Blockade of orexin receptors with Almorexant reduces cardiorespiratory responses evoked from the hypothalamus but not baro- or chemoreceptor reflex responses

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*Running title:* Orexin amplifies responses evoked from the hypothalamus

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ABSTRACT

Orexin neurons form a restricted group in the dorsal hypothalamus. The group is centered on the perifornical area within the classic hypothalamic defense area, an area which when activated produces marked cardiovascular and respiratory effects. Central administration of orexin can produce cardiorespiratory effects, but the extent to which orexin contributes to such responses evoked from the perifornical hypothalamus is not clear. To determine this, we used the dual orexin receptor antagonist Almorexant to challenge the cardiorespiratory effects evoked by disinhibition of the perifornical hypothalamus. Bicuculline (10 and 20 pmol) was microinjected in the perifornical area before and after administration of Almorexant (15 mg/kg, iv) or vehicle in urethane anesthetised rats. Almorexant significantly reduced the pressor, tachycardic, renal sympathoexcitatory and tachypneic responses to bicuculline (10 pmol, by 55%, 53%, 28%, 77%; 20 pmol, by 54%, 27%, 51%, 72%, respectively). Reductions of similar magnitude were observed with bicuculline microinjections centered on more caudal sites just peripheral to the orexin neuron group, which would likely have activated fewer orexin neurons. In contrast, Almorexant had no effect on the cardiorespiratory response of the chemoreflex (sodium cyanide injection) or the sympathetic component of the baroreflex. Thus orexin makes a major contribution to the cardiorespiratory response evoked from the perifornical area even though orexin neurons represent only a fraction of the output of this area. Orexin neurons may also mediate cardiorespiratory responses from non-orexin neurons in the caudal hypothalamus. However, under resting conditions, blockade of orexin receptors does not affect the chemo- and baro-reflexes.

KEY WORDS

hypocretin, perifornical area, sympathetic vasomotor activity, phrenic nerve activity
INTRODUCTION

The importance of orexin (also called hypocretin) in the regulation of arousal is now well established (32). This neuropeptide plays a crucial role in the maintenance of wakefulness during the active phase of the cycle (33) and is also responsible for the increased level of arousal necessary for the expression of behaviors such as drug seeking, food seeking and some forms of stress (2, 12, 17, 42). The neurons that make orexin and mediate these effects are located in the dorsal hypothalamus. They form a restricted group that is centered on the perifornical region (PeF) and covers an area located within the well-known hypothalamic defense area (16, 39). Electrical stimulation or disinhibition of this area with GABA receptor antagonists such as bicuculline evokes marked pressor, tachycardic and respiratory responses and, in the conscious animal, flight responses, or if the stimulus is milder, alerting with increased locomotor activity (8, 27). This hypothalamic defence area is thought to play an important role in somatic/autonomic integration and the expression of a wide range of stress responses (6).

Orexin has potent effects on the central autonomic and respiratory networks, producing hypertension, tachycardic and hyperventilation when injected centrally (3, 23, 24, 35-37, 40, 41). It is therefore likely that orexin contributes to the cardiorespiratory effects evoked by stimulation of the hypothalamic defence area. Indeed, a previous study in orexin knock-out mice showed a marked reduction of the cardiovascular response to bicuculline injections in the PeF (19). However, this needs to be confirmed in normal animals with a more direct approach, such as pharmacological blockade of orexin receptors. There are two orexin receptors (OxR1 and OxR2) on which orexin can act; therefore to test orexin effects, both receptors must be blocked at the same time, ideally with a dual receptor antagonist. Almorexant (formerly known as ACT-078573), which was introduced in 2007 by Brisbare-Roch et al (1), is an ideal candidate, as it is a selective antagonist of both receptors. In their original study describing the properties of Almorexant, Brisbare-Roch et al (1) showed that it reduced wakefulness and locomotor activity in rats, dogs and humans when administered systemically. More recent work in the rat shows that Almorexant can reduce by 40% or more the cardiovascular response associated with exploration and conditioned fear, two behavioural responses that are associated with activation of orexin neurons (12). Almorexant has also been shown to reduce ventilation during the active phase of the sleep-wake cycle but not during the inactive phase in rats, which is consistent with orexin neurons having increased activity during wakefulness (21).
The aim of this study, therefore, was to use Almorexant to challenge the cardiorespiratory effects evoked by bicuculline disinhibition of the PeF in order to evaluate the relative contribution of the orexin peptide to this response. Injections of bicuculline were also made at the caudal periphery of the orexin group to determine if the inhibitory effect of Almorexant would be reduced when fewer orexin neurons are recruited. Finally, we tested the chemo- and baroreflex to determine if Almorexant would have any effect on these basic cardiorespiratory reflexes under resting conditions.

MATERIALS AND METHODS

Experiments were performed on a total of 37 male Sprague-Dawley rats (270-440 g) supplied by the University of Sydney Laboratory Animal Services. All experimental procedures were approved by the Animal Ethics Committee of the University of Sydney and were carried out in accordance with the Guidelines for Animal Experimentation of the National Health and Medical Research Council of Australia.

General Procedures. Anesthesia was initially induced by inhalation of isoflurane (2.0-2.5% in oxygen-enriched air). The trachea was then intubated and body temperature was maintained in the range of 37-38 ºC with a heating pad. Catheters were placed in a femoral artery and a femoral vein for the recording of pulsatile arterial pressure and drug injection, respectively. After the surgery, the isoflurane anesthesia was gradually withdrawn while being replaced by urethane (1.3g/kg, i.v. with supplementary doses of 0.1 g/kg, i.v., if required). The adequacy of anesthesia was verified by the absence of the corneal reflex and a withdrawal response to nociceptive stimulation of a hind paw. A tracheotomy was performed to maintain an unobstructed airway and all animals were allowed to breathe freely.

