNf

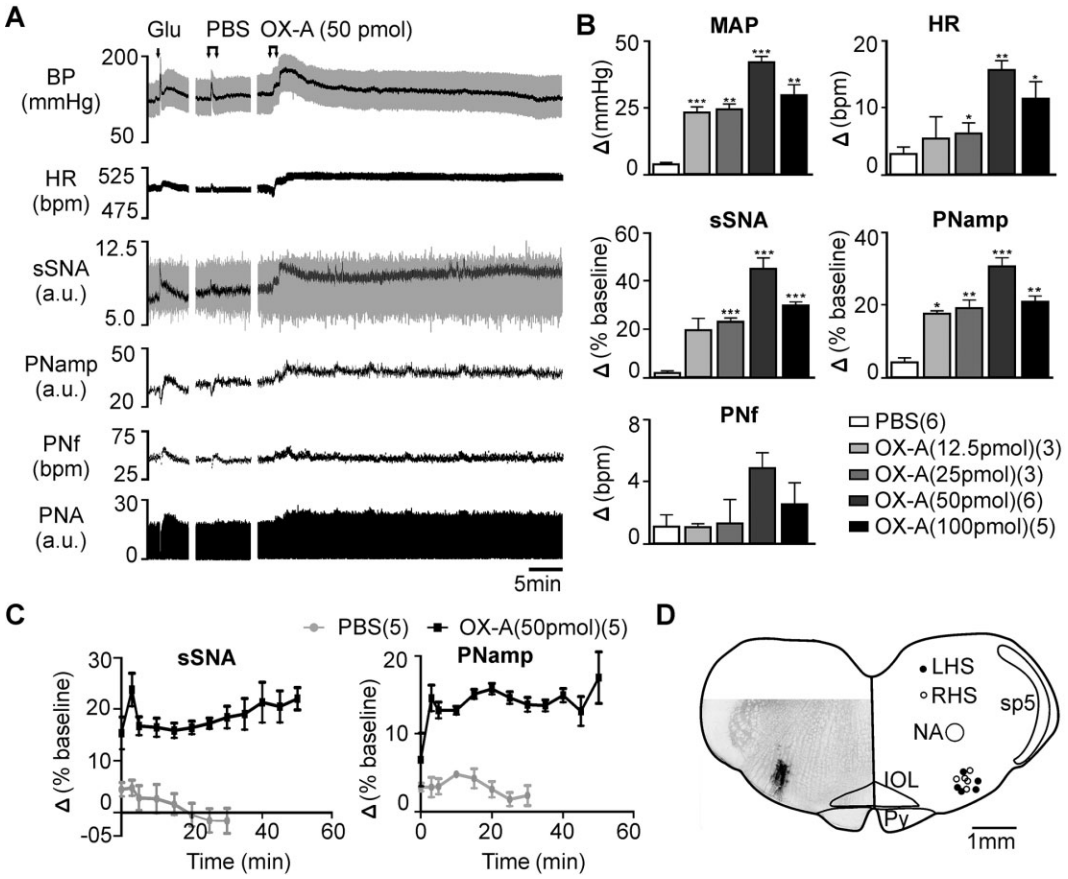

Figure 2

Effect of bilateral microinjection of orexin A (OX-A) in the RVLM. (A) Continuous recording of BP (grey – pulsatile and black – mean), HR, sSNA (grey – raw and black – rectified and integrated) [arbitrary unit (a.u.)], rectified PNA (a.u.), PNf and PNamp before and after injection of glutamate (Glu), PBS or orexin A (50 pmol). (B) Grouped data of maximum cardiovascular and respiratory effects following PBS or orexin A (12.5, 25, 50 and 100 pmol). Peak effects are shown as absolute (MAP, HR, PNf) or percentage (sSNA, PNamp) change from respective basal values. (C) Grouped time course effects of PBS or orexin A (50 pmol) on sSNA and PNamp. (D) Microinjection sites in the RVLM and a section showing an injection site stained following an injection of rhodamine microbeads. LHS, left hand side; RHS;right hand side; NA, nucleus ambiguus; sp5, spinal trigeminal tract; IOL, inferior olive; Py, pyramidal tract. Data are expressed as mean ± SEM. Numbers of animals are shown in parentheses. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, significantly different from PBS. bpm, beats per minute for HR or bursts per minute for PNf.

