Orexins Depolarize Rostral Ventrolateral Medulla Neurons and Increase Arterial Pressure and Heart Rate in Rats Mainly via Orexin 2 Receptors

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ABSTRACT

An injection of orexin A or B into the cisterna magna or the rostral ventrolateral medulla (RVLM), where bulbo-spinal vasomotor neurons are located, elevated arterial pressure (AP) and heart rate (HR). We examined how orexins affected RVLM neurons to regulate cardiovascular functions by using in vitro recordings of neuronal activity of the RVLM and in vivo measurement of cardiovascular functions in rats. Orexin A and B concentration-dependently depolarized RVLM neurons. At 100 nM, both peptides excited 42% of RVLM neurons. Tetrodotoxin failed to block orexin-induced depolarization. In the presence of N-[2-(methyl-6-benzoazoxazolyl)]-N’-1, 5-naphthyridin-4-yl urea (SB-334867), an orexin 1 receptor (OX1R) antagonist, orexin A depolarized 42% of RVLM neurons with a smaller, but not significantly different, amplitude (4.9 ± 0.8 versus 7.2 ± 1.1 mV). In the presence of (2S)-1-(3,4-dihydro-6,7-dimethoxy-2(1H)-isoquinolinyl)-3,3-dimethyl-2-[(4-pyridinylmethyl)amino]-1-butanone hydrochloride (TCS OX2 29), an orexin 2 receptor (OX2R) antagonist, orexin A depolarized 25% of RVLM neurons with a significantly smaller amplitude (1.7 ± 0.5 mV). Coapplication of both antagonists completely eliminated orexin A-induced depolarization. An OX2R agonist, [Ala11,D-Leu15]-orexin B, concentration-dependently depolarized RVLM neurons. Regarding neuronal phenotypes, orexins depolarized 88% of adrenergic, 43% of nonadrenergic, and 36 to 41% of rhythmically firing RVLM neurons. Intracisternal TCS OX2 29 (3 and 10 nmol) suppressed intracisternal orexin A-induced increases of AP and HR, whereas intracisternal SB-334867 (3 and 10 nmol) had no effect on the orexin A-induced increase of HR but suppressed the orexin A-induced pressor response at 10 nmol. We concluded that orexins directly excite RVLM neurons, which include bulbo-spinal vasomotor neurons, and regulate cardiovascular function mainly via the OX2R, with a smaller contribution from the OX1R.
Orexins Excite RVLM and Increase Blood Pressure via OX1R

Materials and Methods

Animals. Adult male and pregnant female Sprague-Dawley (SD) rats were purchased from BioLASCO Taiwan (Taipei, Taiwan) and kept in the animal center of Taipei Medical University. The use of animals was approved by the Institutional Animal Care and Use Committee of Taipei Medical University.

Preparations of Brainstem Slices. Neonatal (8–14 days old) SD rats of either sex were anesthetized with isoflurane and decapitated. The brainstem was removed, and 500-μm coronal brainstem slices were prepared with a Vibratome slicer (Vibratome, St. Louis, MO). Brain slices were incubated in oxygenated Krebs’ solution at room temperature for at least 2 h before being subjected to electrophysiological recordings. The Krebs’ solution consisted of 127 mM NaCl, 1.9 mM KCl, 1.2 mM KH2PO4, 2.4 mM CaCl2, 1.3 mM MgSO4, 26 mM NaHCO3, and 10 mM glucose and was gassed with 95% O2 and 5% CO2.

