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Intrathecal Intermittent Orexin-A Causes Sympathetic Long-Term Facilitation and Sensitizes the Peripheral Chemoreceptor Response to Hypoxia in Rats

Seung Jae Kim, Paul M. Pilowsky, and Melissa M. J. Farnham

Department of Physiology, Sydney Medical School, University of Sydney, and Heart Research Institute, Sydney, New South Wales, Australia

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ABSTRACT

Intermittent hypoxia causes a persistent increase in sympathetic nerve activity (SNA), which progresses to hypertension in conditions such as obstructive sleep apnea. Orexins (A and B) are hypothalamic neurotransmitters with arousal-promoting and sympathoexcitatory effects. We investigated whether the sustained elevation of SNA, termed sympathetic long-term facilitation, after acute intermittent hypoxia (AIH) is caused by endogenous orexin acting on spinal sympathetic preganglionic neurons. The role of orexin in the increased SNA response to AIH was investigated in urethane-anesthetized, vagotomized, and artificially ventilated Sprague-Dawley rats ($n = 58$). A spinally infused subthreshold dose of orexin-A (intermittent; 10 pmol × 10) produced long-term enhancement in SNA (41.4% ± 6.9%) from baseline. This phenomenon was not produced by the same dose of orexin-A administered as a bolus intrathecal infusion (100 pmol; 7.3% ± 2.3%). The dual orexin receptor blocker, Almorexant, attenuated the effect of sympathetic long-term facilitation generated by intermittent orexin-A (20.7% ± 4.5% for Almorexant at 30 mg•kg^{-1} and 18.5% ± 1.2% for 75 mg•kg^{-1}), but not in AIH. The peripheral chemoreflex sympathoexcitatory response to hypoxia was greatly enhanced by intermittent orexin-A and AIH. In both cases, the sympathetic chemoreflex sensitization was reduced by Almorexant. Taken together, spinally acting orexin-A is mechanistically sufficient to evoke sympathetic long-term facilitation. However, AIH-induced sympathetic long-term facilitation appears to rely on mechanisms that are independent of orexin neurotransmission. Our findings further reveal that the activation of spinal orexin receptors is critical to enhance peripheral chemoreceptor responses to hypoxia after AIH.

Introduction

Obstructive sleep apnea is a disorder characterized by episodes of repetitive apnea caused by intermittent collapse of upper airway muscles during sleep (Tilkian et al., 1976; Remmers et al., 1978). The apneic events give rise to frequent hypoxic episodes, and the stimulation of cardiorespiratory neurons in the brainstem and spinal cord (Sun et al., 2011). Consequently, a sequence of reflexes is activated, aimed at relieving the obstruction and promoting the restoration of normal breathing. These counterregulatory mechanisms maintain airway patency and are life-saving responses. However, persistence of intermittent hypoxia gives rise to detrimental changes in cardiorespiratory brain circuits. First, intermittent hypoxia induces a prolonged enhancement in sympathetic discharge from the cardioregulatory neurons, a phenomenon known as sympathetic long-term facilitation. In experimental models, acute intermittent hypoxia (AIH) (10 times 45-second bouts of 10% O_2) causes an immediate augmentation of sympathetic activity for at least 60 minutes (Dick et al., 2007; Xing and Pilowsky, 2010). Prolongation of intermittent hypoxia from an acute to a chronic intermittent hypoxia protocol (intermittent hypoxia for days or weeks) severely exacerbates sympathetic activity, eventually leading to the development of neurogenic hypertension over time in animals (Fletcher et al., 1992a,b; Xing and Pilowsky, 2010) and humans (Caples et al., 2005). Second, AIH and chronic intermittent hypoxia also cause a gradual increase in peripheral chemoreceptor sensitivity to hypoxia (Poon and Siniaia, 2000), another pathophysiologic feature in neurogenic hypertension (Cutler et al., 2004a,b; Leuenberger et al., 2005; Imadojemu et al., 2007). It is now widely accepted that mechanistic understanding of the molecular targets that attenuate or block sympathetic hyperactivity is vital for preventing cardiovascular complications from sympathetic long-term facilitation.

Orexin is a hypothalamic neurotransmitter that promotes arousal (Sakurai et al., 2010; Sakurai and Mieda, 2011; Tsujino and Sakurai, 2013) and sympathoexcitation (Shahid

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ABBREVIATIONS: AUC, area under the curve; AIH, acute intermittent hypoxia; DMSO, dimethylsulfoxide; HR, heart rate; MAP, mean arterial pressure; OXR, orexin receptor; PBS, phosphate-buffered 0.9% saline; PKA, protein kinase A; PKC, protein kinase C; REM, rapid eye movement; SNA, sympathetic nerve activity; SPN, sympathetic preganglionic neuron; sSNA, splanchnic sympathetic nerve activity.

et al., 2011, 2012a). Intermittent hypoxia increases the expression of prepro-orexin mRNA in the orexin neurons of the hypothalamus and orexin receptor (OXR) protein on the brainstem medullary neurons of rats (Liu et al., 2014). Orexin-A and -B enhance sympathetic activity after microinjection into the medullary pressor area or intrathecal infusion into the thoracic spinal region (Chen et al., 2000; Antunes et al., 2001; Machado et al., 2002; Shahid et al., 2011, 2012a). Orexin is extensively implicated in the formation of respiratory plasticity induced by AIH (Yamaguchi et al., 2015).

Respiratory and cardiovascular regulating regions share common sites in the hindbrain and the spinal cord (Pilowsky et al., 1985, 1990, 1993, 1994; Sun et al., 1997, 1998; Miyawaki et al., 2002; Nedoboy et al., 2016). This common morphologic relationship between respiratory and cardiovascular structures as well as common afferent inputs and different output pathways in many cases has led to the proposition that the two systems may interact. These observations indicate that the orexinergic system may also interact with the cardiovascular network, possibly having the potential to induce neuroplasticity in sympathetic neurons after AIH, similar in manner to that seen in the respiratory neurons (Kim et al., 2016). Therefore, orexin and its spinal receptor counterparts on sympathetic preganglionic neurons (SPN) may be an immediate therapeutic target for treating exacerbated sympathetic discharge and enhanced peripheral sympathetic chemoreflex that are robustly evident in AIH models (Xing and Pilowsky, 2010). Understanding the immediate targets that alter sympathetic discharge after AIH will serve to be an effective preventive measure before the hypoxia cycle progresses into a chronic phase (i.e., chronic intermittent hypoxia) with a manifestation of elevated arterial pressure from sympathetic hyperactivity (Lesske et al., 1997; Braga et al., 2006; Zoccal et al., 2008; Fisher and Paton, 2012).

