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Hindbrain orexin 1 receptors blunt intake suppression by gastrointestinal nutrients and cholecystokinin in male rats

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Abstract

Hypothalamic orexin neurons project to many brain areas, including hindbrain structures such as the nucleus of the solitary tract (NTS) and area postrema (AP), where orexin 1 receptors (OX1Rs) are expressed. Hindbrain administration of orexin-A increases feeding and meal size, and blockade of hindbrain OX1Rs with the selective antagonist SB334867 has the opposite effect. Here we asked whether hindbrain OX1R stimulation or blockade alter rats' sensitivity to gastrointestinal satiety signals. Rats received 4th intracerebroventricular (icv) injections of vehicle or orexin-A, at a dose with no effect on its own, prior to an intragastric (IG) infusion of saline or a satiating volume of Ensure. IG Ensure suppressed subsequent chow intake, but orexin-A pretreatment significantly attenuated this IG nutrient-induced satiety at 2 h into the dark phase. In a second experiment, rats received NTS injections of vehicle or orexin-A before intraperitoneal (IP) injection of vehicle or the satiation hormone cholecystokinin (CCK). NTS orexin-A pretreatment completely blocked the intake-suppressive effect of CCK on dark-phase chow intake. Finally, we investigated the role of endogenous hindbrain OX1R activation by pretreating rats with 4th-icv injection of vehicle or SB334867 followed by IG infusion of saline or Ensure just before a chocolate Ensure licking test session. IG nutrient infusion suppressed Ensure intake, and blockade of hindbrain OX1Rs significantly prolonged that intake-suppressive effect. We conclude that hindbrain OX1Rs are a mechanism through which hypothalamic orexin neurons can reduce animals' sensitivity to gastrointestinal nutrient load, allowing them to consume more food.

Keywords

orexin; satiation; satiety; cholecystokinin; hindbrain; brainstem

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1. Introduction

Hypothalamic orexin neurons have been implicated in the control of food intake in many studies (reviewed in Barson, 2018; Goforth and Myers, 2017; Thompson and Borgland, 2011). These cells express a preprogene that is post-translationally modified to yield the isoforms orexin-A and orexin-B. Orexin-A, which binds to the orexin 1 and orexin 2 receptors (OX1R; OX2R), affects food intake when administered into the lateral, 3rd or 4th brain ventricles of rats, whereas orexin-B, which preferentially binds to the orexin 2 receptor (OX2R), has little to no effect on feeding (Ammoun et al., 2003; Dube et al., 1999; Sweet et al., 1999; Haynes et al., 1999). In addition, selective antagonism of the OX1R, via the compound SB334867, suppresses feeding in rats, supporting the idea that endogenous activation of this receptor by orexin-A promotes feeding (Haynes et al., 2000). Orexin neurons in the lateral hypothalamic area (LHA) are robustly activated by cues that predict the availability of positive reinforcers, including highly palatable food (Harris et al., 2005; Choi et al., 2010), and these neurons have extensive projections to other brain areas of relevance to feeding and motivation (Peyron et al., 1998) that express OX1Rs (Hervieu et al., 2001; Marcus et al., 2001). Orexin neurons are thought to play a role in the elevation of high-fat food intake induced by injection of the μ -opioid receptor agonist DAMGO into the nucleus accumbens (Zheng et al., 2003; Zheng et al., 2007). In rats and mice, stimulation of OX1Rs in the brain not only promotes food intake but also increases motivation for food as measured by operant responding on a progressive ratio (PR) schedule of reinforcement, in which the subject must make an increasing number of operant responses to obtain each successive reinforcer, and selective OX1R antagonism reduces PR responding for palatable food in rats (Borgland et al., 2009; Choi et al., 2010; Sharf et al., 2010; Cason and Aston-Jones, 2013). These findings support the idea that orexin neurons are activated by cues that predict palatable food, and the resulting endogenous OX1R activation facilitates obtaining and consuming that palatable food.

Most research attempting to identify the specific brain nuclei in which OX1Rs affect feeding behavior have focused on hypothalamic nuclei and the ventral tegmental area (e.g., Dube et al., 1999; Kotz et al., 2002; Thorpe et al., 2003; Zheng et al., 2007; Terrill et al., 2016). However, orexin neurons project to the caudal brainstem nucleus of the solitary tract (NTS) and area postrema (AP) (Peyron et al., 1998), and there is evidence that OX1Rs in these nuclei increase feeding and motivation for food reward. Fourth intracerebroventricular (4th-icv) or NTS injection of orexin-A increases intake of standard rodent chow, high-fat diet, and sucrose solutions (Zheng et al., 2005; Baird et al., 2009; Parise et al., 2011; Kay et al., 2014), and 4th-icv or NTS injection of SB334867 suppresses intake in rats (Parise et al., 2011; Kay et al., 2014). Moreover, 4th-icv orexin-A increases operant responding for sucrose on a PR schedule, while 4th-icv SB334867 suppresses PR responding in food deprived rats as well as the expression of high-fat food-conditioned place preference (Kay et al., 2014). Because OX1Rs have an established role in arousal and physical activity, it is important to note that the feeding effects of hindbrain orexin-A and SB334867 are observed at doses that have no effect on locomotor behavior (Parise et al., 2011).