The rat was then placed in a stereotaxic apparatus with the incisor bar fixed 3.5 mm below the interaural line. The renal and phrenic nerves were exposed and placed on bipolar recording electrodes as described previously (26). Small portions of the dorsal surface of the cortex were exposed to allow insertion of micropipettes into the dorsal hypothalamus. The signals from the recording electrodes were amplified and filtered (bandwidth 100-2,000 Hz for the renal nerve, and 10-2,000 Hz for the phrenic nerve). These signals were then digitized (1,000 samples per second) and recorded on a computer using a PowerLab system (AD Instruments). Chart software was used to rectify and integrate the renal sympathetic nerve
activity (RSNA) and the phrenic nerve activity (PNA) signals, and to compute the rate and amplitude of the bursts of PNA. The mean arterial pressure (MAP) and heart rate (HR) signals were derived from the pulsatile arterial pressure signal via a low-pass filter and a rate meter, respectively, and also recorded using the PowerLab system.

Microinjections of bicuculline were made into sites in the hypothalamus using a glass micropipette held vertically in a micromanipulator. The vehicle solution was artificial cerebrospinal fluid adjusted to pH 7.4. In all cases the injectate also contained green or red fluorescent latex microspheres (0.5%, Lumafluor), to facilitate the later histological verification of microinjection sites. Microinjections were made by pressure, using a previously described method (11). At the end of each experiment, the rat was euthanized with an overdose of pentobarbital sodium, the brain was removed, and after fixation in 4% paraformaldehyde solution, coronal sections (50µm) were cut on a freezing microtome and mounted onto glass slides. Injection sites were determined using a fluorescence microscope and mapped onto standard sections of the atlas by Paxinos and Watson (29). The injection sites were identified in all experiments except one – in that experiment the tissue near the injection site in the posterior hypothalamus was damaged, but the track was visible more dorsal, so that it was possible to confirm the anterior-posterior level of the injection.

Injection of Almorexant. Almorexant (a gift from Actelion Pharmaceuticals) was dissolved in 10% β-cyclodextrin (2 hydroxypropyl-β-cyclodextrin, Sigma-Aldrich). The dose of Almorexant selected (15 mg/kg, i.v. in 1 ml/kg) was based on a previous study by Brisbare-Roch et al. (1), in which they examined the selectivity and pharmacodynamics of the drug. In 3 preliminary experiments, a dose of 15 mg/kg i.v. was shown to block the pressor effect of orexin-A injected i.c.v. (1 nmol in 5 µL) whereas in 2 other experiments a dose of 3 mg/kg i.v. reduced but did not block the pressor effect of orexin.

Experimental procedures. Hypothalamic microinjections of bicuculline (20 pmol in 40 nl) were made in a total of 19 experiments. Injections were aimed either at the center of the orexin group (2.8mm caudal to bregma, 1.2 mm lateral to the midline, and 8.1-8.4 mm ventral to the dura surface) or at the caudal periphery of the orexin group (3.48 mm caudal to bregma, 0.5mm lateral to the midline, and 8.5-9.0 mm ventral to the dura surface). In most cases (12 out of 19), the first microinjection produced a significant response, which was defined as a
response in which the MAP, HR, RSNA and PNA burst rate all increased by at least 10 mmHg, 40 bpm, 25% of baseline and 50 bursts/min, respectively. In 6 cases one or two additional microinjections, and in 1 case 4 additional microinjections, were required before a significant response was obtained. Once such a site was identified, a waiting period of 40-60 min was allowed for the cardiorespiratory responses to return to their resting levels. A systemic injection of Almorexant (15 mg/kg, i.v, n=13) or vehicle/saline solution (n=6) was then made, and after an additional waiting period of 45-50 min, a repeat microinjection of bicuculline was made at the same site as before Almorexant administration.

In 5 additional experiments, a smaller dose of bicuculline (10 pmol in 20 nl) was injected. These injections were aimed at the center of the orexin group. In this case, because the dose of bicuculline was less, a significant response was defined as a response in which the MAP, HR, RSNA and PNA burst rate all increased by at least 5 mmHg, 35 bpm, 20% of baseline and 25 bursts/min, respectively. The procedure was the same as in the experiments in which 20 pmol was injected, except that 2 or 3 bicuculline microinjections were made into different sites in the PeF before Almorexant administration. A significant response was obtained from a total of 11 sites in these 5 experiments. After Almorexant administration, bicuculline microinjections were made into the same sites (i.e. with the same stereotaxic coordinates) from which a significant response was obtained before Almorexant administration.

In 7 experiments, the effects of Almorexant on the chemoreceptor reflex was tested by determining the responses to stimulation of the peripheral chemoreceptors by bolus injection of sodium cyanide (1 mg/kg, i.v.) before and after injection of Almorexant. In 4 of these experiments, the chemoreceptor reflex was tested in the same rats in which 20 pmol microinjections of bicuculline were made into the hypothalamus, while in the remaining 3 experiments the chemoreceptor reflex only was tested before and after administration of Almorexant.

In 5 of the experiments described above in which bicuculline microinjections were made before and after Almorexant administration, cycle-triggered averaging was used, as described previously (26) to determine the cyclic relationship between arterial pressure pulses and subsequent changes in RSNA, using the minimum diastolic pressure as the trigger point for each cardiac cycle. This procedure was performed on data recorded during the resting period before and after Almorexant administration, just before the bicuculline microinjections were made. The cardiac related changes in sympathetic activity are due to inputs from arterial
baroreceptors (13, 15). The ratio between the magnitude of the averaged cardiac-related change in RSNA and the averaged arterial pressure pulse was taken as a measure of the sensitivity of the baroreceptor-sympathetic reflex. It has been shown previously that this ratio increases under conditions when the gain of the baroreceptor-sympathetic reflex is increased (26, 27).

