Orexins/hypocretins excite rat sympathetic preganglionic neurons in vivo and in vitro


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Received 1 February 2001; accepted in final form 2 August 2001

Orexins/hypocretins excite rat sympathetic preganglionic neurons in vivo and in vitro. Am J Physiol Regulatory Integrative Comp Physiol 281: R1801–R1807, 2001.—The two recently isolated hypothalamic peptides orexin A and orexin B, also known as hypocretin 1 and 2, are reported to be important signaling molecules in feeding and sleep/wakefulness. Orexin-containing neurons in the lateral hypothalamus project to numerous areas of the rat brain and spinal cord including the intermediolateral cell column (IML) of the thoracolumbar spinal cord. An in vivo and in vitro study was undertaken to evaluate the hypothesis that orexins, acting on sympathetic preganglionic neurons (SPNs) in the rat spinal cord, increase sympathetic outflow. First, orexin A (0.3, 1, and 10 nmol) by intrathecal injection increased mean arterial pressure (MAP) and heart rate (HR) by an average of 5, 18, and 30 mmHg and 10, 42, and 85 beats/min in urethane-anesthetized rats. Intrathecal injection of saline had no significant effects. Orexin B (3 nmol) by intrathecal administration increased MAP and HR by an average of 11 mmHg and 40 beats/min. The pressor effects of orexin A were attenuated by prior intrathecal injection of orexin A antibodies (1:500 dilution) but not by normal serum albumin. Intravenous administration of the α1-adrenergic receptor antagonist prazosin (0.5 mg/kg) or the β-adrenergic receptor antagonist propranolol (0.5 mg/kg) markedly diminished, respectively, the orexin A-induced increase of MAP and HR. Second, whole cell patch recordings were made from antidromically identified SPNs of spinal cord slices from 12- to 16-day-old rats. Superfusion of orexin A or orexin B (100 or 300 nM) excited 12 of 17 SPNs, as evidenced by a membrane depolarization and/or increase of neuronal discharges. Orexin A- or B-induced depolarizations persisted in TTX (0.5 μM)-containing Krebs solution, indicating that the peptide acted directly on SPNs. Results from our in vivo and in vitro studies together with the previous observation of the presence of orexin A-immunoreactive fibers in the IML suggest that orexins, when released within the IML, augment sympathetic outflow by acting directly on SPNs.

OREXIN A AND B, ALSO KNOWN AS hypocretin 1 and 2, are 33- and 28-amino acid peptides expressed in neurons of the rat lateral hypothalamus (7, 18). The initial observation that the peptide when injected into the ventricles promoted feeding behaviors, as assessed by food consumption in rats, has generated a considerable interest relative to its role in food intake and obesity (18). Recent studies have revealed that the peptide may also be an important signaling molecule in sleep/wakefulness. Disruption of the hypocretin receptor 2 gene results in a sleep disorder resembling narcolepsy in canines (16), and orexin knockout mice displayed signs and symptoms similar to those of human narcolepsy (2).

Although investigations relative to the role of orexins in feeding and narcolepsy have been the major focus of interest, there is evidence that the peptide may also be an important messenger molecule in the central regulation of autonomic activity including cardiorespiratory. For example, orexin-immunoreactive fibers are noted in areas of the medulla, including the nucleus of the solitary tract and ventral medulla, that are known to influence cardiorespiratory and other autonomic functions (6, 10, 17). Several functional studies have shown that orexins, with intracerebroventricular or intracisternal injection, increased mean arterial pressure (MAP) and heart rate (HR) in anesthetized or conscious rats (4, 19, 22). These studies suggest that orexins may act to increase sympathetic activity at the level of the medulla.

In addition to the medulla, orexin-immunoreactive fibers have been detected in the spinal cord, including the intermediolateral cell column (IML) where the majority of sympathetic preganglionic neurons (SPNs) is located (6, 24). The present study was conducted to evaluate the hypothesis that orexins by acting on SPNs may augment spinal sympathetic outflow, as assessed by a change in the MAP and HR in anesthetized rats and in the membrane potential of individual SPNs in thoracolumbar spinal cord slices.