Electrophysiological Recordings. After equilibrium, one slice was transferred to a recording chamber, held in place between two grids of fine nylon mesh, and perfused with oxygenated Krebs’ solution at a rate of 2 to 3 ml/min. Whole-cell patch-clamp recordings were conducted on RVLM neurons from the first two slices rostral to the area postrema at room temperature as described previously (Hwang and Dun, 1999). Patch electrodes filled with a solution containing 130 mM potassium glutamate, 1 mM MgCl2, 2 mM CaCl2, 4 mM ATP, 0.3 mM GTP, 10 mM EGTA, 10 mM HEPES, and in some cases 0.2% Lucifer yellow had a resistance of 2 to 5 MΩ; the pH of the solution was adjusted to 7.2 with KOH. Signals were recorded with an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA), low pass-filtered at 1 kHz, and acquired by using a personal computer and pClamp software (version 9.0, Molecular Devices) for later analysis. Signals were simultaneously monitored on a two-channel chart recorder (Gould Electronics, Eichstetten, Germany). Membrane potentials were corrected for the liquid-junction potential of ~8 mV. The access resistance was less than 25 MΩ. To minimize any potential interference of previously applied orexin, data were collected from one neuron per slice.

Intracisternal Administration of Orexin A and Antagonists. The recorded neuron was identified with intracellular staining of a fluorescent dye, Lucifer yellow, which was allowed to diffuse from the patch electrode into the recording neuron. At the end of the recording, the slice was immersed in a solution of 4% paraformaldehyde/phosphate-buffered saline (PBS) overnight, and then transferred to a solution of 30% sucrose/PBS until further processing. The fixed slice was then sectioned into 50-μm sections with a cryostat. Sections were viewed under an upright Nikon (Tokyo, Japan) 80i epifluorescence microscope, and the section containing the Lucifer yellow-labeled neuron was selected for immunostaining with phenylethanolamine N-methyltransferase (PNMT), an adrenaline-synthesizing enzyme. Sections were first blocked with 10% normal goat serum and incubated with rabbit polyclonal PNMT antiserum (1:1000) for 2 h and then with Avidin biotinylated goat anti-rabbit immunoglobulin G (1:100) for 1 h, washed with PBS, sections were incubated with biotinylated goat anti-rabbit immunoglobulin G (1:100) for 2 h and then with Avidin Texas red (1:100) for 1 h, washed with PBS, mounted in Vectashield mounting medium, and covered with a coverslip. Sections were then visualized with an epifluorescence microscope. Immunohistochemical control experiments were performed with omission of the primary antiserum from medullary sections. Staining was absent in all control experiments.

Measurements of the Arterial Pressure and Heart Rate. The adult male SD rats were anesthetized with an intraperitoneal injection of chloralose [1,2-O-(2,2,2-trichloroethylidene)-o-D-glucofuranose] (100 mg/kg) and urethane (ethyl carbamate) (500 mg/kg) dissolved in a sodium borate solution (2%). The left femoral artery was cannulated with a polyethylene-50 tube containing heparinized (20 U/ml) saline. The cannula was connected to a pressure transducer with its output signals sent to a data acquisition system (Biopac MP-36; Biopac Systems, Santa Barbara, CA). The signals were acquired and analyzed by using Biopac Pro, version 3.7.3 (Biopac Systems) to obtain the AP, mean AP (MAP), and HR.

Intracisternal Administration of Orexin A and Antagonists. Intracisternal administration was carried out as reported previously (Chen et al., 2000). In brief, a rat’s head was fixed onto a stereotoxic device, and the dura above the cisternal magna of the fourth ventricle was carefully exposed. A 25-gauge needle connected to a polyethylene-20 tube, and a microsyringe was inserted through the dura...
into the cisternal magna. Orexin A and orexin receptor antagonists were dissolved in normal saline or dimethyl sulfoxide (DMSO) and injected into the cisterna magna in a fixed volume of 6 μl over 1 min.

