Orexins in rat dorsal motor nucleus of the vagus potently stimulate gastric motor function

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Orexins are thought to be involved in diverse functions, such as the regulation of the sleep-wake cycle, pain, and arousal; however, many studies have emphasized their role in energy homeostasis. For example, orexin A stimulates feeding when applied to regions of the hypothalamus (5). Orexins of hypothalamic origin have been linked to the dorsal vagal complex (see below). This is where they could integrate energy balance homeostasis with sensory information from the viscera and bloodstream. The vagus nerve conveys both descending influences that prepare and coordinate the gut to receive food and sensory feedback from the gastrointestinal tract about meal size and composition. Integration of vagal afferent-efferent pathways from the gut occurs at the level of the dorsal vagal complex of the hindbrain medulla. This complex comprises the dorsal motor nucleus (DMN) of the vagus, where preganglionic motor neurons innervating the gastrointestinal tract are located, and the nucleus of the solitary tract (NTS), where primary visceral afferents terminate.

The emerging data linking orexins of hypothalamic origin to the dorsal vagal complex are as follows. The terminal field innervation of these orexin-containing cell bodies are nuclei frequently associated with autonomic function (4). Specifically, 15% of orexin A-positive neurons in the lateral hypothalamic area project to the dorsal vagal complex (6). Intracerebroventricular injections of orexins also induce c-Fos expression in the NTS and DMN of the vagus (4). In the latter study, hypoglycemic rats that were fasted showed significantly more c-Fos-positive neurons in the lateral hypothalamus and dorsal vagal complex than fed controls. Interestingly, many of the c-Fos-positive neurons in the lateral hypothalamus contained orexins. These investigators concluded that neurons in the NTS detect the decreasing glucose signal and activate lateral hypothalamic neurons that express orexin.

Although orexins A and B do not stimulate feeding by acting at the level of the dorsal vagal complex (5), there is evidence that orexin A may stimulate feeding-
related functions of the gut. For example, orexin A applied to the dorsal surface of the medulla stimulates gastric acid secretion through vagal pathways (20). Although the site of action of this effect is unknown, it is likely to be via an action in the dorsal vagal complex. In addition, acid secretion is temporally and possibly causally linked to gastric contractile activity; however, little is known about the effects of centrally administered orexin A on gastric contractile activity. Therefore, we microinjected orexins A and B into the dorsal vagal complex in anesthetized rats while monitoring indexes of gastric contractile activity. In addition, we used antibodies to the peptides and receptors and immunocytochemistry to determine the location of the endogenous receptor in the dorsal vagal complex that could account for the observed gastric motor effects.

METHODS

General. Male Sprague-Dawley rats (200–390 g) obtained from Charles River Laboratories (Wilmington, MA) were used in all experiments after an overnight fast with ad libitum access to water. The study was approved by the Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee.

Microinjection experiments. The animals were initially anesthetized with ketamine and xylazine mixture (36 and 3.6 mg/kg im, respectively), and separate indwelling cannulas were placed in the left femoral artery and vein. Afterwards, α-chloralose (60–80 mg/kg) was administered intravenously, and a tracheotomy was performed to connect the animal to a small animal respirator (Kent Scientific, Litchfield, CT). A laparotomy was performed, and an intraluminal latex balloon was inserted into the stomach through an incision in the fundus for recording intragastric pressure (IGP). The imparting pressure within the intragastric balloon was maintained at ~5 cmH₂O before starting microinjection experiments in all animals. A small strain gauge (Warren Research Products, Charleston, SC) was sutured onto the surface of the distal antral region of the stomach for continuous recording of circular smooth muscle. Rectal temperature was kept between 37.0 and 37.5°C by radiant heat.

Animals were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), and the dorsal surfaces of the medulla and obex were exposed by an occipital craniotomy. Seven-barreled micropipettes (20–40 μm total external tip diameter) were prepared from glass capillaries (Dagan, Minneapolis, MN or A-M Systems, Everett, WA) and attached with polyethylene tubing to a pneumatic pico-pump (model PV 830, World Precision Instruments, New Haven, CT). The micropipette tip was stereotaxically placed in the DMN rostral to the obex (coordinates: +0.5–0.9 mm rostral to the obex, 0.4–0.5 mm below the surface of the obex, 0.5 mm lateral) and caudal to the obex (coordinates: −0.5 mm caudal to the obex, 0.5 mm below the surface of the obex, 0.3 mm lateral) according to a stereotaxic atlas (16).

