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RESEARCH PAPER

Intrathecal orexin A increases sympathetic outflow and respiratory drive, enhances baroreflex sensitivity and blocks the somato-sympathetic reflex

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BACKGROUND
Intrathecal (i.t.) injection of orexin A (OX-A) increases blood pressure and heart rate (HR), but the effects of OX-A on sympathetic and phrenic, nerve activity, and the baroreflex(es), somato-sympathetic and hypoxic chemoreflex(es) are unknown.

EXPERIMENTAL APPROACH
Urethane-anaesthetized, vagotomized and artificially ventilated male Sprague-Dawley rats were examined in this study. The effects of i.t. OX-A (20 nmol 10 μL^{-1}) on cardiorespiratory parameters, and responses to stimulation of the sciatic nerve (electrical), arterial baroreceptors (phenylephrine hydrochloride, 0.01 mg kg^{-1} i.v.) and peripheral (hypoxia) chemoreceptors were also investigated.

KEY RESULTS
i.t. OX-A caused a prolonged dose-dependent sympathoexcitation, pressor response and tachycardia. The peak effect was observed at 20 nmol with increases in mean arterial pressure, HR and splanchnic sympathetic nerve activity (sSNA) of 32 mmHg, 52 beats per minute and 100% from baseline respectively. OX-A also dose-dependently increased respiratory drive, as indicated by a rise in phrenic nerve amplitude and a fall in phrenic nerve frequency, an increase in neural minute ventilation, a lengthening of the expiratory period, and a shortening of the inspiratory period. All effects of OX-A (20 nmol) were attenuated by the orexin receptor 1 antagonist SB 334867. OX-A significantly reduced both sympathoexcitatory peaks of somato-sympathetic reflex while increasing baroreflex sensitivity. OX-A increased the amplitude of the pressor response and markedly amplified the effect of hypoxia on sSNA.

CONCLUSIONS
Thus, activation of OX receptors in rat spinal cord alters cardiorespiratory function and differentially modulates sympathetic reflexes.

Abbreviations
AIH, acute intermittent hypoxia; AUC, area under the curve; ECG, electrocardiogram; HR, heart rate; ICM, intracisternomagna; ICV, intracerebroventricular; IML, intermediolateral cell column; i.t., intrathecal; LTF, long term facilitation; MAP, mean arterial pressure; OX, orexin; OX-A, orexin A; OX-B, orexin B; OX_1, orexin receptor 1; OX_2, orexin receptor 2; PE, phenylephrine hydrochloride; PNA, phrenic nerve activity; PNamp, phrenic nerve amplitude; PNf, phrenic nerve frequency; RVLM, rostral ventrolateral medulla; SAP, systemic arterial pressure; SB 334867 (N-(2-methyl-6-benzoxazolyl)-N-1,5-naphthyridin-4-yl-urea; SNP, sodium nitroprusside; SPN, sympathetic preganglionic neurones; sSNA, splanchnic sympathetic nerve activity; T_E, expiratory period; T_I, inspiratory period

Introduction

Orexin A (OX-A) and orexin B (OX-B), also referred to as hypocretin-1 and -2, are neuropeptides that are cleaved from a common precursor, prepro-orexin. The amino acid sequences of the 33-residue peptide OX-A and the 28-residue peptide OX-B are encoded by a single gene localized on human chromosome 17q21 and share 46% homology (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998). The actions of these peptides are mediated by two G-protein coupled receptors, orexin receptor 1 (OX_1) and orexin receptor 2 (OX_2). OX-A is 10 times more selective for OX_1 than OX-B, while OX-A and OX-B have an equal affinity for OX_2 (Sakurai *et al.*, 1998). Activation of OX_1 results in the activation of $G\alpha_{q/11}$, which induces the elevation of intracellular Ca^{2+}, and the OX_2 couples to both $G\alpha_{q/11}$ and inhibitory $G\alpha_i$ G proteins (Zhu *et al.*, 2003).

Immunohistochemical and *in situ* hybridization studies have revealed that OX-containing cell bodies are restricted to the lateral hypothalamus, perifornical area and dorsomedial hypothalamus. OX-containing nerve terminals and receptors, on the other hand, are widely distributed in the hypothalamus, thalamus, cerebral cortex, circumventricular organs, brainstem and spinal cord (Elias *et al.*, 1998; Nambu *et al.*, 1999; Llewellyn-Smith *et al.*, 2003). This distribution of OX-nerve terminals establishes a basis for the contributions by OX to the control of multiple physiological functions, including control of energy homeostasis, feeding behaviour, sleep–wake state, stress response, reward and nociception (Kukkonen *et al.*, 2002; Sakurai, 2007; Tsujino and Sakurai, 2009).

There is a growing body of evidence to suggest that OX is involved in central cardiovascular and respiratory control. Intracerebroventricular (third ventricle) or intracisternomagnal application of OX-A or OX-B augments sympathetic outflow and catecholamine release, and a dose-dependent increase in systemic arterial pressure and heart rate (HR) (Shirasaka *et al.*, 1999; Matsumura *et al.*, 2001; Zhang *et al.*, 2005). On the other hand, prepro-orexin knockout mice with a complete lack of both OX-A and OX-B manifest lower baseline arterial pressure than the wild-type controls (Kayaba *et al.*, 2003). However, the effects of intrathecal OX-A on *in vivo* splanchnic sympathetic nerve activity (sSNA), phrenic nerve activity (PNA) as well as on sympathetic reflexes are unknown.

Sympathetic preganglionic neurones (SPN), located in the intermediolateral cell column (IML) of the spinal cord, receive inputs from different brain regions and regulate the cardiovascular responses through their projections to the adrenal medulla and sympathetic autonomic ganglia in periphery (Pilowsky and Goodchild, 2002; Guyenet, 2006). Orexinergic fibres and receptors are distributed throughout the spinal cord, including IML. OX fibres have also been found in the dorsal and ventral horn neurones of the spinal cord (van den Pol, 1999; Date *et al.*, 2000; Cluderay *et al.*, 2002). The dense innervation of all spinal cord regions by OX fibres, expression of OX receptors on SPN and the depolarizing action of OX on spinal neurones (van den Top *et al.*, 2003) also suggest that OX is a neuropeptide in the spinal cord. Therefore, it is likely that OX not only regulates cardiovascular function acting as a neurotransmitter in the spinal cord but also modulates sympathetic reflexes.

Because OX-A has a greater selectivity for the OX_1, we first evaluated the hypothesis that OX-A, by acting on SPN, might influence spinal sympathetic outflow, as assessed by a change in mean arterial pressure (MAP), HR, sSNA when delivered intrathecally (i.t.). Additionally, the effects of i.t. OX-A on PNA and cardiovascular responses to stimulation of somatosympathetic, arterial baroreceptor and peripheral chemoreceptor reflexes were examined. Our principal findings are that i.t. OX-A causes a dose-dependent increase in MAP, HR and sSNA, and an increase in phrenic nerve discharge. Adaptive reflexes are differentially affected: barosensitivity is enhanced, the somato-sympathetic reflex is attenuated, and the hypoxic chemoreflex is enhanced for MAP and sSNA. The data reveal that OX-A has pleiotropic effects on cardiorespiratory functions and reflexes that warrant further investigation. Part of this work was presented to the Australian Neuroscience Society (Shahid and Pilowsky, 2010).

Methods

All animal experiments in this study 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/publications/synopses/eal6syn.htm) and were approved by the Animal Ethics Committee of Macquarie University, Sydney, Australia.

