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Anterior Insula Activity Regulates the Associated Behaviors of High Fat Food Binge Intake and Cue Reactivity in Male Rats

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Abstract

Binge eating episodes are characterized by uncontrollable, excessive intake of food and are associated with binge eating disorder and some subtypes of obesity. One factor thought to contribute to binge episodes is a high level of reactivity to food-associated cues (i.e., cue reactivity). The insula is a neural node poised to regulate both binge eating and cue reactivity because of its prominent role in interpretation of internal and external cues. This work established a positive association between high fat food (HFF) binge intake and cue reactivity in male rats. Furthermore, we demonstrated that activation of the anterior insula suppressed both HFF binge intake and cue reactivity, without altering homeostatic intake of food. We further show that attenuation of HFF binge intake and cue reactivity is not due to decreased food-reward efficacy or deficits in motivation. Together, these data establish a key role for the anterior insula in the control of binge eating related-behaviors and support novel avenues for the treatment of binge eating.

Keywords

Insula; binge eating; cue reactivity; high fat food; DREADD

Introduction:

Binge eating is defined by uncontrolled excessive intake of food. Repeated episodes of binge eating is the characteristic behavior underlying binge eating disorder and is also associated with obesity (American Psychiatric Association, 2013). Multiple psychological processes,

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Competing Interests:

The authors declare no conflicts of interest.

including reactivity to food cues and craving, are proposed to drive binge eating (Kober & Boswell, 2018). These behaviors are tightly interwoven in that exposure to food cues produces craving (Kober & Boswell, 2018). Of note, binge eaters experience a higher level of cue-induced food craving which further associates with elevated food consumption relative to non-binge eaters (Ng & Davis, 2013). Thus, binge eating and cue reactivity may be interlocked behaviors.

The insula is a multimodal integration center for interoceptive, perceptive, motivational, and gustatory information that is poised to regulate binge eating (Frank, Kullmann, & Veit, 2013; Naqvi, Gaznick, Tranel, & Bechara, 2014). Food cue exposure is associated with insula activation in healthy individuals and binge eaters (Schienle, Schafer, Hermann, & Vaitl, 2009). However, only subjects who engage in dysregulated eating exhibit altered functional connectivity between the insula and anterior cingulate cortex and striatum upon food cue exposure, which may represent disruption in salience neurocircuitry (Frank et al., 2013; Geliebter, Benson, Pantazatos, Hirsch, & Carnell, 2016). Although these findings suggest that altered insular outflow of information may be responsible for some forms of dysregulated eating, it is unclear if insula activity, specifically via neurons in the anterior output subregion (insula_{ant}), causally regulates binge eating and cue reactivity. In the present study, we examined the relationship between binge eating and cue reactivity and further established the role of the insula_{ant} on these behaviors in rats.

Methods:

Animals:

Male, outbred Sprague-Dawley rats (n=92; Envigo, Indianapolis, IN, USA) weighing 250–275 g at arrival were housed two per cage (except where noted below) under a 12-hour light-dark cycle (lights on between 0600–1800h) with controlled temperature (21–23°C) and humidity (40–50%). Animals were acclimated for one week to the colony room before the start of handling and experimental procedures. Standard food (SF) and water were available to rats *ad libitum* except during daily operant sessions and where noted below. Sample sizes were determined based on a power analysis (G*Power, Germany) (Faul, Erdfelder, Lang, & Buchner, 2007) of pilot data collected from previous cohorts. All experiments were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (2011) and with the University of Texas Medical Branch Institutional Animal Care and Use Committee approval.

Food:

SF (LM-485 Mouse/Rat Sterilizable Diet; Teklad Diets, Madison, WI, USA; 3.1 kcal/g) consisted of 25% protein, 58% carbohydrate, and 17% fat (by kcal). HFF (D12451, Research Diets, New Brunswick, NJ, USA; 4.73 kcal/g) contained 20% protein, 35% carbohydrate, and 45% fat (by kcal). HFF pellets used in operant conditioning assays (#F06162, BioServ, Flemington, NJ, USA; 4.60 kcal/g) consisted of 16% protein, 38% carbohydrate, and 46% fat (by kcal).