Data analysis. The resting values of MAP, HR, RSNA, PNA burst rate and PNA burst amplitude, and the changes in these variables evoked by microinjections of bicuculline (either 10 or 20 pmol) under control conditions and after systemic injection of Almorexant or vehicle solution were compared using the paired t-test. A similar procedure was used to compare the chemoreflex-evoked changes in these variables, and baroreflex sensitivity before and after Almorexant administration. Responses (before and after Almorexant administration) evoked from sites centered within the region containing a high density of orexin neurons and those evoked from other sites in the hypothalamus were compared using the unpaired t-test. A P value of <0.05 was regarded as statistically significant. All values are presented as mean ± SE. In 5 of the 27 rats used for physiological experiments, RSNA could not be measured due to technical problems.

Identification of orexin neurons. In a separate group of 5 rats, the locations of orexin neurons were mapped at 12 levels of the hypothalamus, 0.2 mm apart, extending from the level 1.6 mm to 3.8 mm caudal to bregma. In these experiments, the rats were euthanized by overdose of sodium pentobarbitone (120 mg/kg, i.p.), and perfused transcardially with 0.9% saline (500 ml) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (500 ml). Following cryoprotection in 30% sucrose-phosphate buffer, the hypothalamus was cut into 40 µm thick transverse sections using a CO2 freezing microtome, and free floating sections were processed immunohistochemically for orexin-A using an avidin-biotin-HRP protocol (12). The antibodies used for these procedures were mouse anti-orexin A (Hypocretin-1) IgG (dilution 1:20,000, Phoenix Pharmaceuticals, Burlingame, CA, USA), biotinylated donkey anti rabbit IgG (1 in 500, Vector Laboratories) and ExtrAvidin-HRP ™ (1:1000, Sigma). Following HRP visualization using a standard DAB reaction, the sections were mounted onto gelatin-coated slides, dried and coverslipped. Sections containing orexin-labeled neurons were identified throughout the rostro-caudal extent of the hypothalamus and photographed.
using the 5X and 10X objectives. The images were processed using Metamorph (Molecular Devices, Sunnyvale, CA, USA) to discriminate labeled neurons. The number of labeled neurons was calculated for each section and their distribution mapped onto standard hypothalamic sections from a rat brain atlas (29). The accuracy of cell counts using Metamorph was confirmed by performing manual counts on a random selection of sections.

RESULTS

Since the main aim was to target the area that contains orexin neurons, we first analysed their distribution in the dorsal hypothalamus to determine the optimal coordinates for the microinjections. As can be seen in Fig 1A & B the highest density of orexin neurons was found in the PeF, dorsal to the fornix. The group was well defined and circumscribed, particularly in the rostro-caudal direction, extending from 1.8 to 3.8 mm caudal to bregma, but with the large majority of neurons located between 2.4 and 3.2 mm caudal to bregma. Figure 1C shows the sites, identified histologically, at which bicuculline was injected, according to these coordinates. Those that were injected with the small dose (10 pmol) and aimed at the center of the orexin group are represented on the left of the plates. Those that were injected with the large dose (20 pmol) and aimed at the center or the caudal periphery of the orexin group are represented on the right of the plates.

Effect of Almorexant on cardiorespiratory variables at rest.

Systemic injection of Almorexant (15 mg/kg, iv) resulted in initial decreases in MAP and HR, and in slightly delayed (~ 2 min) decreases in RSNA. PNA burst rate was reduced in 14 out of 21 cases, while PNA burst amplitude was unchanged in the majority of cases (16 out of 21), with only a slight (~10%) and transient (~ 2 min) increase in the remaining 5 cases. These variables returned to close to their pre-injection resting values within 30 min after the injection. Comparison of pre-injection and 30 min post-injection resting values revealed no significant change in MAP and RSNA, but there were significant though modest reductions in HR and PNA burst rate (of 19 bpm and 7 bursts/min, Table 1). Changes in the five variables were also measured before and 30 min after vehicle (β-cyclodextrin) or saline injections in 6 animals. Small reductions in HR and PNA burst rate were again observed (of 8 bpm and 13 bursts/min, respectively, Table 1), but pre-and post-injection comparisons revealed no significant difference for any of the variables. Changes in resting cardiorespiratory values
after Almorexant were also compared to those after vehicle/saline with no difference observed in any variable (ΔMAP, p = 0.46; ΔHR, p=0.32; ΔRSNA p=0.09; and ΔPNA burst rate, p=0.28). Thus, after 30 min, the effect of Almorexant administration on cardiorespiratory variables at rest was not significantly different to that of vehicle or saline administration.