We and others have reported that hindbrain application of orexin-A increases meal size (Baird et al., 2009; Parise et al., 2011), and 4th-icv SB334867 reduces meal size in rats

(Parise et al., 2011). In the influential framework developed by Smith (1996), all controls of meal size can be categorized as either “direct” or “indirect”. Direct controls are signals released in response to food stimulation of the gastrointestinal tract, and all other factors that affect meal size are indirect controls, which exert their effects by increasing or decreasing sensitivity to direct controls. Thus, we proposed that orexin-A action at OX1 Rs in the hindbrain function as an indirect control of meal size, increasing meal size by suppressing the brain’s response to gastrointestinal nutrient-induced satiation signals. Initial support for this hypothesis was provided by our finding that 4th-icv orexin-A, at a dose that had no effect on feeding alone, eliminated the meal size suppression induced by intraperitoneal (IP) injection with the pancreatic satiation hormone amylin (Parise et al., 2011). Here, we further investigate this hypothesis by determining if 4th-icv orexin-A can blunt the intake-suppressive effects of intragastric (IG) nutrient infusion, and if intra-NTS orexin-A treatment can attenuate the hypophagic response to IP injection of the intestinal satiation signal cholecystokinin octapeptide (CCK).

While the effects of pharmacologic stimulation of hindbrain OX1 Rs provide information about how these receptors can affect behavior, these effects do not necessarily reflect outcomes of endogenous OX1R activation. Orexin neuron terminals have been observed within the NTS and AP in close apposition to hindbrain neurons that are activated (as measured by c-Fos) by gastrointestinal nutrient load and IP amylin injection (Zheng et al., 2005; Parise et al., 2011), supporting the idea that endogenous orexin-A can modulate caudal brainstem processing of gastrointestinal signals. Mahler and colleagues (2014) recently proposed the “motivational activation” hypothesis of brain orexin action, suggesting that the diversity of orexin effects on behavior and physiology can all be attributed to orexin neurons facilitating the appropriate response in high motivation situations. We hypothesized that the orexin projection to the hindbrain is playing just this role: when orexin neurons are activated by cues that predict rewarding food, release of orexin-A and activation of OX1Rs in the hindbrain reduces sensitivity to gastrointestinal signals that normally suppress feeding, thus promoting increased consumption of that food. We test this possibility here by asking whether 4th-icv pretreatment with SB334867 enhances IG nutrient-induced intake suppression in a setting intended to promote activation of orexin neurons. For this test, we trained rats to lick for highly palatable chocolate Ensure in a test chamber context, and we included a light cue over the spout when Ensure was available. Taken together, our results support the idea that hindbrain OX1Rs suppress the response to IG nutrients, but this effect is not necessarily mediated through modulation of meal size.

2. Methods

2.1. Subjects

Naïve adult male Wistar rats (Harlan, Indianapolis, IN) were individually housed in plastic cages with food hoppers in a temperature controlled-vivarium maintained on a 12:12 hour light-dark cycle. Distilled water and rat chow (5001; Purina, St. Louis, MO) were available *ad libitum* except where otherwise noted. Rats were handled daily and habituated to experimental procedures before the studies began. All experimental procedures were approved by the Florida State University Institutional Animal Care and Use Committee and

conform to the standards of the Guide for the Care and Use of Laboratory Animals (National Research Council 1996).

2.2. Surgery

Under isoflurane anesthesia (2-4% isoflurane in oxygen delivered at a rate of 1 L/minute), rats were implanted with 26G guide cannulas (Plastics One, Roanoke, VA) targeting either the 4th ventricle or the NTS as described previously (Kay et al. 2014). Stereotaxic coordinates for 4th-icv cannulas were on the midline, 2.5 mm anterior to the occipital suture and 5.2 mm ventral to the skull surface. Unilateral NTS cannulas were implanted at 0.6 mm to the right of the midline, on the occipital suture and 7 mm ventral to surface of the skull. Injectors (33G) extending 2.0 mm below the end of the guide cannula were used in all experiments. Rats in Experiments 1 and 3 also received intragastric catheters, implanted in the same surgical session as the brain cannulas, as described previously (Maske et al., 2018). The catheter was connected to a 10 mm 20G back-mount infusion port (Plastics One, Roanoke, VA) that was sutured to the interscapular muscle to promote adhesion. IG catheters were flushed daily with 1 ml of 0.9% saline to maintain patency. Body weight and food intake were monitored while rats recovered for at least 7 days before experimental procedures began.

For Experiment 1, 4th-icv cannula placements were verified before the start of the study by the measurement of a sympathetically mediated increase in plasma glucose 45-60 min after injection of 5-thio-D-glucose (MP Biomedicals, Santa Ana, CA) at a dose of 210 µg/2 µl for the 4th ventricle. Fourth-icv cannula placements for rats in Experiment 3 were verified post-mortem via 2 µl black India ink injection. Rats were anesthetized (180 mg/kg ketamine and 30 mg/kg xylazine, i.p.) immediately before the 4th-icv injection, then they were transcardially perfused with 10mM PBS and 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Brains were removed and cut with a razor blade in the coronal plane at the mid-cerebellum anterior-posterior level to visualize the 4th ventricle. A placement was considered a hit if the ventricle was ink-filled, and this was the case for 100% of subjects. For Experiment 2, NTS cannula placements were verified histologically based on identification of injection-related damage within the boundaries in the NTS as drawn in the atlas of Paxinos and Watson (2007). Rats were anesthetized and perfused as described above, then brains were sectioned. Thionine-stained sections were inspected on an Olympus BX41 microscope and digital images were acquired with a Retiga EXI Aqua camera and Q-Capture software (Hunt Optics, Pittsburgh, PA). Only subjects with correct NTS injection placements were included in the analysis presented here (incorrect n = 2; hit rate 80%).