Surgical preparation

Surgical preparation was performed as described previously (Burke *et al.*, 2008; Farnham *et al.*, 2008; Gaede *et al.*, 2009). Briefly, male Sprague-Dawley rats (n = 38, 350–600 g) were anaesthetized with urethane (1.2–1.4 $g \cdot kg^{-1}$, i.p.). The depth of anaesthesia was assessed approximately every 30 min by monitoring changes in arterial pressure in response to pinching a hind paw; supplemental doses of urethane (20–30 mg, i.v.) were given if blood pressure rose more than 10 mmHg. Animals were placed on a feedback-controlled heating blanket for the duration of the experiment to maintain body temperature between 36°C and 37°C (Harvard Apparatus, Holliston, MA, USA).

The right jugular vein and carotid artery were cannulated with polyethylene tubing [internal diamter (I.D.) = 0.58 mm; outer diameter (O.D. = 0.96 mm] for administration of drugs and fluids, and for measurement of arterial blood pressure (AP) respectively. In some experiments, both femoral veins were cannulated to enable administration of sodium nitroprusside (SNP) or phenylephrine hydrochloride (PE). HR was derived from AP. A tracheal cannula permitted artificial ventilation. Ventilation was adjusted so that phrenic nerve discharge was just above the apnoeic threshold. Nerve recordings were made of PNA and sSNA. The left phrenic nerve was approached dorsally and the left greater splanchnic nerve was dissected using a retro-peritoneal approach. The distal end of the respective nerves were tied with silk thread and cut to permit recording of efferent nerve activity. In an additional subset of animals, the sciatic nerve was isolated, tied and cut. Once the nerves were isolated, they were covered with saline-soaked cotton wool for the duration of the remainder of surgical preparation to prevent desiccation. The neurograms

were amplified (×10 000, CWE Inc., Ardmore, PA, USA), band-pass 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, Cambridge, UK).

Rats were secured in a stereotaxic frame, paralysed (pancuronium bromide; 0.8 mg i.v. initially, then 0.4 mg·h^{-1} i.v.) and artificially ventilated with oxygen-enriched room air. End-tidal CO_2 was monitored and maintained between 4.0% and 4.5%. Arterial blood gas was monitored, and the rate and depth of ventilation was adjusted to maintain pH at an optimum range (7.35–7.45, pH 7.4 ± 0.01, P_aCO_2 40.9 ± 0.8). Animals were infused with 5% glucose in saline (1.0–2.0 mL·h^{-1}) to ensure hydration. Three needle electrodes were placed under the skin at the right and left arm and the right hind limb to record an electrocardiogram. Nerve recordings were made with bipolar silver wire electrodes. The recording electrodes were immersed in a pool of liquid paraffin oil to prevent dehydration and for electrical insulation. After being placed on the recording electrodes, the rat was allowed to stabilize for 30–60 min.

Activation of sympathetic reflexes

Reflexes were evoked as described previously (Miyawaki *et al.*, 2002a; Makeham *et al.*, 2004). Activation of somato-sympathetic reflex was achieved by electrical stimulation (10–25 V, 100, 0.2 ms pulses at 1 Hz) of the sciatic nerve with bipolar electrodes. Sympathetic baroreflex function curves were generated by sequential intravenous injection of SNP (0.01 mg·kg^{-1}) and PE (0.01 mg·kg^{-1}) via two different vein lines. Peripheral chemoreceptors were stimulated with a brief period of hypoxia induced by ventilating the animals with 100% N_2 for 12–14 s.

i.t. Drug administration

The occipital crest of the skull was exposed, and the atlanto-occipital membrane was incised. A polyethylene catheter (I.D. = 0.4 mm; O.D. = 0.8 mm) was inserted through this slit into the i.t. space and advanced caudally to the levels of T6–T8. The slit was left open to prevent increases in i.t. pressure caused by the injection of agents or by flushing. The volume of each catheter was measured before insertion (range 6–7 μL), and this volume was then used to flush the catheter. A 25-μL Hamilton syringe was used to inject drugs (OX-A; 100 μM, 500 μM, 1 mM and 2 mM equivalent to 1, 5, 10 and 20 nmol, respectively) or vehicle [10 mM phosphate-buffered saline (PBS); pH 7.4] in a total volume of 10 μL. Injections were made over a 15- to 20-s period. In 11 animals, a selective OX_1 antagonist, N-(2-methyl-6-benzoxazolyl)-N-1,5-naphthyridin-4-yl-urea 200 nmol (SB 334867; Tocris Bioscience, Bristol, UK), was injected i.t. 20 min prior to the i.t. injection of OX-A (20 nmol): six with OX-A and five without. Successful catheterization into the i.t. space was confirmed by administering L-glutamate (100 mM, 10 μL) and observing sharp increases in blood pressure (~20 mmHg), HR [~30 beats per minute (bpm)] and sSNA (~30%) (e.g. Hong and Henry, 1992).

The following reflexes were activated before and 40 or 15 min after i.t. injection of OX-A (20 nmol) or PBS, respectively: (i) somato-sympathetic reflex; (ii) baroreflex; and (iii) peripheral chemoreceptor reflex. The spread of injectate was determined by observing the distribution of a 10 μL injection of India ink. Following the death of the animals (3 M KCl, 0.5 mL, i.v.), a laminectomy was performed to verify the location of the catheter tip. To avoid possible confounding effects of drug interactions, each animal received only one treatment of OX-A.

Temporary spinal blockade by microinjection of bupivacaine anaesthetic at the C8 spinal level

The spinal cord was anaesthetized at the C8 level by microinjection of a local anaesthetic, bupivacaine (500 nL, AstraZeneca, Australia) into the middle of each hemi-spinal cord in four animals. These injections were always adequate to cause blood pressure and sympathetic nerve activity to fall to levels equivalent to that seen following spinal transection at the C8 level (Goodchild *et al.*, 2008). Following local anaesthetic injection at C8, OX-A (20 nmol) or PBS was injected i.t.

Data acquisition and analysis

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 6.01). To analyse blood pressure, HR, sSNA, phrenic nerve amplitude (PNamp), phrenic nerve frequency (PNf), neural minute ventilation (=PNamp × PNf), duration of inspiratory burst (T_I) and duration of expiratory period (T_E), baseline values were obtained by averaging 60 s of data, 5 min prior to drug or PBS injection. Maximum responses were expressed as absolute changes in MAP, HR and PNf, and percent changes in sSNA, PNamp and neural minute ventilation from baseline values. Time course analysis averaged 60 s of data every 5 min from 0 min to 60 or 70 min post-injection for OX-A. To evaluate cardiorespiratory coupling, phrenic-triggered ensemble averages of sSNA were generated from 60-s portions of data. 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 sympathoexcitatory peaks was analysed. The maximum response to stimulation was then expressed as a percentage change from the baseline (control). 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 percentage changes were calculated according to the formulae below.

$$\frac{OXAHR - CHR}{CHR} \times 100$$

$$\frac{PBS - CHR}{CHR} \times 100$$

Where OXAHR is the response to hypoxia following OX-A, PBSHR is the response to hypoxia following PBS injection.

CHR is the response to hypoxia in the absence of any i.t. injection.

Analysis was conducted with GraphPad Prism (version 5.0) (GraphPad, La Jolla, CA, USA). All values are expressed as mean ± standard error. Results are presented as control versus OX-A. Paired *t*-test was used to analyse peak effects and reflexes. A one-way repeated-measures analysis of variance with Dunnett's *post hoc* multiple comparison test was used to compare values after OX-A (20 nmol) administration with the baseline value. $P < 0.05$ was considered significant.