Operant Conditioning for HFF Self-Administration:

Self-administration studies took place between 1000–1500h in standard operant chambers housed within ventilated and sound-attenuated chambers; each chamber is equipped with two retractable levers, a stimulus light above each lever, and a houselight opposite the levers (MedAssociates, Georgia, VT, USA). HFF self-administration studies consisted of daily 30-minute sessions during which rats were trained to lever press for a HFF pellet. Rats were food restricted to 85–90% of free-feeding levels of SF for four days until acquisition of a fixed ratio (FR) FR1 schedule of reinforcement at which point they were provided with *ad libitum* access to SF. Rats remained on an FR1 schedule of reinforcement for three additional days before moving to an FR5 schedule of reinforcement, which was maintained for the duration of testing except on progressive ratio (PR) and cue reactivity test days. Schedule completion on the active lever resulted in HFF pellet delivery and presentation of the discrete cue complex, which consisted of visual (flashing light), olfactory (scent of HFF pellet), and auditory (sound of pellet delivery) cues. There were no scheduled consequences for lever presses on the inactive lever. Rats were required to display stability prior to being assessed in test sessions. The criterion for stability on the FR schedule was less than 25% variability in the number of pellets earned over three consecutive days. Motivation for HFF was assessed using a PR schedule of reinforcement. In this session, the number of active lever presses required to earn the subsequent reinforcer progressively increased (1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95) (McCue, Kasper, & Hommel, 2017; Richardson & Roberts, 1996). The PR session ended after the rat had not earned a reinforcer in 10 minutes. HFF cue reactivity was assessed in a single test session. During this session, responding on the previously active lever resulted in delivery of the discrete cue complex on an FR1 schedule of reinforcement; however, no pellets were delivered as they were diverted outside the chamber.

Association Between HFF Binge Intake and Cue Reactivity:

The binge eating paradigm used throughout this study was adapted from a well-established limited access palatable food protocol (R. L. Corwin, Avena, & Boggiano, 2011; R.L. Corwin & Wojnicki, 2006) and has been validated to induce binge eating behavior (i.e., greater intake of HFF after limited access compared to intake of HFF or SF after continuous access) in previous publications (Benzon et al., 2014; Price, Anastasio, Stutz, Hommel, & Cunningham, 2018). Single-housed rats (Cohort 1; n=12) received exclusive access to HFF for one week which was then replaced by exclusive access to SF. At the beginning of the dark cycle (1800h) one week after the last exposure to HFF, SF was replaced with 40 g of HFF. At the end of two hours, the HFF was removed and weighed to determine binge intake, and rats were given *ad libitum* access to SF. Rats were subsequently acclimated to pair housing and trained to self-administer HFF pellets using operant conditioning. After 29 days of self-administration, rats were subjected to a HFF cue reactivity session in which schedule completion resulted in delivery of the discrete cue complex, but no pellet delivery (Figure 1, **Top**). Rats which did not meet stability criteria on day 29 of self-administration (n=5) were excluded from analyses.

Viral-Mediated Gene Transfer:

Rats (n=58) were anesthetized intramuscularly (i.m.) with a cocktail containing xylazine (8.6 mg/kg), acepromazine (1.5 mg/kg), and ketamine (43 mg/kg) in bacteriostatic saline and placed in a stereotaxic apparatus with the upper incisor bar at -3.8 mm below the interaural line. Two microsyringes (28 gauge, Hamilton Company, Reno, NV, USA) were lowered bilaterally to target the insula_{ant}; the coordinates targeted were anteroposterior +3.0 mm, mediolateral +3.9 mm, and dorsoventral -5.6 mm from skull in relation to bregma (Paxinos & Watson, 2005). The AAV8-CAMKII α -hM3D(G_q)-mCherry excitatory DREADD (Designer Receptors Exclusively Activated by Designer Drugs; UNC Vector Core, Chapel Hill, NC, USA; lot #AV5361h, 1.8×10^{-12} vg/mL) was infused bilaterally at 0.1 μ L/min over eight minutes for a total of 0.8 μ L per infusion. The microsyringes remained in place for an additional 10 minutes to allow for adequate diffusion prior to removal. The CAMKII α promoter was employed to enrich hM3D expression in insula_{ant} glutamatergic efferent neurons (Nathanson, Yanagawa, Obata, & Callaway, 2009). A subset of rats (n=10) were used in initial pilot behavioral studies and to biochemically validate the hM3D system. The remainder of rats were divided into two cohorts (n=24/cohort). Cohort 2 was designated for SF and HFF intake studies while HFF self-administration and cue reactivity were assessed in Cohort 3. Activation of hM3D receptors was achieved via 1 mL/kg intraperitoneal (i.p.) injection of clozapine N-oxide (CNO; BML-NS105, Enzo Life Sciences, Farmingdale, NY, USA) dissolved in saline at a concentration of 2 mg/mL (for a final concentration of 2 mg/kg) 20 minutes prior to testing.