Microinjections were delivered (at 30 lbs./in.²) in a volume of 20 nl for 10–15 s. Initially, L-glutamate (7.5 nmol) was microinjected to precisely locate the rostral DMN, where

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Fig. 1. Representative chart recording showing the effect of microinjection of L-glutamate (7.5 nmol) and orexin A (10 pmol) into the rostral (A) and caudal (B) dorsal motor nucleus (DMN) on intragastric pressure (IGP) and antral motility.

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brief (<2 mins) but marked increases in intragastric pressure and antral motility were observed. The placement of the pipette tip in the DMN was assured by the expected gastric motor responses. Microinjections of the peptides were then performed after an interval of at least 15 min. This protocol has reliably resulted in microinjections in which the tip of the pipette is located within the DMN rostral to the obex (10). We used similar injections of L-glutamate into the DMN caudal to the obex to evoke gastric relaxation before microinjection of orexin A. The relaxation evoked by stimulation of DMN neurons caudal to the obex is thought to be due to activation of vagal neurons that innervate inhibitory nonadrenergic–noncholinergic (NANC) motor neurons (1, 18).

Orexins A and B (Bachem, CA) were dissolved in saline with 0.1% bovine serum albumen and 2% ascorbic acid (as an antioxidant). At 15- to 30-min intervals, vehicle and orexin A were microinjected unilaterally into the DMN. Generally, only one microinjection site was tested in this nucleus, although microinjections may have been made into other medullary nuclei, such as the nucleus ambiguus (0.7 mm rostral, 1.5 mm lateral, and 1.3 mm ventral) and nucleus raphe obscurus (0.7 mm rostral, in the midline, and 1.3 mm ventral). A dose range of 1–100 pmol orexin A was selected, and several animals received microinjections of different doses of the agent. On the basis of preliminary results, 10 pmol were selected as the maximal dose for the gastric response in the DMN. The same dose of orexin B was microinjected in the DMN rostral to the obex in a limited number of experiments (n = 3). Orexin A (10 pmol) was microinjected into the DMN before and after midcervical vagotomy, which was performed by avulsion during the experiment.

At the end of each experiment, a similar volume of 1% pontamine sky blue was microinjected into the DMN, and the animal was euthanized by pentobarbital sodium (60–80 mg/kg). Brains were removed, fixed in 4% paraformaldehyde, sectioned at 50 μm, and counterstained with neutral red. The placement of the microinjection tips in the DMN was confirmed histologically.

Peak decreases (nadir) and increases in intragastric pressure were determined after microinjection and compared with pre-injection levels. The area under the curve was not calculated, because the responses to microinjections involved striking increases in contractile activity, with little difference in baseline “tone” between contractions. Minute motility index (MMI) was calculated for antral motility for a 2-min period before microinjection and during the peak response (15).

The differences between groups were assessed by one-way ANOVA followed by Student Newman-Keuls multiple-comparisons test. P values <0.05 were considered to be statistically significant.

Immunocytochemistry. Animals were deeply anesthetized by overdose of pentobarbital before cardiac perfusion with saline followed by 4% paraformaldehyde. The hindbrains were postfixed and then placed in 20% sucrose at 4°C for 24–48 h. Coronal sections at 40 μm were cut through the medulla by either vibratome or a cryostat from approximately −1.5 to +2.00 mm relative to the obex, and the sections were sequentially collected in four wells of Tris·HCl at pH 7.5. Sections were incubated in antibodies raised against OR1, OR2, orexin A, or orexin B (all from Alpha Diagnostic International, San Antonio, TX; 1:1,000–1:4,000) and stained using a tyramide system amplification kit tagged with fluorescein (New England Nuclear, Boston, MA), a protocol that amplifies the signal by repeated exposure to biotin-tyramide (3). These antibodies have been well characterized for use in immunocytochemistry. For example, preadsorption of antisera with the respective immunizing peptides completely abolished staining in rat central nervous system (antisera to orexin A and OR1; see Ref. 2) and guinea pig enteric neurons (all four antisera; see Ref. 8). Brains analyzed after staining for each antiserum were as follows: eight for orexin A, four for orexin B, eight for OR1, and two for OR2. In some brains, adjacent sections were stained for more than one antiserum, so that 16 brains were used for final analysis.

