Orexin-A enhances feeding in male rats by activating hindbrain catecholamine neurons

Ai-Jun Li, Qing Wang, Hana Davis, Rong Wang, and Sue Ritter

Programs in Neuroscience, Washington State University, Pullman, Washington

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Both lateral hypothalamic orexinergic neurons and hindbrain catecholaminergic neurons contribute to control of feeding behavior. Orexin fibers and terminals are present in close proximity to hindbrain catecholaminergic neurons, and fourth ventricular (4V) orexin injections that increase food intake also increase c-Fos expression in hindbrain catecholamine neurons, suggesting that orexin neurons may stimulate feeding by activating catecholamine neurons. Here we examine that hypothesis in more detail. We found that 4V injection of orexin-A (0.5 nmol/rat) produced widespread activation of c-Fos in hindbrain catecholamine cell groups. In the A1 and C1 cell groups in the ventrolateral medulla, where most c-Fos-positive neurons were also dopamine β hydroxylase (DBH) positive, direct injections of a lower dose (67 pmol/200 nl) of orexin-A also increased food intake in intact rats. Then, with the use of the retrogradely transported immunotoxin, anti-DBH conjugated to saporin (DSAP), which targets and destroys DBH-expressing catecholamine neurons, we examined the hypothesis that catecholamine neurons are required for orexin-induced feeding. Rats given paraventricular hypothalamic injections of DSAP, or unconjugated saporin (SAP) as control, were implanted with 4V or lateral ventricular (LV) cannulae and tested for feeding in response to ventricular injection of orexin-A (0.5 nmol/rat). Both LV and 4V orexin-A stimulated feeding in SAP controls, but DSAP abolished these responses. These results reveal for the first time that catecholamine neurons are required for feeding induced by injection of orexin-A into either LV or 4V.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats were purchased from Simonsen Laboratories (Gilroy, CA) and housed individually in an animal care facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Rats were maintained on a 12:12-h light/dark cycle with ad libitum access to pelleted rodent food (Roden diet 5001; LabDiet, St. Louis, MO; 3.36 kcal/g; 28.5% protein, 13.5% fat, and 58.0% carbohydrates) and tap water. All experimental procedures were approved by Washington State Univer-

Address for reprint requests and other correspondence: A.-J. Li, Dept. of Integrative Physiology and Neuroscience, College of Veterinary Medicine, Washington State Univ., Pullman, Washington 99164-7620 (e-mail: aijunli@vetmed.wsu.edu).
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placed in 4% formaldehyde/PBS overnight at 4°C, then transferred to 12.5% and 25% sucrose in PBS for 24 h each, then sectioned coronally on a cryostat at 40-μm thickness. Brain sections were collected into two or three serial sets for immunohistochemical staining, depending on the cell group and its distribution, as described below. For each area, the same number of sections were collected for each rat.

For double immunofluorescence staining of brain tissues, sections were incubated with mouse monoclonal anti-DBH (1:10,000; Millipore, San Jose, CA) and goat anti-c-Fos (1:1,000; Santa Cruz Biotech, Santa Cruz, CA) antibodies for 2 to 3 days (at room temperature), then washed and sequentially incubated with donkey anti-mouse IgG-Alexa 488 and donkey anti-goat-Cy3 antibodies (all at 1:500; Jackson ImmunoResearch Laboratories) for 4 h, and then coverslipped with ProLong Gold medium (Life Technology, Grand Island, NY). Slides were examined using a Zeiss Axiomat microscope, and images were collected for quantification of immunoreactive neurons. Catecholamine cell groups were defined as described by The Rat Brain in Stereotaxic Coordinates (35). DBH- and c-Fos-positive cells were counted bilaterally in two (for A2 subregions and A5v) or three (for all other regions) consecutive sections from the following regions for each rat. Anatomical levels for each region were (distance in mm caudal from bregma) A1, 14.4-14.1; A1/C1, 13.7-13.4; C1m, 13.0-12.7; C1r (rostral C1),