METHODS

A breeding colony of Sprague-Dawley rats, purchased from Harlan (Indianapolis, IN), was established at the Division of Laboratory Animal Resources, East Tennessee State Univer-

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sity. Animals were housed two per cage in a room maintained at 22 ± 1°C with an alternating 12:12-h light-dark cycle; food and water were available ad libitum. Male rats weighing 325–350 g were used in the in vivo experiments, and 12- to 16-day-old rats were used in the in vitro study. Animal protocols were reviewed and approved by the Institution Animal Care and Use Committee.

In vivo experiments. Procedures for intrathecal injection to anesthetized rats were similar to those described earlier (14, 15). Under urethane anesthesia (1.2 g/kg ip), the right femoral artery was cannulated for intravenous injection of adrenergic receptor antagonists in one series of experiments. For intrathecal injection, a polyethylene tubing (Intramedic PE-10) was passed through a slit in the dura at the atlantooccipital junction to the T2-T3 segments; the position of the tubing was visually verified at the end of each experiment. Orexins were dissolved in physiological saline to desired concentrations. Intrathecal injections were made at a volume of 10 μl followed by 10 μl saline in wash in the peptide. Orexin A antiserum (1:500 dilution) or normal rabbit serum albumin (1:500 dilution) was injected intrathecally at a volume of 10 μl followed by 10 μl saline in a manner similar to that described for orexin injections. The protocol for the above series of experiments is as follows. First, saline was injected and followed by orexin A (1 nmol). After MAP and HR had returned to the basal level, orexin A antiserum and orexin A (1 nmol) were administered in succession, with a 10-min interval between injections. Lastly, orexin A was administered to determine whether or not the response to the peptide had recovered from the blocking effects of antiserum. MAP was calculated using the equation [S – D]/3 + D, where S is systolic pressure and D is diastolic pressure, whereas saline (20 μl) injection had no significant effects. Orexin B (3 nmol; n = 4) caused a significant increase of MAP and HR of 11.4 ± 6 mmHg and 16.3 ± 4.4 beats/min, respectively, above basal values. Orexin B (3 nmol; n = 4) caused a significant increase of MAP and HR of 11.4 ± 6 mmHg and 40.2 ± 5.7 beats/min over basal values.

RESULTS

Effects of intrathecal orexins on MAP and HR. The mean MAP and HR in anesthetized rats were 100.8 ± 10.7 mmHg and 335.7 ± 33.1 beats/min (n = 21). Orexin A and B consistently increased MAP and HR in all rats tested. The increase developed slowly, reached a peak in 3–5 min, and lasted for 15 to over 60 min. Generally, MAP and HR recovered to the basal level within 60 min after orexin A (1 nmol) injection; a representative experiment is shown in Fig. 1. The mean increases of MAP and HR by 0.3 nmol (n = 4), 1 nmol (n = 10), and 10 nmol (n = 3) orexin A were 5.3 ± 1.6, 18.4 ± 1.8, and 32.0 ± 4.4 mmHg, and 10.3 ± 1.1, 42.3 ± 6.1, and 85.7 ± 16.3 beats/min, respectively, above basal values. Orexin B (3 nmol; n = 4) caused a significant increase of MAP and HR of 11.4 ± 1.7 mmHg and 40.2 ± 5.7 beats/min over basal values. Figure 2 illustrates the change of MAP and HR induced by orexin A (1 nmol) and orexin B (3 nmol) relative to time. Intrathecal injection of saline before or after orexin A or B consistently increased MAP and HR in all rats tested. The increase developed slowly, reached a peak in 3–5 min, and lasted for 15 to over 60 min. Generally, MAP and HR recovered to the basal level within 60 min after orexin A (1 nmol) injection; a representative experiment is shown in Fig. 1. The mean increases of MAP and HR by 0.3 nmol (n = 4), 1 nmol (n = 10), and 10 nmol (n = 3) orexin A were 5.3 ± 1.6, 18.4 ± 1.8, and 32.0 ± 4.4 mmHg, and 10.3 ± 1.1, 42.3 ± 6.1, and 85.7 ± 16.3 beats/min, respectively, above basal values. Orexin B (3 nmol; n = 4) caused a significant increase of MAP and HR of 11.4 ± 1.7 mmHg and 40.2 ± 5.7 beats/min over basal values. Figure 2 illustrates the change of MAP and HR induced by orexin A (1 nmol) and orexin B (3 nmol) relative to time. Intrathecal injection of saline before or after orexin A or B consistently increased MAP and HR in all rats tested. The increase developed slowly, reached a peak in 3–5 min, and lasted for 15 to over 60 min. Generally, MAP and HR recovered to the basal level within 60 min after orexin A (1 nmol) injection; a representative experiment is shown in Fig. 1. The mean increases of MAP and HR by 0.3 nmol (n = 4), 1 nmol (n = 10), and 10 nmol (n = 3) orexin A were 5.3 ± 1.6, 18.4 ± 1.8, and 32.0 ± 4.4 mmHg, and 10.3 ± 1.1, 42.3 ± 6.1, and 85.7 ± 16.3 beats/min, respectively, above basal values. Orexin B (3 nmol; n = 4) caused a significant increase of MAP and HR of 11.4 ± 1.7 mmHg and 40.2 ± 5.7 beats/min over basal values. Figure 2 illustrates the change of MAP and HR induced by orexin A (1 nmol) and orexin B (3 nmol) relative to time. Intrathecal injection of saline before or
after orexin A or B injection caused no significant change of MAP and HR in any of the rats tested (Figs. 1 and 2).