**Effect of Almorexant on cardiorespiratory response evoked by 10 pmol bicuculline.**

Microinjections of 10 pmol of bicuculline were made at 11 sites aimed at the center of the orexin group. The histology confirmed that the centers of 7 of these sites were located inside the orexin group while the remaining 4 were located on the periphery of this group either dorsally or caudally (Fig 1C, left side of plates). The first injections before administration of Almorexant (control) evoked clear cardiorespiratory responses with increases in all variables (MAP, +14.6 ± 2.4 mmHg; HR, +73.4 ± 10.3 bpm; RSNA, +34.4 ± 4.2 %; PNA burst rate, +66.5 ± 8.9 bursts/min, and PNA burst amplitude, +17.0 ± 3.9%, peak responses averages, Fig 2A, B). When the injections were repeated after administration of Almorexant, the evoked increases in MAP, HR, RSNA and PNA burst rate were all significantly reduced (to 44.8 ± 6.4%, 47.3 ± 6.4%, 72.5 ± 10.6%, and 22.9 ± 3.4% of control, respectively)(Fig 2A&B). In contrast, administration of Almorexant had variable effects on the bicuculline-evoked increase in the PNA burst amplitude, resulting in a reduction in 6 cases, an increase in 4 cases, and no change in the remaining case. Overall, the bicuculline-evoked increase in PNA burst amplitude after Almorexant was 8.3 ± 3.0%, which was not statistically significantly different from the control response (P > 0.14).

**Effect of Almorexant on cardiorespiratory response evoked by 20 pmol bicuculline microinjections inside the orexin group.**

Microinjections of 20 pmol bicuculline were made at 6 sites aimed at the center of the orexin group. The histology confirmed that all 6 sites of injection were within the orexin group (Fig 1C, right side of plates). As expected, this larger dose of bicuculline evoked larger and more long-lasting responses than the 10 pmol dose (Fig 3A, control), with higher average peak increases (MAP, +22.4 ± 1.9 mmHg; HR, +128.4 ± 12.9 bpm; RSNA, +49.4 ± 7.7 % and PNA burst rate, +99.6 ± 16.9 bursts/min, Fig 4A&B, left). As with the response to 10 pmol bicuculline, after administration of Almorexant the evoked increases in MAP, HR, RSNA and PNA burst rate evoked by 20 pmol bicuculline were all significantly reduced (to 46.5 ± 7.0%,...
73.4 ± 12.7%, 49.3 ± 9.3% and 28.4 ± 5.1% of control, respectively)(Fig. 3A, Fig 4A&B, left panels, Fig 4C). In contrast, administration of Almorexant had no significant effect on the increase in the PNA burst amplitude evoked by 20 pmol bicuculline (peak increase 15.8 ± 2.6% baseline before and 27.0 ± 8.0% after Almorexant, P > 0.12).

Effect of Almorexant on cardiorespiratory response evoked by 20 pmol bicuculline microinjections at the periphery of the orexin group.

Microinjections of 20 pmol bicuculline were made at 7 more caudal sites, aimed such that the centers of the sites were just peripheral to the orexin group. The histology confirmed that 4 of the sites of injection were medial and caudal to the orexin group, one was medial and dorsal and one was in the caudal PeF (Fig 1C, right side of plates). One site could not be plotted due to damage to the tissue near the tip of the cannula track, however the track was visible more dorsally. It was located medially and caudally to the orexin group, at the level 3.48 mm caudal to bregma. An example of the response evoked at one of these sites is shown on Fig 3B (control). The average MAP and HR responses evoked at these sites were smaller than after injections within the orexin group (MAP, +13.8 ± 1.2 mmHg; HR, +86.8 ± 13.7 bpm, peak increase, Fig 4A&B, right), however the average RSNA and PNA burst rate responses were similar to the responses to microinjections within the orexin group (RSNA, +57.5 ± 9.9% and PNA burst rate, +90.4 ± 17.5 bursts/min, peak increases, Fig 4A&B, right). After administration of Almorexant the evoked increases in MAP, HR, RSNA and PNA burst rate evoked by 20 pmol bicuculline were all significantly reduced (to 48.9 ± 6.4%, 58.1 ± 11.2%, 42.8 ± 5.5% and 43.7 ± 5.3% of control, respectively)(Fig. 3B, Fig 4A&B, right panels, Fig 4C). For all these variables, the magnitudes of the reductions in the response after Almorexant were not significantly different to those observed when the sites were centered within the orexin group (p= 0.06-0.75, Fig 4C). As observed for responses evoked by bicuculline microinjections into the orexin group, administration of Almorexant did not have a significant effect on the increase in the PNA burst amplitude evoked by 20 pmol bicuculline microinjections centered on sites just peripheral to the orexin group (peak increase 22.1 ± 4.6% baseline before and 14.4 ± 2.2% after Almorexant, P > 0.25).
**Effect of vehicle on the cardiorespiratory response evoked by 20 pmol bicuculline microinjections.**

To verify that repeating bicuculline injections at the same site produced reproducible responses, bicuculline injections were made into 6 sites before and after intravenous injections of vehicle (n=4) or saline (n=2) instead of Almorexant. Three sites were centered within the orexin group and 3 were on the periphery (Fig 1A, right side of plates). As can be seen on Fig 5 (A, B&C), the responses to the first and second microinjections of 20 pmol bicuculline were not significantly different.

**Effect of Almorexant on the cardiorespiratory response evoked by chemoreceptor stimulation.**

To determine if orexin is involved in the arterial chemoreceptor reflex under resting conditions, intravenous injections of sodium cyanide (NaCN) were given before and after Almorexant in 7 rats. NaCN evoked the expected sympathoexcitatory and hyperventilation response as can be seen by the increases in MAP, HR, RSNA and PNA burst rate (Fig 6A&B). The same response was observed when the NaCN injection was repeated after Almorexant, indicating that the chemoreflex was not affected by the dual receptor antagonist (Fig 6A&B).