Fig. 1. Dopamine β hydroxylase (DBH) and c-Fos double-immunofluorescence staining in ventrolateral medulla (VLM) catecholamine cell groups. Coronal sections at A1, A1/C1 overlap, C1m, and C1r regions from saline- or orexin-A-treated rats are shown. Green labeling indicates DBH-immunoreactive (DBH-ir) and red indicates c-Fos-immunoreactive (c-Fos-ir). Orexin-A (0.5 nmol/rat) significantly stimulated c-Fos expression in these VLM areas, primarily in DBH-positive cells. Schematic representations of coronal brain sections showing each region are presented at left, and distance (in mm) caudal to bregma is shown. Bar, 25 μm.
12.2-11.9; A2m (middle A2), 14.3-14.1; A2r (rostral extent of A2) from saline- or orexin-A-treated rats. Orexin-A (0.5 nmol/rat) significantly stimulates c-Fos expression in A2, but cells expressing immunoreactivity for both c-Fos and DBH were observed primarily in A2r. To assess the lesion produced by DSAP injection, DBH-ir cells were quantified in the above catecholamine groups, except as noted. The presence of DBH terminals in PVH and other hypothalamic sites was examined, but not quantified. However, all DSAP rats with significant loss of catecholamine neurons in the above areas also had significant loss of DBH terminals in the hypothalamus, as reported previously (40, 43).

Fig. 2. DBH and c-Fos double-immunofluorescence staining in A2 region. Coronal sections showing A2m (middle rostro-caudal region of A2) and A2r (rostral extent of A2) from saline- or orexin-A-treated rats. Orexin-A (0.5 nmol/rat) significantly stimulates c-Fos expression in A2, but cells expressing immunoreactivity for both c-Fos and DBH were observed primarily in A2r. Schematic representations of coronal brain sections showing each region are presented at left, and distance (in mm) caudal to bregma is shown. Bar, 25 μm.

Fig. 3. DBH and c-Fos double-immunofluorescence staining in C2 region. Coronal sections from C2 (caudal and medial parts) from saline- or orexin-A-treated rats are shown. In caudal C2, orexin-A (0.5 nmol/rat) significantly stimulated c-Fos expression primarily in non-DBH cells. Schematic representations of coronal brain sections showing each region are presented at left, and distance (in mm) caudal to bregma is shown. Bar = 25 μm.
Statistical analysis. All results are presented as means ± SE. For statistical analysis of data, we used t-tests, one-way ANOVAs, or two-way repeated-measures ANOVAs, as appropriate. After significance was determined by ANOVA, multiple comparisons between individual groups were tested using a post hoc Fisher least significant difference test. Confidence limits for significance were set at $P < 0.05$.

RESULTS

Fourth ventricular orexin-A injection increased c-Fos expression in hindbrain catecholamine neurons. After preliminary tests, a dose of 0.5 nmol/rat orexin-A, which significantly increases food intake during the 4 h after the injection, was selected for 4V or LV injections throughout this study.

Activation of hindbrain sites by 4V orexin-A injection was investigated. Orexin-A (0.5 nmol/rat) or saline was injected into the 4V, and rats were euthanized 90 min later ($n = 5$/group). Brain tissue was processed to detect c-Fos- and DBH-ir. In ventrolateral medulla, numbers of neurons expressing c-Fos-ir and c-Fos- plus DBH-ir were increased in A1 through C1 regions ($P < 0.001$; Table 1 and Fig. 1). With the exception of rostral C1, 42% to 43% of DBH-ir neurons were c-Fos-positive and most (68–72%) of the c-Fos-positive cells were DBH-ir in A1–C1 regions. In rostral C1, 44% of c-Fos-positive neurons was DBH-ir and only 14% of DBH-ir neurons were c-Fos positive. c-Fos-positive cells were also found throughout the NTS. However, cells colabeled for c-Fos/DBH were present mainly in rostral A2 ($P < 0.001$; Table 1 and Fig. 2) and were sparse in C2 (Fig. 3). In rostral A2, 66% of c-Fos-ir cells were DBH-ir but only 26% of DBH-ir neurons were c-Fos-positive. In the mid rostro-caudal level of A2, 30% of c-Fos-ir cells were DBH-ir but only 11% of DBH-ir neurons were c-Fos-positive. At the same coronal plane as A2m, more c-Fos-positive cells were found in DMV area ($P < 0.001$; Table 1). DBH and c-Fos in cell groups A5–A7 were not quantified. However, about half of the DBH-positive cells in ventral A5 region were activated by orexin-A (Fig. 4), but no c-Fos-positive cells were found in the dorsal part of A5. In A6, many or most DBH-positive neurons appeared to be activated by orexin-A (Fig. 4). Only a few c-Fos-ir cells were found in the vicinity of A7, but these did not express DBH (Fig. 4). Some C3 neurons also expressed c-Fos, but because it was close to the injection site, c-Fos in C3 area was not quantified.