**Antagonism of orexin A by orexin A antibodies.** As specific orexin A receptor antagonists are currently unavailable, the effects of orexin A antiserum were evaluated in relation to the pressor effects induced by the peptide. The specificity of orexin A antiserum has been verified in our immunohistochemical studies in which the immunostaining of orexin A antiserum was completely blocked by preabsorbing the antiserum with orexin A (3, 10).

In this series of experiments, the first injection of orexin A (1 nmol) caused a pressor response. Administration of orexin A antiserum caused a slight but nonsignificant change of MAP and HR (0.9 ± 0.2 mmHg and 1.1 ± 0.6 beats/min) in the five rats tested, whereas the orexin A-induced pressor response was significantly attenuated. Cardiovascular responses to orexin A fully recovered after 30–45 min. The results from five experiments are shown in Fig. 3.

As a negative control, substitution of orexin A antiserum by normal rabbit serum albumin (1:500 dilu-
tion) in the injection sequence did not prevent the pressor response of subsequent administrations of orexin A. Thus orexin A (1 nmol) produced a mean increase of MAP and HR of 16 ± 2.2 mmHg and 34 ± 5.2 beats/min before and 18.4 ± 3.4 mmHg and 31.6 ± 6.2 beats/min after injection of normal rabbit serum albumin (n = 3; P > 0.05).

Effects of adrenergic receptor antagonists. This series of experiments evaluated whether or not the orexin A-induced pressor response is mediated via the activation of the sympathetic nervous system. Propranolol (0.5 mg/kg) and prazosin (0.5 mg/kg) were selected as the β-adrenergic and α1-adrenergic receptor antagonists. Because intravenous administration of prazosin and propranolol caused a fall in MAP and HR, the second injection of orexin A was made after MAP and HR had stabilized to a new level, which was 65.5 ± 8.3 mmHg and 320.2 ± 7.4 beats/min in prazosin-treated rats and 97.3 ± 19.2 mmHg and 259.4 ± 17.3 beats/min in propranolol-treated rats. Propranolol and prazosin attenuated, respectively, the increase in HR and MAP caused by subsequent intrathecal administration of orexin A (Fig. 4).