(Figure 7A). Peak effects occurred near the end of stimulus and recovered rapidly to baseline. Bilateral injection of orexin A (50 pmol per side) in the RVLM significantly increased the sympatho-excitatory response by 23% while attenuating the tachycardia by 43%, without any significant alteration in the pressor response ($P < 0.01$ for either; $n = 4$, Figure 7B). Orexin A caused significant attenuation of peak amplitude of PNamp and peak PNf during hypoxia by 33% and 24% of baseline, respectively ($P < 0.001$;$n = 4$; Figure 7B).

Activation of central chemoreceptors with hypercapnia evoked an increase in MAP, sSNA, PNamp and a decrease in HR (Figure 7C). Orexin A (50 pmol per side) markedly attenuated the effect of hypercapnia on MAP by 143% ($P < 0.01$) and sSNA by 82% ($P < 0.01$) without any significant alteration in the bradycardia response ($n = 5$, Figure 7D). The effects of hypercapnia on peak PNamp was also reduced following orexin A (50 pmol per side) injection in the RVLM ($P < 0.05$; $n = 5$, Figure 7D).

Discussion

The principal new findings of the present study are: (i) OX_1 and OX_2 receptors are expressed in both C1 and non-C1 neurones of the RVLM; (ii) microinjection of orexin A into the RVLM causes sympatho-excitation, hypertension and tachycardia; (iii) orexin A microinjection significantly increases PNamp without affecting PNf; (iv) the respiratory modulation of sSNA is dramatically increased after microinjection of orexin A; (v) both OX_1 and OX_2 receptors are involved in the cardio-respiratory responses to orexin A; (vi) both peaks of somato-sympathetic reflex are attenuated by orexin A; (vii) sympathetic baroreflex sensitivity is increased following orexin A microinjection; (viii) the sympatho-excitatory response to hypoxia is potentiated, but there is a decrease in the HR, and PNA, response; and (ix) the cardiovascular and respiratory responses to hypercapnia are attenuated by orexin A.

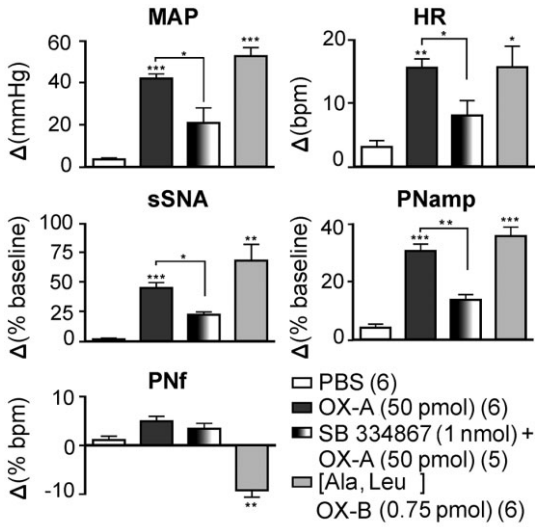

Figure 3

Effects of SB334867 and [Ala[11], D-Leu[15]]orexin B ([Ala,Leu]OX-B) on orexin A-induced cardiorespiratory effects in the RVLM. Bar charts showing group data of maximum cardiovascular and respiratory effects following injection of PBS, orexin A (OX-A, 50 pmol), SB334867 [1 nmol + orexin A (50 pmol)] or [Ala[11], D-Leu[15]]orexin B (0.75 pmol). Peak effects are shown as absolute (MAP, HR, PNf) or percentage (sSNA, PNamp) change from respective basal values. Data are expressed as mean ± SEM. Numbers of animals are shown in parentheses. ***P < 0.001, **P < 0.01, *P < 0.05 significantly different from PBS [except SB334867 (1 nmol) + orexin A (50 pmol)], which was compared with orexin A (50 pmol)). bpm, beats per minute for HR or bursts per minute for PNf.

