Hypocretin/orexin type 1 receptor in brain: role in cardiovascular control and the neuroendocrine response to immobilization stress

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Samson WK, Bagley SL, Ferguson AV, White MM. Hypocretin/orexin type 1 receptor in brain: role in cardiovascular control and the neuroendocrine response to immobilization stress. Am J Physiol Regul Integr Comp Physiol 292: R382–R387, 2007. First published August 10, 2006; doi:10.1152/ajpregu.00496.2006.—Hypocretin/orexin acts pharmacologically in the hypothalamus to stimulate stress hormone secretion at least in part by an action in the hypothalamic paraventricular nucleus, where the peptide’s receptors have been localized. In addition, orexin acts in the brain to increase sympathetic tone and, therefore, mean arterial pressure and heart rate. We provide evidence for the role of endogenously produced hypocretin/orexin in the physiological response to immobilization stress and identify the receptor subtype responsible for this action of the peptide. Antagonism of the orexin type 1 receptor (OX1R) in the brain prevented the ACTH-stimulating effect of centrally administered hypocretin/orexin. Furthermore, pretreatment of animals with the OX1R antagonist blocked the ACTH response to immobilization/restress stress. The OX1R antagonist did not, however, block the pharmacological or physiological release of prolactin in these two models. Antagonism of the OX1R also blocked the central action of orexin to elevate mean arterial pressures and heart rates in conscious rats. These data suggest receptor subtype-selective responses to hypocretin/orexin and provide further evidence for the importance of endogenously produced peptide in the physiological control of stress hormone secretion.

IN ADDITION TO THE WELL-CHRONICLED pharmacological actions of the hypocretins/orexins on arousal state (6, 23, 29, 36), the peptides (referred to here simply as orexins) exert a variety of behavioral (24), autonomic (7, 15, 34), and endocrine (1, 5, 7, 13, 22, 26–28, 31, 32) actions mediated by the two characteristic binding proteins, the orexin type 1 (OX1R) and the orexin type 2 (OX2R) receptors (29). Although orexin A and orexin B have many common pharmacological effects, some actions unique to one and not the other have been reported (7, 36). Since orexin A binds with higher affinity to the OX1R than orexin B and both isoforms bind with apparently equal affinity to the OX2R, many investigators have narrowed their attention to the pharmacological effects of orexin A. Our original studies focused on endocrine, cardiovascular, and behavioral actions of orexin A in the hypothalamus and brain stem (7, 32, 37, 38). We demonstrated direct neuronal actions of orexin in the hypothalamic paraventricular nucleus (PVN) and established that the action of exogenous orexin to stimulate the hypothalamic-pituitary-adrenal (HPA) axis was mediated at least in part by the release of corticotrophin-releasing hormone (CRH) (32). We have attempted to identify the receptor subtype responsible for the cardiovascular and neuroendocrine actions of these peptides and provide evidence for the involvement of endogenously produced orexin in the HPA axis response to immobilization stress. These studies were made possible by the commercial availability of relatively selective OX1R antagonists (19) and the prior descriptions of the pharmacological actions of exogenously administered orexin.

MATERIALS AND METHODS

Animals. All procedures were approved by the Saint Louis University Animal Care and Use Committee. Adult male rats (Harlan Sprague Dawley; 250–300 g body wt) were individually housed under controlled conditions (lights on 0600–1800, 23–25°C) with free access to food and water. An indwelling lateral cerebroventricular cannula (23 gauge, stainless steel, 17 mm) was implanted and the aid of a stereotaxic device into rats anesthetized with a mixture of ketamine (60 mg/ml; Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (8 mg/ml; Tranquiled, Vedco, St. Joseph, MO) at 0.1 ml/100 g body wt, as previously described (30). Animals were allowed ≥5 days to recover and were observed daily for general vigor and return to presurgery body weight. For the neuroendocrine studies, a second surgery was conducted under isoflurane-induced anesthesia (3% in O2 for induction, 2% in O2 for maintenance; IsoSol, Vedco), during which an indwelling jugular vein cannula was implanted and exteriorized on the back of the neck, as previously described (11). These animals were used for neuroendocrine studies 2 days later. For the cardiovascular studies, a second surgery was conducted under ketamine-xylazine anesthesia to implant a carotid cannula (PE-50), as previously described (30). The cannula was exteriorized at the back of the neck and sealed with heparinized saline (200 U heparin/ml 0.9% NaCl). Blood pressure was monitored 2 days later in these animals.