Validation of Cellular Activation by CNO:

The ability of CNO to elicit cellular activation of the insula_{ant} was assessed in a pilot cohort of rats receiving viral-mediated transfer of the hM3D system (n=10). This group underwent an extensive behavioral battery of tests that included cocaine self-administration, HFF binge intake, SF non-binge intake, novel environment-induced hypophagia, and elevated plus maze testing over a period of three months. Twenty minutes prior to the final behavioral test (elevated plus maze) rats received i.p. injections of 1 mL/kg vehicle (VEH, 0.9% NaCl) or 2 mg/kg CNO. Upon completion of behavioral testing, rats were returned to the home cage. Ninety minutes after injection of VEH or CNO, rats were anesthetized (400 mg/kg of chloral hydrate, i.p.) and decapitated. Brains were extracted and sliced into 1 mm thick coronal sections to determine hM3D placement via visualization of mCherry expression. A DFP-1 Dual Fluorescent Protein Flashlight and VG2 barrier filter glasses were used to visualize red fluorescence (Nightsea, Bedford, MA, USA). Photographs were taken with a Canon Rebel XSi digital single lens reflex (DSLR) camera equipped with a macro lens and red filter. The insula_{ant} was sub-dissected from rats displaying bilateral expression of mCherry (n=6), flash frozen in liquid nitrogen, and stored at -80°C for subsequent protein extraction.

Dissected tissue was extracted by homogenizing samples in 10 mM HEPES (pH 7.4) and 320 mM sucrose. Total homogenate was centrifuged at 1000 x g for 10 minutes at 4°C. The supernatant was removed and the pellet (containing the nuclear-enriched fraction) was resuspended in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 10 μ L/mL protease inhibitor and phosphatase inhibitor 2 and 3 cocktails (Sigma-Aldrich, St. Louis, MO, USA). Lysates were

probed for specific protein expression using the Wes™ automated western blotting system (ProteinSimple, San Jose, CA, USA), which utilizes capillary electrophoresis-based immunodetection for higher resolution, sensitivity, and reproducibility (even at low sample concentrations) relative to traditional immunoblotting techniques (Anastasio et al., 2015). Wes™ reagents (biotinylated molecular weight marker, streptavidin-HRP fluorescent standards, luminol-S, hydrogen peroxide, sample buffer, DTT, stacking matrix, separation matrix, running buffer, wash buffer, matrix removal buffer, secondary antibodies, antibody diluent, and capillaries) were obtained from the manufacturer (ProteinSimple) and used according to the manufacturer's recommendations. Levels of phosphorylated c-Fos (pcFos) expression were assessed using a monoclonal rabbit antibody directed against pcFos Ser32 (D82C12, #5384, Cell Signaling Technology, Danvers, MA, USA) at a concentration of 1:50 which was multiplexed to a monoclonal rabbit antibody directed against the loading control, β -actin (D6A8, #8457, Cell Signaling Technology), at a concentration of 1:50.