Drugs

OX-A (molecular weight = 3561.2) was obtained from Bachem AG (Bubendorf, Switzerland), SB 334867 from Tocris Bioscience. Urethane, L-glutamate, PE and SNP were purchased from Sigma-Aldrich (St. Louis, MO, USA); pancuronium bromide and bupivacaine from AstraZeneca Pty Ltd (Sydney, NSW, Australia); and PBS (10 mM in 0.9% NaCl) tablets from AMRESCO Inc. (Solon, OH, USA). OX-A was dissolved and further diluted in PBS (10 mM; pH 7.4). SB 334867 was dissolved and further diluted in 10% dimethyl sulphoxide. PBS, PE and SNP were prepared in de-ionized water. Urethane was dissolved in 0.9% NaCl, and L-glutamate was dissolved in PBS.

Results

Effects of i.t. OX-A on resting cardio-respiratory parameters

To test the hypothesis that exogenous application of OX-A to the spinal cord modulates cardiorespiratory responses, OX-A (1, 5, 10 and 20 nmol) was administered i.t., and the effects on MAP, HR, sSNA, PNf, PNamp, neural minute ventilation, T_E and T_I were evaluated ($n = 34$). OX-A evoked a dose-dependent and significant increase in MAP, HR and sSNA (Figure 1A, B). The maximum elevation occurred after injection of 20 nmol OX-A, and the highest levels attained were 32 ± 5 mmHg (MAP, $n = 6$, $P < 0.01$), 52 ± 6 bpm (HR, $n = 6$, $P < 0.001$) and 100 ± 9% (sSNA, $n = 6$, $P < 0.001$) from the baseline (Figure 1A, B). Following administration of OX-A (20 nmol, i.t.), the peak pressor effect was reached after approximately 20 min (Figure 1A, C). Blood pressure was monitored for about 70 min, and remained elevated (Figure 1C). Both HR and sSNA increased progressively after 20 nmol OX-A injection, reaching a maximum change of 35 bpm and 97%, respectively, at the end of the recording period (Figure 1A, C). Injection of PBS (vehicle) into the spinal cord was without effect on MAP (5 ± 1 mmHg, $n = 6$), HR (5 ± 1 bpm, $n = 6$) or sSNA (8 ± 1%, $n = 6$) above basal values (Figure 1A–C).

Injection of OX-A (1, 5, 10 and 20 nmol) evoked a dose-dependent increase in PNamp, and neural minute ventilation and a decrease in PNf (Figures 2A, B and 3A). The maximum decrease in PNf of 18 ± 2 bursts·min^{-1} ($n = 6$, $P < 0.01$) was elicited by OX-A (20 nmol). OX-A (20 nmol) also caused a peak increase in PNamp of 62 ± 9% ($n = 6$, $P < 0.01$) and neural minute ventilation of 53.47 ± 12% ($n = 6$, $P < 0.05$) from the baseline (Figure 3A). Following OX-A (20 nmol) administration, PNf decreased, PNamp and neural minute ventilation increased gradually reaching peak levels after about 30 min (Figure 2A, B). There is a clear dose–response relationship between the concentration of OX-A and increasing PNamp (Figure 3A). A dose–response relationship is also seen between the concentration of OX-A and the fall in PNf (Figure 3A). However, the maximum effect on PNamp is seen at 20 nmol, while the effect on PNf is maximal at 5 nmol; this means that beyond 5 nmol, there is an increasing effect on phrenic neural minute ventilation. OX-A (20 nmol) caused a lengthening of T_E and a shortening of T_I, reaching a peak level of 0.75 ± 0.1 s ($n = 5$, $P < 0.05$) and –0.122 ± 0.03 s ($n = 3$, $P < 0.05$) respectively (Figures 2A, B and 3B). No significant changes in PNf, PNamp, neural minute ventilation, T_E or T_I were observed after injection of PBS (vehicle) (Figures 2A, B and 3A, B).

In the absence of OX-A, SB 334867 (200 nmol) did not affect MAP, HR, sSNA, PNamp, PNf or neural minute ventilation ($n = 5$) (data not shown). On the other hand, the cardio-respiratory effects of OX-A (20 nmol) were significantly reduced by prior i.t. injection of SB 334867 [MAP: 9 ± 2 vs. 32 ± 5 mmHg, $P < 0.01$; HR: 16 ± 6 vs. 52 ± 6 bpm, $P < 0.01$; sSNA: 28 ± 6 vs. 100 ± 9%, $P < 0.01$; PNamp: 34 ± 3 vs. 62 ± 8%, not significant (ns); PNf: –8 ± 1 vs. –18 ± 2 bursts·min^{-1}, $P < 0.05$; neural minute ventilation: 23 ± 6 vs. 53 ± 12%, ns, of baseline; $n = 6$, Figures 1B and 3A].

Peri-phrenic averaging of the sympathetic nerve activity reveals an inspiratory (I) and post-inspiratory (P-I) peak of sSNA (Figure 4A). The amplitude of P-I peak of sSNA was significantly increased by i.t. injection of 20 nmol OX-A (153 ± 41% vs. 13 ± 7% of PBS; $P < 0.05$; $n = 5$, Figure 4A, C) over baseline (control). OX-A showed a gradual increase in P-I peak over time (Figure 4B). In contrast, no significant change in the amplitude of I peak was observed (43 ± 9% vs. 27 ± 3% of PBS; ns; $n = 5$, Figure 4A, C).

Effects of bupivacaine anaesthesia at the C8 spinal level on OX-A activity

C8 anaesthesia caused a fall in MAP (from 95 ± 7 to 59 ± 3 mmHg), HR (from 459 ± 8 to 408 ± 19 bpm) and sSNA (–45 ± 12%) without any change in PNA ($n = 3$, Figure 5B). Following C8 transection, i.t. OX-A (20 nmol) caused a greater increase in MAP, HR and sSNA, but for a shorter period when compared with intact animals (MAP: 74 ± 7 vs. 32 ± 5 mmHg, $P < 0.05$; HR: 51 ± 10 vs. 52 ± 6 beats·min^{-1}, ns; sSNA: 292 ± 26 vs. 100 ± 9%, $P < 0.05$, of the baseline, Figure 5A–C). Conversely, the effects of OX-A on PNA were abolished when compared with the response prior to C8 anaesthesia (PNamp: 10 ± 4 vs. 62 ± 8%, $P < 0.05$; PNf: 0.7 ± 1 vs. –18 ± 2 bursts·min^{-1}, $P < 0.05$, of the baseline; Figure 5A–C). PBS injection after C8 anaesthesia induced no change in MAP, HR, sSNA or PNA ($n = 1$; data not shown).