**Chemicals.** Orexin A and B were from Phoenix Pharmacuetics Inc. (Belmont, CA), and N-(2-methyl-6-benzoxazolyl)-N'-(1,5-naphthyridin-4-yl urea (SB-334867), [Ala¹¹,D-Leu¹⁵] orexin B (ALOXB), (2S)-1-(3,4-dihydro-6,7-dimethoxy-2H-isoquinolinyl)-3,3-dimethyl-2-[(4-pyridinylmethyl)amino]-1-butane hydrochloride (TCS OX2 29), and tetrodotoxin (TTX) were from Tocris Cookson (Ellisville, MO). 6-Cyano-7-nitroquinolxalene-2,3-dione (CNQX), 2-amino-5-phosphopentanoic acid (AP-5), Lucifer yellow CH dilithium salt, chloralose, urethane, sodium borate, dextrose (D-glucose), sodium bicarbonate (NaHCO₃), oleandomycin (OAM), cyanide (CN), and AgCl were from Sigma-Aldrich (St. Louis, MO). PNMT antiserum was from Millipore Corporation (Billerica, MA), and all other reagents for immunohistochemistry were from Vector Laboratories (Burlingame, CA). For the in vitro electrophysiological experiments, all reagents, except SB-334867, were dissolved in water to form stock solutions and diluted in Krebs’ solution. SB-334867 was dissolved in DMSO, and the final concentration of DMSO in the bath solution was 0.1%. Vehicle control experiments were performed (n = 5), and no significant effect of DMSO was found. For in vivo experiments, SB-334867 was dissolved in DMSO, whereas orexin A and TCS OX2 29 were dissolved in normal saline.

**Statistical Data Analysis.** Data are expressed as the mean ± S.E.M. and were analyzed statistically by Student’s t test or one-factor analysis of variance (ANOVA) followed by multiple comparisons using the Student-Newman-Keuls test at p < 0.05.

**Results**

**In Vitro Recording of Neuronal Activity in the RVLM**

**Membrane Properties of RVLM Neurons.** Whole-cell patch-clamp recordings were made from RVLM neurons of neonatal rat brainstem slices. Approximately 50% of RVLM neurons fired spontaneously. Because some of these neurons fired at high frequency, the resting membrane potential and whole-cell input resistance were not measured in this group of neurons. Averaged resting membrane potentials and whole-cell input resistance were −59 ± 1 mV and 499 ± 26 MΩ, respectively, from 150 neurons exhibiting no or low spontaneous firing activity. Both silent and spontaneously firing neurons with action potentials in excess of 50 mV were included in the study.

**Effects of Orexin A and B on RVLM Neurons.** Orexin A and B were perfused into bath solutions at concentrations ranging from 30 to ~300 nM. Orexin A and B (100 nM) produced membrane depolarization accompanied sometimes by intense firing (Fig. 1, A and B, upper traces) in 34 of 80 (42%) and 35 of 84 (42%) of RVLM neurons, respectively. In the 34 orexin A-responsive neurons, 20 were silent and 14 were spontaneously firing. In the 35 orexin B-responsive neurons, 20 were silent and 15 were spontaneously firing. Orexin A- or orexin B-induced depolarization ranged from 3 to ~20 mV. The effects of orexins were concentration-dependent (Fig. 1C). Orexin A- and B-induced depolarization persisted in a TTX (0.5 μM)-containing Krebs’ solution (Fig. 1, A and B, lower traces) in 12 and 6 neurons tested, respectively, indicating that orexins directly depolarized RVLM neurons.

**Effects of Orexin Receptor Subtype-Specific Antagonists and Agonist.** To elucidate the contribution of OX₁R and OX₂R in orexin-induced depolarizations, we examined the effects of an OX₁R antagonist, SB-334867 (Smart et al., 2001), and an OX₂R antagonist, TCS OX2 29 (Hirose et al., 2003), in relation to orexin A-induced depolarizations. The effect of an OX₂R agonist, [Ala¹¹,D-Leu¹⁵]-orexin B, was also examined in RVLM neurons. The antagonist (10 μM) was continuously applied in a bath solution 10 min before, during, and 20 min after the administration of orexin A.