Equal amounts of protein (4 μ g) were combined with 0.1X sample buffer and 5X master mix (200 mM DTT, 5X sample buffer, 5X fluorescent standards), gently mixed, and then denatured at 90°C for 10 minutes. The denatured samples, biotinylated ladder, antibody diluent, primary antibodies, HRP-conjugated secondary antibodies, chemiluminescent substrate, and wash buffer were dispensed to designated wells in a pre-filled microplate (ProteinSimple). Separation electrophoresis (375 volts, 31 minutes, 25°C) and immunodetection in the capillaries were fully automated using the following settings: separation matrix load for 200 seconds, stacking matrix load for 14 seconds, sample load for 7 seconds, antibody diluent for 30 minutes, primary antibody incubation for 60 minutes, secondary antibody incubation for 30 minutes, and chemiluminescent signal exposure for 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 seconds. Data analyses were performed using the Compass Software (ProteinSimple). Relative pcFos expression in the insula_{ant} after VEH or CNO administration was normalized to β -actin.

Insula_{ant} Activation Effect on SF and HFF Intake:

Two weeks after surgical implantation of the AAV8-CAMKIIa-hM3D(G_q)-mCherry excitatory DREADD, rats in Cohort 2 were single-housed and given exclusive access to HFF for 1 week to reduce food neophobia (acclimation), followed by SF access for the remainder of the study except during 2-hour HFF intake test sessions and during the 24 hours preceding intake test sessions under the food-restricted condition. Rats underwent HFF or SF intake testing in the home cage every week for two hours at the beginning of the dark cycle (1800–2000h) starting one week after HFF acclimation. Tests were conducted under both the freely-fed and food-restricted condition. Rats received both VEH and CNO (in a counterbalanced manner) prior to the tests listed in the following order: HFF freely-fed intake (i.e., binge intake), HFF food-restricted intake, SF freely-fed intake, and SF food-restricted intake. Under freely-fed conditions, rats were injected i.p. with 1 mL/kg VEH or 2 mg/kg CNO at 1740h (20 minutes prior to the intake test). At 1800h, the SF in the home cage was replaced with 40 grams of HFF or SF. At 2000h, the remaining food was removed and weighed to determine 2-hour intake. For food-restricted conditions, SF was removed from the home cage 24 hours prior to testing. Rats were injected i.p. with 1 mL/kg VEH or 2 mg/kg CNO at 1740h the following day (20 minutes prior to the intake test). At 1800h,

investigators administered 40 grams of HFF or SF to the home cages. At 2000h, the remaining food was removed and weighed to determine 2-hour intake. Following all tests, SF was then returned to the home cage for *ad libitum* feeding (Figure 2, **Middle**).

One week after completion of intake studies, rats were anesthetized (400 mg/kg of chloral hydrate, i.p.) and decapitated. Brains were extracted and sliced into 1 mm thick coronal sections to determine hM3D placement via visualization of mCherry expression. Visualization and photography was achieved as described above. Rats with bilateral expression of mCherry in the insula_{ant}, without spread to adjacent cortical regions, were included in the data analysis as judged by a blinded experimenter (n=9). Rats which exhibited no evidence of mCherry expression, unilateral mCherry expression, or inappropriately localized mCherry expression were excluded from behavioral analyses (n=14). An additional rat was excluded due to surgical complications resulting in early euthanasia (n=1).

To assess CNO specificity to the hM3D system in HFF binge intake, 1 mL/kg VEH or 2 mg/kg CNO was administered i.p. to a separate group of surgically naïve rats (n=11/group) 20 minutes prior to HFF binge intake testing (1740h). At 1800h, the SF in the home cage was replaced with 40 grams of HFF. At 2000h, the remaining food was removed and weighed to determine 2-hour HFF intake, and SF was returned to the home cage for *ad libitum* feeding.