Microinjections of orexin-A into A1/C1 stimulated food intake. To determine whether orexin-A innervation stimulates feeding by activating neurons in A1/C1 area, a dose of

![DBH and c-Fos double-immunofluorescence staining in A5-A7 regions.](http://ajpregu.physiology.org/)
orexin-A (67 pmol/200 nl), lower than our ventricular dose, was bilaterally injected directly into the A1/C1 area. As shown in Fig. 5, food intake was significantly enhanced by local orexin-A injection (P < 0.05; n = 9). In the same rats, 5TG injection (24 μg/200 nl) into A1/C1 also enhanced feeding (P < 0.05).

**DISAP abolished feeding induced by 4V and LV orexin-A.** Effects of 4V or LV orexin-A on feeding were investigated in DSAP-lesioned and SAP control rats. Orexin-A (0.5 nmol/rat), injected into 4V in SAP rats (n = 8), stimulated feeding significantly compared with intake after saline injection (P < 0.01; Fig. 6A). However, orexin-A did not increase feeding in DSAP rats (P > 0.7; n = 8). Glucoprivic feeding induced by 2DG (200 mg/kg sc) was also abolished in DSAP rats compared with SAP rats (P < 0.001: Fig. 6B), confirming the DSAP lesion. Similarly, orexin-A (0.5 nmol/rat) injected into LV stimulated feeding in SAP rats (n = 5) above their intake after saline injection (P < 0.05; Fig. 6C), and this response was abolished in DSAP rats (P > 0.5; n = 6). Lesions of hindbrain catecholamine neurons in LV-DSAP rats were confirmed by 2DG-induced glucoprivic feeding (P < 0.001; Fig. 6D).

At the end of the experimentation, DBH cells in each hindbrain catecholamine region were counted. As shown in Table 2, PVH DSAP treatment resulted in significant loss of DBH-ir neurons in the ventrolateral cell groups, A1, A1/C1, and C1m (Table 2), and also in the dorsomedial cell groups, A2, C2, and C3 (not shown) in both 4V- or LV-implanted rats (Ps < 0.001; n = 5–8). DBH immunoreactivity was not reduced in A5 and A7 (not shown) and was only slightly reduced in C1r of DSAP-treated rats, since these cell groups do not project to the PVH DSAP injection site. Loss of neurons in A6 was apparent, but because of the density of DBH neurons in A6, cell loss was not quantified.

**DISCUSSION**

Abundant evidence over the past four decades has shown that the hindbrain catecholamines, NE and E, potently stimulate food intake (6, 22, 24, 31, 38, 41, 44). Previous work (59) also has shown that 4V injection of orexin-A stimulates feeding, that orexin terminals are present in the vicinity of hindbrain catecholamine cell groups, and that ventricular administration of orexin-A increases c-Fos expression in catecholamine neurons. Our c-Fos experiments confirm and extend this previous work showing that significant numbers of catecholamine neurons are activated by 4V administration of orexin-A. We observed orexin-A-induced c-Fos expression in all NE and E cell groups except A7, with regional differences in the prevalence of c-Fos/dbh coexpression within each group. With the exception of A6, which was highly activated by orexin-A, the cell groups with the greatest proportion of orexin-A-activated DBH neurons were A1 and C1 in the ventrolateral medulla. In the A1/C1 and C1m regions, ~40% of DBH-positive neurons were activated by orexin-A and ~70% of c-Fos-ir neurons were also DBH positive. Fewer DBH-ir neurons (14%) in C1r were activated.

Although results showing that activation of catecholamine neurons by ventricular orexin-A injection provide confirming evidence for the role of orexin-A in feeding, further studies are needed to determine whether these neurons provide the sole source of orexinergic input to the ventrolateral hypothalamus, and how other orexinergic inputs into this brain region contribute to food intake regulation.
We have shown previously that the lesion produced by hypo-
induced feeding in DSAP-lesioned rats is not likely to be due
of the circuitry for orexin-induced feeding. Loss of orexin-A-
thalamus are not only involved in but are required components
that hindbrain catecholamine neurons projecting to the hypo-
responses to both LV and 4V orexin-A injections, indicating
PVH. We report here that this treatment abolished feeding
DSAP, the retrogradely transported saporin conjugate that
responses (20).