Our study investigated whether endogenous orexin is mechanistically necessary for evoking immediate sympathetic long-term facilitation, and chemoreflex sensitization induced by AIH. For this purpose, we used a combination of pharmacologic and electrophysiologic approaches to confirm the mechanistic role of orexin-A on SPNs in urethane-anesthetized, vagotomized, paralyzed, and artificially ventilated rats. Orexin-A was intermittently infused into the spinal cord at a subthreshold dose (10 pmol × 10), which otherwise would not elicit sympathoexcitation as a single dose (100 pmol). Almorexant was intraperitoneally injected at either 30 or 75 $mg \cdot kg^{-1}$ before intermittent orexin-A or AIH. Peripheral chemoreflex was activated by a single bout of hypoxia (10% O_2) immediately before drug administration or AIH and immediately after all nerve recordings.

Materials and Methods

Animals. All experimental procedures were executed in strict accordance with the guidelines set in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, endorsed by the National Health and Medical Research Council of Australia. This study was approved by the Sydney Local Health District Animal Welfare Committee. The animals were housed in cages and were allowed to acclimatize for 7 days with environmental enrichment and access to food and water ad libitum for their basic behavioral and physiologic requirements.

Surgical Preparations. Animal preparation was performed as previously described elsewhere (Farnham et al., 2008). Briefly, male Sprague Dawley rats (n = 58; 250–400 g) were anesthetized with urethane (1.2–1.4 $g \cdot kg^{-1}$, i.p., with additional doses of 30–40 mg in 10% solution, as required) to suppress nociceptive reflexes. Anesthetic depth was monitored throughout the experiment by observing for changes in blood pressure by greater than 10 mm Hg in response to a tail pinch. The animal was laid on an electric heating pad (Harvard Apparatus, Holliston, MA) for controlled thermoregulation. The body temperature was maintained at 37.0 ± 0.5°C.

General Surgical Procedure. The right carotid artery and jugular vein were cannulated using polyethylene tubing (outer diameter: 0.90 mm × inner diameter: 0.50 mm; Microtube Extrusions, North Rocks, Australia) for the purpose of recording arterial blood pressure and infusing drugs and fluids, respectively. Electrocardiograms were recorded from leads connected to the forepaws of the rat, and heart rate (HR) was derived from it. Tracheal intubation was performed for artificial ventilation (rodent ventilator; Ugo Basile, Varese, Italy) and the recording of expired CO_2 (CapStar 100 CO_2 analyzer; CWE, Inc., Ardmore, PA). A tracheostomy was created, and a shortened 14 gauge cannula (length: 50 mm φ_o: 2.20 mm, Optiva I.V. Catheter Radiopaque; Smiths Medical Australasia, Bella Vista, Australia) was inserted. The rats were vagotomized, artificially ventilated with 100% oxygen-enriched room air, and paralyzed using pancuronium bromide (0.4 mg administered as a 0.2 ml bolus i.v. injection, followed by an infusion of 10% pancuronium diluted in 0.9% saline at a rate of 2 $ml \cdot hour^{-1}$; AstraZeneca, London, United Kingdom). Arterial blood gases were analyzed with an electrolyte and blood gas analyzer (VetStat; IDEXX Laboratories, Westbrook, ME). $PaCO_2$ was maintained at 40.0 ± 5 mm Hg and pH between 7.35 and 7.45.

A retroperitoneal approach gives access to the left greater splanchnic nerve at a site proximal to the celiac ganglion and a dorsal approach allows for the left phrenic nerve to be isolated, dissected, and tied using 5/0 silk thread. Nerve activity was recorded using bipolar silver electrodes. The neurograms were amplified 10-fold by a very-low-noise preamplifier (CWE, Inc.), band-pass filtered (0.1–3 kHz), and amplified a further 1000 times for phrenic and 2000 times for splanchnic by a scaling amplifier (BMA-400 AC/DC Bioamplifier; CWE Inc.). The analog signal was then digitized (A/D converter 1401; Cambridge Electronic Design, Cambridge, United Kingdom), sampled at 5 kHz (1401 plus; Cambridge Electronic Design), and displayed using Spike 2 software (version 8; Cambridge Electronic Design).

Intrathecal Catheter Insertion. The occipital musculature and connective tissue were removed from the occipital bone, which exposes the atlanto-occipital junction. A catheter (polyethylene, outer diameter: 0.50 mm × inner diameter: 0.20 mm; Microtube Extrusions) with a dead space of ~6 μl was inserted into the intrathecal space through a slit in the dura and advanced caudally to the level of $T_{5/6}$.

Acute Intermittent Hypoxia. The experimental protocol in this study adopted the hypoxia protocol used in a previous study (Xing and Pilowsky, 2010). Intermittent bouts of hypoxia gas composed of 10% oxygen balanced with nitrogen (Coregas, Yennora, Australia) were given for 45 seconds 10 times. There were 5-minute intervals between each challenge. The gas tank was connected to the ventilator via a tube, and it was ensured that the normal 100% oxygen gas tap was turned off during each hypoxia challenge.

Intrathecal Drug Administration. Depending on the variables being tested, control experiments were performed either by infusing 10 μl of 10 mM phosphate-buffered 0.9% saline (PBS), which was washed in with an additional 5 μl of PBS, 10 minutes before challenging the physiologic system by hypoxia or by infusing 10 μl of 10 mM PBS intermittently 10 times with 5-minute intervals between each infusion. Intrathecal drug administration comprised infusing 10 μl orexin-A (Sigma-Aldrich, Castle Hill, Australia) as a single 100 pmol 10 μl^{-1} (10 μM) infusion or 10 times repeated at 10 pmol 10 μl^{-1} (1 μM) per infusion, and the nerve response was recorded for a period of 60 minutes. Orexin-A was used because it targets both OX_1Rs and OX_2Rs whereas orexin-B is specific to OX_2Rs (Shahid et al., 2012b). The solutions were infused using a 25-μl glass Hamilton

microsyringe (AIS, Ringoes, NJ) over a time period of 10–15 seconds, as described previously elsewhere (Farnham et al., 2008), with 5-minute intervals between individual infusions.

Intraperitoneal Drug Injection. Dimethylsulfoxide (DMSO) (Sigma-Aldrich) at 200 μl was injected using a 1-ml syringe (Terumo Australia, Macquarie Park, Australia) intraperitoneally as a vehicle control. Almorexant-HCl was dissolved in DMSO to make solutions at 30 mg·kg^{-1} or 75 mg·kg^{-1} (Selleck Chemicals, Houston, TX) which were injected intraperitoneally 10 minutes before performing either the intermittent orexin-A injection or the AIH protocol. Almorexant is capable of inducing a transient and reversible blockade of OXRs specifically in the central nervous system. Almorexant effectively moves through the blood–brain barrier and centrally promotes sleep at 30 mg·kg^{-1} when the drug is injected via the intraperitoneal route (Morairty et al., 2012).