Orexin A-stimulated SPNs. Whole cell recordings were made from SPNs located in the IML of lower thoracic and upper lumbar spinal cord slices, as described previously (25). SPNs had a mean resting potential of −57 ± 2 mV and input resistance of 670 ± 52 MΩ (n = 17), which were comparable to those reported earlier (14, 15, 25). Also, as reported earlier (21), some of the SPNs in spinal cord slices were found to discharge spontaneously (n = 7) (Fig. 5A). Five of seven (70%) spontaneous active cells responded to superfusion of orexin A or B (100 nM) with an increase in discharge frequency and/or a small depolarization (Fig. 5A). The peptide caused a slow membrane depolarization and/or increase of neuronal discharges in 7 of 10 (70%) silent SPNs tested (Fig. 5, B and D). Orexin A- or B-induced hyperpolarization was not observed in any of the SPNs studied. At the higher dose of 300 nM, orexin A or B caused a larger depolarization and intense neuronal discharges in four of five SPNs (Fig. 5, B and D); the mean depolarization was 6.2 ± 0.8 mV. Superfusion of the slices with a Krebs solution containing TTX (0.5 μM) blocked the neuronal discharge, but not the depolarization, induced by orexin A (Fig. 5C). The depolarization was associated with either a small increase (10–25%) or no apparent change in input resistance in responsive neurons.

![Figure 4](http://ajpregu.physiology.org/)

**Fig. 4.** Histograms showing the peak percent change in MAP and HR induced by orexin A (1 nmol) before and after intravenous administration of prazosin (0.5 mg/kg) and propranolol (0.5 mg/kg). Anesthetized rats in the prazosin-treated group had a mean basal MAP and HR of 113.8 ± 14.2 mmHg and 340 ± 22.4 beats/min (n = 5), and the mean basal MAP and HR of propranolol-treated rats were 100.3 ± 18.3 mmHg and 315.6 ± 28.5 beats/min. **Top:** saline injection had no appreciable effects on MAP and HR, whereas prazosin significantly attenuated the MAP increase induced by intrathecal injection of orexin A. The second injection of orexin A was made after MAP and HR had stabilized to a new level, which was 65.5 ± 8.3 mmHg and 320.2 ± 7.4 beats/min. **Bottom:** saline injection had no appreciable effects on MAP and HR, whereas propranolol significantly attenuated the HR increase induced by intrathecal injection of orexin A. The second injection of orexin A was made after MAP and HR had stabilized to a new level (97.3 ± 19.2 mmHg and 259.4 ± 17.3 beats/min). *Statistically significant difference between orexin A and saline-injected groups, and ** denotes a statistically significant difference between orexin A- and orexin A + prazosin or propranolol-treated groups. Vertical bars are means ± SE.
In vivo and in vitro experiments were conducted in the rats to evaluate the hypothesis that the peptide orexin modulates spinal sympathetic outflow by interacting with SPNs of the thoracolumbar spinal cord.

Orexin occurs in two forms: A and B (or hypocretin 1 and 2). In our earlier study, orexin A or B by intracisternal injection or microinjection to the rostral ventrolateral medulla elicited a qualitatively similar increase in MAP and HR in urethane-anesthetized rats (4). Here, our results indicate that intrathecal injections of orexin A or B produced qualitatively similar increases of MAP and HR. Orexin A, however, appears to be more potent than orexin B in eliciting a pressor response. For these reasons, orexin A was selected as the representative of the two peptides whose effects were more closely scrutinized in the present study.

Results from the in vivo experiments showed that orexin A upon intrathecal injection consistently caused a significant and prolonged increase of MAP and HR, whereas saline injection had no appreciable effects. The pressor response was markedly attenuated by prior injection of orexin A antiserum, which presumably neutralized the orexin A action by binding to the peptide. The specificity of orexin A antiserum used in our study has been verified in our previous studies in which preabsorption of the antiserum with the peptide orexin A rendered the former ineffective (3, 10). Intrathecal injection of normal serum albumin resulted in no appreciable change of the pressor response induced by orexin A, indicating that the antagonizing effect of orexin A antiserum was specific. Furthermore, the observation that the α₁-adrenoceptor and β-adrenoceptor antagonists prazosin and propranolol differentially attenuated the increase of MAP and HR supports the contention that the pressor response induced by intrathecal administration of orexin A is mediated by the activation of the sympathetic nervous system.