Two neuroendocrine protocols were conducted. In the first, rats were moved to a quiet room at 0600 and left undisturbed for 1 h. Then extension tubing (PE-50) was attached to the jugular cannula, which was flushed with heparinized saline (200 U heparin/ml 0.9% NaCl; MP Biochemicals, Aurora, OH). After 2 h, a baseline (time 0) blood sample (0.3 ml) was removed via the cannula, and the withdrawn volume was replaced with sterile isotonic saline (Abbott Laboratories, North Chicago, IL). Then saline vehicle (2 μl of isotonic saline) alone or vehicle containing 3.0 nmol of the OX1R antagonist was injected via the indwelling lateral ventricular cannula. After 15 min, a second blood sample was collected as described above, and the second intracebroventricular injection was completed, with all animals receiving 2 μl of saline vehicle containing 3.0 nmol orexin A, a dose previously demonstrated to result in ACTH secretion in vivo (27, 32). Additional blood samples were removed 30, 45, and 60 min after this second intracebroventricular injection. Blood samples were collected into heparin-washed syringes, maintained on ice until the end of

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the experiment, and then centrifuged to separate plasma for subsequent hormone determinations. In the second protocol, additional animals were prepared as described above. After removal of the time 0 blood sample, animals received an intracerebroventricular injection of saline vehicle (2 μl of isotonic saline) or vehicle containing 3.0 nmol of the OX1R antagonist. After 5 min, a second blood sample was collected, and the animals were placed in Plexiglas restrainer tubes (6.2 cm ID; Harvard Apparatus, Boston, MA), and the nose cones were tightened so that the animal was held immobile for 10 min. Two more blood samples were collected while the animals were immobilized, 5 and 10 min into the period of immobilization; then the rats were released into their home cages, and two more samples withdrawn 5 and 15 min later.

For the cardiovascular studies, animals were moved to a quiet room, where they were allowed to habituate for 1 h. Then the exteriorized carotid cannula was flushed with heparinized saline and attached to a blood pressure transducer (Digi-Med Blood Pressure Analyzer, Micro-Med, Louisville, KY). The rats were left undisturbed for an additional 1 h before experimentation. Mean arterial pressure and heart rate were digitally averaged and recorded every 60 s during the test period, which consisted of a 5-min baseline recording period followed by intracerebroventricular injection of 2 μl of sterile 0.9% NaCl vehicle (group 1) or vehicle containing 3.0 nmol of the OX1R antagonist (groups 2 and 3). After 5 min, animals in group 2 (antagonist pretreated) received an intracerebroventricular injection of 2 μl of sterile 0.9% NaCl vehicle. At that time, animals in group 1 (vehicle pretreated) and group 3 (antagonist pretreated) received intracerebroventricular injections of 2 μl of sterile 0.9% NaCl vehicle containing 3.0 nmol of orexin A. After the injections, blood pressure and heart rate were monitored for an additional 30 min. Data are presented as mean values for each group during the rest period and then as absolute changes in mean pressure and heart rate after intracerebroventricular administration of orexin A (groups 1 and 3) or saline vehicle (group 2).

Synthetic rat orexin A was purchased from Phoenix Pharmaceuticals (Belmont, CA) and the OX1R antagonist (catalog no. SB-408124) from Sigma (St. Louis, MO). The dose of orexin was based on our previous findings on the neuroendocrine and cardiovascular effects of central peptide administration (30, 32). An equimolar dose of the OX1R antagonist was chosen on the basis of dose-response data we obtained in separate behavioral studies conducted before these experiments (unpublished observations). An RIA kit (Peninsula Labs, San Carlos, CA) was used to determine ACTH levels in unextracted plasma. Minimum detectable level of ACTH was 1.0 pg/ml (defined as <90% B/B0 and the intra- and interassay coefficients of variability were <9%. Plasma levels of prolactin (PRL) were determined using the kit materials obtained from the National Hormone and Pituitary Program (rPRL-RP-3 standard). The minimum detectable hormone level in plasma for PRL was 0.5 ng/ml, and the intra- and interassay coefficients of variability were <8%. All plasma samples (20 μl for PRL and 33 μl for ACTH) for each individual protocol were included in one respective hormone assay.