Data Acquisition and Analysis. Data were obtained using an ADC system (CED 1401; Cambridge Electronic Design) and Spike 2 acquisition/analysis software (version 8.04; Cambridge Electronic Design). The splanchnic sympathetic nerve activity (sSNA) raw data were rectified and smoothed (τ 1 second) and normalized to zero by subtracting the residual activity present 5 minutes after animal death.

Sympathetic nerve activity (SNA) was analyzed by obtaining the % sSNA range, where the activity level given as a mean value over a 1-minute interval at 60 minutes was subtracted by the mean at baseline. The nerve activity was recorded after treatment. The spike area under the curve (AUC) triggered by the very first hypoxic bout (before any drug treatment or AIH) and the final hypoxia (after the treatment and waiting period of 60 minutes) were subtracted, and compared against each treatment groups.

Phrenic nerve activity was rectified and smoothed (τ 0.05 seconds), and phrenic nerve frequency, and phrenic minute activity were all measured at the time points of 10 and 1 minutes before treatment and at the time points of 15, 30, 45, and 60 minutes after treatment. Mean arterial pressure (MAP) and HR (mean value across a 1-minute interval) were recorded at the 10- and 1-minute time points before the treatment (hypoxia, Almorexant, or intrathecal orexin-A). After the treatment, the activity was measured at the time points of 15, 30, 45, and 60 minutes to observe any changes. If the antagonist was used, the effect of the drug was taken as baseline. The temperature and CO_2 were measured at time points of 10 and 1 minutes before treatment and at the time points of 15, 30, 45, and 60 minutes after treatment. However, the results for MAP, HR, and phrenic nerve activity are not provided in this study. These parameters were mainly used as indicators to check the vitality of the animal (phrenic, MAP, HR, and CO_2 grouped data were not included).

Arterial blood gas levels (P_aCO_2 and pH) were measured immediately after the surgical procedure to check the status of the animal. Blood gas was measured again 5 minutes before the starting time to ensure optimal physiologic conditions of the animals, then at both 30 minutes and 60 minutes after treatment/AIH to maintain the condition of the animal.

Statistics. Statistical analyses were performed using GraphPad Prism software (version 6.04; GraphPad Software, San Diego, CA). All grouped data are shown as mean ± S.E.M. Statistical significance of the treatment responses were measured by one-way analysis of variance, using multiple comparisons and Holm-Sidak correction. In all cases, the responses and differences in mean values were considered statistically significant if $P < 0.05$.

Results

Intermittent Spinally Administered Orexin-A Is Sufficient To Elicit Sympathetic Long-Term Facilitation

A subthreshold dose (100 pmol) of orexin-A was divided into 10 individual doses (10 pmol each) and infused intermittently into the intrathecal space. Similarly, an intermittent suprathreshold dose (2 nmol × 10) of orexin-A was infused into the intrathecal space. This dose was selected as a positive control (Shahid et al., 2011). Intermittent orexin-A enhances SNA during the 60-minute recording period (Fig. 1 and 2). The subthreshold dose does not elicit a sympathoexcitatory response when administered as a bolus infusion (Δ7.3% ± 2.3%; $P > 0.05$ versus control; Fig. 1A and Fig. 2). Pretreatment with Almorexant (75 mg·kg^{-1}) before orexin-A (100 pmol) also had no effect on sSNA (Δ10.7% ± 2.5%; $P > 0.05$ versus control; Fig. 2C).

Intraperitoneal injection of Almorexant at 75 mg·kg^{-1} alone did not affect the baseline sympathetic response (−Δ2.9% ± 4.9%) when compared with intermittent PBS control (Δ4.5% ± 1.8%; Fig. 2D). There was a statistically significant elevation in sSNA caused by both the intermittent subthreshold dose of orexin-A (10 pmol × 10; Δ41.4% ± 6.9%; $P < 0.005$) and the suprathreshold dose of orexin-A (2 nmol; Δ66.2 ± 8.8%; $P < 0.001$) compared with control (Δ4.5% ± 1.8%; Fig. 2D). Administering Almorexant before intermittent orexin-A (10 pmol) significantly attenuated these effects; the range was reduced to Δ20.7% ± 4.5% ($P < 0.05$) by Almorexant 30 mg·kg^{-1} and to Δ18.5% ± 1.2% ($P < 0.05$) by Almorexant 75 mg·kg^{-1} (Fig. 2D).

Sympathetic Long-Term Facilitation after Acute Intermittent Hypoxia Is Not Mediated by Orexin

A robust increase in SNA is present after AIH (hypoxia administered for 45 seconds with 5-minute intervals, repeated 10 times), as previously described elsewhere (Xing and Pilowsky, 2010). Almorexant did not affect the sympathetic long-term facilitation evoked by AIH (Fig. 3). Acute intermittent hypoxia induced sympathetic long-term facilitation in all cases, regardless of prior injection of Almorexant at both 30 (Δ52.9% ± 9.9%; $P > 0.05$ versus DMSO +AIH) and 75 mg·kg^{-1} (Δ47.8% ± 12.7%; $P > 0.05$ versus DMSO + AIH; Fig. 3). To determine whether DMSO (solvent of Almorexant) alone affects nerve activity, studies were conducted on animals that had intrathecal PBS infusion and intraperitoneal administration of DMSO before AIH. The change in %sSNA, was Δ22.7% ± 2.0% for PBS control and Δ30.6% ± 5.3% for DMSO control after AIH (Fig. 3).

Orexin Receptor Antagonism by Almorexant Desensitizes Chemoreflex Response

A hypoxic stimulus was given to animals for all treatment groups at their baseline nerve recordings and at the end of the 60-minute recording period. The AUC of the final and baseline sympathetic responses to hypoxia was subtracted to determine the effect of treatment on the peripheral chemoreflex.

Orexin-A. Intermittent intrathecal administration of orexin-A (10 pmol per infusion) sensitizes the sympathetic nerve response to hypoxia. The difference in AUC between the first hypoxia before intermittent treatment and the final hypoxia after intermittent orexin-A is significantly greater than intermittent PBS control (Δ707% ± 47% μV·t versus Δ213% ± 21% μV·t; $P < 0.005$; Fig. 4A). Almorexant, at both 30 mg·kg^{-1} (Δ294% ± 51% μV·t; $P < 0.05$ versus intermittent orexin-A) and 75 mg·kg^{-1} (Δ81% ± 90% μV·t; $P < 0.005$ versus intermittent orexin-A) doses, ablates the sympathetic response to the final hypoxia (Fig. 4A).