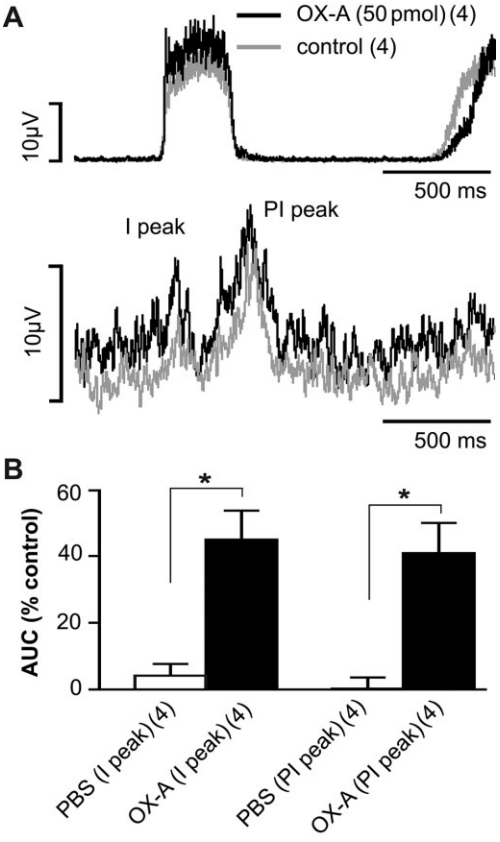

Figure 4

Effect of orexin A on phrenic nerve discharge-related rhythmicity of sSNA in the RVLM. (A) Phrenic-triggered average of sSNA before and after bilateral orexin A (OX-A; 50 pmol, each side) injection. (B) Grouped data illustrating the effects of PBS or orexin A (50 pmol, n = 4) on I and PI peaks of sSNA. The AUC of both inspiratory (I) and post- inspiratory (PI) peaks are increased following bilateral orexin A injection. Values are expressed as mean ± SEM. Numbers of animals are shown in parentheses. *P < 0.05, significantly different from PBS. Both PBS and orexin A values were normalized to the control period before injections.

Both C1 and non-C1 neurones in the RVLM play a role in the tonic and reflex control of sympathetic vasomotor tone (Lipski *et al.*, 1995; 1996; Pilowsky and Goodchild, 2002; Guyenet, 2006; Pilowsky *et al.*, 2009). The results of this study reveal that both OX_1 and OX_2 receptors are expressed in a discrete population of C1 and non-C1 neurones throughout the rostro-caudal pole of the RVLM, some of which may be bulbospinal and barosensitive. This study also demonstrates that varicose orexin A-immunoreactive fibres are present in the RVLM and are in close proximity to TH-immunoreactive neurones, consistent with the findings of Machado *et al.* (2002). The findings suggest that OX_1 and OX_2 receptors are activated under physiological or pathological conditions to modulate the activity of cardio-respiratory neurones in the RVLM. OX_1 and OX_2 receptors are found throughout the brainstem in regions involved in cardio-respiratory regulation, including: the NTS, RVMM, nucleus ambiguus, pre-Bötzinger complex (Trivedi *et al.*, 1998; Marcus *et al.*, 2001; Sunter *et al.*, 2001; Cluderay *et al.*, 2002; Ciriello and de Oliveira, 2003). The presence of OX_1 and OX_2 receptors within C1 neurones in the rostral 600 μm of the RVLM is consistent with the idea that these receptors are expressed by bulbospinal baro-sensitive, sympatho-excitatory neurones (Brown and Guyenet, 1984; Lipski *et al.*, 1995; 1996; Schreihofer and Guyenet, 1997), and that orexin A is involved in regulating sympathetic outflow.

We suggest that the hypertensive and tachycardiac effects of centrally administered orexin A, as reported in previous studies (Chen *et al.*, 2000; Machado *et al.*, 2002), are likely to be due to an increase in sympathetic vasomotor tone mediated by the RVLM. In previous studies, orexin A microinjection into the RVLM resulted in hypertension and tachycardia for 25–30 min in anaesthetized rats (Chen *et al.*, 2000) and for about 7 min in an conscious rats (Machado *et al.*, 2002). We observed a significant, sympathetically mediated, hypertension and tachycardia after bilateral microinjection of orexin A into the RVLM that lasted for ~10 min. There were several differences between this study and earlier work; firstly, the rats used in this study were vagotomized, secondly, the rats used here were significantly larger (~425 g vs. ~280 g), and thirdly, the doses used in this study, based on an initial dose-response study, were higher (1 mM vs. ~250 μM.). The doses and weights used here were similar to those used in our earlier study of the effects of orexin A in the spinal cord (Shahid *et al.*, 2011).