**Effect of Almorexant on the baroreceptor-sympathetic reflex.**

To determine if orexin is involved in the sympathetic component of the baroreceptor reflex under resting conditions, we determined the size of the arterial pulse-synchronous changes in RSNA before and after Almorexant using cycle-triggered averaging, in 5 experiments (Fig 7A). The ratio of the change in RSNA to the magnitude of the arterial pressure pulse was not significantly different before and after Almorexant (Fig 7B), indicating that the sensitivity of the baroreceptor-sympathetic reflex was not affected by the antagonist, at least at resting levels of arterial pressure.
DISCUSSION

The results show that systemic administration of Almorexant reduced very substantially the cardiovascular response (peak increases in MAP, HR and RSNA reduced by 28-55%) and the respiratory response (peak increase in PNA burst rate reduced by 72-77%) evoked by disinhibition of the PeF. This demonstrates that orexin makes a significant contribution to the cardiorespiratory effects evoked from the PeF. In contrast, Almorexant had no effect on the cardiorespiratory and sympathetic changes evoked by stimulation of peripheral chemoreceptors or baroreflex modulation of sympathetic discharge at rest. This indicates that orexin receptors are not essential components of the neural pathways subserving these reflexes, and that Almorexant itself does not interfere with the expression of baroreceptor and chemoreceptor reflexes under resting conditions.

Contribution of orexin to cardiorespiratory responses evoked from PeF in the center of the orexin group.

Almorexant (or ACT-078573) is a dual orexin receptor antagonist that competitively antagonizes the binding of orexin to both its receptors OxR1 and OxR2, and which also effectively crosses the blood-brain barrier (1). As described in the Methods, the dose of Almorexant used (15 mg/kg i.v.) was selected on the basis of a previous study by Brisbare-Roch et al. (1), in which they examined the selectivity and pharmacodynamics of the drug. In preliminary experiments we found that this dose was sufficient to block the pressor response evoked by orexin-A injected icv, whereas a lower dose did not block this pressor response. At the same time, we cannot rule out the possibility that the dose of Almorexant used was insufficient to cause complete blockade of the effects of synaptically released orexin on OxR1 and OxR2. If that were the case, however, then the contribution of orexin receptors to the cardiorespiratory responses evoked from the hypothalamus in the present study would be even greater than the observed effects.

The present study showed that systemic administration of Almorexant can produce marked reductions of the cardiorespiratory changes evoked by bicuculline disinhibition of the PeF, the area in which the density of orexin neurons is greatest. Similarly, in prepro-orexin knock-out mice, Kayaba et al (19) also reported marked reductions of cardiorespiratory responses to perifornical bicuculline microinjections. In fact the extent of the reductions in MAP, HR and PNA burst rate that they reported with their moderate dose of bicuculline is
comparable to those we observed with our two doses of bicuculline (half to more than half for MAP and HR; two-thirds or more for PNA burst rate). Our results therefore are entirely consistent with the observations of Kayaba et al (19) in knock-out mice, and demonstrate for the first time that acute blockade of orexin receptors in normal rats greatly reduces the cardiorespiratory response evoked from the PeF.

The output pathways from the PeF that mediate the cardiovascular and respiratory responses to disinhibition of this region are not known, although there are projections to the Kölliker-Fuse nucleus, solitary nucleus/dorsal vagal complex, raphe pallidus/parapyramidal area, rostral ventrolateral medulla, and sympathetic preganglionic nuclei and phrenic motor nucleus in the spinal cord (4, 22, 28, 30, 36, 43). All of these regions contain neurons regulating cardiovascular and/or respiratory function (5, 10) and also have orexin receptors (3, 4, 7, 22-25, 36, 38, 40). Systemic administration of Almorexant would be expected to antagonize orexin receptors in all these regions, and so it is not possible to determine, on the basis of the present results, the specific sites at which antagonism of orexin receptors resulted in the observed reductions in sympathoexcitatory, cardiac and respiratory responses to activation of PeF neurons. Nevertheless, when considered together with the results of previous studies, some conclusions can be made. First, it has been shown that both intrathecal injection and microinjection into the rostral ventrolateral medulla of orexin-A (which activates both OxR1 and OxR2 (32)) evokes an increase in arterial pressure, HR and sympathetic activity (3, 24, 35, 36), consistent with the possibility that orexin, released in response to disinhibition of PeF neurons, activates sympathetic preganglionic neurons in the spinal cord and/or sympathetic premotor neurons in the RVLM to increase arterial pressure, HR and RSNA.

As mentioned above, the raphe pallidus/parapyramidal area (also known as the rostral ventromedial medulla) is also a major target for orexinergic inputs from the PeF (4, 30, 38). Microinjection of orexin-A into the raphe pallidus, parapyramidal area and other sites within the rostral ventromedial medulla increases HR and arterial pressure (4, 23, 38). Thus, orexin receptors within these regions may also contribute to the increase in HR and MAP evoked by activation of neurons within the PeF.

On the other hand, in regard to respiratory effects, Shahid et al. (35, 36) and Young et al. (40) found that both intrathecal injection and microinjection into the RVLM (including the pre-Bötzinger region) of orexin-A evokes an increase in PNA burst amplitude accompanied by either no significant change or else a decrease in PNA burst frequency (35, 36, 40).
Similarly, Zhang et al. (41) found that intracisternal administration of orexin-A evoked an increase in respiratory tidal volume with no change in respiratory frequency. This is in contrast to our results which indicate that, in response to disinhibition of PeF neurons, orexin receptors contribute to the evoked increase in PNA burst frequency but not PNA burst amplitude. We therefore propose that a more likely explanation is that orexin, released in response to PeF stimulation, acts at a higher level of the central respiratory network to increase PNA burst frequency. In fact, microinjection of orexin-B into the Kölliker-Fuse nucleus in the pons evokes a significant increase in PNA burst frequency without significant change in PNA burst amplitude, arterial pressure and HR (9). Furthermore, the Kölliker-Fuse nucleus is a major target of orexinergic neurons from the PeF (30). Therefore, our results, together with these previous findings, suggest that the Kölliker-Fuse nucleus may be an important site at which orexin, released in response to activation of PeF neurons, causes a significant increase in respiratory frequency.