Acute Intermittent Hypoxia. There is no significant difference between PBS and DMSO on the sympathetic chemoreflex response to a single burst of hypoxia. Almorexant

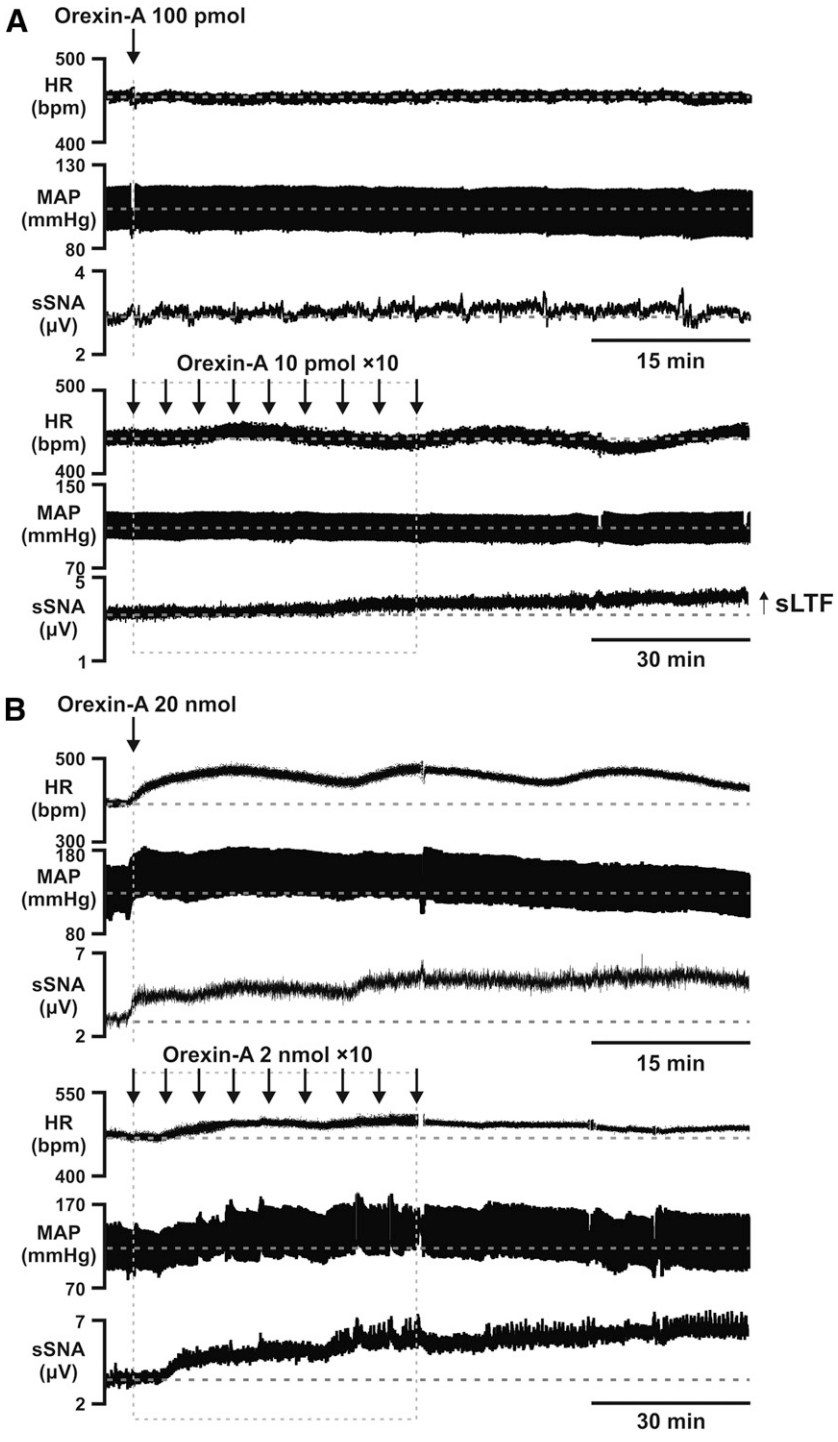

Fig. 1. Effect of intrathecal orexin-A at (A) subthreshold bolus (100 pmol)/intermittent (10 pmol × 10) and (B) suprathreshold bolus (20 nmol)/intermittent (2 nmol × 10) doses in a urethane-anesthetized rat on (from the top) HR (beats per minute), MAP (mm Hg), and sSNA (μV). Time of administration of intrathecal orexin-A (both single and intermittent) are indicated by the arrow(s). Note that intermittent subthreshold (10 pmol) orexin-A causes the generation of sympathetic long-term facilitation.

at both doses significantly reduced the difference in AUC between the first and final hypoxia compared with the vehicle control group. A difference of $-\Delta 708\% \pm 285\%$ $\mu V{\cdot}t$ ($P < 0.01$ versus DMSO) for 30 mg·kg^{-1} and $-\Delta 879\% \pm 124\%$ $\mu V{\cdot}t$ ($P < 0.001$ versus DMSO) for 75 mg·kg^{-1} was observed (Fig. 4B).

Discussion

This is the first study to demonstrate that intermittent stimulation of spinal OXRs using subthreshold doses of orexin-A causes an immediate and prolonged increase in SNA. In this study we show that orexin neurotransmission is not essential for AIH-induced sympathetic long-term facilitation because the dual OXR antagonist Almorexant did not block the facilitatory response. Our study is also the first to show that intermittent orexin-A, like AIH, enhances the sensitivity of the peripheral chemoreflex. Sympathetic chemoreflex responses to brief hypoxia were intensified after spinal SPNs were intermittently stimulated by orexin-A, as we had previously shown for AIH (Xing and Pilowsky 2010). In our present study, heightened sensitivity to hypoxia required contributions from the orexinergic system because