Pre-sympathetic neurones in the RVLM region are close to, and intermingled with, neurones in the Bötzinger region

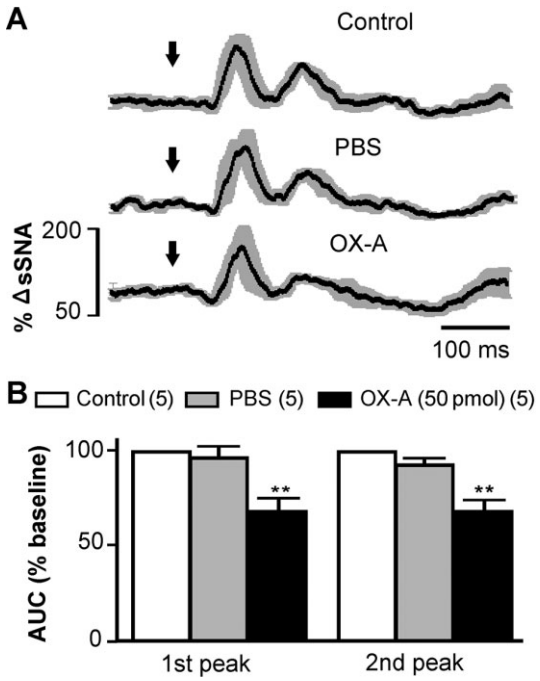

Figure 5

Effect of bilateral microinjection of orexin A (OX-A) on somato-sympathetic reflex. (A) Grouped effect of sciatic nerve-evoked stimulation of sSNA at control period and after injection of PBS and orexin A. Data are mean (black) ± SE (grey). Arrows indicate the time of stimulation. (B) Group data illustrating the effects of PBS or orexin A (50 pmol) on the AUC of 1st and 2nd sympatho-excitatory peaks. Numbers of animals are shown in parentheses. $^{**}P < 0.01$, significantly different from control.

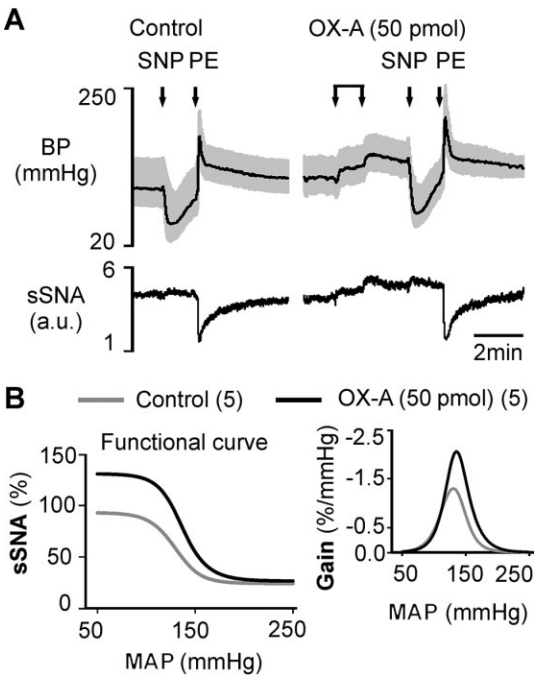

Figure 6

Effect of bilateral orexin A (OX-A) injection in the RVLM on the arterial baroreflex evoked by i.v. injection of sodium nitroprusside (SNP) or phenylephrine hydrochloride (PE). (A) Representative experimental recording of the effect of changes in BP on sSNA due to SNP or phenylephrine before (control) or after orexin A injection. (B) Average sympathetic baroreflex function curves generated for data before (control) or after orexin A (50 pmol) injection (numbers of animals are shown in parentheses). Trace at right represents baroreflex gain for sSNA (error bars are omitted for clarity – see Table 1). The range and gain of the reflex are significantly increased.

and pre-Bötzinger complex (Pilowsky *et al.*, 1990; Sun *et al.*, 1994; Kanjhan *et al.*, 1995), and receive projections from the respiratory column (Sun *et al.*, 1994.). Here we find that PNamp is increased following orexin A microinjection into the RVLM, while PNf remained unchanged. This result agrees with previous studies demonstrating that orexin A in the pre-Bötzinger complex increased amplitude without affecting breathing rate (Young *et al.*, 2005). We also observed a long term facilitation (LTF) of sSNA and PNA in some of the experiments following orexin A microinjection into the RVLM which supports earlier studies suggesting a role for orexin A in LTF in the brain (Mileykovskiy *et al.*, 2002; Walling *et al.*, 2004; Wayner *et al.*, 2004; Terada *et al.*, 2008; Toyama *et al.*, 2009). One possible explanation is that orexin A triggers the release of excitatory neurotransmitters (glutamate or noradrenaline) in the spinal cord and respiratory column, to cause LTF of SNA and PNA (Shi *et al.*, 1988).