2.3. Drugs and injections.

Orexin-A (Bachem, Torrance, CA) was dissolved in 0.9% sterile saline, whereas the OX1R antagonist SB334867 (Tocris, Ellisville, MO) was dissolved in 100% DMSO. This concentration of DMSO was required to maintain the drug in solution at the dose used here, and we and others have previously demonstrated that this vehicle does not adversely affect behavior or brain tissue (Blevins et al., 2002; Kay et al., 2014). The corresponding vehicle was used for comparison with drug in each study. Injections were made with a 10 µl syringe connected to a 33G injector, and vehicle or drug injections were delivered at a rate of 1

µl/min for the 4th ventricle or 0.25 µl/min for the NTS. CCK octapeptide (Bachem, Torrence, CA) was dissolved in 0.1% BSA in sterile 0.9% saline and delivered via intraperitoneal (IP) injection. Doses of orexin-A and SB334867 were chosen based on published dose response studies from our lab (Parise et al., 2011; Kay et al., 2014) and unpublished preliminary data, and the goal was that orexin-A and SB334867 would have little to no effect on their own in these experiments. CCK dose was chosen based on the literature and our previous experience with this drug (e.g., Williams et al., 2009; McMinn et al., 2000; Riedy et al., 1995).

2.4. Experiment 1: Effect of 4th-icv orexin-A on the feeding response to an IG nutrient preload

Rats (n = 7) in this experiment were 4 months of age and an average of 435 g body weight at the time the experiment began. All subjects underwent each of the 4 treatment conditions in this experiment in counterbalanced order: icv vehicle with IG saline; icv vehicle with IG Ensure; icv orexin-A with IG saline; and icv orexin-A with IG Ensure. We used 5 ml chocolate Ensure (4.65 kcal) as the IG stimulus because it is a mixed macronutrient load that should stimulate a variety of gastrointestinal satiation and satiety signals, and effectively suppresses dark-phase chow intake in our experience (unpublished pilot data). Experimental conditions were separated by 48 – 72 h. The 48-h minimum interval was selected so that we could confirm the health and normal feeding behavior of rats on non-treatment days, and conditions were sometimes separated by 72 h to accommodate experimenter schedules.

On infusion days, food was removed from home cages 3 h before dark phase onset to eliminate the possibility that some subjects would eat more than others during the immediate period before IG infusions and food intake measurements. The 3-h duration for food restriction was chosen based on our previous experience examining meal patterns in ad lib fed rats suggesting that rats sometimes consume meals of significant size during the last few hours of the light phase. Rats received 4th-icv injections of either 2 µl 0.9% saline vehicle or orexin-A (0.05 nmol in 2 µl) between 30 – 45 min before IG infusions. The IG infusions began 15 min before dark onset, and were done in Habitest operant chambers enclosed in sound-dampening boxes (Coulbourn Instruments, Holliston, MA). Each chamber was fitted an infusion line consisting of polyethylene (PE) tubing (VWR, Radnor, PA) protected by a stainless steel spring. The PE tubing was connected to an infusion swivel (Instech Solomon, Plymouth Meeting, PA) mounted on a counterbalanced lever at the top of the chamber, allowing the rats to move freely around the chamber while the infusion line was connected to the back-mounted IG catheter port. All infusions were 5 ml in volume and occurred at a rate of 1 ml/min, chosen to approximate rats' average rate of ingestion of liquids (e.g., Williams et al., 2002; Spector & Smith, 1984; Smith et al., 1980). Rats were left connected to the infusion line for 5 minutes after the infusion ended, then they were disconnected. To ensure that all infusate reached the stomach, and to prevent any Ensure from remaining in the catheter and potentially clogging the line, rats had their catheters flushed with 0.5 ml saline before the port was closed and they were returned to their home cages. This 0.5 ml flush was done regardless of infusion condition.

After the rats were returned to their home cages, immediately before dark onset, pre-weighed food hoppers were returned. Hoppers were then weighed by an experimenter at 30, 60, and 120 min after dark onset. Cages were inspected with a dim red flashlight for any spillage, which was collected and weighed if found. Body weights and overnight food intake were measured daily throughout the experiment.

Before the experimental treatments occurred for this study, rats were habituated to the ICV injection procedure, the IG infusion chamber, and connection of infusion lines, and we determined that their dark phase chow intake was not impacted by these procedures (see Table 1). This ensures that any effects on food intake observed here can be attributed to the drug or IG nutrient infusion manipulations.