Double-labelling studies indicate that orexin neurons form only a minority of the total number of projection neurons in the PeF. For example, in the case of the solitary nucleus/dorsal vagal complex and raphe pallidus/parapyramidal area, orexin neurons are estimated to constitute 20% of PeF neurons projecting to those targets (38, 43). Similarly, we have also found that only 28% of PeF neurons projecting to the upper thoracic cord are orexinergic (Carrive, unpublished observations). The fact that pharmacological blockade of orexin receptors or permanent loss of orexin has such marked effects on the cardiorespiratory response evoked from the PeF, even though orexin neurons appear to be only a minority of the projection neurons, could be explained by the possibility that orexin acts as a neuromodulator, amplifying the excitatory effects of other neurotransmitters such as glutamate that are released from non-orexin neurons or are released as a co-transmitter from orexin neurons (31). Consistent with this hypothesis, Tupone et al (38) have demonstrated that microinjection of orexin-A into the raphe pallidus or parapyramidal area in the medulla, regions that contain sympathetic premotor neurons regulating brown adipose tissue (BAT), evoked large increases in BAT sympathetic activity when BAT sympathetic premotor neurons were active, but not under conditions when these neurons were inactive. Thus, our results suggest that this may be a general property of orexin neurons, such that they act as a gain controller that can amplify inputs to neurons regulating sympathetic vasomotor activity, heart rate, and respiratory activity.
This important role of orexin is consistent with previous work by Furlong et al (12) in the conscious rat, which showed that Almorexant can reduce by nearly half or more the cardiovascular response to novelty and conditioned fear, two natural stimuli that activate orexin neurons (12). Kayaba et al (19) reported similar findings with psychosocial stress (resident-intruder paradigm) in knock-out mice, thus further demonstrating the importance of orexin neurons in the PeF in the expression of these responses.

Apart from the PeF, orexin neurons are also located more laterally in the lateral hypothalamus and more medially in the dorsomedial hypothalamus. There is some evidence that orexin neurons are not functionally homogeneous, but consist of two functionally distinct groups (14). Specifically, it has been proposed that orexin neurons located in the PeF and DMH are primarily involved in arousal and stress, whereas those located more laterally in the lateral hypothalamus are primarily involved in reward processing. In our study the microinjections of bicuculline were centered on sites within the PeF (Fig. 1C), and so would be expected to activate primarily orexin neurons that modulate stress or arousal responses. It is possible, however, that orexin neurons located more laterally in the lateral hypothalamus, or perhaps more medially in the DMH, may have different modulatory roles on cardiorespiratory responses evoked from these regions, compared with that from thePeF. Future studies would be required to test that possibility.

**Contribution of orexin to baroreceptor and peripheral chemoreceptor reflexes at rest.**

Intrathecal injection of orexin A or microinjection of orexin A into the rostral ventrolateral medulla results in an enhancement of the sympathoexcitatory response to chemoreceptor stimulation, and an increase in the sensitivity of the baroreceptor-sympathetic reflex (36). Thus, our finding that Almorexant had no effect on the chemoreceptor reflex and on the baroreceptor-sympathetic reflex at rest suggests that there was little or no tonic release of orexin at these sites in our anesthetised preparation, at least not enough to affect these reflexes. It is possible that there may have been a weak tonic release of orexin at sites modulating HR, because baseline values of HR were slightly reduced after Almorexant. However, these effects were not significantly different to effects observed after vehicle or saline administration, and are therefore probably negligible.
Although our results indicate that orexin does not significantly affect baroreceptor and chemoreceptor reflexes under resting conditions in the anesthetized state, it is entirely possible that orexin does have a significant effect on these reflexes under conditions where orexin neurons in the PeF are activated, such as during psychological stress or arousal (12, 17, 42). In conscious rats, the baroreceptor reflex control of sympathetic activity is reset, such that the operating range for the reflex is increased to higher levels together with an increase in the gain of the reflex (18). Very similar effects occur when the PeF is activated (6, 27), and this may be due, at least in part, to activation of orexin neurons within this region. Further studies will be required to test this possibility.

**Contribution of orexin to cardiorespiratory responses evoked at the caudal periphery of the orexin group.**

In one series of experiments injections of bicuculline were made into sites at the caudal periphery of the orexin group, to see if the inhibitory effect of Almorexant would be reduced when fewer orexin neurons are recruited. We found, however, that Almorexant also substantially reduced the cardiorespiratory response to bicuculline microinjections made into these caudal peripheral sites, and in fact the magnitudes of these effects were not significantly different to the effects of Almorexant on responses evoked from sites within the orexin group. It has been shown that 90% of the bicuculline within a 50 nl microinjection into the hypothalamus is contained within a sphere of 0.6 mm radius (34). It is therefore very unlikely that the bicuculline microinjections made at the periphery of the orexin group would have spread to the center of the orexin group (0.7 mm away) to a sufficient degree to activate as many orexin neurons as microinjections made into the center of this group. It is also unlikely that this effect was due to blockade of a tonic release of orexin in our preparation, since the chemoreceptor and baroreceptor reflexes were not affected, as we have discussed above.