Insula_{ant} Activation Effect on HFF Self-Administration and Cue Reactivity:

Two weeks after surgical implantation of the AAV8-CAMKIIa-hM3D(G_q)-mCherry excitatory DREADD in Cohort 3, rats were trained to self-administer HFF as described above. Rats were injected i.p. with 1 mL/kg VEH 20 minutes prior to each FR5 session unless being tested with CNO. Once rats demonstrated stability on HFF self-administration, one FR5 test was completed. Rats were injected i.p. with 2 mg/kg CNO 20 minutes prior to an FR5 session. Responses after insula_{ant} activation were compared to the prior VEH-treated FR5 session. Rats were restabilized on FR5 responding and then tested in two PR test sessions. Rats were injected i.p. with 1 mL/kg VEH or 2 mg/kg CNO 20 minutes prior to PR testing in a counterbalanced manner and were required to exhibit stable responding between test sessions. Rats were restabilized on FR5 responding and then tested in one cue test 20 minutes after i.p. injection of 1 mL/kg VEH or 2 mg/kg CNO (Figure 1, **Bottom**). Treatments were counterbalanced so that the average lifetime pellets earned in the VEH and CNO groups were not significantly different.

One week after completion of cue testing, most rats (n=15) were anesthetized (400 mg/kg of chloral hydrate, i.p.) and decapitated. Brains were extracted and sliced into 1 mm thick coronal sections to determine hM3D placement via visualization of mCherry expression. A DFP-1 Dual Fluorescent Protein Flashlight and VG2 barrier filter glasses were used to visualize red fluorescence. Photographs were taken with a Canon Rebel XSi DSLR camera equipped with a macro lens and red filter. A subset of rats (n=8) were anesthetized (100 mg/kg sodium pentobarbital, i.p.) and transcardially perfused with 3% paraformaldehyde for visualization of mCherry using fluorescent microscopy. Brains were removed, post-fixed in 3% paraformaldehyde for four hours at 4°C, cryoprotected in 30% sucrose for 72 hours at

4°C, and stored at -80°C until further use. Coronal sections (30 µm) at the level of the insula_{ant} were mounted, and slides were coverslipped with Vectashield fluorescent mounting media with 4',6-diamidino-2-phenylindole (DAPI) to stain cellular nuclei for fluorescence microscopy (Vector Laboratories, Burlingame, CA, USA). Visualization of mCherry was achieved using a Leica DFC3000 wide field camera and Leica Application Suite (Leica Microsystems, Wetzlar, Germany). Rats with bilateral expression of mCherry in the insula_{ant}, without spread to adjacent cortical regions as visualized using either a DFP-1 Dual Fluorescent Protein Flashlight or fluorescent microscopy, were included in the data analysis as judged by a blinded experimenter (n=7). Rats which exhibited no evidence of mCherry expression (n=8) were analyzed separately as a control group to assess specificity of CNO to the hM3D system. (This same control analysis was not completed in Cohort 2 because only two rats total displayed no evidence of mCherry expression; thus, surgically naïve rats were used instead.) Rats which exhibited unilateral mCherry expression or inappropriately localized mCherry expression were excluded from data analyses (n=8). An additional rat was excluded due to surgical complications resulting in early euthanasia (n=1).

We completed qualitative assessment of pcFos expression 90 minutes after administration of VEH or CNO in rats from Cohort 3 who underwent transcatheter perfusion and demonstrated hM3D expression in the insula_{ant} (n=2). Free-floating coronal sections (30 µm) at the level of the insula_{ant} were washed with phosphate-buffered saline (PBS), permeabilized with 0.4% Triton PBS for 60 minutes at room temperature, blocked with 3% normal donkey serum in 0.4% Triton PBS for 60 minutes at room temperature, and incubated with primary antibody (rabbit x pcFos Ser32; D82C12, #5384, Cell Signaling Technology) 1:800 in 0.4% Triton PBS overnight at 4°C. The next day, sections were rinsed 3X with PBS for 10 minutes each, incubated in secondary antibody (Alexa Fluor 488 donkey anti-rabbit; A21206 Life Technologies, Carlsbad, CA) 1:2000 in 0.4% Triton PBS for 2 hours at 4°C, and washed 3X with PBS for 5 minutes each. Sections were mounted, and slides were coverslipped with Vectashield fluorescent mounting media with DAPI (Vector Laboratories). Images were acquired using a Leica DFC3000 wide field camera and Leica Application Suite (Leica Microsystems).