2.5 Experiment 2: Effect of intra-NTS orexin-A on the hypophagic response to CCK

Rats ($n = 8$) in this study were 4 months of age and an average of 428 g when the experiment began. All subjects underwent each of the 4 treatment conditions in this experiment in counterbalanced order, separated by 48 h: icv vehicle with IP vehicle; icv vehicle with IP CCK; icv orexin-A with IP vehicle; and icv orexin-A with IP CCK.

On treatment days, food was removed from home cages 3 hours before dark onset, as in Experiment 1. Rats received intra-NTS injections of either 0.5 μ l 0.9% saline vehicle or orexin-A (0.025 nmol in 0.5 μ l) between 30 – 45 min before IP injections. The IP injections of either 1 ml 0.9% saline or CCK (2 μ g/kg in 1 ml) were delivered immediately before dark onset. This timing was chosen to hold constant the duration between brain injection and the peripheral manipulation between Experiments 1 and 2. Pre-weighed food hoppers were returned to the home cages at that point, and food intake was measured as described above at 30, 60, and 120 min into the dark phase, as well as overnight. Body weight was monitored daily.

2.6. Experiment 3: Effect of 4th-icv SB334867 on the feeding response to an IG nutrient preload

Rats ($n = 5$) in this experiment were 5 months of age and an average of 425 g body weight at the time the experiment began. All subjects underwent each of the 4 treatment conditions in this experiment in counterbalanced order, separated by 48 h: icv vehicle with IG saline; icv vehicle with IG Ensure; icv SB334867 with IG saline; and icv SB334867 with IG Ensure. We used 5 ml Ensure (4.65 kcal) as the IG stimulus for reasons explained above, however, we anticipated that it would not suppress subsequent intake as robustly in this experiment because chocolate Ensure is a highly palatable food for rats and they were conditioned to consume a large amount during these mid light-phase test sessions.

For this study, rats were trained to lick for chocolate Ensure during daily infusion and licking test sessions that took place in the mid-light phase, and experimental treatments began after rats showed stable day-to-day total Ensure intake during licking test sessions. Stable intake was defined as an average day-to-day variation of 10% or less over at least 3 consecutive days, which was reached at 16 days post-surgery. These tests occurred in the Habitest chambers described above, which were fitted with photobeam lickometers in addition to the infusion lines for this experiment. On experimental treatment days, food was

removed from rats' home cages 1 h before test sessions, rather than 3 h as in Experiments 1 and 2 because we expect little/no spontaneous chow intake for male rats in the mid-light phase. Fourth-icv injections of either DMSO or 10 nmol SB334867 (2 μ l volume) were made 45 min before the IG infusions began. Rats remained in their home cages until that time, and then they were placed into the chamber for infusion line connection to their IG catheter port. Infusions of either saline or Ensure were made as described above for Experiment 1, however, rats in this experiment remained in this chamber for their 60-min licking test session, which began 5 min after the end of the IG infusions. Just before the start of the licking session, rats' infusion lines were disconnected and flushed as described in Experiment 1. Immediately before Ensure access began, an LED cue light above the spout flashed for 15 s, then remained illuminated for the duration of the session. This Ensure-predictive cue was included in attempt to maximally drive endogenous activation of orexin neurons. Graphic State software recorded the timestamp for each lick, and the session ended after 60 minutes. Bottles of Ensure for the licking test were weighed before and after the licking session to determine amount consumed. After the licking session, rats were returned to their home cages with a pre-weighed hopper of chow. On days between experimental treatment conditions, rats were placed in the chambers for licking test sessions but did not receive infusions. Body weight and overnight chow intake was recorded daily.

2.7. Statistical Analysis

Data for all experiments were analyzed by 2-factor (brain injection X IG infusion or CCK) repeated measures ANOVA done in Prism v6 (Graphpad, San Diego, CA), followed by Holm-Bonferonni pairwise planned comparisons. $P < 0.05$ was taken as significant.

In Experiment 3, licks were recorded by Graphic State 3.03 software (Coulbourn Instruments, Allentown, PA) and stored on the computer for later analysis. Licking data were then analyzed by a custom macro. A meal was defined as at least three licks, and the criterion for the end of a meal was a pause of 600 or more seconds (Baird et al., 1999; Spector et al., 1998). Within a meal, a licking burst was defined as series of licks separated by an interlick interval (ILI) of >1 s (Spector et al., 1998). Variables we analyzed from this custom macro included meal size (number of licks), meal duration, within-meal number of bursts, mean within-meal burst duration, and mean licks/minute during the meal. Within-burst ILI was calculated as an average of ILIs below 250 ms, because this captures more than 95% of interlick intervals (Davis and Smith, 1992).