In another series of experiments, six rats were pentobarbital anesthetized and the stomach was exposed by midline laparotomy. Then, 25 μl (n = 4) of 0.1% cholora toxin B subunit (CTB) conjugated to rhodamine tetramethylrhodamine isothiocyanate (TRITC) or FITC (List Biological, Campbell, CA) was administered in multiple (5–10 μl) injections via a Hamilton syringe into the muscular wall of the stomach. After 3 days, animals were perfused intracardially and hindbrains were stained for OR1 using TSA kit tagged with either CY3 or fluorescein to determine colocalization of OR1 with CTB-FITC and CTB-TRITC, respectively. Sections were analyzed by Nikon Optiphot 2 microscope using appropriate excitation wavelengths for each fluorescent probe (540–550 nm for rhodamine/CY3 and 490–525 nm for fluorescein).

Images were captured using a Nikon Optiphot microscope with a digital camera (Magnafire, Optronics) attached to a Dell Pentium 3 computer. Color or black and white images were imported unmodified into Adobe Photoshop. With the use of this software, the images were reversed (if necessary)

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Fig. 2. Location of the injection site (arrow) in intermediate (A) and caudal (B) DMN for the responses illustrated in Fig. 3, A and B, respectively. Please note that in A, histological processing resulted in tissue tearing at the pipette tract. AP, area postrema; cc, central canal; cNTS, commissural nucleus of the solitary tract; mNTS, medial NTS; XII, hypoglossal nucleus; TS, tractus solitarius. Bar = 100 μm.
and adjusted for brightness and contrast. To combine fluorescent images, the fluorescent (green) image was superimposed on the rhodamine/CY3 (red) image using the “screen” command. Double-labeled cells appeared orange/yellow. No alterations in color were made. Background fluorescence was not abolished because this provided information on anatomical landmarks to aid the reader in the localization of labeled structures.

RESULTS

Figure 1A is a sample chart recording demonstrating the gastric motor responses elicited on microinjection of L-glutamate and 10 pmol orexin A into the DMN rostral to the obex. Within 20 s after L-glutamate microinjection, there was a marked but brief increase in antral contractility and phasic changes in IGP. This is a typical response on excitation of vagal neurons that increase contractility via cholinergic postganglionic/enteric neurons. Within 1 min after microinjection of orexin A at a dose of 10 pmol into the same site, there was a marked and prolonged increase in antral contractility and phasic increases in IGP. The location of the microinjection site in this example is within the DMN at the level where the area postrema is prominent (Fig. 2A).

Whereas a majority of microinjections of orexin A into the DMN evoked increases in gastric contractility, some were ineffective. Therefore, we mapped the location of the microinjection sites in terms of the responses to L-glutamate and orexin A (Fig. 3). Only those sites within the DMN at the level of the area postrema resulted in increases in response to orexin A (10 pmol). Indeed, at more rostral sites, there was absolutely no response to the highest dose of orexin A tested (100 pmol). This suggests that the response to orexin A is quite site specific and that the responses obtained are unlikely to be due to the diffusion of orexin A to adjacent structures. In one case, an ineffective microinjection of orexin A (10 pmol) was located at the border of the DMN and NTS. In addition, microinjection of orexin A (100 pmol) into the midline nucleus raphe obscurus did not result in significant changes in gastric contractility (change in peak IGP +0.8 ± 0.4 cmH2O; antral MMI +1.7 ± 0.8, n = 5).