Effects of OX-A on somato-sympathetic reflex

The average sSNA response to intermittent stimulation of the sciatic nerve was evaluated (somato-sympathetic reflex) before and after i.t. injection of PBS (vehicle) or OX-A (20 nmol). In five animals, intermittent stimulation of the sciatic nerve resulted in two characteristic excitatory peaks in sSNA with latencies of 93 ± 3 ms and 188 ± 2 ms ($n = 5$, Figure 6A). The latencies were not significantly altered by PBS

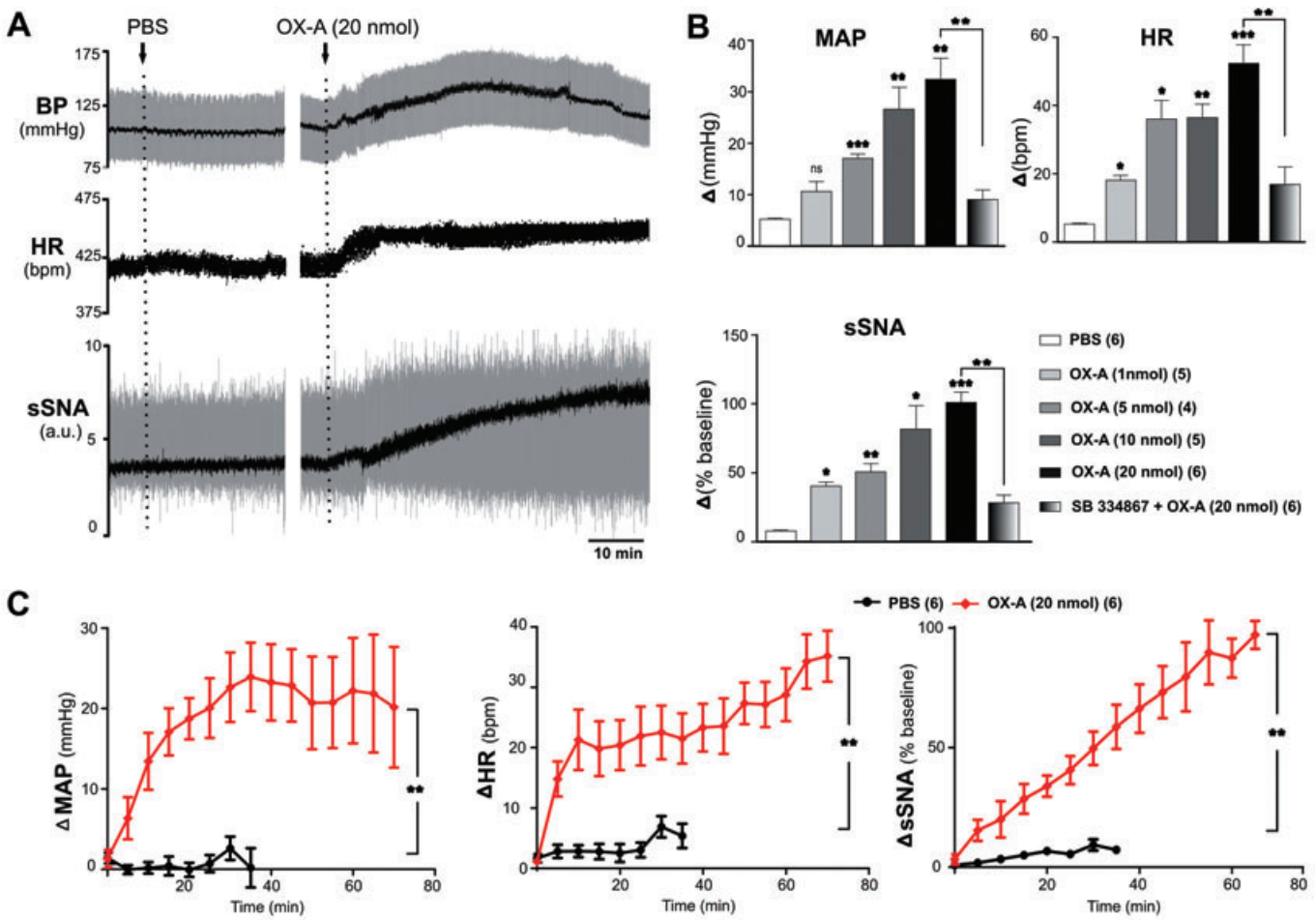

Figure 1

Effect of intrathecal injection of orexin A (OX-A) on mean arterial pressure (MAP), heart rate (HR) and splanchnic sympathetic nerve activity (sSNA). (A) Representative trace of data from a recording of blood pressure (BP), HR and sSNA (arbitrary unit, a.u.) before and after injection of phosphate-buffered saline (PBS) or OX-A (20 nmol). Rectified and integrated sSNA (black) is superimposed over raw sSNA (grey). MAP (black) is superimposed over BP (grey). (B) Comparison of peak cardiovascular effects produced by PBS, OX-A (1, 5, 10 and 20 nmol) or SB334867 (200 nmol) + OX-A (20 nmol). Peak effects are shown as absolute (BP, HR) or percentage (sSNA) change from their respective basal values. (C) Grouped time course effects of PBS (black) or OX-A (20 nmol) (red) on MAP, HR and sSNA. Values are expressed as mean ± standard error. Number of animals are shown in parentheses. bpm, beats per minute; ns, non-significant; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared with PBS [except SB 334867 (200 nmol) + OX-A (20 nmol) that was compared with OX-A (20 nmol)].

(91 ± 3 ms and 186 ± 1 ms, $n = 5$, ns) or by OX-A (89 ± 2 ms and 191 ± 4 ms, $n = 5$, ns) injection. OX-A significantly increased the basal sSNA to 221 ± 22% ($n = 5$, $P < 0.05$) as compared with PBS (100 ± 7%). OX-A markedly attenuated the amplitude of both excitatory peaks. The first and second sympatho-excitatory peaks were attenuated by 25 ± 10% and 72 ± 13% of the baseline ($n = 5$, $P < 0.05$, Figure 6A), as compared with the effect seen following PBS injection (Figure 6A).

Effects of OX-A on baroreflex

In five animals, the changes in sSNA were plotted against the changes in MAP evoked by i.v. injection of SNP and PE. The changes in AP following PE injection were of nearly equal magnitude (71 ± 7 mmHg following PBS injection, and 65 ± 8 mmHg following OX-A injection). In fact, the blood pressure increase following PE after OX-A injection was, if anything, slightly smaller than the increase that followed PBS injection. OX-A (20 nmol, i.t.) significantly enhanced the reflex sympathoexcitatory and inhibitory responses evoked by these equipotent doses of SNP and PE (Figure 6B). OX-A significantly increased the upper plateau, range of sSNA and maximum gain of the sSNA without significantly altering lower plateau, the threshold level, midpoint, the saturation levels of MAP and the operating range as compared with PBS (Table 1).

Effects of OX-A on peripheral chemoreflex

Activation of peripheral chemoreceptors with brief hypoxia evoked an increase in MAP, sSNA and HR (Figure 6C). Peak effects occurred near the end of stimulus and recovered rapidly to baseline. Injection of OX-A (20 nmol) into the