3. Results

3.1. Experiment 1: Effect of 4th-icv Orexin-A on the feeding response to an IG nutrient preload

As expected, the IG Ensure preload significantly suppressed dark phase chow intake relative to IG saline at 30, 60, and 120 minutes (see Figure 1, main effect at 30 min $F(1,6) = 33.93$, $p < 0.01$; 60 min $F(1,6) = 8.89$, $p < 0.05$; 120 min $F(1,6) = 35.58$, $p < 0.01$). Contrary to our expectations, there was no effect of orexin-A nor an interaction at 30 or 60 min; the IG nutrient load suppressed chow intake similarly regardless of whether rats had been pre-treated with saline or orexin-A (at both timepoints, pair-wise planned comparison p 's < 0.05

for icv vehicle IG saline vs IG Ensure, and icv orexin-A IG saline vs IG Ensure). At 120 min, however, a significant interaction between orexin-A and IG Ensure ($F(1,6) = 19.6$, $p < 0.01$), such that IG Ensure only suppressed 120-min chow intake when rats had been pre-treated with saline ($p < 0.001$) with no effect of IG Ensure when rats were pre-treated with orexin-A. Intake at this timepoint after 4th-icv vehicle and IG Ensure was significantly lower than intake after 4th-icv orexin-A and IG Ensure ($p < 0.05$). At no time did orexin-A increase intake when rats received IG saline infusions. There were no effects of orexin-A nor IG nutrient load on overnight chow intake (see Table 2) or body weight.

3.2 Experiment 2: Effect of intra-NTS Orexin-A on the hypophagic response to CCK

Orexin-A pretreatment significantly attenuated the ability of CCK to suppress intake in this experiment (Figure 2A). There were main effects of both orexin-A ($F(1,7) = 9.41$, $p < 0.05$) and CCK ($F(1,7) = 31.14$, $p < 0.01$) at 30 min, and a main effect of orexin-A at 60 min ($F(1,7) = 19.55$, $p < 0.01$), but importantly, ANOVA revealed significant interactions between orexin-A and CCK at both timepoints (30 min $F(1,7) = 13.44$, $p < 0.01$; 60 min $F(1,7) = 6.23$, $p < 0.05$). Pairwise planned comparisons showed that at both 30 and 60 min, CCK significantly suppressed intake relative to IP vehicle only when rats were pretreated with NTS vehicle (p 's < 0.01), and there was no effect of CCK after rats received NTS orexin-A. At both timepoints, intake after NTS vehicle and CCK was significantly lower than intake after NTS orexin-A and CCK (p 's < 0.05). Intra-NTS Orexin-A did not increase intake relative to NTS vehicle treatment when followed by IP vehicle. There were no effects of either treatment on food intake at 120 min nor overnight (see Table 3) and no effects on body weight. NTS injection placements are depicted in Figure 2B.

3.3. Experiment 3: Effect of 4th-icv SB334867 on the feeding response to an IG nutrient preload

IG Ensure significantly suppressed the size of the first meal of the licking test session, regardless of the 4th-icv pre-treatment (Figure 3A). There was a significant main effect of IG infusion ($F(1,4) = 73.5$, $p < 0.01$), and pairwise planned comparisons showed that the number of licks in the first meal was reduced by IG Ensure vs IG saline when rats received 4th-icv vehicle ($p < 0.01$) or 4th-icv SB334867 ($p < 0.01$). Within this first meal, there were no treatment effects on any licking microstructural variable assessed, except for an observed main effect of IG infusion on mean burst duration ($F(1,4) = 13.4$, $p < 0.05$) (see Table 4). However, despite this tendency toward reduction in burst duration after IG Ensure, pairwise comparisons failed to reach significance. The dose of SB334867 we used in this experiment did not suppress meal size nor did it have any effect on licking microstructure when delivered before IG saline.

The ability of OX1R blockade to enhance the intake-suppressive effects of IG nutrient load was observed for total number of licks in the test session (Figure 3B). There was a main effect for IG infusion ($F(1,4) = 11.93$, $p < 0.05$), and the interaction approached significance ($F(1,4) = 7.13$, $p = 0.05$). Rats that received 4th-icv vehicle appeared to compensate for the reduction in first meal size by additional licking later in the 60-min session, such that total licks were not suppressed by IG Ensure, however, when rats received 4th-icv SB334867, rats showed a significant suppress in total licks ($p < 0.01$). This compensation, and lack thereof

after SB334867 injection is suggested by the observation that in the 4th-icv/IG Ensure condition, 4 rats out of 5 took a second meal later in the session averaging 1494 $\pm$ 353 licks in size, whereas only 2 out of 5 rats took a second meal after 4th-icv SB334867/IG Ensure and those meals were far smaller, averaging 371.5 $\pm$ 72.8 licks in size. The number of rats that took second meals was too low and inconsistent across conditions for a within-subjects statistical analysis of this meal (n's of 2-4/condition, and not the same rats in each condition), but average number of meals in the session is reported in Table 4. The pattern of results for total grams of Ensure consumed during the test session (Table 4) was similar to that observed for total licks in the session, though here the interaction did not approach significance. Planned pairwise comparisons again showed that IG Ensure only suppressed Ensure consumed when rats were pre-treated with 4th-icv SB334867 ($p < 0.01$).

There was a significant interaction between IG infusion and SB334867 for overnight chow intake ($F(1,4) = 26.13$, $p < 0.01$) (Figure 3C). IG Ensure did not suppress overnight chow intake when rats received 4th-icv vehicle, but chow intake was significantly suppressed when rats received both 4th-icv SB334867 and IG Ensure ($p < 0.01$). The Ensure consumed in the licking test session accounts for a substantial amount of total kcal consumed for each day, so we also examined total kcal consumed from both Ensure and chow throughout the 24-h period after treatment (Figure 3D). Again we observed a significant interaction ($F(1,4) = 21.6$, $p < 0.01$), such that total kcal consumed was significantly suppressed only when rats received both 4th-icv SB334867 and IG Ensure ($p < 0.01$).