Statistical Analyses:

The relationship between HFF binge intake and cue reactivity was assessed using Pearson's correlation. Comparison of pcFos expression 90 minutes after VEH or CNO administration was completed using a two-tailed unpaired Student's *t*-test. A two-way repeated measures analysis of variance (ANOVA) was used to assess SF and HFF intake under freely-fed and food-restricted conditions. Planned comparisons were conducted using Bonferroni's multiple comparisons test. The specificity of CNO to the hM3D system on HFF binge intake was analyzed using a two-tailed unpaired Student's *t*-test. HFF cue reactivity was assessed using a two-tailed unpaired Student's *t*-test and a two-way mixed model ANOVA. FR and PR responding for HFF pellets was assessed using a two-tailed paired Student's *t*-test. All statistical analyses were performed in GraphPad Prism (Version 7) with an experimentwise error rate of $\alpha=0.05$.

Results:

Association Between HFF Binge Intake and Cue Reactivity:

The relationship between HFF binge intake and cue reactivity was assessed in Cohort 1. Pearson's correlation revealed a positive correlation between HFF binge intake and cue reactivity ($r=0.8585$; $p=0.0134$; Figure 2), indicating that these behaviors are associated.

Localization of hM3D within the Insula_{ant}:

Accurate localization of hM3D within the insula_{ant} was determined via visualization of mCherry using either a DFP-1 Dual Fluorescent Protein Flashlight (Figure 3A) or fluorescent microscopy (Figure 3B). Rats in Cohort 2 and Cohort 3 exhibiting bilateral mCherry expression exclusively in the insula_{ant} ($n=16$) were included in data analyses (Figure 3C).

Validation of Cellular Activation by CNO:

The ability of CNO to induce activation of insula_{ant} was assessed in a pilot study assessing the behavioral and biochemical effects of CNO in rats exhibiting accurate localization of hM3D in the insula_{ant}. A two-tailed unpaired Student's *t*-test demonstrated administration of CNO (2 mg/kg, i.p.) 90 minutes prior to euthanasia and insula_{ant} dissection resulted in a significantly higher level of pcFos protein expression normalized to β -actin compared to VEH administration (1 mL/kg, i.p.) on western blot analysis ($t_4=3.057$; $p=0.0378$; Figure 4A). Immunohistochemical qualitative analyses also indicated greater evidence of pcFos expression in rats from Cohort 3 that exhibited accurate localization of hM3D in the insula_{ant} who were treated with CNO (2 mg/kg, i.p.) 90 minutes prior to transcardial perfusion compared to VEH (1 mL/kg, i.p.) administration (Figure 4B). Interestingly, about 40% of the cells demonstrating pcFos expression after CNO treatment did not exhibit mCherry expression, which may be due to reciprocal connections with other regions (Noori et al., 2012) that may result in positive feedback circuits of neuronal activation.

Insula_{ant} Activation Effect on SF and HFF Intake:

The role of insula_{ant} activation on HFF and SF 2-hour intake was assessed under freely-fed and food-restricted conditions in Cohort 2. HFF intake in the freely-fed condition represented binge intake. Effects on caloric intake were assessed in a two-way repeated measures ANOVA for each feeding condition. A main effect of food ($F_{1,8}=206.7$; $p<0.0001$) and treatment ($F_{1,8}=10.93$; $p=0.0107$), but no food x treatment interaction ($F_{1,8}=4.089$; $p=0.0778$), was observed in the freely-fed condition (Figure 5A). Bonferroni's multiple comparisons test showed insula_{ant} activation did not alter SF intake ($p=0.4157$), but significantly suppressed HFF intake ($p=0.0058$), suggesting that the insula_{ant} regulates hedonic feeding. A main effect of food ($F_{1,8}=104.1$; $p<0.0001$), but not of treatment ($F_{1,8}=2.131$; $p=0.1825$) or a food x treatment interaction ($F_{1,8}=0.02668$; $p=0.8743$), was observed in the food-restricted condition (Figure 5B), suggesting the insula_{ant} does not modulate homeostatic feeding. To demonstrate CNO specificity for the hM3D system, the effects of CNO administration (2 mg/kg, i.p.) on binge intake were compared to VEH administration in a group of surgically naïve rats. A two-tailed unpaired Student's *t*-test

indicated no difference between VEH and CNO administration ($t_{20}=0.566$; $p=0.5777$; Figure 5C), thus supporting that CNO specifically suppresses HFF binge intake via activation of hM3D.