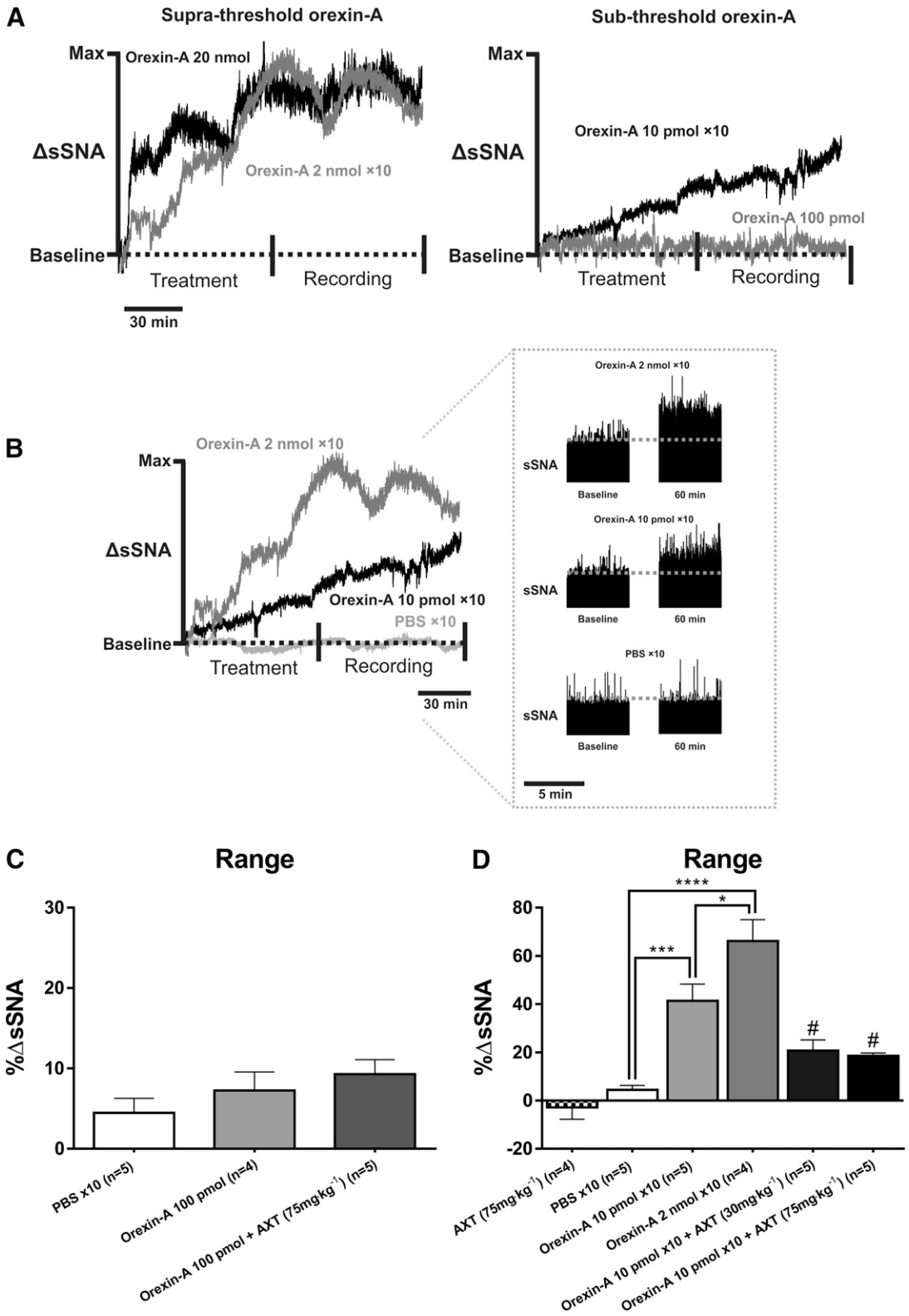

Fig. 2. A comparison of the sympathoexcitatory response elicited by bolus versus intermittent orexin-A at both (A) suprathreshold and (B) subthreshold doses. Single administration of orexin-A (100 pmol; $n = 4$) and orexin-A (100 pmol) + Almorexant (AXT) at 75 mg·kg^{-1} ($n = 5$) did not elevate sympathetic activity. (C) No significant changes were observed compared with PBS control ($n = 5$) when sSNA is measured as % range. Grouped data for the % range from every treatment group are compared. AXT at 75 mg·kg^{-1} ($n = 4$) produced no effect on baseline sympathetic activity. (D) AXT at 30 mg·kg^{-1} ($n = 5$) and 75 mg·kg^{-1} ($n = 5$) attenuated the effect of intermittent orexin-A (10 pmol × 10; $n = 5$) on sSNA. Statistical significance was determined using one-way analysis of variance followed by Holm-Sidak correction to compare the effects with the control. Data are expressed as mean ± S.E.M. ****$P < 0.0001$; ***$P < 0.001$; *$P < 0.05$. #$P < 0.05$ compared with intermittent orexin-A (10 pmol × 10).