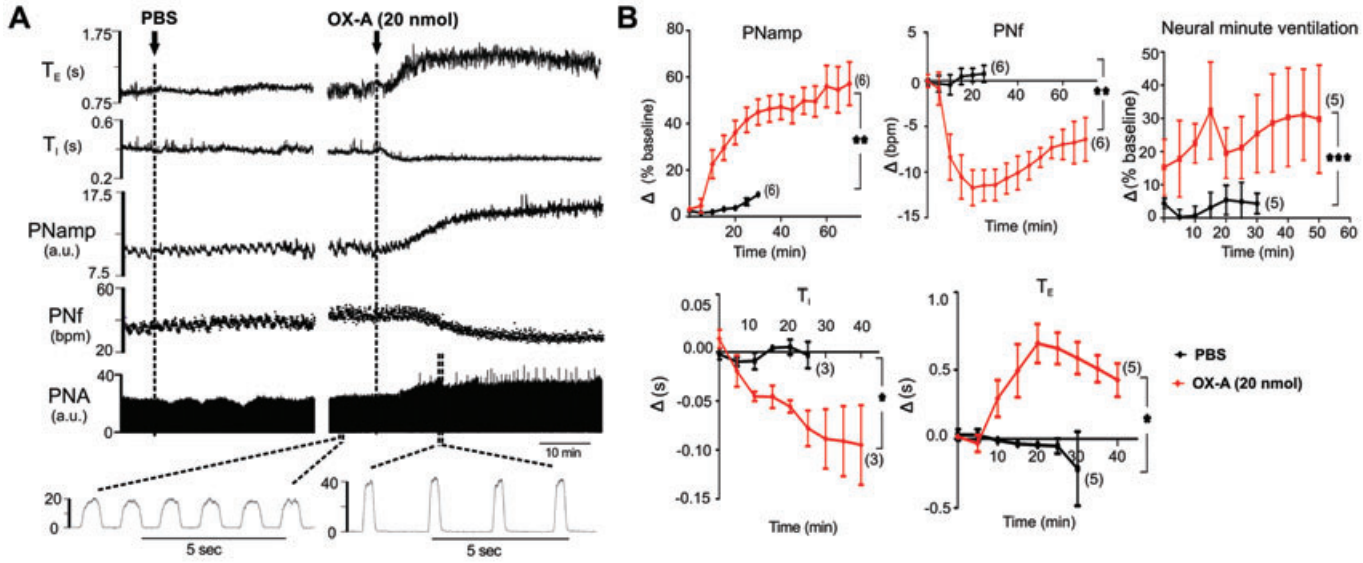

Figure 2

Effect of intrathecal injection of orexin A (OX-A)on phrenic nerve activity (PNA). (A) Representative trace of data from a recordings of rectified PNA (arbitrary unit, a.u.), phrenic nerve frequency (PNf), phrenic nerve amplitude (PNamp), inspiratory period (T_I) and expiratory period (T_E) before and after injection of phosphate-buffered saline (PBS) or OX-A. (B) Grouped time course effects of PBS (black) or OX-A (20 nmol) (red) on PNamp, PNf, neural minute ventilation, T_I and T_E. Following injection of OX-A there is an increase in PNamp associated with a bradypnoea that is due to both an increase in T_E and a decrease in T_I. There is an overall increase in neural minute ventilation over the period of the response. Values are expressed as mean ± standard error. Number of animals is shown in parentheses. bpm, beats per minute; ns, non-significant; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared with PBS.

spinal cord significantly increased the pressor response which was 25 ± 4% greater than control, compared with the PBS response which was only 1 ± 1% greater than control ($n = 5$, $P < 0.01$, Figure 6C). The sympathoexcitatory effect was also markedly increased by OX-A compared with the response following PBS (94 ± 7% vs. 6 ± 7%, $n = 5$, $P < 0.001$, Figure 6C). The effect on HR was unchanged by OX-A (–25 ± 10 vs. –10 ± 11%, $n = 5$, ns, Figure 6C). OX-A increased peak PNf, but had no effect on the peak amplitude of PNamp and neural minute ventilation during hypoxia [PNf: 21 ± 5 vs. 1 ± 2%, $P < 0.05$; peak PNamp: –35 ± 11 vs. –21 ± 12%, ns; neural minute ventilation: 7 ± 5 vs. –4 ± 7%, ns, of control baseline ($n = 5$)].

Discussion

This study is the first to investigate the *in vivo* effects of i.t. OX-A on sympathetic outflow, phrenic burst discharge and sympathetic physiological reflexes. The major findings are that i.t. injection of OX-A in the 1–20 nmol range (i) elicits sympathetically mediated hypertension and tachycardia, confirming an earlier report (Antunes *et al.*, 2001); (ii) modulates respiratory drive by increasing PNamp and decreasing PNf; (iii) reduces both sympathoexcitatory peaks in response to sciatic nerve stimulation; (iv) increases sympathetic baroreflex sensitivity; and (v) potentiates the pressor responses and sympathoexcitation to hypoxia.

SPN integrate the central nervous system output to vasomotor pathways and chromaffin cells (Gilbey, 1997). L-Glutamate, or a related amino acid, is likely to be the major excitatory neurotransmitter to these neurones (Pilowsky and Goodchild, 2002; Pilowsky *et al.*, 2009). Extensive evidence supports the view that the excitatory cardiovascular effects in the spinal cord are modulated by a number of neuropeptides (Pilowsky *et al.*, 2009), including vasopressin (Riphagen and Pittman, 1989), angiotensin (Yashpal *et al.*, 1989), substance P (Yashpal *et al.*, 1987) and pituitary adenylate cyclase activating polypeptide (PACAP) (Farnham *et al.*, 2008).

OX-A fibres and nerve terminals are widely distributed throughout the spinal cord in rat, mouse and human (Cutler *et al.*, 1999; van den Pol, 1999; Date *et al.*, 2000). OX-A directly, and reversibly, depolarizes SPN by activation of pertussis toxin-sensitive G-proteins and closure of a K^+ conductance via a protein kinase A-dependent pathway (Antunes *et al.*, 2001; van den Top *et al.*, 2003). Pretreatment with α_1 and β-adrenoceptor antagonists attenuates the pressor and tachycardiac response to i.t. OX-A. Intravenous OX-A does not affect blood pressure or HR (Chen *et al.*, 2000; Antunes *et al.*, 2001). These findings support the idea that the pressor and tachycardiac effects induced by i.t. OX-A are mediated centrally. In addition, mRNA for OX_1 and OX_2 are expressed on the majority of SPN and neurones in the dorsal and ventral horns of the spinal cord (Cluderay *et al.*, 2002; van den Top *et al.*, 2003; Guan *et al.*, 2004). OX_1 is selective for OX-A and OX_2 interacts with both OX-A and OX-B (Sakurai *et al.*, 1998). As a selective OX_1 antagonist was unable to abolish the responses to OX-A completely, it seems likely that the excitatory effects of OX-A observed in our study may be due to both OX_1 and OX_2.