In the present experiments, we destroyed catecholamine
ners in glucoprivic feeding. Our previous work has
demonstrated a critical role for the A1/C1 catecholamine neu-
ners in glucoprivic feeding. Localized nanoinjections of 5TG
in this area stimulate food intake (1, 42), glucoprivation in-
creases expression of both neuropeptide Y (NPY) (25) and
DBH genes (27) in this region, and simultaneous silencing of
copposed genes for NPY and DBH at this site reversibly
suppresses glucoprivic feeding (26). Moreover, the possibility
that orexin neurons are involved in glucoregulatory feeding is
enhanced by their known responsiveness to glucose availability
(7, 8, 18). However, the fact that destruction of hindbrain
catecholamine neurons abolishes the glucoprivic control of
feeding does not preclude orexin-catecholamine interaction in
nonglucoprivic controls of feeding in intact rats. Our
PVH DSAP injection damages a large proportion of the total
population of both dorsomedial and ventrolateral medullary
catecholamine neurons with hypothalamic projections (40,
43), some of which mediate nonglucoprivic feeding responses
(20).

In the present experiments, we destroyed catecholamine
ners with projections to the hypothalamus by injecting
DSAP, the retrogradely transported saporin conjugate that
selectively targets DBH-expressing neurons (4, 56), into the
PVH. We report here that this treatment abolished feeding
responses to both LV and 4V orexin-A injections, indicating
that hindbrain catecholamine neurons projecting to the hypo-
thalamus are not only involved in but are required components
of the circuitry for orexin-induced feeding. Loss of orexin-A-
induced feeding in DSAP-lesioned rats is not likely to be due
to nonspecific behavioral disruption arising from the lesion.
We have shown previously that the lesion produced by hypo-
thalamic DSAP injection abolishes glucoregulatory responses,
including loss of feeding responses to systemic and central
glucoprivation, but does not produce nonspecific behavioral

anatomical evidence that catecholamine neurons constitute a
potential hindbrain substrate for orexin-induced stimulation of
feeding, they do not in themselves reveal the pertinent sites of
orexin action specifically for the feeding response. However, the
fact that injection of orexin-A directly into the A1-C1m
region was effective in stimulating a robust feeding response,
at a much lower dose than used for 4V or LV injections,
identifies ventrolateral medullary catecholamine neurons as
key contributors to orexin-induced feeding. In addition, these
results implicate orexin neurons specifically (but not exclus-
vively) in glucoregulatory feeding. Our previous work has
represented a rats increase consummatory feeding responses to sys-

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<td>2.4 ± 0.3*</td>
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<td>15.1 ± 0.7*</td>
<td>19.9 ± 1.0</td>
<td>15.8 ± 0.9#</td>
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Values are means ± SE. Numbers of DBH-positive cells per side in each hindbrain region in paraventricular hypothalamic nucleus SAP and DSAP rats, implanted with a 4V or lateral ventricular (LV) cannula, are shown. Numbers were averages for each region (counted 2 or 3 sections per rat for each region).

#P < 0.01, *P < 0.001 vs. SAP rats.

impairment. Under normal laboratory conditions, DSAP-
treated rats remain healthy, maintain normal body weights,
increase their food intake in response to overnight food depre-
vation and lipoprivic challenge, and have normal daily caloric
intake (40, 43) and normal circadian distribution of feeding
behavior (unpublished). Similarly, the PVH injection of DSAP
would not have damaged orexin neurons or their terminals
nonspecifically. In previous work, we reported that injection of
DSAP into the PVH did not damage CRH neurons located in
the PVH injection site or impair their function in response to
nonglucoprivic stimulation (43).

Although orexin-A activates catecholamine neurons and
stimulates food intake, and may facilitate aspects of gluco-
privic feeding, orexinergic input is not required for activation
of catecholamine neurons involved in feeding responses to
glucoprivation. Localized glucoprivation induced by nanoliter
injections of the glucoprivic agent, 5TG, into hindbrain cate-
cholamine cell groups stimulates feeding (1, 42). Although
local glucoprivation could conceivably increase release of
orexins from terminals at a hindbrain injection site in intact
rats, it has also been shown that unanesthetized chronic decer-
brate rats increase consummatory feeding responses to sys-
temic glucoprivation (12, 16). In this chronic decerebrate
preparation, orexin terminals in the hindbrain would be elim-
nated. In addition, LV administration of 5TG is ineffective in
stimulating feeding when confined to the rostral ventricular
space by an acute aqueduct plug, a preparation that would not be
expected to interfere with activation of orexin neurons by
5TG or with orexin projections to the hindbrain (39). In
contrast, 4V injections of 5TG remain effective in aqueduct-
occluded animals (39). Thus, it seems clear that while orexin
ners may be a sufficient source, they are not the only source
of catecholamine activation leading to food intake and are not
required for activation of these neurons in response to gluco-
privic feeding.