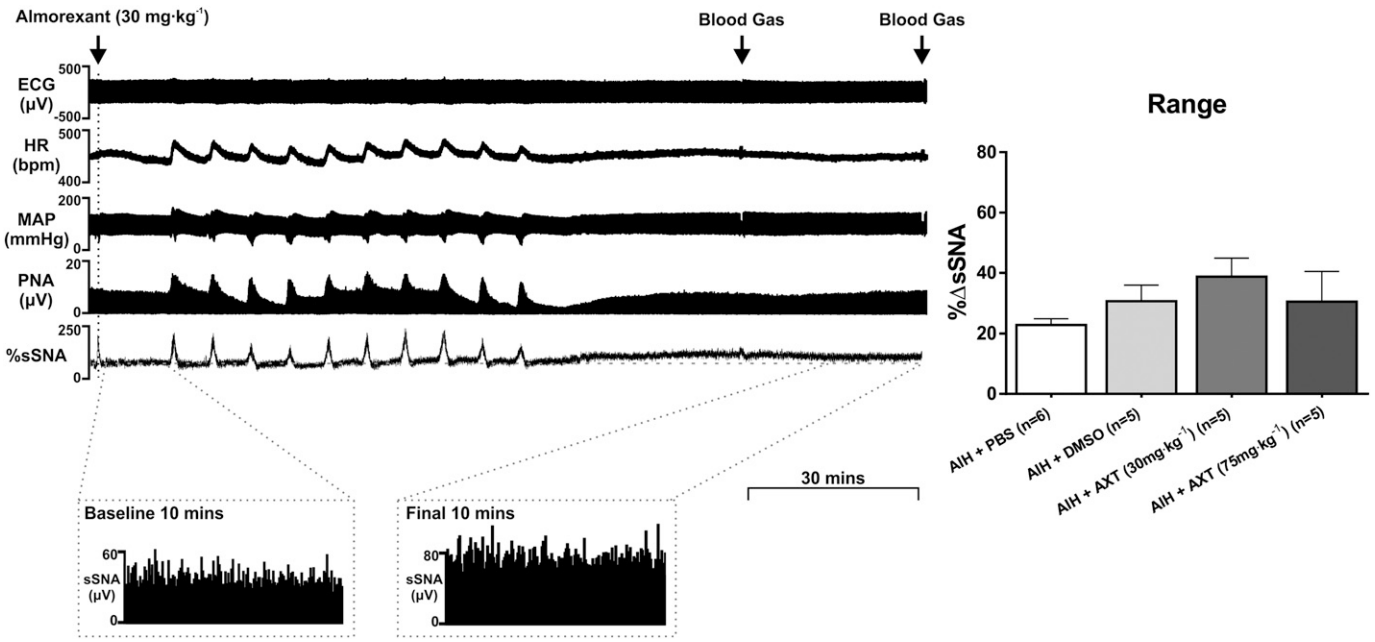

Fig. 3. In vivo effects of acute intermittent hypoxia (AIH, 10 bouts of 10% oxygen interspersed by 5-minute intervals) and intraperitoneal injection of Almorexant (AXT). (A) Experimental trace displaying the effect of AXT 30 mg·kg^{-1} (n = 5) in AIH, recording the changes in electrocardiogram (ECG), HR, MAP, phrenic nerve activity (PNA), and sSNA. Blood gas was analyzed at 30 and 60 minutes after AIH as indicated by the arrows. A 10-minute recording of pre-AIH sSNA and post-AIH sSNA is referred by the expanded period as indicated. (B) Comparison between the change in sSNA between PBS-treated AIH (n = 6) and DMSO-treated AIH (n = 5). All AXT-treated groups are compared with the DMSO-treated AIH group, where both AXT 30 mg·kg^{-1} and 75 mg·kg^{-1} (n = 5) AXT injections were made 10 minutes before AIH. (C) Change in sSNA shown as % range. Statistical significance was determined using one-way analysis of variance followed by Holm-Sidak correction. Data are expressed as mean ± S.E.M.

Almorexant completely abolished the sensitization of the sympathetic response to hypoxia.

Technical Considerations. Orexin-A infusion into the thoracic ($T_{5/6}$) subarachnoid space evokes sympathoexcitation, tachycardia, and pressor responses by activating OX_1Rs and OX_2Rs expressed on SPNs of the intermediolateral (Shahid et al., 2011). Spinal orexin-A also enhances the sympathetic baroreflex but blocks the somatosympathetic reflex (Shahid et al., 2011). Orexin-B, another endogenously released orexin, is also known to act on OX_2Rs of the spinal SPNs to elicit sympathoexcitation (Antunes et al., 2001). The development of AIH-induced sympathetic long-term facilitation and heightened sympathetic chemoreflex sensitivity may have an endogenous orexin-B component that was not investigated in this study. OX_2Rs that are stimulated specifically by orexin-B are coupled to either an excitatory phospholipase C–mediated intracellular pathway or an inhibitory G-protein that leads to the opening of K^+ channels and hyperpolarization (Spinazzi et al., 2006; Shahid et al., 2012b). Although the principal effect of OX_2Rs is excitatory (Antunes et al., 2001; van den Top et al., 2003), it is possible that inhibitory pathways activated by orexin-B may have important mechanistic contributions in regulating the sympathetic responses seen after intermittent hypoxia. Nevertheless, spinal SPNs are densely populated by OX_1Rs (van den Top et al., 2003; Beig et al., 2015), and endogenous orexins are most likely to exert their effect primarily through OX_1Rs.

Almorexant administered intraperitoneally is highly lipid soluble and acts at all OXRs. Morairty et al. (2012) demonstrated that intraperitoneal injection of Almorexant at 30 mg·kg^{-1} and 100 mg·kg^{-1} effectively antagonized but did not completely abolish central orexin receptor-mediated rapid eye movement (REM) and non-REM activity in rats without any dose-dependent differences. A recent study investigating the effect of stress on locomotor and cardiovascular activity (Beig et al., 2015) demonstrated that intraperitoneal Almorexant at 30 mg·kg^{-1} and 100 mg·kg^{-1} caused attenuations, but did not completely antagonize, behavioral responses related to orexin release with no significant dose-dependent changes. Centrally mediated sleep and stress are only partially blocked by Almorexant; thus Almorexant may have side effects in other central areas. The testes contain low levels of OXR mRNA expression (Jöhren et al., 2001), and orexin and OX_2R mRNA is detectable in adrenal cortex (Randeva et al., 2001). Thus, it is possible that sympathetic long-term facilitation evoked by intermittent orexin-A was not completely blocked by Almorexant because OXRs on SPNs were not completely antagonized.

Another reason for choosing intraperitoneal injection over direct intrathecal injection is that the DMSO agent (the only available solvent for Almorexant) can be neurotoxic. DMSO can adversely affect astrocyte function (Yuan et al., 2014), which may lead to unknown off-target effects. For this reason, many neuropharmacologic studies requiring DMSO to dissolve their neuroprotectant or neurotoxic drugs dilute the DMSO concentration. However, Almorexant is only soluble in 100% DMSO, so the drug was injected intraperitoneally for all experiments.

The pharmacokinetic profile of Almorexant is characterized by rapidly decreasing concentrations to approximately 20% of the maximum concentration over 8 hours, with a terminal half-life of 32 hours in humans (Hoever et al., 2013). In male canines, oral administration of Almorexant at a dose of 100 mg·kg^{-1} induced clinical signs of somnolence for up to