4. Discussion

We hypothesized that orexin-A action at hindbrain OX1R reduces sensitivity to gastrointestinal nutrient-induced satiation signals, and increases meal size through this mechanism. We tested this hypothesis using 4th-icv administration of orexin-A and SB334867 because cerebrospinal fluid flows in a rostro-caudal direction, so this approach delivers these compounds to caudal brainstem nuclei including the NTS and AP. We examined the effect of site-specific injection of orexin-A into the NTS in Experiment 2 because we previously found evidence that the NTS is a site for OX1R effects on feeding (Kay et al., 2014), but we do not rule out the contribution of other nearby hindbrain areas where OX1Rs are expressed. The AP, in particular, likely plays a role in OX1R effects on behavior because lesion of this nucleus along with partial lesion of the NTS attenuates the effects of 3rd-icv orexin-A injection on licking for sucrose (Baird et al., 2009). Whether this could be a direct effect of receptors in the AP is unclear, because although orexin fibers innervate the AP (Cutler et al., 1999; Parise et al., 2011), papers describing brain expression and distribution of orexin receptors have not reported data for the AP (Marcus et al., 2001; Hervieu et al., 2001). OX1R mRNA is expressed in the neighboring dorsal motor nucleus of the vagus nerve (DMX)(Marcus et al., 2001), which likely plays a role in autonomic effects of orexin-A treatment. Based on the known functions of DMX neurons, we consider orexin action in the DMX unlikely to have a direct impact on behavior, though it remains plausible that changes in feeding could occur as a consequence of other physiological effects.

The results of the present experiments partially support our original hypothesis. In Experiment 1, we saw that 4th-icv pre-treatment with orexin-A did attenuate the intake

suppression induced by IG nutrient infusion, however, this effect occurred over a longer timeframe (2 hours) and nutrient-induced intake suppression was unaffected by orexin-A during the time when the first meal of the dark cycle would have occurred. Experiment 2 more narrowly tested the role of NTS orexin-A treatment, and instead of a gut nutrient load that would stimulate many satiation signals, we tested whether orexin-A interacts with one specific satiation signal, CCK. Here, we saw that NTS orexin-A did completely prevent intake suppression by CCK within the first 30 and 60 minutes of the dark phase. It is well established that the form of CCK used here reduces feeding via selective reduction in meal size (Gibbs et al., 1977; Overduin et al., 2014), so one could speculate that NTS orexin-A prevented that meal size effect. However, we cannot draw this conclusion because in this experiment, we only measured intake at discrete timepoints and cannot conduct a meal pattern analysis. It therefore remains possible that NTS orexin-A pretreatment caused rats to quickly compensate for CCK-induced suppression of the first meal by initiating a second meal earlier than usual.

Experiment 3 addressed the question of whether endogenous stimulation of hindbrain OX1Rs has effects similar to those observed for pharmacologic stimulation. We hypothesized that 4th-icv pretreatment with the OX1R antagonist SB334867 would render rats more sensitive to the satiating effects of IG nutrients, and we observed that indeed, the greatest intake suppression was obtained with a combination of SB334867 pretreatment and IG Ensure infusion, both within the 60-min test session and overnight in the home cage. It is important to point out that this dose of SB334867 had no effect on intake when delivered with IG saline, so this significantly greater total session intake suppression is not simply an additive effect. We also know that a higher dose of SB334867 than used here, also delivered 4th-icv, does not suppress locomotor activity (Parise et al., 2011), and therefore it is unlikely that the effects of OX1R antagonism observed here are secondary to motor or arousal suppression. We also saw no effect of SB334867 on licking microstructural variables that would suggest a motor mechanism, such as mean within-burst inter-lick interval, although it is worth noting that we may have seen an effect or interaction with IG nutrient infusion for variables such as meal duration if this experiment had a larger n. Others have shown that this compound does not appear to suppress feeding through nausea or malaise (Ishii et al., 2004). In addition to effects on behavior, OX1R stimulation in the NTS affects autonomic activity and can modulate cardiovascular responses in anesthetized animals (Carrive, 2013), but we view it as unlikely that such effects are responsible for the feeding behavioral changes observed in the present experiments. For these reasons, we conclude that that endogenous activity of orexin-A at hindbrain OX1Rs is a physiological mechanism for limiting the hypophagic response to gastrointestinal nutrient detection.