Within the sympathetic nervous system, SPNs in the thoracolumbar spinal cord provide the known output to the sympathetic ganglia, which in turn, innervate the heart and blood vessels. The increase in HR and blood pressure after intrathecal orexin A should reflect an excitation of SPNs innervating the respective organs. The possibility that the peptide following intrathecal injection may enter the circulation and produce a pressor response via a peripheral action appears to be unlikely, because intravenous administration of orexin A or orexin B at higher doses has been found to be ineffective in eliciting a pressor response in urethane-anesthetized rats (4).

More importantly, results from in vitro experiments directly demonstrated an excitatory action of orexin on SPNs. Superfusion of orexin A or orexin B caused a depolarization and/or neuronal discharge in the majority of SPNs tested. The membrane depolarization, but...
not the neuronal discharge, persisted in a TTX-containing solution, indicating that the peptide acted directly on SPNs. The depolarization and associated increase of neuronal discharges of SPNs observed in vitro could explain the sympathoexcitatory effect of orexin A in vivo.

At the concentrations tested here, orexin A or B had no apparent membrane effects on a number of SPNs. A simple explanation is that SPNs that bear orexin receptors are target specific. Alternatively, the concentrations used here may not be optimal to some of the SPNs tested. Two subtypes of orexin receptors,OX1R and OX2R, have been characterized (18). The OX1R shows high affinity for orexin A, and orexin A and B bind with about equal affinity to OX2R (18). Both types of orexin receptors are G protein coupled (18). Activation of orexin receptors by orexin A or B on SPNs produced a membrane depolarization of relatively slow time course, which is characteristic to activation of other known G protein-coupled receptors.

The ionic mechanism underlying orexin A-induced depolarizations in SPNs has not been elucidated. The depolarization was accompanied by either an increase of input resistance in some neurons or no apparent change in others. Orexin-induced depolarizations in the rat dorsal motor nucleus of vagus neurons were associated with an increase of input resistance or no apparent change, which is attributed to an increase of nonspecific cation conductance and a decrease of potassium conductance (11). In view of the similarity of membrane resistance change induced by orexin A in SPNs on one hand and dorsal motor nucleus of vagus neurons on the other, the ionic mechanism underlying the depolarization in these two types of neurons is expected to be similar. In the case of rat locus coeruleus neurons, the orexin-induced depolarization appears to be caused by a decrease of potassium conductance (12).

The question of whether endogenously released orexin A in the spinal cord may play a role in regulating sympathetic outflow remains to be addressed. Orexin A- and orexin B-immunoreactive fibers are present in the IML area of the rat thoracolumbar spinal cord (5, 24). The physiological and/or pathological conditions under which the peptide may be released within the IML area are not known. Another question that needs to be addressed in future studies is whether or not activation of orexin-containing neurons in the lateral hypothalamus produces a pressor response similar to that observed here. An earlier study showed that electrical stimulation applied to various sites in the forebrain and midbrain including areas of the lateral hypothalamus, where orexin-containing neurons are located, elicited vasoconstriction, as assessed by lumen diameter changes of mesenteric vascular beds (9). Both pressor and depressor responses were observed in more recent studies where L-glutamate was microinjected to the lateral hypothalamus (1, 23). The development of specific orexin receptor antagonists would be crucial in defining a physiological role of orexins in central regulation of sympathetic outflow.

Perspectives

The hypothalamic peptides orexin A and B or hypocretin 1 and 2, discovered independently by two groups (7, 18), have been implicated to play a major role in food consumption and sleep/arousal behaviors (8, 13). More recent studies suggest that the peptides elevate blood pressure and HR by acting at a site(s) in the medulla (4, 19, 22). Results from the present study show that the peptide increases blood pressure and HR by stimulating sympathetic preganglionic neurons in the spinal cord. This observation together with earlier studies supports the thesis that orexins/hypocretins may play a significant role in cardiovascular regulation by interacting with receptors at different levels of the neuroaxis. As the lateral hypothalamus is the only site where orexin-containing neurons are found in the mammalian brain (3, 7, 18), the orexin projection to the medulla and IML may represent two parallel pathways through which the lateral hypothalamus may influence the sympathetic nerve output to the target organ.

This study was supported by National Institute of Neurological Disorders and Stroke Grant NS-18710 and National Heart, Lung, and Blood Institute Grant HL-51314.

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