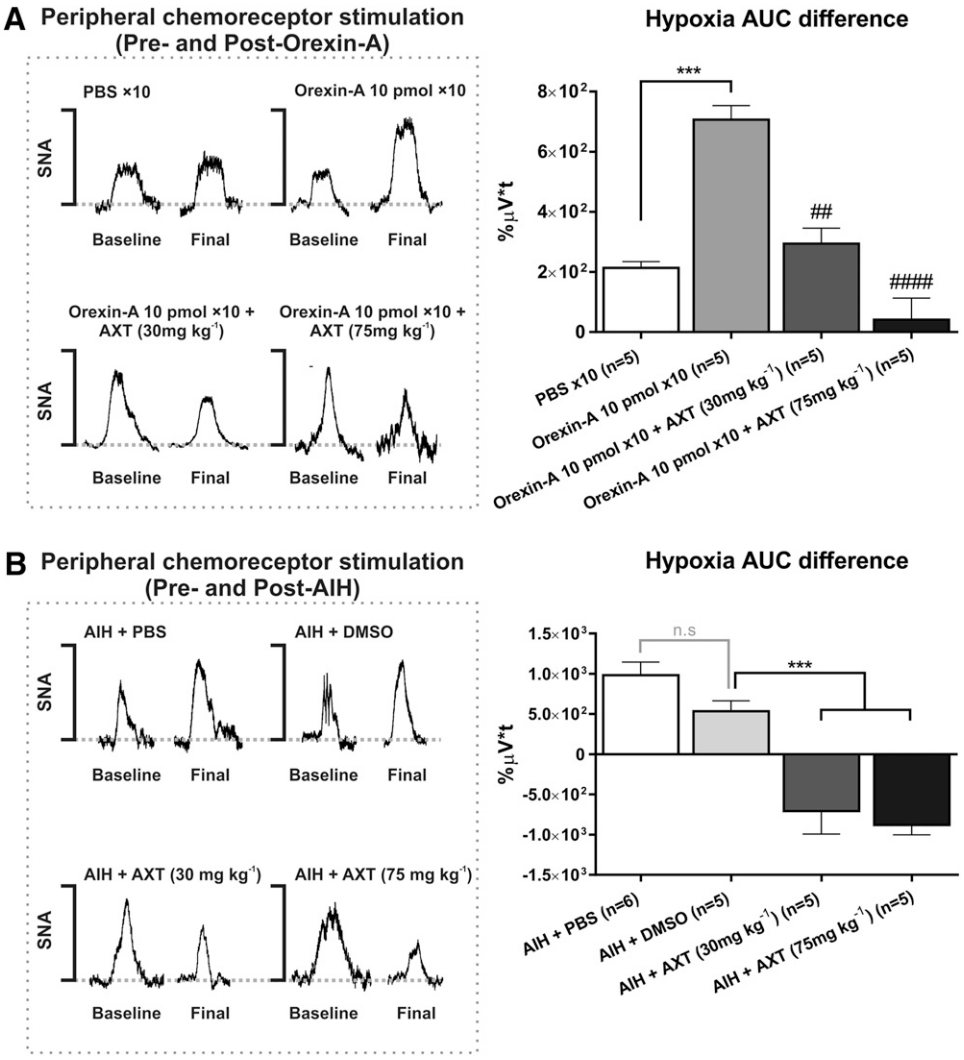

Fig. 4. Difference in the AUC between the initial and final sympathetic responses to hypoxia-induced chemoreceptor activation. The net AUC difference in the sympathetic response to hypoxia was identified for the baseline activity and the response at 60 minutes after treatment: intermittent orexin-A or acute intermittent hypoxia (AIH). (A) The value for the net difference in AUC (final minus initial hypoxia) compared for the treatments groups involving PBS ($n = 5$), intermittent orexin-A 10 pmol × 10 ($n = 5$), and Almorexant (AXT) at 30 ($n = 5$) and 75 mg·kg^{-1} ($n = 5$). (B) Similarly, net difference in AUC between the baseline and final sympathetic response to hypoxia after AIH + PBS ($n = 6$), AIH + DMSO ($n = 5$), AIH + AXT 30 mg·kg^{-1} ($n = 5$), and AIH + AXT 75 mg·kg^{-1} ($n = 5$). For grouped data, statistical significance was determined using one-way analysis of variance followed by multiple comparisons with a Holm-Sidak correction to compare the effects with the control. All values are expressed as mean ± S.E.M. ***$P < 0.005$ for significance. ##$P < 0.01$ and ####$P < 0.0001$ compared with intermittent orexin-A (10 pmol × 10).

6 hours (Brisbare-Roch et al., 2007). Stress-induced cardiovascular responses in Wistar rats were reduced by intraperitoneal Almorexant at 30 mg·kg^{-1} 4 hours after the injection (Beig et al., 2015). The time course of our study does not exceed 3 hours after the administration of the drug, so systemic injection of Almorexant would manifest its full effect throughout the span of the experiments.

The baseline level for all cardiovascular parameters and sSNA in this study was established under hyperoxic and normocapnic conditions ($P_aO_2 > 140$ mm Hg, P_aCO_2 maintained at 40 ± 5 mm Hg) by adjusting the ventilation (tidal volume and pump frequency) without determination of apneic and recruitment thresholds. Olson et al. (2001) suggested that hypocapnia may restrain the development of facilitation in cardiorespiratory activity. A consistent monitoring of isocapnic and metabolic conditions in all groups tested ensures that no central chemoreceptor drive caused alterations to central presympathetic inputs to the spinal cord.

Potential Mechanisms Underlying the Formation of Sympathetic Long-Term Facilitation. An earlier study demonstrated that exogenously applied orexin-A strengthens glutamatergic transmission in SPNs (Van Den Pol et al., 1998). Previous evidence has demonstrated that orexin neuron activation is necessary for the expression of phrenic facilitation after AIH (Toyama et al., 2009; Yamaguchi et al., 2015). Phrenic facilitation is evoked in the cervical phrenic motor nucleus by intracellular pathways activated by serotonergic G protein-coupled receptors coupled to protein kinase Cθ (PKCθ) and protein kinase A (PKA) effector proteins. PKCθ activates extracellular signal–regulated kinase/mitogen-activated protein kinase pathways (Devinney et al., 2015), whereas PKA phosphorylates phosphatidylinositol-3-kinase/protein kinase B pathways (Fields et al., 2015). Both mechanisms strengthen excitatory responses mediated by postsynaptic glutamatergic signals (Hoffman et al., 2012). Orexin-A activates OXRs 1 and 2 that are coupled to PKC and PKA proteins (Spinazzi et al., 2006; Shahid et al., 2012b). We speculate that the generation of sympathetic long-term facilitation in spinal thoracic SPNs may be similar in mechanism to phrenic facilitation.

Another potential mechanism for generating sympathetic long-term facilitation is via calcium-mediated mechanisms. Intracellular calcium stores in neurons regulate synaptic plasticity and signaling cascades that are important for long-term potentiation in memory (Baker et al., 2013). Intracellular ryanodine receptor activation leads to calcium-induced

calcium release, causing calcium signal propagation that promotes the consolidation of labile memory into long-term memory (Raymond and Redman, 2002). Several studies have shown that ryanodine receptor antagonism, which prevents the release of Ca^{2+}, inhibits the formation of long-term potentiation (Wang et al., 1996).

OX_1R activation by orexin-A also induced calcium influx. OX_1R up-regulates PLC proteins to hydrolyze phosphatidylinositol 4,5-biphosphate into inositol triphosphate and diacylglycerol (Shahid et al., 2012b), both of which increase intracellular Ca^{2+}, thus evoking synaptic plasticity in SPNs.