The compiled data from the microinjections of orexin A (1 and 10 pmol) in the DMN at the level of the area postrema are shown in Fig. 4. This figure demonstrates that, similar to L-glutamate microinjection, there is a significant increase in peak IGP (orexin A: 1 and 10 pmol) and antral motility index (orexin A: 10 pmol) into this region of the DMN. In two additional animals, a dose of 100 pmol microinjected into this region of the DMN evoked a similar increase in peak IGP (4.3 ± 0.3 cmH2O; P < 0.05 compared with vehicle). Thus 10 pmol probably evoke a maximal response at this site. With the use of this dose, the time to peak response is 5.5 ± 1.5 min, and there is a long duration of the gastric effects evoked by these microinjections of 27.2 ± 5.7 min. In three cases, 10 pmol orexin B were microinjected into the DMN rostral to the obex. This resulted in a peak IGP increase of 3.8 ± 0.7 cmH2O, a
vagal complex, with no noticeable subnuclear organization. Immunocytochemical staining of OR1 consistently indicated that the receptor is highly expressed in the majority of neurons in the DMN (Fig. 5B). Neuronal cell bodies in the hypoglossal nucleus also express the receptor (not shown). In general, OR2 immunostaining was weaker than OR1, but it was observed within cell bodies within the DMN of the vagus (not shown).

Because OR1 was highly expressed in the DMN, we performed double-labeling experiments to combined OR1 and retrograde tracer (CTB) injected into the stomach. Figure 6 illustrates these results and shows that OR1 is expressed in the majority of preganglionic neurons innervating the stomach.

To further investigate the regional responses to orexin A within the DMN, we performed additional microinjections into the DMN at the level of, and caudal to, the obex. Direct excitation of cell bodies by L-glutamate microinjection in this region decreased motility index increase of 10.3 ± 1.8 MMI, and the effects lasted for 33–47 min.

Excitation of gastric motor function evoked by microinjection of orexin A (10 pmol) into the DMN was completely abolished by ipsilateral vagotomy (peak IGP was 3.4 ± 0.7 cmH₂O before and 0.2 ± 0 cmH₂O after vagotomy, \( P < 0.05 \); MMI was 5.8 ± 1.2 before vagotomy and 0.5 ± 0.2 after vagotomy \( P < 0.05 \)). In a few cases, two doses of orexin A were microinjected into the same sites in some of the animals, after allowing 15–30 min after recovery to baseline before the second injection. We were able to evoke a gastric excitatory response to the second microinjection, and, therefore, it is unlikely that the inability to evoke responses in vagotomized animals was due to tachyphylaxis.

Immunostaining of orexins and their receptors was very consistent in all brains analyzed, and examples are shown for orexin A, OR1, and orexin B in the dorsal vagal complex at the level of the area postrema (Fig. 5, A–C). Orexins A (Fig. 5A) and B (Fig. 5C) immunoreactive varicosities were noted throughout the dorsal...
IGP (Fig. 7), a response that would be expected after activation of vagal output to inhibitory (nonadrenergic-noncholinergic) postganglionic/enteric neurons. However, microinjections of 10 pmol orexin A into the same sites resulted in either no response (1 case) or a modest increase in gastric motor function (Fig. 7). The compiled data, using only those cases in which microinjection of L-glutamate evoked decreases in IGP, demonstrated that there was no significant effect of orexin A microinjected into the caudal DMN (Fig. 4B). A sample chart recording (Fig. 1B) shows that L-glutamate microinjection evokes a brief decrease in IGP and slight inhibition of antral contractility. Orexin A (10 pmol) microinjected into the same site did not decrease intragastric pressure but rather evoked a modest, though prolonged, increase in phasic IGP oscillations and antral motility. The site of microinjection in this case is clearly in the DMN caudal to the obex (Fig. 2B).

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Fig. 6. Cholera toxin B (CTB)-FITC labeled (A), OR1-immunostained (B), and double-labeled neurons (C) in the DMN rostral to the obex at the level of the AP. This demonstrates that OR1 is expressed in a majority of preganglionic neurons innervating the stomach. * in C indicates an OR1-positive neuron that does not project to the stomach. Bar = 50 μm.