Cardiovascular data were analyzed by one-way ANOVA (within and among groups across time) followed by Scheffé’s multiple comparison testing. Neuroendocrine data were analyzed by ANOVA within each treatment group across time or unpaired t-test, examining differences between the two treatment groups at any time point. Homogeneity of variance was established using the F test. Significance was assigned to results that occurred with <5% probability.

RESULTS

Plasma levels of ACTH and PRL did not differ significantly between groups before treatment with the OX1R antagonist. Administration of the antagonist or saline vehicle did not result in any significant differences in hormone levels 15 min after their intracerebroventricular administration (Table 1). Subsequent intracerebroventricular administration of 3.0 nmol of orexin A resulted in a significant elevation in plasma ACTH levels in saline vehicle-pretreated controls (F4,49 = 6.45, P < 0.001; Table 1). However, in rats pretreated with the OX1R antagonist, plasma levels of ACTH did not differ across all time points (F4,39 = 0.78, P > 0.50). At 45 min after intracerebroventricular administration of orexin A, plasma ACTH levels were significantly greater in saline vehicle- than in antagonist-pretreated animals. Exogenously administered orexin A also stimulated PRL secretion in animals pretreated with saline vehicle (F4,49 = 3.12, P < 0.05) and the OX1R antagonist (F4,39 = 4.77, P < 0.01).

Immobilization resulted in significantly elevated ACTH (F5,72 = 10.92, P < 0.001) levels in saline vehicle-pretreated animals (Fig. 1). Pretreatment with the OX1R antagonist prevented the ACTH response to immobilization. No significant differences in plasma ACTH levels were observed at any time within this treatment group (F5,78 = 1.92, P = 0.10; Fig. 1). Animals pretreated with the saline vehicle (F5,72 = 10.43, P < 0.001) or the OX1R antagonist (F5,78 = 9.66, P < 0.001) experienced significant elevations in plasma PRL levels 5 and 10 min into the immobilization period (Fig. 2). Plasma levels of ACTH and PRL did not differ between groups after the animals were released from immobilization and returned to their home cages.

Resting mean arterial pressures and heart rates did not significantly differ among groups before administration of the test substance: 135 ± 3 mmHg and 374 ± 13 beats/min (n = 15) for animals treated with 3.0 nmol of orexin A, 134 ± 5 mmHg and 355 ± 12 beats/min (n = 10) for animals treated with 3.0 nmol of the OX1R antagonist, and 138 ± 5 mmHg and 377 ± 16 beats/min (n = 9) for animals treated with orexin A and the OX1R antagonist. No significant effects of vehicle

Table 1. Blockade of orexin A stimulation of ACTH, but not PRL, in vivo by intracerebroventricular OX1R antagonist

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 min + pretreatment</th>
<th>15 min + ORX</th>
<th>45 min</th>
<th>60 min</th>
<th>75 min</th>
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<tbody>
<tr>
<td>Plasma ACTH, pg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Saline Vehicle</td>
<td>11.5 ± 1.3</td>
<td>11.3 ± 1.1</td>
<td>15.9 ± 1.3</td>
<td>19.4 ± 1.3</td>
<td>17.9 ± 2.0</td>
</tr>
<tr>
<td>OX1R antagonist</td>
<td>12.9 ± 1.8</td>
<td>13.3 ± 1.5</td>
<td>12.9 ± 1.9</td>
<td>13.4 ± 1.4</td>
<td>16.7 ± 2.3</td>
</tr>
<tr>
<td>Plasma PRL, ng/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Saline Vehicle</td>
<td>35.5 ± 1.9</td>
<td>37.4 ± 2.9</td>
<td>50.9 ± 2.9</td>
<td>48.1 ± 4.5</td>
<td>45.6 ± 5.6</td>
</tr>
<tr>
<td>OX1R antagonist</td>
<td>28.9 ± 3.5</td>
<td>30.9 ± 4.2</td>
<td>54.9 ± 4.9</td>
<td>52.2 ± 5.9</td>
<td>41.4 ± 7.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. OX1R, orexin type 1 receptor; PRL, prolactin. All animals received pretreatments [saline vehicle (n = 10) or antagonist, (n = 8)] after removal of the initial blood sample; after 15 min, all received 3.0 nmol of orexin A (ORX) (after removal of the 2nd blood sample). ∗P < 0.05; †P < 0.01 vs. baseline values before ORX administration (within-group ANOVA).
administration (group 1) or OX₁R antagonist administration (groups 2 and 3) on mean arterial pressures (F_{2,31} = 0.163, P > 0.05) or heart rates (F_{2,31} = 0.671, P > 0.05) were observed during the 5-min period before the second intracerebroventricular injections (data not shown). Intracerebroventricular administration of 3.0 nmol of orexin A in saline vehicle-pretreated rats (group 1) resulted in significant elevations (Figs. 3 and 4) in mean arterial pressure and heart rate (compared with groups 2 and 3: F_{2,31} = 3.98, P < 0.05 for mean arterial pressure; F_{2,31} = 4.35, P < 0.05 for heart rate). The OX₁R antagonist alone did not significantly alter mean arterial pressure or heart rate over the entire experimental period. Administration of 3.0 nmol of orexin A to OX₁R antagonist-pretreated animals (group 3) resulted in mean arterial pressures and heart rates that did not differ from those in the antagonist-only group (group 2). The antagonist significantly abrogated the effects of orexin A on mean arterial pressure and heart rate.