In the presence of SB-334867, orexin A (100 nM) induced membrane depolarizations in 10 of 24 (42%) RVLM neurons. This incidence did not differ from that when orexin A was given alone. The amplitude of depolarization induced by orexin A in the presence of SB-334867 was slightly, although insignificantly, smaller than that by orexin A alone (4.9 ± 0.8 mV, n = 10 versus 7.2 ± 1.1 mV, n = 12, p = 0.054) (Fig. 2, A and D). On the other hand, TCS OX2 29 significantly suppressed the incidence and magnitude of orexin A (100 nM)-induced depolarizations. In the presence of TCS OX2 29, the percentage of orexin A (100 nM)-excited neurons decreased to 25% (4/16) from 42% (34/80) by orexin A alone, and...
the depolarization amplitudes were significantly reduced to 1.7 \pm 0.5 \text{ mV} (n = 4) (Fig. 2, B and D). In four orexin A-responsive neurons, orexin A failed to induce any depolarization in the presence of SB-334867 and TCS OX2 29 (Fig. 2C, center trace). Similar to orexins, [Ala\textsuperscript{11},D-Leu\textsuperscript{15}]-orexin B (30–300 nM), an OX\textsubscript{2}R-selective agonist, also caused membrane depolarizations in RVLM neurons in a concentration-dependent manner (Fig. 3). Together, these results suggest that the OX\textsubscript{2}R is the major receptor subtype that mediates the membrane depolarization effect of orexins on RVLM neurons, whereas OX\textsubscript{1}R may make a minor contribution to the effects.

**Phenotypes of Orexin A-Excited RVLM Neurons.** By using intracellular staining and immunohistochemical techniques, we examined whether orexin A-excited RVLM neurons are PNMT-containing adrenergic neurons. Among 62 recorded neurons, 8 were PNMT-immunoreactive neurons and the remaining 54 were not. Almost all (7 of 8 neurons) PNMT-positive neurons were orexin A-sensitive, whereas approximately 43\% (23 of 54 neurons) of PNMT-negative neurons were orexin A-sensitive (Fig. 4).

**Effects of Orexins in Rhythmically Firing RVLM Neurons.** We examined the effects of orexin A and B on rhythmically firing RVLM neurons. These neurons had rhythmically firing activity and gradual interspike membrane depolarization (Fig. 5Aa). The rhythmically firing pattern remained in the presence of glutamate receptor antagonists CNQX (10 \text{ M}) and AP-5 (10 \text{ M}) (Fig. 5A, bottom trace). Orexin A and B (100 nM) increased the frequency of firing activity in 7 of 17 (41\%) and 5 of 14 (36\%) rhythmically firing RVLM neurons, respectively (Fig. 5A, top and middle traces, and b). In the presence of TTX (0.5 \text{ M}), orexin A (100 nM) caused membrane depolarizations of 4 and 5 mV, respectively, in two orexin-sensitive rhythmically firing RVLM neurons (Fig. 5B).

**Effects of Orexin Receptor Antagonists on Orexin A-Induced Cardiovascular Responses**

The pharmacological characteristics and the involved neuronal phenotypes of the in vitro excitatory effects of orexins in the RVLM suggest that orexins can regulate cardiovascular functions mainly by acting on the OX\textsubscript{2}R in...
the RVLM. Evidence from previous reports indicates that the RVLM may be the major brainstem region responsible for the long-lasting pressor and positive chronotropic effects of intracisternal orexin A (Chen et al., 2000; Machado et al., 2002; Smith et al., 2002; Ciriello and de Oliveira, 2003; Ciriello et al., 2003; de Oliveira et al., 2003). We therefore hypothesized that the RVLM and the medullary OX₁R play an important role in regulating cardiovascular functions. To verify this hypothesis, we examined the effects of OX₁R and OX₂R antagonists in relation to intracisternal orexin A-induced cardiovascular responses. Similar to the results of our previous report (Chen et al., 2000), intracisternal orexin A (3 nmol) significantly increased the AP and HR in anesthetized rats. The OX₁R antagonist, SB-334867, or OX₂R antagonist, TCS OX2 29, were applied by intracisternal injection 30 min before the administration of orexin A. SB-334867 at 3 nmol did not affect orexin A-induced cardiovascular responses, whereas at 10 nmol it significantly reduced the elevation of MAP, but not HR, induced by intracisternal orexin A (Fig. 6A). On the other hand, TCS OX2 29, effectively suppressed orexin A-induced increases in the MAP and HR at both 3 and 10 nmol (Fig. 6B). SB-334867 or TCS OX2 29 (3–10 nmol) alone did not cause significant changes in the MAP or HR (Table 1).