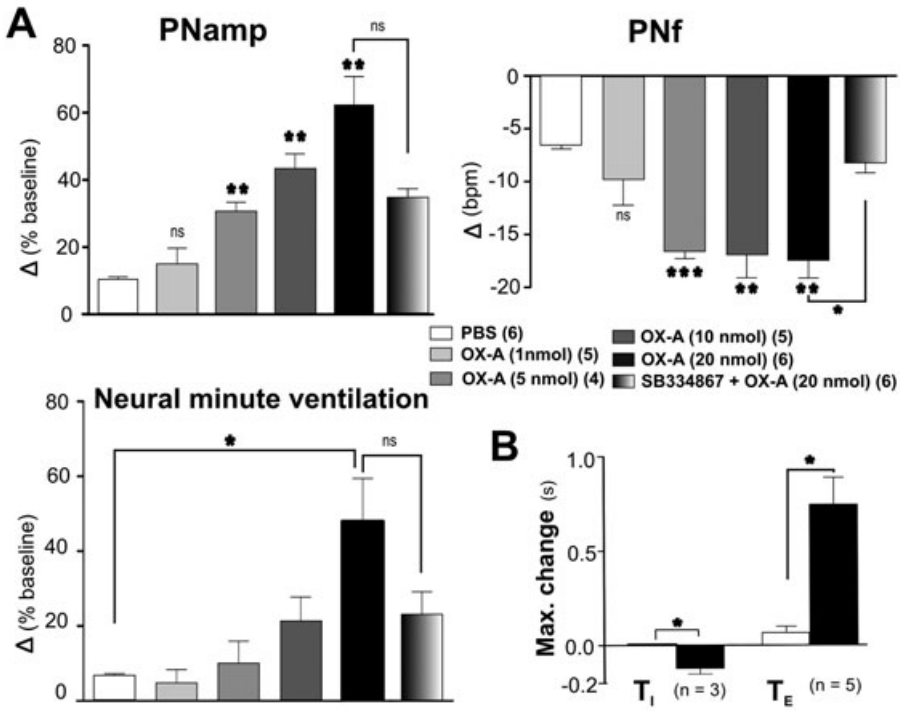

Figure 3

Effect of intrathecal injection of orexin A (OX-A)on phrenic nerve activity (PNA). (A) Comparison of peak effects produced by phosphate-buffered saline (PBS), OX-A (1, 5, 10 and 20 nmol) or SB 334867 (200 nmol) + OX-A (20 nmol) on PNamp, PNf and neural minute ventilation. Peak effects are shown as absolute or percentage change from respective basal values. (B) Grouped data illustrating the effects of PBS and OX-A (20 nmol) on T_I and T_E. Values are expressed as mean ± standard error. Number of animals is shown in parentheses. bpm, beats per minute; ns, non-significant; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared with PBS [except SB 334867 (200 nmol) + OX-A (20 nmol) that was compared with OX-A (20 nmol)].

An unanticipated finding of this study is that PNA increased after OX-A injection at T6–T8 spinal levels. The respiratory effects of i.t. OX-A do not appear to be due to either retrograde diffusion of the peptide into supra-spinal structures or diffusion to C3–C5 level, because (i) C8 anaesthesia abolished the effects of OX-A on PNA; and (ii) India ink injected i.t. at the end of the experiments only spread as far rostral as T2. Moreover, in our previous study, we reported that transection at the C1 level leaves the effects of i.t. bicuculline infusion unaltered (Goodchild *et al.*, 2000; 2008) and i.t. administration of drugs appear not to spread more than a few segments away from the site of administration (Yaksh and Rudy, 1976) and are able to penetrate around 2 mm (Yamada *et al.*, 1984). Furthermore, it is known that in an adult cat, SPN have dendrites that reach the dorsal pial surface of the spinal cord (Pilowsky *et al.*, 1994); in the neonatal rat, dendrites of SPN are reported to project towards the dorsolateral funiculus (Shen and Dun, 1990), but it is not yet known if they reach the sub-pial area in the adult. Paralysis, vagotomy and artificial ventilation of the animals rule out the possibility that OX-A acts directly on intercostal and abdominal motor neurones, thereby limiting lung inflation leading to an increase in phrenic nerve output. The mechanisms underlying the increase in PNamp, decrease in PNf and increase in neural minute ventilation following i.t. OX-A injection are currently unknown. Conceivably, activation of spinal OX receptors might affect sensory and intraspinal pathways, thereby modulating spinal pattern generators. On the other hand, the decrease in PNf is likely to be mediated by spino-bulbar connections modulating the respiratory rhythm generator.

The very long-lasting increase in PNA and sympathetic nerve activity (SNA) following i.t. OX-A seen here mimics the long-term facilitation (LTF) of PNA and SNA induced by acute intermittent hypoxia (AIH) (Xing and Pilowsky, 2010). A possible role for OX-A in AIH/LTF is supported by the finding that OX neurone ablated mice have a reduced propensity to develop AIH/LTF (Toyama *et al.*, 2009).

SNA activity displays a rhythmic fluctuation in relation to PNA (Adrian *et al.*, 1932; Miyawaki *et al.*, 2002b) that is dependent on the level of arterial CO_2. The significant increase in post-inspiratory peak, as well as change in baseline, activity of sSNA by i.t. OX-A may be due to the depolarization and increase in sensitivity of SPN (Date *et al.*, 2000; Antunes *et al.*, 2001; van den Top *et al.*, 2003). A large part of the enhancement of SNA seen here was clearly related to a marked increase in phrenic discharge-related activity in SNA.

Lamina I of spinal cord receives inputs from myelinated and unmyelinated nociceptors and transfers the information to brainstem including rostral ventrolateral medulla (RVLM) and higher regions of the brain (Sato and Schmidt, 1973;

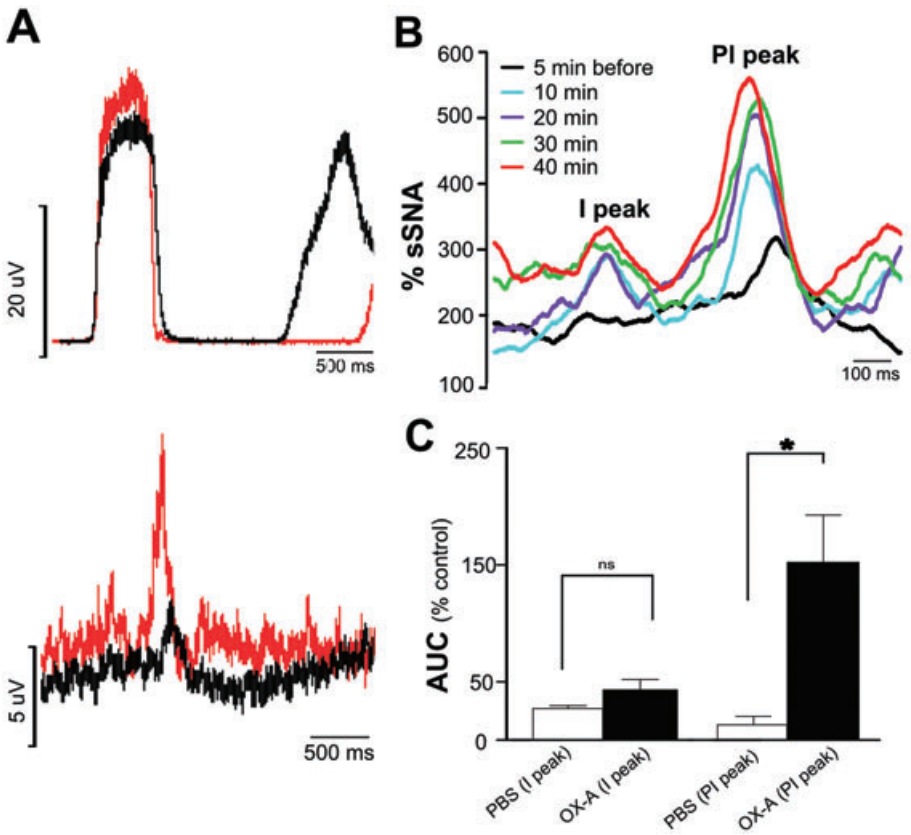

Figure 4

Effect of orexin A (OX-A) on phrenic nerve discharge-related rhythmicity of sSNA. (A) Phrenic-triggered average of sSNA before (black) and after (red) intrathecal injection of OX-A (20 nmol). (B) Time course effect of OX-A (20 nmol) on inspiratory (I) and post-inspiratory (PI) related activity of sSNA. (C) Grouped data illustrating the effects of OX-A (20 nmol, $n = 5$) on I and PI peaks of sSNA. Note that there is a marked increase in PI following OX-A injection. Values are expressed as mean ± standard error. ns, non-significant; * $P < 0.05$ compared with phosphate-buffered saline (PBS). Both PBS and OX-A values were normalized to the control period prior to injections.