**DISCUSSION**

Hypocretin/orexin-producing neurons located in the perifornical region of the lateral hypothalamus project to brain sites important in cardiovascular, behavioral, and endocrine function (25). Importantly, neuroendocrine centers, such as the hypothalamic PVN and arcuate (ARC) nuclei, receive innervation from orexin-positive cells (5, 25), central administration of exogenous orexin induces c-fos expression in both nuclei (5), and orexin receptors are expressed on neurons in these nuclear groups (2, 3, 12, 20, 21). Indeed, OX₁R and OX₂R are abundantly expressed in the PVN (12, 20, 21), whereas, with the exception of one group (2), most investigators report only the OX₂R to be present in the ARC (12, 20, 21). These two nuclear groups are known to be involved in the neuroendocrine regulation of stress hormone secretion (4, 35). The PVN is thought to be the primary integrative site controlling ACTH release.
secretion (35), whereas the ARC is the preeminent controller of PRL release (9).

Pharmacological administration of synthetic orexin in rats resulted in increased release of ACTH and PRL (1, 5, 13, 14, 27, 28, 32), and it is clear that the ability of orexin to stimulate the HPA axis is due primarily to an action in the brain that causes release of corticotrophin-releasing factor (CRF) into the median eminence (and subsequent diffusion into the hypophyseal portal vessels for access to the corticotrphs of the anterior pituitary gland) from parvocellular neurons located in the PVN (1, 13, 14, 27, 28, 32). If this pharmacological action of exogenously administered orexin has physiological relevance, then antagonism of the actions of the endogenous peptide should alter the HPA axis response to physiological stressors. We have demonstrated here that the OX1R antagonist not only blocks the ability of exogenously administered orexin to stimulate, by a brain action, ACTH secretion in conscious unrestrained rats but also that antagonism of the OX1R abrogates the ACTH response to immobilization stress. We conclude that immobilization stress-induced activation of the CRF cells in the PVN requires the input of orexin neurons of the lateral hypothalamus and that the stimulatory effect of orexin is mediated via the OX1R. We and others have provided evidence for direct cellular actions of orexin on identified PVN neurons (8, 33), cells reported by others to express orexin receptors (3, 12, 20); however, direct evidence for involvement of the OX1R in the control of identified CRF neurons has not been presented. We hypothesize that CRF-producing parvocellular cells in the PVN express the OX1R and that endogenous orexin controls the activity of those neurons during immobilization stress. Indeed, immobilization stress resulted in increased Fos immunoreactivity in orexin neurons (28), and orexin administration induced expression of the c-fos gene and Fos protein in parvocellular neurons of the PVN (18). We anticipate that future single-cell RT-PCR experiments will allow us to directly identify the phenotype of the neurons in the PVN that responds to orexin and, thus, determine whether the stimulation of ACTH secretion in vivo is the result of orexin action directly on CRF-producing cells or interneurons within the PVN.