Fig. 7. Map of the location of microinjection sites for orexin A (left) and L-glutamate (right) in the DMN relative to obex by −0.5 mm (top), 0.0 mm (middle), and +0.2 mm (bottom). The approximate location of the DMN is outlined. ▲, increases in IGP in response to 10 pmol orexin A and L-glutamate. ▼, decreases in IGP in response to L-glutamate. ◄, no discernable change in IGP. cDMN, caudal DMN; comNTS, commissural nucleus of the NTS; fg, fasciculus gracilis; px, Decussation of the pyramids.
DISCUSSION

The major emphasis of the present study was on orexin A, and our results indicated that this peptide evokes prolonged and marked increases in gastric contractility when microinjected into the DMN rostral to the obex. Supportive of this, there is a high level of expression of OR1 in vagal preganglionic motor neurons that project to the stomach. Therefore, one mechanism of action for the excitatory gastric motor effect of orexin A could be via OR1 on vagal preganglionic neurons innervating the stomach. We were not able to visualize OR2 in neurons in the DMN to the same extent as OR1, but it is highly likely that both peptides act on their respective receptors to evoke the observed gastric motor effects. We base this conclusion on the following findings: 1) mRNA for both OR1 and OR2 is present in the DMN and NTS (11); 2) orexin B microinjected into the DMN evoked a marked gastric excitatory effect; and 3) orexin B-immunostained fibers are visualized in this region.

The only other report of the direct effects of orexin A on central vagal circuitry controlling gastric function suggests that dorsal application of the peptide increases gastric acid output (20). The time course of orexin A on the dorsal vagal complex to increase gastric acid is very comparable with the increase in gastric motor function when microinjected directly into the DMN. Our mapping study demonstrates that microinjection of orexin A into the DMN rostral to the obex at the level of the area postrema is the most effective site for the stimulatory gastric effects of this peptide. In the DMN caudal to the obex, where L-glutamate evokes inhibition of gastric motor function, orexin A did not significantly increase motility. However, OR1 expression is evident in DMN neurons throughout the rostrocaudal extent of the nucleus. We cannot explain why activation of postsynaptic OR1 does not seem to be effective in the caudal DMN. Possibly, it is related to the dose of orexin A microinjected; however, increasing the dose microinjected into the caudal DMN would increase the likelihood of diffusion of high concentrations of the peptide into the adjacent commissural NTS and compromise the selectivity of the stimulus.

Preganglionic neurons in the DMN may be functionally divided into a group rostral to the obex, where stimulation results in upper gastrointestinal excitation, and a more caudal area located behind the obex, where stimulation results in upper gastrointestinal muscle (lower esophageal sphincter) relaxation (1, 18). Gastric motor excitation evoked by stimulation of the DMN is abolished by atropine and involves cholinergic preganglionic neurons that synapse onto postganglionic cholinergic neurons. The data presented here illustrate that L-glutamate microinjected into the caudal DMN evokes gastric relaxation. This response probably mediated cholinergic preganglionic neurons that synapse onto postganglionic/enteric NANC motor inhibitory neurons (21). If orexin A were activating postsynaptic OR1 on vagal preganglionic motor neurons in the caudal DMN, then inhibition of gastric motor function would be predicted. In contrast, gastric relaxation was never observed after microinjection of orexin A into this site. Thus our data in the caudal DMN do not support a postsynaptic site of action of OR1. Preliminary data in whole cell patch clamp in a brain stem slice preparation show that OR1 activates inhibitory inputs onto vagal neurons (19). Therefore, a presynaptic site of action of OR1 to influence vagal output is likely, but we cannot determine this based on the techniques used in the present study. However, we can conclude that orexin A is similar to thyrotropin-releasing hormone, a peptide that potently increases contractility in DMN rostral to the obex but has no effect in the caudal DMN, where vagal-NANC inhibitory neurons are located (14).

These data suggest that orexins act on sites in the DMN to promote gastric contractility. This is consistent with the observation that orexin A given centrally increases c-Fos expression in neurons of the DMN (4). The action of orexin A on vagal pathways may contribute to the gastrointestinal changes associated with feeding. For example, similar to hypoglycemia-induced feeding (which is associated with c-Fos expression in the DMN and increased gastric motility), orexins could act on vagal motor neurons to increase both motility and gastric emptying. The result is that the stomach empties faster, and this could contribute to the orexigenic effects of the peptide.

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