RVLM neurones are influenced by central respiratory activity (Guyenet *et al.*, 1990; Miyawaki *et al.*, 1996; Pilowsky *et al.*, 1996), and sympathetic nerve discharge shows a respiratory rhythmogenicity in relation to PNA (Adrian *et al.*, 1932; Guyenet, 2000). orexin receptor activation caused a significant increase in the inspiratory and post-inspiratory burst in sSNA with an increase on baseline activity suggesting that orexin receptor activation enhanced the respiratory-related, excitatory synaptic drive to RVLM neurones.

In order to determine if orexin A was involved in the tonic regulation of neurones in the RVLM, and to find out which type of orexin receptor mediated the central effects of orexin A, the selective OX_1 receptor antagonist, SB334867 (pK_b $OX_1 = 7.2$), and the selective OX_2 (400 times more potent compared with OX_1) receptor agonist, [Ala^{11}, D-Leu^{15}]orexin B, were injected into the RVLM. SB334867 itself did not affect baseline cardio-respiratory parameters, suggesting that OX_1 receptors are not constitutively, or tonically, active. SB334867 reduced orexin A-induced cardio-respiratory responses by about 50%. Furthermore, [Ala^{11}, D-Leu^{15}]orexin B induced sympatho-excitation, hypertension, tachycardia and increases respiratory drive, in a similar manner to orexin A. Taken together, the results suggest that both OX_1 and OX_2 receptors mediated the central effects of orexin A, confirming results from a previous *in vitro* study (Huang *et al.*, 2010). Huang *et al.* (2010) suggested a minor role of OX_1 receptors on orexin A-induced depolarization of RVLM neurones in the brainstem slice preparation. This discrepancy may be due to the lower dose of SB334867 used compared with other studies (Deng *et al.*, 2007; Shih and Chuang, 2007), or developmental differences between the neonate and the adult animal. In this study we also found that activation of OX_2 receptors increased PNamp, but decreased PNf. We speculate that acti-

Table 1

Parameters describing baroreflex control of sSNA after bilateral microinjection of orexin A (OX-A) (50 pmol)

	Lower plateau (%)	Upper plateau (%)	Mid point (mmHg)	Maximum gain (%/mmHg)	Range of SNA (%)	Threshold level (mmHg)	Saturation level (mmHg)	Operating range (mmHg)
Control	24.1 ± 6.9	93.2 ± 9.1	131.2 ± 3.2	−1.4 ± 0.2	57.3 ± 8.5	93.7 ± 6.5	168.8 ± 5.3	68.3 ± 10.2
OX-A (50 pmol)	26.5 ± 7.6 (ns)	131.0 ± 4.6**	138.0 ± 3.1 (ns)	−2.1 ± 0.3**	100.4 ± 5.2*	99.3 ± 3.6 (ns)	176.6 ± 7.5 (ns)	104.7 ± 16.2*

Values are mean ± SEM ($n = 5$). Maximum gain is the slope of the sigmoid curve of best fit at the MAP corresponding to steepest part of the curve. $^{**}P < 0.01$, $^{*}P < 0.05$ significantly different from control. ns, non-significant.

vation of orexin receptors decreases the activity of inhibitory Bötzinger neurons, resulting in an increase in PNamp. This increase in amplitude may be counteracted by a reflex decrease in frequency by the unaffected pre-Bötzinger complex which controls respiratory rhythm. Further studies will be required to clarify this issue.

In order to maintain cardiovascular homoeostasis, RVLM neurones integrate information from many peripheral afferent neurones, including: somatic receptors, baroreceptors and chemoreceptors (Dampney, 1994; Sun, 1995; Pilowsky *et al.*, 2009). Peptide neurotransmitters modulate the different reflex responses of RVLM neurones via pre- or post-synaptic pathways (Miyawaki *et al.*, 2002; Makeham *et al.*, 2005; Burke *et al.*, 2008; Kashihara *et al.*, 2008; Abbott and Pilowsky, 2009). The somato-sympathetic reflex evoked by sciatic nerve stimulation was significantly attenuated by orexin A microinjection into the RVLM. This result suggests that the attenuation of somato-sympathetic reflex was not an indirect effect of raised sympathetic activity following orexin A injection into the RVLM. The mechanism by which orexin A exerts its effect on the somato-sympathetic reflex is likely to be complicated, as OX_1 receptors were found to be localized both pre- and post-synaptically.