There is controversy in the literature over the action of orexin on the hypothalamic control of PRL secretion. Russell and colleagues (27) reported that intracerebroventricular administration of orexin inhibited PRL secretion and that this suppressive action was even present in the domperidone-treated animals, suggesting a nondopaminergic mechanism of action for the peptide. This would imply an action of orexin outside the ARC. On the other hand, Kohsaka and co-workers (17) demonstrated that food deprivation inhibited steroid-induced PRL surges in female rats and that this inhibition could be reversed by orexin administration. Antiiorexin A antiserum abrogated steroid-induced surges in normally fed ovariectomized rats, suggesting a role for endogenous orexin in the PRL response to steroid priming.

Thus there is evidence for inhibitory and stimulatory actions of orexin in the hypothalamic control of PRL secretion. In our hands, only a modest PRL stimulatory effect of intracerebroventricular administration of 3.0 nmol of orexin A was observed in male rats. This may be nonspecific, reflecting the increased behavioral arousal that accompanies central administration of orexin (10). Furthermore, a dose of the OX1R antagonist that was capable of abrogating the ACTH response to immobilization stress was without effect on the PRL response in those animals. Thus our results do not support a major role for endogenously produced orexin, acting at least via the OX1R, in the PRL response to stress. It may be necessary to inject orexin into a specific site and to inject orexin antagonist directly into the ARC to support that possibility. The role of the OX2R in these cardiovascular and neuroendocrine responses, in particular in the PRL response to immobilization stress, cannot be ascertained, because a selective OX2R antagonist is not available.

It is possible that endogenous orexin is an important factor in neuronal pathways that coordinate stress responses. To be sure, the physical responses to administration of exogenous orexin mirror stress/anxiety-related behaviors. Our data may reflect that central role for orexin in the neuroendocrine and cardiovascular responses to stress. Alternatively, our data could be interpreted to indicate that the neuroendocrine and cardiovascular responses to orexin are secondary to the behavioral actions of the peptide. This may indeed be true in terms of the neuroendocrine actions; however, the cardiovascular effects of the peptide have been demonstrated in anesthetized animals (7), arguing against the secondary hypothesis.

We (30, 32) and others (34) have demonstrated that exogenously administered orexin activates the sympathetic arm of the autonomic nervous system, resulting in increased blood pressure and heart rate. As with the action of orexin to activate the HPA axis in conscious unrestrained rats, the central action of orexin to raise blood pressure and heart rate appears to be mediated via the OX1R, since the antagonist abrogated both actions of the peptide. As mentioned above, the OX1R is broadly expressed in the PVN (12). Furthermore, parvocellular neurons in the PVN project to brain stem sites known to be important in the regulation of sympathetic outflow, and it is tempting to speculate that orexin activates the autonomic nervous system by binding to the OX1R in the PVN. Indeed, direct administration of orexin into the PVN results in increases in spontaneous physical activity, an effect that was compromised by pretreatment with an OX1R antagonist (16). Site-specific antagonist administration into the PVN is required to establish that the results we have observed reflect a direct action of the antagonist via the OX1R on cells in that nucleus.

In a broader perspective, does this mean that endogenous orexin-producing cells are necessarily the primary gatekeepers communicating the perception of stress to the cells of the PVN? No, it may be that numerous other peptides and neurotransmitters are required for an appropriate neuroendocrine response to a variety of stressors; however, at least in the case of the model examined here (immobilization), endogenous orexin does appear to be an important part of the neuroendocrine response to stress. It may be that psychogenic stressors (e.g., novel environment, anticipatory stress) do not require the interaction of orexinergic cells of the lateral hypothalamic areas for the activation of CRF-producing neurons in the PVN. Is the action of orexin needed for an appropriate cardiovascular response to stress? Although the experiments reported here identify only the OX1R as the mediator of the effect of orexin A on cardiovascular function, it should be noted that orexin-knockout...
mice (15) demonstrated a compromised cardiovascular response to resident-intruder stress. Thus it may be that, similar to the case with HPA axis activation during immobilization stress, the cardiovascular response to a perceived stress requires signaling via the OX₁R. It will be important now to examine other modalities of stress for the possible involvement of endogenously produced orexin in mediation of the neuroendocrine regulation of hormone secretion (e.g., emotional as opposed to physical stress) and cardiovascular function (enteroceptively as opposed to efferent manipulations).

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