There are three possible explanations, however, for our observation. The first is that orexin neurons that are critical for cardiorespiratory function are located at the caudal end of the orexin group, such that they would be disinhibited to a similar degree by microinjections into the center of the group or into the peripheral caudal region. The second possibility is that the effect of activating orexin neurons quickly reaches a ceiling when only a small number of orexin neurons are activated, wherever they are located. The third explanation is that the output of this caudal hypothalamic region projects to and activates the orexin neurons located
more rostrally. In support of this last possibility, the posterior hypothalamus is one of the major sources of afferent input to orexin neurons, and it has been shown that an anterograde tracer injected into the posterior hypothalamus resulted in labeled appositions in 32-58% of orexin neurons (39).

In conclusion, the main finding of the present study is that Almorexant can markedly reduce cardiorespiratory responses evoked from or mediated by the perifornical hypothalamus. This demonstrates the important contribution of orexin to the output of the perifornical and caudal hypothalamus, at least with respect to cardiorespiratory regulation. The study also shows that Almorexant does not affect baroreceptor and chemoreceptor reflexes under resting conditions.

**Perspectives and significance**

There is considerable evidence that at least in many cases the origin of essential hypertension is a stressor, acute or chronic, that engages the hypothalamic defense area (20). Our findings raise the possibility that dual orexin receptor antagonists such as Almorexant may be effective as anti-hypertensive drugs for some forms of hypertension. Such antagonists may reduce cardiorespiratory responses evoked from the hypothalamus without having a deleterious effect on basic homeostatic cardiovascular reflexes.
ACKNOWLEDGMENTS

We wish to thank Dr Francois Jenck and Actelion Pharmaceuticals for the gift of Almorexant.

GRANTS

This work was supported by research grants from the National Health and Medical Research Council of Australia.

DISCLOSURES

None of the authors have any conflicts of interest.

AUTHORS CONTRIBUTIONS

Kamon Iigaya and Jouji Horiuchi performed all the experiments in anesthetized rats, and analysed the experimental data. Alex Lam, Yusuf Sediqi and Jaimie Polson performed and were responsible for the immunohistochemical labeling and mapping of orexin neurons. Lachlan McDowall performed the analysis for the determination of baroreceptor reflex sensitivity. Pascal Carrive and Roger Dampney designed and planned the studies and had the main responsibility for the drafting of the manuscript. All of the authors contributed to drafting the manuscript and approved the final version of the manuscript.
REFERENCES


FIGURE LEGENDS

Figure 1. A, Distribution of orexin neurons (amalgamation of results from 5 rats), mapped onto a standard section at the level 2.76 mm caudal to bregma. B, Graph showing the number of orexin neurons at different levels of the hypothalamus, as determined from these 5 rats. Mean ± SE. C, Locations of centers of bicuculline microinjection sites for all experiments, drawn onto two standard sections of the hypothalamus at the rostro-caudal levels indicated in mm caudal (-) to bregma. 10 pmol injections are represented on the left of the plates. 20 pmol injections are represented on the right. Sections are from the atlas of Paxinos and Watson (29). Abbreviations: f: fornix; DA, dorsal hypothalamus; DMN, dorsomedial nucleus of the hypothalamus; mt, mammillothalamic tract; PeF, perifornical area; PH, posterior hypothalamus.

Figure 2. A, Example from one experiment showing changes in arterial pressure (AP), heart rate (HR), integrated renal sympathetic nerve activity (RSNA), and phrenic nerve activity (PNA) burst rate and burst amplitude evoked by microinjection of bicuculline (10 pmol) into the orexin group in the PeF, before and after Almorexant administration. B, Group results showing peak increases in the various cardiorespiratory variables evoked by bicuculline (10 pmol) before and after Almorexant administration. Technical problems precluded RSNA recording at 2 of the sites. *P < 0.05, ***P < 0.001.

Figure 3. Examples of chart recordings showing changes in AP, HR, integrated RSNA and PNA burst rate evoked by microinjection of bicuculline (20 pmol) into (A) a site within the orexin group, and (B) a site at the caudal periphery of the orexin group, before and after Almorexant administration.
Figure 4. A, Averaged time courses of responses to microinjections of bicuculline (20 pmol) centered on sites within the orexin group and for those centered on caudal sites peripheral to the orexin group. B, Group results showing peak increases in the various cardiorespiratory variables evoked by 20 pmol of bicuculline, before (control) and after Almorexant administration, into sites within and at the caudal periphery of the orexin group. Mean ± SE. C, Grouped results comparing response ratio (i.e. peak increase in each cardiorespiratory variable after Almorexant administration relative to the peak control response) evoked by bicuculline into sites within and at the caudal periphery of the orexin group. *P < 0.05; **P < 0.01, ***P < 0.001.

Figure 5. A, Example from one experiment showing changes in AP, HR, integrated RSNA and PNA burst rate evoked by microinjection of bicuculline (20 pmol) into the PeF, before and after vehicle administration. B, Averaged time courses for all experiments in which microinjections of bicuculline (20 pmol) were made into sites before and after vehicle or saline administration. C, Group results showing peak increases in the various cardiorespiratory variables evoked by 20 pmol of bicuculline, before (control) and after vehicle or saline administration. The P-values represent comparisons of control vs after vehicle or saline administration.

Figure 6. A, Example from one experiment showing changes in AP, HR, integrated RSNA and PNA burst rate evoked by peripheral chemoreceptor stimulation with sodium cyanide (NaCN), before and after Almorexant administration. B, Group results showing peak increases in the various cardiorespiratory variables reflexly evoked by chemoreceptor stimulation, before (control) and after Almorexant administration. The P-values represent comparisons of control vs after Almorexant.