It is interesting that orexin neurons are not only able to
activate catecholamine subpopulations but are themselves tar-
geted and activated by catecholamine neurons, indicating a
reciprocal relationship between these cell types. Catechol-
amine neurons from the A1 and C1 cell group project to
orexergic areas of the hypothalamus (9, 20), and the majority
of these express phenethanolamine N-methyl transferase
(PNMT) (5). Recent work using neuron-selective viral tracing,
immunohistochemistry, and electron microscopy reports that

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these PNMT-expressing neurons (i.e., C1 neurons) form excitatory synaptic contacts (predominantly asymmetric synapses) with orexin cell bodies and dendrites (5). PNMT synaptic profiles in orexinergic sites also contain a predominance of small, clear synaptic vesicles typical of those that release glutamate, and C1 neurons are known to express vesicular glutamate transporter 2-ir (50). Therefore, it is possible that (at least under some conditions) glutamate is the predominant excitatory neurotransmitter at catecholaminergic synapses on orexin neurons.

In addition to glutamate, rostrally projecting catecholamine neurons in the A1/C1 group also coexpress NPY (15, 47), a peptide that is potently orexigenic when injected into the cerebroventricles (49, 52, 57) and into various hypothalamic sites, including the rostral perifornical hypothalamus (48). In addition, simultaneous blockade of Dbh and Npy in A1/C1 impairs the feeding response to systemic glucoprivation (26), suggesting that both NPY and catecholamines mediate orexigenic effects. However, the effects of NPY and catecholamines on orexin neurons themselves are complex. Inhibitory effects of NE, E, and NPY on orexin neurons have been demonstrated (17, 28, 58). This inhibition is puzzling in light of the increased c-Fos expression apparently arising from activation of catecholamine neurons in our experiment. This may reflect the differential modulation of orexin neuron activation state by physiological variables (such as glucoprivation), the receptor subtype that predominates at the particular sites of transmitter release, or the specific cotransmitter that predominates at this site and under the conditions tested. A reasonable explanation is that glutamate is the excitatory neurotransmitter released from catecholamine neurons that is responsible for activation of orexin neurons at this site, as discussed above. Clearly, additional work will be required to resolve this issue.

The dorsomedial medulla, which contains primary input and output sites for vagal sensory and motor neurons, respectively, contains catecholamine neurons comprising cell groups A2 and C2 and is associated with multiple controls of feeding and metabolism (19). Orexin fibers make close contact with catecholamine neurons in the dorsomedial medulla (59), and neurons in this area were also activated in our experiment following 4V injection of orexin-A, as described previously (59). We did not inject orexin directly into the NTS in this experiment. However, earlier work by Zheng et al. (59), by using a number of feeding paradigms, reported that injection of orexin-A into the NTS itself did not increase food intake. These investigators reported that orexin briefly stimulated intake of a high-fat diet, but the effect was significant only at 30 min, and higher doses suppressed food intake. Intake of 15% sucrose was not increased by NTS orexin injection. NTS orexin injections, however, were effective in increasing various autonomic responses that may have interfered with the feeding response. Alternatively, orexin terminals in the NTS may not be involved in food intake or may mediate suppression of food intake.

Perspectives and Significance. It is clear from our results that orexin-A stimulates feeding and that hindbrain catecholamine neurons are a required substrate for the feeding response. The glucoprivic control of feeding is an important, but not the only, control of feeding and energy homeostasis that uses catecholamine neurons (37). Thus there are various ways in which the orexin and catecholamine neurons may influence food intake by their reciprocal interactions. The contribution of orexin neurons to glucoprivic feeding is a particularly interesting possibility given electrophysiological evidence revealing their seemingly unique glucose-sensing strategies (7, 8, 18) and the reciprocal innervation between orexin and catecholamine neurons that we show here to be a component of the feeding circuitry. A reasonable speculation that is consistent with their widespread innervation of the brain and spinal cord is that the role of orexin neurons in the glucoregulatory feeding is to coordinate anticipated and ongoing levels of arousal and activity with appropriate behavioral, metabolic, and autonomic adjustments (54, 55). Similarly, it is both reasonable and provocative to consider that the catecholaminergic glucoregulatory system, which appears to be organized for rapid and unconditional responses to acute glucose deficit, would be wired to activate an arousal system that facilitates these adjustments. On the basis of current data, mechanisms of cooperativity between these two widely projecting systems would appear to be a rich field for future studies of glucoregulatory circuitry, food intake, and autonomic controls.

REFERENCES