The reflex responses of sSNA to baroreceptor loading and unloading induced by vasoactive drug administration were markedly enhanced by orexin A microinjection into the RVLM. This response is not due to an increase in sSNA following orexin A injection, as injection of phenylephrine was equally able to reduce sSNA to the same low levels seen in the control situation. Exogenous orexin A increased the upper plateau of the baroreflex curve as well as the maximum gain and operating range, suggesting that orexin A in the RVLM regulates baroreflex control of sSNA by increasing disinhibition of RVLM neurones or by activation of barosensitive RVLM neurones.

Neurones in the ventral respiratory column and the RVLM are activated directly, and indirectly, by hypoxia. (Sun and Reis, 1994; De Paula and Branco, 2005; Alheid and McCrimmon, 2008). Orexin A microinjection into the RVLM enhanced the sympatho-excitatory response to hypoxia while reducing tachycardiac and phrenic responses. Enhanced sympatho-excitatory, but reduced tachycardiac effects, suggest that orexin A activates different population of presympathetic RVLM neurones projecting to different levels of the spinal cord. On the other hand, attenuated respiratory responses to hypoxia following orexin A microinjection into the RVLM suggest that orexin A may act on inhibitory Bötzinger neurones that project to other respiratory nuclei in the brainstem and spinal cord.

The reflex sympathetic response to hypercapnia is initiated by central chemoreceptors in the RTN, which then activate presympathetic neurones in the RVLM (Guyenet, 2000; Guyenet *et al.*, 2010). Orexin A microinjection in the RVLM markedly attenuated cardio-respiratory responses to hypercapnia. It is not known whether chemosensitive RTN neurones make monosynaptic or polysynaptic connection with bulbospinal RVLM neurones. The inhibition of cardiovascular response to hypercapnia suggests the possible activation of GABAergic interneurones in the RVLM by orexin A.

Our findings show that OX_1 and OX_2 receptors are present in both C1 and non-C1 neurones in the RVLM and activation