Similar to the time-course observed in Experiment 1, we saw no effect on the first meal of the test session in Experiment 3. The effect of 4th-icv SB334867 to enhance the response to IG Ensure emerged later in the 60-minute test session, and surprisingly, remained apparent in overnight chow intake. This duration of effect is particularly interesting because the previously reported effects of acute hindbrain orexin-A or SB334867 were short lived, lasting between 30 min to 3 h post-injection at most (Zheng et al., 2005; Parise et al., 2011; Kay et al., 2014), although considerably longer duration effects have been reported after orexin-A or SB334867 injected into other brain areas or systemically. For example, Sarihi

and colleagues (2005) showed elevation in feeding up to 8 hours after relatively high-dose orexin-A injection into the medial preoptic area in rats, and several studies have shown that IP injection of SB334867 can suppress feeding for 4 – 24 h after treatment (e.g., Haynes et al., 2000; Ishii et al., 2005). In combination with the lack of attenuation of IG Ensure-induced satiety by orexin-A at 30 or 60 minutes in Experiment 1, the lack of enhancement of IG Ensure-induced satiety by SB334867 in the first meal of the test session in Experiment 3 is contrary to our initial hypothesis that hindbrain OX1Rs function as indirect controls of meal size. These findings are also in contrast with our previously reported observations that higher doses of 4th-icv orexin-A and SB334867 respectively increase and decrease the size of the first meal of the dark phase (Parise et al., 2011). We previously showed that the same dose of 4th-icv orexin-A used here (0.05 nmol) reversed IP amylin's ability to suppress the size of the first meal of the dark phase (Parise et al., 2011), again seemingly inconsistent with the results reported here. IG Ensure infusion-induced suppression of immediately subsequent meal size is likely mediated by numerous gastrointestinal signals, including distention, CCK, amylin, and several other gut hormones and neural signals arising from intestinal detection of nutrients. It may be that hindbrain OX1R interact with only some of these gastrointestinal signals, and are therefore unable to modulate the substantial satiation response to all of these signals at once.

Information about duration of receptor binding in the brain after 4th-icv or intra-NTS treatment is unavailable, but we speculate based on measured half-life in the plasma (acknowledging that this may differ from brain clearance) that both orexin-A (half-life of 27 minutes; Ehrström et al., 2004) and SB334867 (half-life of 24 minutes; Porter et al., 2001) remained present at the time behavioral testing started in our experiments. However, these drugs should be cleared within a few hours. Therefore, the effects of orexin-A at 2 h into the dark phase in Experiment 1 and SB334867 at 24 h in Experiment 3 are likely mediated through mechanisms downstream of the OX1R. Recent work examining persistent effects of OX1R antagonist treatment on motivation for drugs of abuse suggests that acute blockade of these receptors alters mesolimbic dopamine neuron function up to 24 h later (Mohammadkhani et al., 2020; Brodnik et al., 2020). Whether hindbrain-specific application of these compounds indirectly impacts the dopamine system or acts through other neural pathways remains to be investigated.

One limitation of our studies is that we examined male rats only, and we do not know that these results would generalize to females. The vast majority of studies on the anatomy of orexin neuron projections, distribution of orexin receptors in brain, and feeding effects of orexins and orexin receptors in rats were performed only in male rats, so the likelihood of sex differences here is difficult to estimate. However, a few findings suggest that orexins have broadly similar effects females. Haynes and colleagues (2000) showed that lateral-icv orexin-A increased food intake similarly and IP SB334867 suppressed food intake similarly in both male and female rats. Another group showed that IP injection of a different selective OX1R antagonist, GSK1059865, suppressed eating in female rats in a binge eating model involving a combination of stress and access to highly palatable food (Piccoli et al., 2012), confirming a role for OX1Rs in feeding in females. Nonetheless, sex differences in the orexin innervation or receptor expression in the caudal brainstem may exist, as may

differences in the behavioral effects of orexin receptors located there, and this deserves further investigation.

Another limitation to note is that the pharmacologic manipulations used here cannot definitively rule out a contribution of the OX2R to the observed effects. Orexin A effectively binds and activates the OX2R, and OX2R mRNA expression has been reported in the NTS, though at lower density than the OX1R (Marcus et al., 2001). We consider it unlikely that the OX2R plays a significant role here in part because orexin B preferentially activates the OX2R yet has little to no effect on feeding (Ammoun et al., 2003; Dube et al., 1999; Sweet et al., 1999; Haynes et al., 1999). SB334867 is widely considered a selective OX1R antagonist, with 50-fold higher affinity for the OX1R than OX2R, but this does leave open the possibility that some portion of the effects of SB334867 are mediated by OX2R blockade, especially at higher doses. Investigation of feeding effects of other selective compounds has been limited, but Piccoli and colleagues (2012) showed no effect of a selective OX2R antagonist (JNJ-10397049) on feeding while the selective OX1R antagonist GSK1059865 did suppress intake in their binge eating model. These data further support our contention that the OX1R is the primary mediator of the effects we report here, although it would be valuable to test whether there are any feeding effects of the OX2R-selective antagonist when applied directly to the hindbrain.

Taken together, the findings presented here support our overall hypothesis that activation of hypothalamic orexin neurons that project to the NTS and AP is a physiological mechanism to reduce sensitivity to the presence of nutrients in the gastrointestinal tract, and thereby allow further food consumption. In accordance with the motivational activation hypothesis of orexin function (Mahler et al., 2014), this pathway may only become active in situations when motivation for food is very high, such as in Experiment 3 where rats were conditioned to expect highly palatable Ensure in a salient context. Under normal physiological conditions (i.e., in the absence of brain site-specific drug manipulations), the dorsal hindbrain is likely to be just one of several orexin neuron projection targets participating in this process. For example, we have previously shown that OX1R activation in the VTA attenuates intragastric nutrient-induced hypophagia (Terrill et al., 2016). It will be valuable to determine which cells within the NTS and other neighboring hindbrain regions mediate the effects we observed in these studies, and how these circuits interact with other signals that control food intake.