Figure 7. A, Example from one experiment showing the results of cycle-triggered averaging of AP and RSNA (100 cycles), where each cycle is triggered by the minimum diastolic pressure. B, Histogram showing the grouped results for the ratio of the average change in RSNA to the average pulse pressure as determined using cycle-triggered averaging. The P-value represents the comparison of control vs after Almorexant.
**TABLE**

*Table 1.* Resting values of cardiovascular and respiratory variables before and 30 min after Almorexant injection compared to before and after vehicle or saline

<table>
<thead>
<tr>
<th></th>
<th>Before Almorexant</th>
<th>After Almorexant</th>
<th>Before Vehicle/Saline</th>
<th>After Vehicle/Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MAP (mmHg)</strong></td>
<td>95 ± 1</td>
<td>92 ± 2</td>
<td>92 ± 5</td>
<td>94 ± 2</td>
</tr>
<tr>
<td><strong>HR (bpm)</strong></td>
<td>334 ± 9</td>
<td>315 ± 9**</td>
<td>350 ± 12</td>
<td>342 ± 10</td>
</tr>
<tr>
<td><strong>RSNA (%baseline)</strong></td>
<td>100</td>
<td>91 ± 7</td>
<td>100</td>
<td>117 ± 15</td>
</tr>
<tr>
<td><strong>PNA burst rate (bursts/min)</strong></td>
<td>102 ± 4</td>
<td>95 ± 2*</td>
<td>117 ± 7</td>
<td>104 ± 4</td>
</tr>
<tr>
<td><strong>PNA burst amp (% baseline)</strong></td>
<td>100</td>
<td>103 ± 2</td>
<td>100</td>
<td>104 ± 3</td>
</tr>
</tbody>
</table>

*amp, amplitude; MAP, mean arterial pressure; RSNA, renal sympathetic nerve activity; PNA, phrenic nerve activity. *P < 0.05 vs Before Almorexant. **P < 0.01 vs Before Almorexant.*
Fig 1

A. Orexin neurons

B. Orexin neurons (number per section)

C. Orexin neurons (number per section)

- Bic 10 pmol
- + Almorexant

- Bic 20 pmol
- + Almorexant (inj in orexin group)
- + Almorexant (inj peripheral to orexin group)
- + vehicle/saline
Fig 2

A

Control

After Almorexant

AP (mmHg)

HR (beats min⁻¹)

Integrated RSNA (% baseline)

PNA burst rate (burst min⁻¹)

PNA burst amplitude (% baseline)

B

\[ \Delta \text{MAP (mmHg)} \]

\[ \Delta \text{HR (beats min⁻¹)} \]

\[ \Delta \text{RSNA (% baseline)} \]

\[ \Delta \text{PNA burst rate (burst min⁻¹)} \]

\[ \Delta \text{PNA burst amplitude (% baseline)} \]

\[ n=11 \]

\[ n=11 \]

\[ n=9 \]

\[ n=11 \]

\[ n=11 \]
Fig 3
**Fig 4**

A. **In orexin group**

- **ΔMAP (mmHg)**
  - $n=6$
  - Time after injection (min) from -2 to 25

- **ΔHR (beats min⁻¹)**
  - $n=6$
  - Time after injection (min) from -2 to 25

- **ΔRSNA (% baseline)**
  - $n=5$
  - Time after injection (min) from -2 to 25

- **ΔPNA burst rate (burst min⁻¹)**
  - $n=6$
  - Time after injection (min) from -2 to 25

B. **In orexin group**

- **ΔMAP (mmHg)**
  - $n=6$

- **ΔHR (beats min⁻¹)**
  - $n=6$

- **ΔRSNA (% baseline)**
  - $n=5$

- **ΔPNA burst rate (burst min⁻¹)**
  - $n=6$

C. **Peripheral to orexin group**

- **ΔMAP (mmHg)**
  - $n=7$

- **ΔHR (beats min⁻¹)**
  - $n=7$

- **ΔRSNA (% baseline)**
  - $n=5$

- **ΔPNA burst rate (burst min⁻¹)**
  - $n=7$

**Legend:**
- control
- after Almorexant
- Peripheral to orexin group

Statistical significance:
- $P > 0.35$
- $P > 0.50$
- $P > 0.06$
- $P > 0.75$
Fig 5

A

Control

After vehicle

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>After vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (beats min⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integrated RSNA (% baseline)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNA burst rate (beats min⁻¹)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

\[
\begin{align*}
\Delta MAP (mmHg) & \quad (n=6) \\
\Delta HR (beats min⁻¹) & \quad (n=6) \\
\Delta RSNA (% baseline) & \quad (n=5) \\
\Delta PNA burst rate (beats min⁻¹) & \quad (n=6)
\end{align*}
\]

C

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>After vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>P &gt; 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P &gt; 0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P &gt; 0.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Time after injection (min): 0, 5, 10, 15, 20, 25

Legend:
- control
- after vehicle or saline
Fig 6

A

Control
After Almorexant

AP (mmHg)

160
120
80
40
0

100
80
60
40
20
0

HR (beats min⁻¹)

350
300
250
200
150
100
50
0

Integrated RSNA (% baseline)

140
120
100
80
60
40
20
0

PNA burst rate (burst min⁻¹)

220
200
180
160
140
120
100
80
60
40
20
0

NaCN
NaCN

40 sec

B

n=7  P > 0.15

n=5  P > 0.5

n=7  P > 0.26

n=7  P > 0.13

ΔMAP (mmHg)

15
10
5
0

ΔHR (beats min⁻¹)

100
80
60
40
20
0

ΔRSNA (% baseline)

100
80
60
40
20
0

ΔPNA burst rate (burst min⁻¹)

80
60
40
20
0

control
after Almorexant
Fig 7

A

Control

After almorexant

AP (mmHg)

RSNA (% baseline)

100 msec

100 msec

B

Ratio (% baseline / mmHg)

n=5

P > 0.6

control

after Almorexant