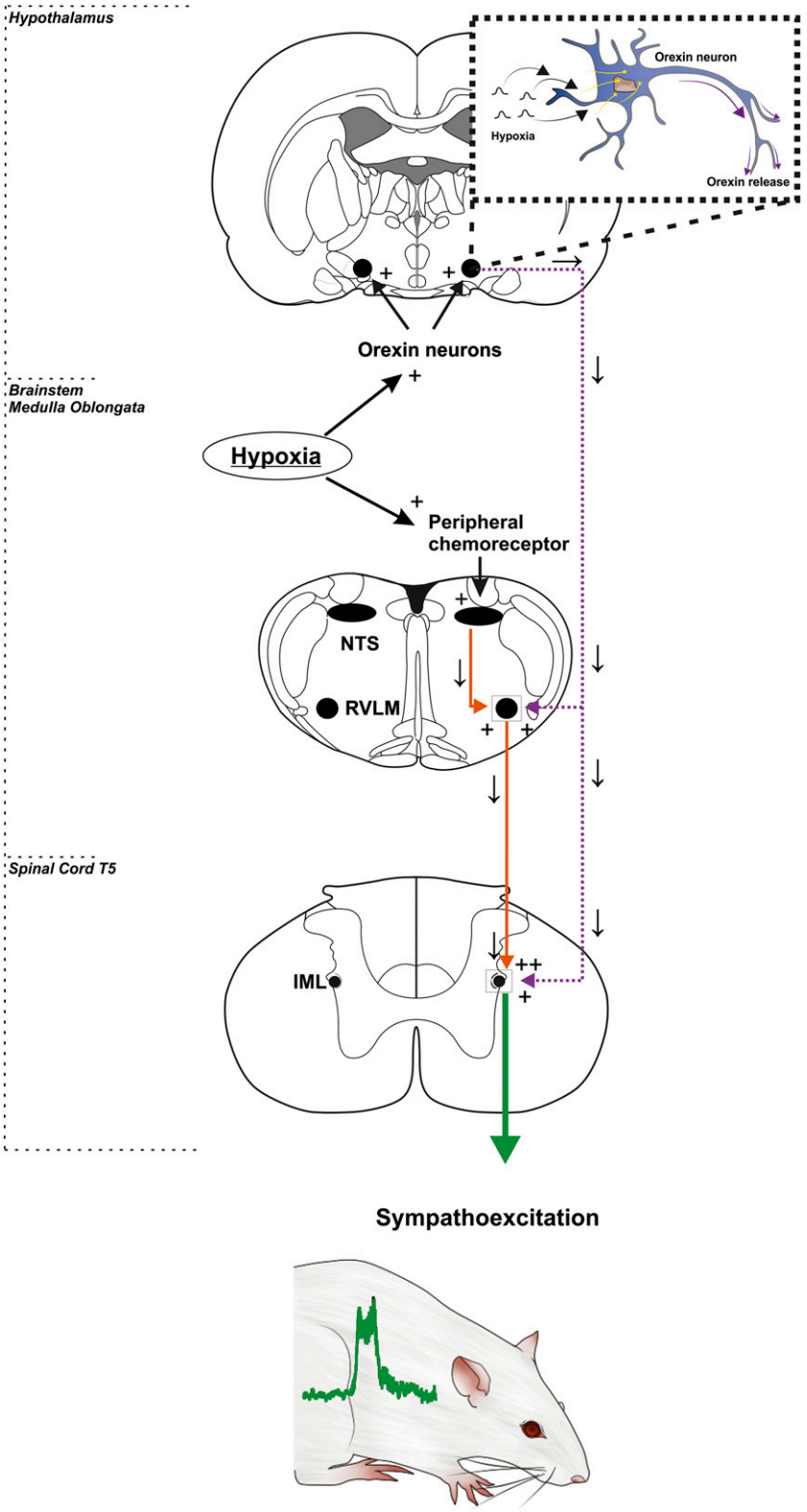

Fig. 5. Sympathetic outflow from hypoxia-induced peripheral chemoreceptor activation requires a contribution from hypothalamic orexin neurons. Hypoxia directly activates the peripheral chemoreceptors of the carotid body, which sends afferent excitatory signals to the brainstem medulla. The nerves synapse to the nucleus tractus solitarii (NTS), which excite the rostral ventrolateral medulla (RVLM) nerves and further relay these signals to the intermediolateral (IML) of the spinal $T_{5/6}$ level. Simultaneously, orexin neurons are excited by repetitive hypoxia. Both orexin receptor subtypes (OX_1R and OX_2R) are found on SPNs in the intermediolateral and are necessary to sensitize the sympathetic chemoreflex response to hypoxia.

Peripheral Chemoreceptor Sensitization Is Mediated by Orexin-A. Our study is first to demonstrate that an increase in cardiorespiratory sensitivity to hypoxia after AIH is mediated by orexin-A. Enhanced chemoreflex sensitivity is pathogenic to neurogenic hypertension in obstructive sleep apnea (Leuenberger et al., 2005; Imadojemu et al., 2007; Xing and Pilowsky, 2010; Paton et al., 2013). Repetitive hypoxia causes a cumulative elevation in chemoreflex-mediated sympathoexcitatory response, gradually increasing the excitatory response for every succeeding stimulus during AIH or chronic intermittent hypoxia. Administration of Almorexant before intermittent orexin-A and AIH completely abolished the enhanced sensitivity to a brief hypoxic stimulus 60 minutes after the intermittent stimuli. These observations indicate two mechanistic features of the chemoreflex pathway: 1) AIH activates orexin neurons to exert excitatory effects on the sympathetic circuitry, and 2) the physiologic sympathoexcitatory responses to hypoxia require orexin neuronal inputs to the peripheral chemoreflex pathway.

It remains unknown as to how orexin neurons are activated in response to hypoxia. One recent study demonstrated that C1 neurons of the rostral ventrolateral medulla, which are intrinsically hypoxia-sensitive neurons, have afferent inputs to the orexinergic neurons (Bochorishvili et al., 2014). C1 neurons respond to severe reductions in brain PaO_2 (Sun and Reis, 1994). A large proportion of C1 neurons express Fos protein in conscious mammals exposed to hypoxia (Erickson and Millhorn, 1994), and sympathetic nerve activation elicited by carotid body stimulation is severely depressed after selective lesions of these cells (Schreihofer and Guyenet, 2000). The C1 neurons establish asymmetric synapses with orexin-immunoreactive cell bodies or dendrites (Bochorishvili et al., 2014). Anterograde tracing with viral vectors has revealed C1 cell projections to the lateral hypothalamus (Bochorishvili et al., 2014); the rostral ventrolateral medulla may be a key nucleus regulating both descending presympathetic neurons and afferents projections to orexin nuclei to activate state-dependent control of sympathetic responses to hypoxia (Fig. 5).

Conclusion

In conclusion, our present study has determined a functional role for orexin in the cardiorespiratory regulation of SNA. Intermittent orexin-A infusion and AIH significantly enhance the activity of the sympathetic pathway and evoke sympathetic long-term facilitation. Peripheral chemoreflex sensitivity was also enhanced by intermittent orexin-A and AIH. This sympathetic response was completely abolished by OXRs antagonism using Almorexant. This is the first study to relate the significance of intermittent hypoxia-induced sympathetic elevation with orexin in the cardiorespiratory system. In future, targeting the OXRs may serve as an immediate option for preventing the acute symptoms of abnormal sympathetic hyperactivity that precede neurogenic hypertension.

Authorship Contributions

Participated in research design: Kim, Pilowsky, Farnham.

Conducted experiments: Kim.

Contributed new reagents or analytic tools: Kim, Pilowsky, Farnham.

Performed data analysis: Kim, Pilowsky, Farnham.

Wrote or contributed to the writing of the manuscript: Kim, Pilowsky, Farnham.

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Address correspondence to: Paul M Pilowsky, Heart Research Institute, 7 Eliza Street, Sydney, 2042, Australia. E-mail: paul.pilowsky@hri.org.au