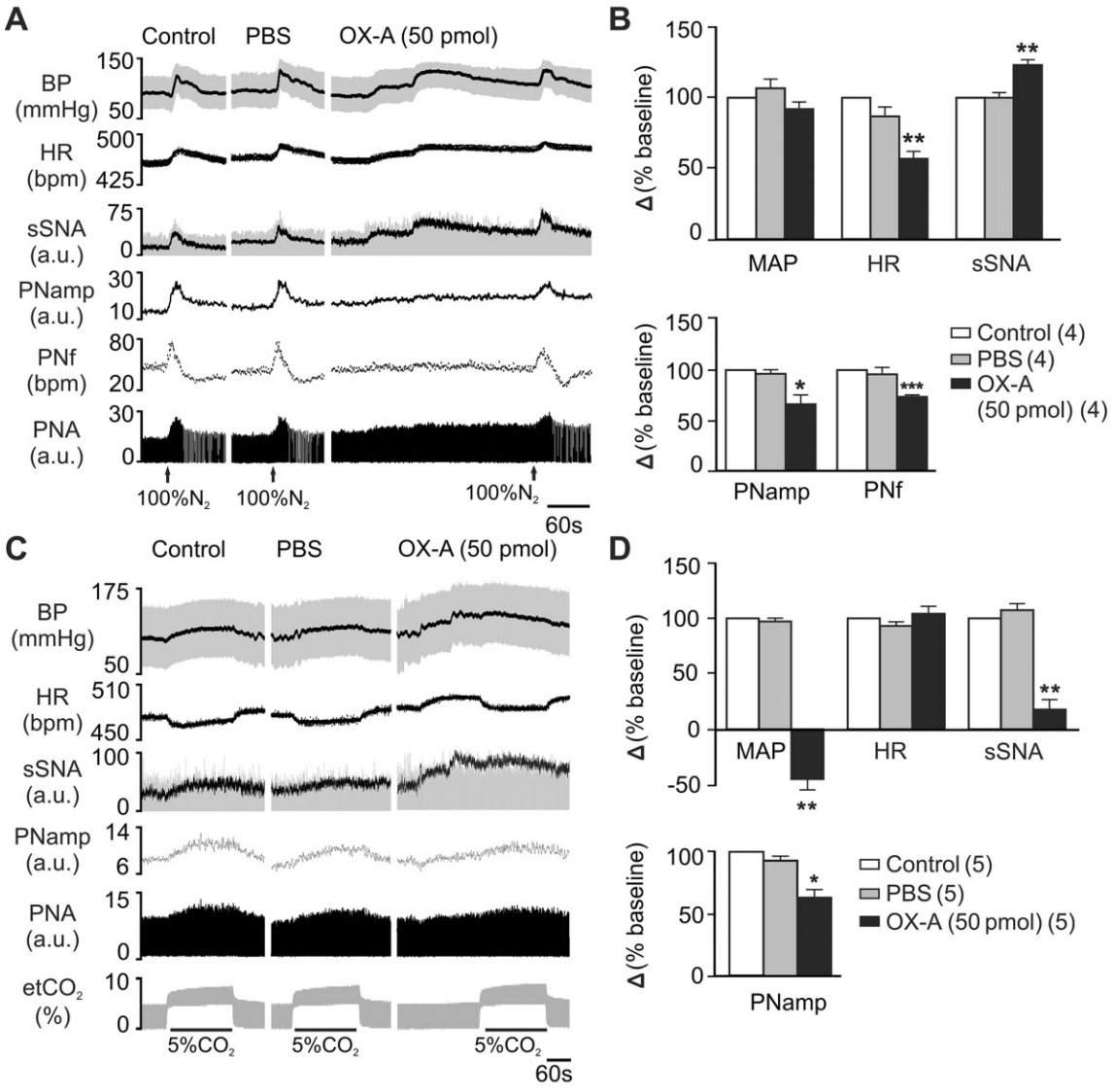

Figure 7

Effect of bilateral orexin A (OX-A) injection in RVLM on the cardiovascular and respiratory response to hypoxia and hypercapnia. (A) Effect on the peripheral chemoreceptor reflex activated by brief hypoxia with 100% N_2 for 12–14 s – experimental recording of hypoxic episodes at control period and after PBS or orexin A injection. (B) Grouped data of peak effects on cardiovascular (MAP, HR and sSNA) (top bar chart) and respiratory function (PNamp and PNf) (bottom bar chart) after injection of PBS and orexin A (50 pmol, $n = 4$) in response to brief hypoxia. (C) Effect on central chemoreceptor reflex activated by hypercapnia with 5% CO_2 in O_2 for 3 min – experimental recording of hypercapnic episodes at control period and after PBS or orexin A injection. (D) Comparison of peak effects on cardiovascular (MAP, HR and sSNA) and respiratory function (PNamp) after injection of PBS and orexin A (50 pmol, $n = 5$) in response to hypercapnia. Values are expressed as mean ± SE. Numbers of animals are shown in parentheses. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, significantly different from control. bpm, beats per minute for HR or bursts per minute for PNf.

of these receptors in the RVLM causes sympathetically mediated hypertension, tachycardia and increase in respiratory drive. Orexin receptor activation in the RVLM reduces the somato-sympathetic reflex, but increases barosensitivity. Sympatho-excitatory responses to hypoxia and hypercapnia are enhanced and inhibited, respectively, by orexin receptor activation in the RVLM. Orexin A and orexin B play a key role in the stabilization of wakefulness, and are thought to be arousal-promoting peptides. Orexin ablated mice have disrupted sleep patterns (Sakurai, 2007)and orexin neurones are activated just before awakening, remain activated during wakefulness and cease firing during sleep (Kuwaki and Zhang, 2010). Active waking causes increase in HR, MAP, respiration and locomotor activity. However, the neural mechanisms and pathways that mediate the pressor and tachycardic responses to arousal are poorly understood. The presence of orexin receptors in C1 bulbospinal sympatho-excitatory neurones of the rostral RVLM and the effects of orexin A in the RVLM on basal cardio-respiratory parameters and adaptive reflexes suggest that orexin receptor activation plays a key role in mediating the sympatho-excitatory responses to arousal. Finally, the results suggest that drugs affecting central orex-

inergic pathways may be a useful target for the pharmacological treatment of disorders such as hypertension.

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Conflict of interest

The authors have no conflict of interest to declare.

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