McMullan *et al.*, 2008). The somato-sympathetic response, integrated in the RVLM, is relayed to SPN (Stornetta *et al.*, 1989; Miyawaki *et al.*, 2002a; Makeham *et al.*, 2005). The present study demonstrates that the activation of OX receptors in the spinal cord inhibited both the first and second sympatho-excitatory peaks of the somato-sympathetic reflex induced by sciatic nerve stimulation. This reflex inhibition was unrelated to OX-A-induced sympathoexcitation. The distribution of OX fibres as well as receptors in dorsal horn, including superficial lamina (van den Pol, 1999; Date *et al.*, 2000; Grudt *et al.*, 2002) and in dorsal root ganglionic cells (Bingham *et al.*, 2001) suggest that OX-A might modulate somato-sympahetic reflex at the spinal level. Activation of spinal γ-aminobutyric acid – 'A' subtype receptors is hypothesized to decrease nociceptive traffic in the spinothalamic tract. OX receptors have been found to increase pre-synaptic release of GABA in the lateral hypothalamus (van den Pol *et al.*, 1998). If this is also the case in spinal cord, i.t. OX-A-induced inhibition of somato-sympathetic reflex might be due to the modulation of sensory information from primary afferents by increasing the release of GABA. However, the precise mechanism is yet to be established.

RVLM neurones that are inhibited by baroreceptors project to SPN and provide the major descending excitatory input responsible for baroreflex activity (Pilowsky and Goodchild, 2002; Pilowsky *et al.*, 2009). Two pathways may mediate baroreceptor-induced sympathoinhibition: (i) inhibition of tonically active descending excitatory pathway, which is believed to be the principal mode of action (Pilowsky and Goodchild, 2002); and (ii) a direct spinal inhibitory mechanism (Wang *et al.*, 2008). The present study reveals an increase in the upper plateau, range and maximum gain of the baroreflex function curves obtained from sSNA, indicating that i.t. OX-A significantly increases baroreflex sensitivity. OX-A may modulate either the disfacilitation or the direct inhibition at the spinal level to enhance the baroreflex sensitivity.

Hypoxia causes a rapid and reversible excitation of reticulospinal sympathoexcitatory RVLM neurones that monosynaptically excite SPN. i.t. Kynurenate blocks the sympathetic excitation elicited by hypoxia, revealing the involvement of glutamatergic transmission (Sun and Reis, 1994). i.t. OX-A increases the amplitude of the pressor response and markedly amplifies the chemoreflex effect on sSNA. PNf was increased,

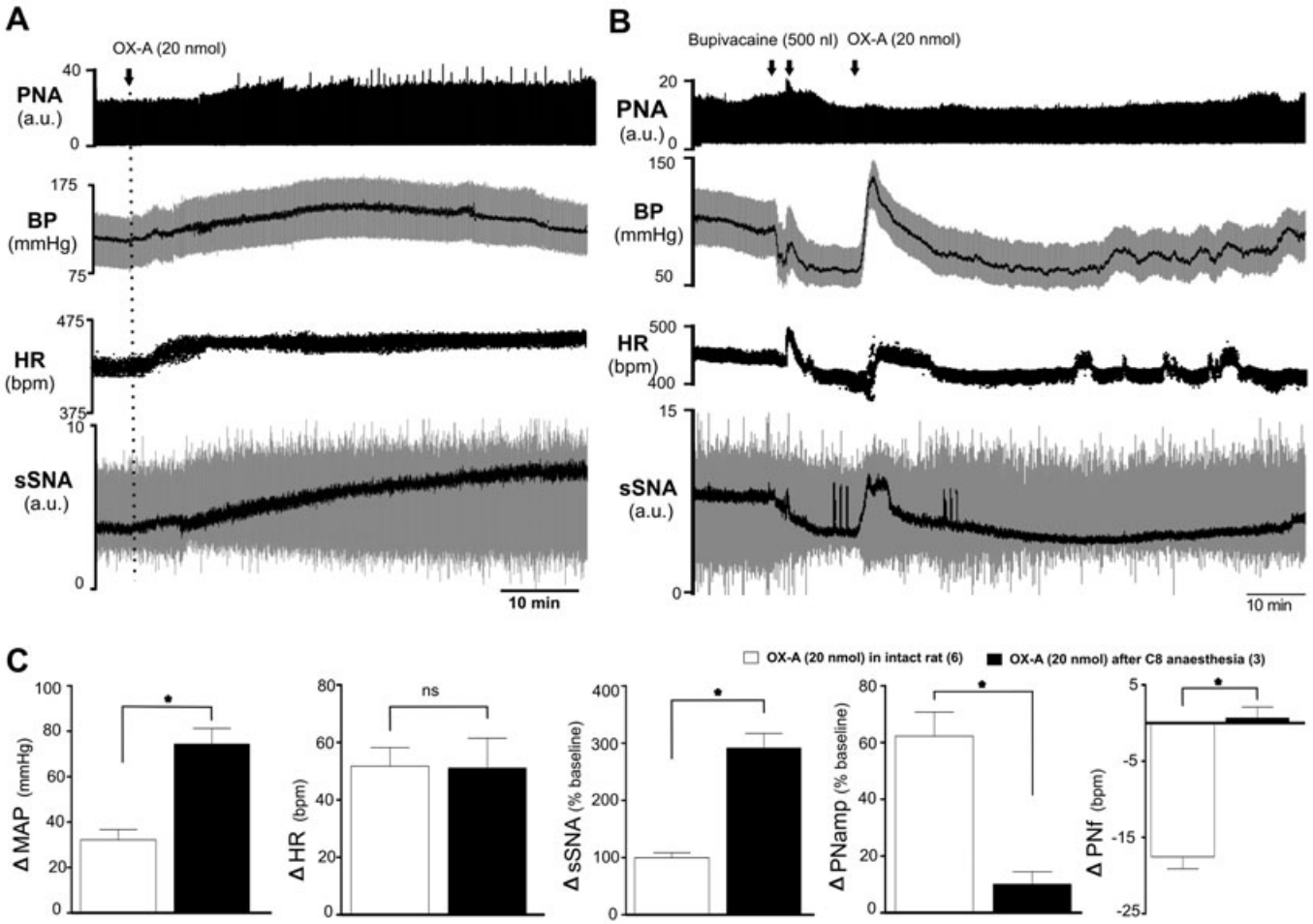

Figure 5

Effect of intrathecal orexin A (OX-A; 20 nmol) on mean arterial pressure (MAP), heart rate (HR), splanchnic sympathetic nerve activity (sSNA) and phrenic nerve activity (PNA) in an intact rat (A) and a C8 anaesthetized rat (B). (A) Representative trace of data from a recording of PNA, blood pressure (BP), HR and sSNA (arbitrary unit, a.u.) in intact rat. Integrated sSNA (black) is superimposed over raw sSNA (grey). MAP (black) is superimposed over blood pressure (BP) (grey). (B) Representative trace of data from a recording of PNA, BP, HR and sSNA in a C8 anaesthetized rat. (C) Comparison of peak cardio-respiratory effects produced by OX-A (20 nmol) in intact ($n = 6$) and C8 anaesthetized rat ($n = 3$). Peak effects are shown as absolute or percentage change from respective basal values. Values are expressed as mean ± standard error. Note that OX-A causes a greater increase in MAP, HR and sSNA for a shorter period when compared with the response prior to C8 anaesthesia. The effect on PNA was abolished. Number of animals is shown in parentheses. bpm, beats·per minute (HR) or bursts·per minute (PNf); ns, non-significant; *$P < 0.05$ compared with OX-response in intact animal.

but PNamp was not affected, and neural minute ventilation was unchanged following OX-A.