Discussion

The present study provides three major findings. First, orexins directly excited RVLM neurons. Second, the majority (88%) of adrenergic neurons, 43% of nonadrenergic neurons, and 36 to 41% of rhythmically firing neurons in the RVLM were sensitive to orexins. Third, the excitatory effects of orexins in the RVLM and the pressor effects of intracisternal orexin A were mediated mainly by the OX₂R and to a lesser extent by the OX₁R.

The RVLM plays an important role in tonic and phasic controls of sympathetic vasomotor outflow. The bulbospinal vasomotor neurons of the RVLM provide the main supraspinal excitatory input for sympathetic vasomotor control (reviewed in Guyenet, 1990 and Dampney, 1994). In addition to bulbospinal vasomotor neurons, the RVLM contains neurons regulating other physiological functions, such as respiratory functions (reviewed in Ballanyi et al., 1999). We are unable to conclude whether orexins affected these noncardiovascular neurons in the RVLM. However, our results together with several previous reports show that orexin-sensitive RVLM neurons probably include bulbospinal vasomotor neurons. First, orexins excited most (88%) of the RVLM adrenergic neurons, which are an important element of bulbospinal vasomotor neurons. Second, orexins depolarized rhythmically firing RVLM neurons, which are believed to be crucial in the generation of sympathetic vasomotor tone. Third, a microinjection of orexin A or B into the RVLM elicited an increase in the AP and HR in both anesthetized (Chen et al., 2000) and conscious rats (Machado et al., 2002). Therefore, the excitatory effects of orexins on putative bulbospinal vasomotor neurons within the RVLM may contribute to the pressor effects of orexins in vivo.

Both OX₁ and OX₂ receptor subtypes exist in the RVLM (Trivedi et al., 1998; Cluderay et al., 2002). Our findings that the OX₂R antagonist significantly suppressed orexin A-induced depolarization and the OX₁R-selective agonist mimicked the excitatory effects of orexins suggest a critical role for the OX₂R in the excitatory effect of orexins on RVLM neurons. On the other hand, the OX₁R antagonist, SB-334867, did not significantly suppress orexin A-induced depolarization at a concentration 100 times higher than that of orexin A. This concentration of SB-334867 should be sufficient to block the action of orexin A at the OX₁R because there is only a 4- to 5-fold difference in the OX₁R binding affinity between SB-334867 and orexin A (Kᵢ values: orexin A, 16–20 nM; SB-334867, 67 nM) (Sakurai et al., 1998; Smart et al., 2001). Therefore, the OX₁R might not contribute much to the excitatory effect of orexins in the RVLM. Nevertheless, coapplication of OX₁R and OX₂R antagonists completely abolished orexin A-induced depolarizations (Fig. 2, C and D), whereas the OX₂R antagonist alone did not (Fig. 2, B and D). Therefore, the OX₁R may play a minor role in orexin A-induced depolarization of RVLM neurons.

The present in vivo finding further confirmed an essential role of the OX₁R in the medullary regulation of cardiovascular functions because the selective OX₁R antagonist, TCS OX2 29, effectively antagonized the pressor and positive chronotropic responses induced by an equimolar dose of orexin A. At the same doses, the selective OX₂R antagonist, SB-334867, was less effective in suppressing the cardiovascular effects of orexin A. These two antagonists exhibited comparable potencies at their specific receptor subtypes in antagonizing orexin A-induced calcium mobilization in vitro. The pKᵢ values of SB-334867 and TCS OX2 29 to the OX₁R and OX₂R, respectively, are 7.3 (Smart et al., 2001) and 7.4 (estimated from the IC₅₀ value reported by Hirose et al., 2003). The higher potency of TCS OX2 29 compared with SB-334867 in suppressing intracisternal orexin A-induced depolarization of RVLM neurons may be important in vivo.
cardiovascular responses might be caused by the more important role of the OX2R in this regard.