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Highlights

- Hindbrain orexin-A blunts the satiety response to intragastric nutrient infusion.
- Orexin-A in the nucleus of the solitary tract blocks IP cholecystokinin effects.
- Orexin 1 receptor blockade increases sensitivity to intragastric nutrients.

Figure 1.

Cumulative chow intake (g) at 30, 60, and 120 min after dark phase onset, when rats were either injected 4th-icv with vehicle or orexin-A, followed by IG infusion of either saline or Ensure. Data are means $\pm$ SEM. * p < 0.05 for comparison of IG saline vs Ensure within 4th-icv injection condition; # p < 0.05 for comparison of 4th-icv vehicle/IG Ensure vs 4th-icv orexin-A/IG Ensure.

Figure 2.

A) Cumulative chow intake (g) at 30 and 60 min after dark phase onset, when rats were injected into the NTS with vehicle or orexin-A, followed by IP vehicle or CCK. Data are means $\pm$ SEM. * $p < 0.05$ for comparison of IP vehicle vs CCK within NTS injection condition; # $p < 0.05$ for comparison of NTS vehicle/IP CCK vs NTS orexin-A/IP CCK. B) Diagrams indicating the estimated center of each rat's NTS injection site (black dots), based on the atlas of Paxinos and Watson (2007), ranging from -13.44 to 14.16 mm relative to bregma. 4V, 4th-ventricle.

Figure 3.

Licking behavior and subsequent intake in Experiment 3, when rats were either injected 4th-icv with vehicle or SB334867, followed by IG infusion of either saline or Ensure. A) Number of licks in the first meal of the test session. B) Total number of licks in the 60-min test session. C) Overnight chow intake after the licking test session. D) Total kcal consumed, including both Ensure ingested during the licking test session and overnight chow intake. Data are means $\pm$ SEM. * $p < 0.05$ for comparison of IG saline vs Ensure within 4th-icv injection condition.

Table 1

Dark phase cumulative chow intake for rats in Experiment 1, measured on habituation days before the experiment began. On the “Handling” day, rats were food restricted starting 3 h before dark phase onset, and handled for body weight measurement and for removal and replacement of the ICV cannula obturator. On the “Chamber” day, rats were treated as on the handling day, but also spent the 15 minutes before the dark phase in the IG infusion chamber connected to the infusion lines. On the “Injection/Infusion” day, rats were treated as on the Chamber day, but were also given an ICV injection of saline and received IG infusion of saline. Data are mean g ($\pm$ SEM).

Min	Day of measurement		
	Handling	Chamber	Injection/Infusion
30	4.50 (0.42)	4.47 (0.44)	5.20 (0.29)
60	5.17 (0.48)	5.24 (0.49)	5.90 (0.31)
120	6.98 (0.29)	6.83 (0.44)	6.91 (0.45)

Table 2

Cumulative 24-h chow intake for rats in Experiment 1. Data are mean g ($\pm$ SEM).

Time	ICV Vehicle		ICV Orexin-A	
	IG saline	IG Ensure	IG saline	IG Ensure
24 h	26.51 (0.47)	26.03 (0.96)	26.03 (0.74)	25.91 (0.85)

Table 3

Cumulative 2 and 24-h chow intake for rats in Experiment 2. Data are mean g ($\pm$ SEM).

Time	NTS Vehicle		NTS Orexin-A	
	IP Vehicle	IP CCK	IP Vehicle	IP CCK
2 h	5.85 (0.55)	5.05 (0.43)	5.18 (0.48)	5.88 (0.75)
24 h	29.33 (0.86)	27.74 (1.16)	26.99 (1.24)	28.24 (0.91)

Table 4

Variables measured in Experiment 3, in which rats licking for Ensure were injected 4th-icv with either vehicle or 10 nmol SB334867 before IG infusion of either saline or Ensure. Total Ensure intake and mean number of meals are for the entire 1-h test session; all other variables are for only the first meal of the session. ILI = inter-lick interval. Data are mean (+/- SEM).

Variable	Vehicle		SB334867	
	Saline	Ensure	Saline	Ensure
Mean burst duration (s)	25.59 (9.02)	11.50 (3.60)	22.12 (8.18)	10.76 (4.64)
Mean licks/min	277.66 (32.74)	269.17 (69.80)	225.52 (44.22)	215.09 (31.49)
Number of bursts	30.8 (8.76)	36.4 (16.66)	39.0 (16.31)	46.1 (18.5)
Mean within-burst ILI (ms)	161.01 (5.80)	159.54 (4.84)	161.58 (4.58)	163.88 (7.87)
Mean meal duration (min)	10.23 (0.98)	8.56 (1.46)	12.70 (2.30)	9.12 (1.32)
Total Ensure intake (g)	20.6 (2.31)	19.34 (1.79)	20.84 (1.81)	15.64* (1.11)
Mean number of meals	1.4 (0.27)	1.8 (0.22)	1.6 (0.27)	1.4 (0.27)

* p < 0.05 for pairwise comparison between IG saline vs Ensure within 4th-icv condition.