In conclusion, the present study reveals a direct excitatory effect of OX-A on SPN that is presumably mediated by the activation of OX_1 and OX_2 in the rat spinal cord and a potent modulation of respiratory drive to SPN. Our results also show that OX-A differentially modulates behavioural reflexes. Collectively, these data help to elucidate the role of the OX-A system in the regulation of autonomic nervous system and physiological reflexes.

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Conflict of interest

The authors have no conflicts of interest to declare.

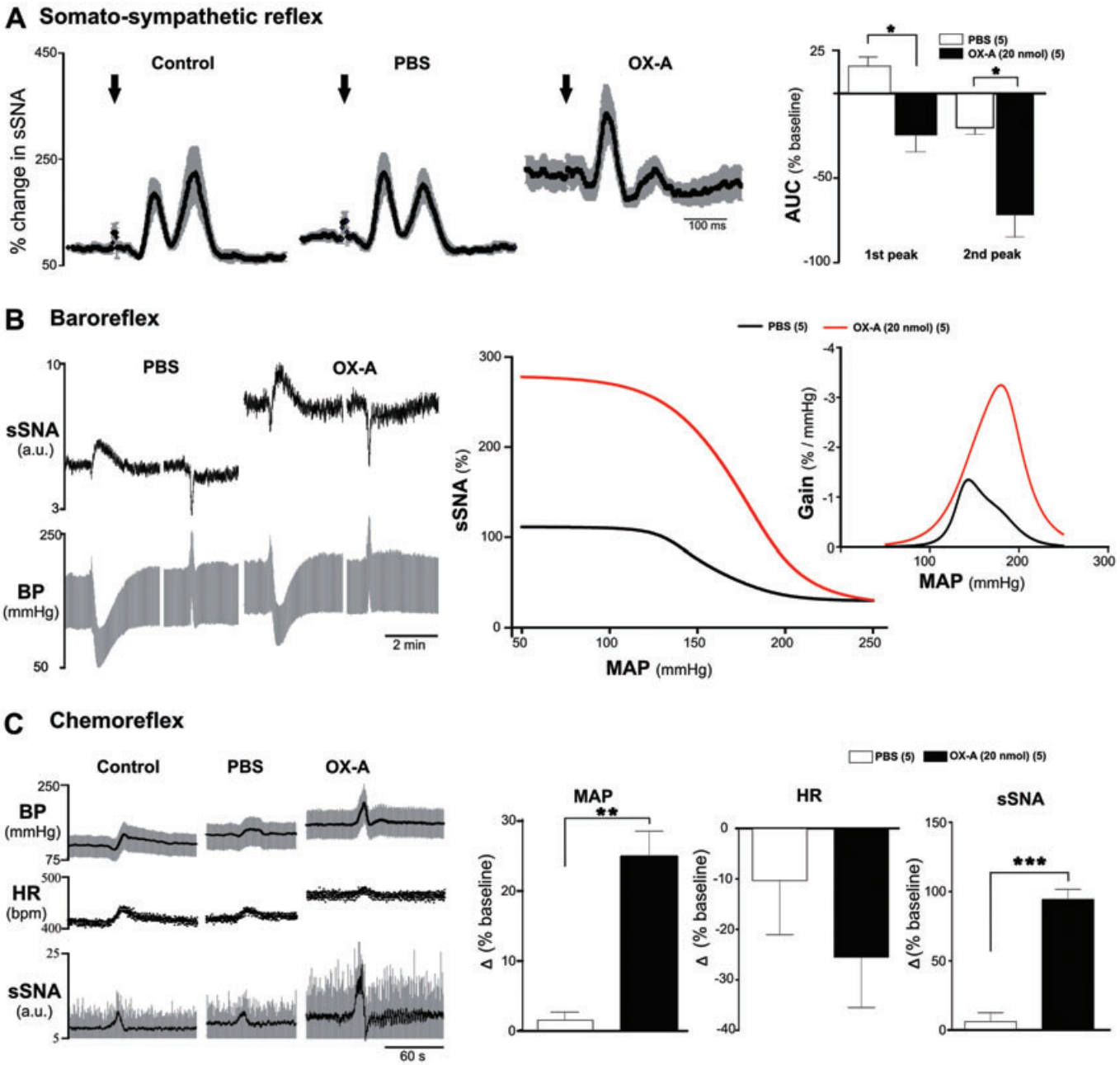

Figure 6

Effect of intrathecal injection of orexin A (OX-A) on somato-sympathetic, baroreceptor and peripheral chemoreceptor reflex. (A) Effect on somato-sympathetic reflex evoked by sciatic nerve (SN) stimulation. Left 3 traces represent grouped effect of SN-evoked stimulation of sSNA at control period and after injection of phosphate-buffered saline and OX-A. Data are mean (black) ± standard error (grey). Arrows indicate the time of stimulation. Trace at right represents group data illustrating the heights of 1st and 2nd sympathoexcitatory peaks. (B) Effect on baroreceptor reflex evoked by intravenous injection of SNP and PE. Left trace represents experimental recording of the effect of changes in BP on sSNA due to SNP or PE after phosphate-buffered saline (PBS) and OX-A injection. Middle trace shows average sympathetic baroreflex functional curves generated for data after PBS (black) or OX-A (red) injection. Trace at right represents baroreflex gain for sSNA (error bars are omitted for clarity). (C) Effect of peripheral chemoreceptor reflex activated by brief hypoxia with 100% N2 for 12–14 s. Left trace shows experimental recording of hypoxic episodes at control period and after PBS or OX-A injection. 3 traces of right represent comparison of peak effects on MAP, HR and sSNA after intrathecal injection of PBS and OX-A in response to brief hypoxia. Values are expressed as mean ± standard error. Number of animals is shown in parentheses. bpm, beats per minute; ns, non-significant; $^{**}P < 0.01$, $^{*}P < 0.05$ compared with PBS.

Table 1
Parameters describing baroreflex control of sSNA after intrathecal injection of PBS or OX-A (20 nmol)

	Lower plateau (%)	Upper plateau (%)	Mid-point (mmHg)	Max. gain (%/mmHg)	Range of SNA (%)	Threshold level (mmHg)	Saturation level (mmHg)	Operating range (mmHg)
PBS	29.7 ± 8.7	111.2 ± 2.3	157.9 ± 7.0	−1.8 ± 0.4	81.6 ± 9.4	118.0 ± 6.6	197.8 ± 12.9	79.7 ± 14.9
OX-A (20 nmol)	24.6 ± 16.9 (ns)	279.1 ± 38.0 ($P < 0.01$)	175.2 ± 6.2 (ns)	−3.6 ± 0.6 ($P < 0.05$)	254.6 ± 48 ($P < 0.01$)	121.4 ± 10.4 (ns)	228.9 ± 12.1 (ns)	107.5 ± 18.8 (ns)

Values are means ± standard error ($n = 5$). Maximum (Max.) gain is the slope of the sigmoid curve of best fit at the MAP corresponding to steepest part of the curve.
ns, non-significant; OX-A, orexin A; PBS, phosphate-buffered saline; SNA, sympathetic nerve activity.

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