Although SB-334867 did not suppress orexin A-induced cardiovascular responses at 3 nmol, it significantly reduced orexin A-induced elevation of the MAP at 10 nmol. In addition, our previous report revealed that intracisternal orexin B produced smaller changes in the MAP and HR compared with orexin A (Chen et al., 2000). Therefore, the OX1R may also contribute to the medullary regulation of cardiovascular functions. Taken together, we conclude that the OX2R may be the major receptor subtype responsible for the cardiovascular effects of intracisternal orexin A, whereas the OX1R may have a minor contribution.

Different subtypes of orexin receptors might contribute to different functional roles of orexins. For example, the OX1R in the ventral tegmental area plays an important role in orexin A-induced, cue-induced reinforcement (Borgland et al., 2009), and the OX2R in the ventrolateral periaqueductal gray mediates the supraspinal antinociceptive effect of orexin A (L. C. Chiou, H. J. Lee, Y. C. Ho, S. P. Chen, Y. Y. Liao, C. H. Ma, P. C. Fan, J. L. Fuh, and S. J. Wang, submitted for publication), whereas the OX2R in the tuberomammillary nucleus is crucial in the sleep–wake regulatory role of orexins (Eriksson et al., 2001). In this study, we found that the OX2R in the RVLM plays an important role in the pressor response of orexins.

Both adrenergic and nonadrenergic neurons in the RVLM contribute to cardiovascular regulation. Schreihofer et al. (2000) introduced an immunotoxin to selectively deplete bulbo spinal adrenergic neurons and concluded that these neurons do not contribute to the maintenance of sympathetic activity and AP in anesthetized rats, but are important in sympathoexcitatory and pressor responses evoked by stimulating RVLM or chemoreceptors (Schreihofer and Guyenet, 2000; Schreihofer et al., 2000). Schreihofer et al.’s studies also imply a crucial role for nonadrenergic RVLM neurons in the maintenance of the basal AP. In the present study, we found that both adrenergic and nonadrenergic neurons were excited by orexins. Our in vivo study showed that intracisternal TCS OX2 29 or SB-334867 did not affect the basal AP or HR, implying a minor contribution of orexins in the medullary control of basal blood pressure in rats. However, the

![Fig. 5. Orexins excited rhythmically firing RVLM neurons. A, the continuous recording traces show that orexin A and B increased the firing rate of a rhythmically firing RVLM neuron. The rhythmically firing activity persisted in the presence of CNQX and AP-5 of 10 μM (bottom trace). a and b, recording traces shown at a different time scale taken at times a and b, indicated on the continuously recording traces. B, in the presence of TTX (0.5 μM), orexin A (100 nM) caused membrane depolarization in this RVLM neuron. Recordings (A and B) were made from the same RVLM neuron. Labels are defined as in Fig. 1.](image-url)
lar effect of intracisternal orexin A in the present study is a concern. However, promotion of respiratory function usually does not result in an increase in blood pressure. In addition, Zhang et al. (2005) have demonstrated that intracerebroventricular, compared with intracisternal, administration of orexin A caused a similar increase in the tidal volume but smaller increases in the AP and HR, implying independent mechanisms mediating the cardiovascular and respiratory excitation by orexin A. This notion is further supported by that microinjection of orexin A into pre-Bötzinger, which is involved in the generation of inspiratory rhythmic activity and located very close to the RVLM, increased diaphragm electromyographic activity without significant effects on the AP or HR (Young et al., 2005). Therefore, the cardiovascular effects of intracisternal orexin A observed in the present study may have resulted mainly from the direct activation of cardiovascular centers in the RVLM with little influence from the change of respiratory activity.

In summary, the present study shows that orexins directly depolarized RVLM vasomotor neurons mainly via the OX2R. This effect of orexins may contribute to their promotion of sympathetic outflow from the RVLM to the cardiovascular system and their pressor effects. As a corollary, the RVLM is probably one of the important brain regions through which orexins exert their pressor effects.

### References


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