Insula_{ant} Activation Effect on HFF Cue Reactivity:

The role of insula_{ant} activation on HFF cue reactivity was assessed in Cohort 3. An unpaired Student's *t*-test demonstrated insula_{ant} activation suppressed previously active lever presses ($t_5=2.99$; $p=0.0304$; Figure 6A), but not inactive lever presses ($t_5=1.673$; $p=0.1551$; Figure 6B), suggesting that the insula_{ant} modulates HFF cue reactivity. To better understand the time course of cue reactivity, a two-way mixed model ANOVA was run on previously active lever presses using the repeated measures factor of time (broken down into 5-minute bins) and the factor of treatment (Figure 6C). There was a main effect of time ($F_{5,25}=29.79$; $p<0.0001$) and treatment ($F_{1,5}=8.943$; $p=0.0304$), but no time x treatment interaction ($F_{5,25}=0.5361$; $p=0.7470$). There was no difference in previously active lever presses ($t_6=0.4876$; $p=0.6432$; Figure 6D) or inactive lever presses ($t_6=0.6202$; $p=0.5579$; Figure 6E) between vehicle treated and CNO treated rats that did not express hM3D in the insula_{ant}, thus suggesting the effects of CNO on cue reactivity are specific to hM3D activation. A two-way mixed model ANOVA demonstrated a main effect of time ($F_{5,30}=32.3$; $p<0.0001$) but not of treatment ($F_{1,6}=0.2377$; $p=0.6532$) or a time x treatment interaction ($F_{5,30}=0.1378$; $p=0.9822$) on previously active lever presses in rats not exhibiting hM3D (Figure 6F).

Insula_{ant} Activation Effect on FR Responding for HFF:

To better assess behavioral mechanisms underlying altered levels of cue reactivity, we assessed the reinforcing efficacy of HFF, and motivation to work for HFF, prior to assessment of cue reactivity. The role of insula_{ant} activation on FR responding for HFF pellets was assessed in Cohort 3. A paired Student's *t*-test indicated a trend to increase FR active lever presses ($t_6=2.385$; $p=0.0544$; Figure 7A), but no effect on inactive lever presses ($t_6=1.098$; $p=0.3144$; Figure 7B) after insula_{ant} activation, suggesting that insula_{ant} activation does not decrease HFF-reward efficacy, as a decrease in FR responding is often interpreted as a decrease in the perceived reinforcing efficacy (Cunningham et al., 2011). A two-tailed paired Student's *t*-test demonstrated no difference between VEH and CNO administration on active lever presses ($t_7=0.6867$; $p=0.5144$; Figure 7C) or inactive lever presses ($t_7=0.3689$; $p=0.7231$; Figure 7D) in rats exhibiting no evidence of hM3D expression.

Insula_{ant} Activation Effect on PR Responding for HFF:

A paired Student's *t*-test indicated insula_{ant} activation did not alter PR active lever responding ($t_6=0.1649$; $p=0.8745$; Figure 8A), inactive lever responding ($t_6=1.048$; $p=0.3352$; Figure 8B), or breakpoint ($t_6=0.281$; $p=0.7882$; Figure 8C). These data, together with no change in homeostatic food intake observed in Cohort 2, indicate insula_{ant} activation does not disrupt global motivation. A two-tailed paired Student's *t*-test also demonstrated no difference between VEH and CNO administration on active lever responding ($t_7=0.1621$; $p=0.8758$; Figure 8D), inactive lever responding ($t_7=0.1956$; $p=0.8505$; Figure 8E), or breakpoint ($t_7=0.6637$; $p=0.5281$; Figure 8F) in rats exhibiting no evidence of hM3D expression.

Discussion:

The present work established a positive association between HFF binge intake and cue reactivity, similar to studies demonstrating higher cue reactivity levels in patients with dysregulated eating (van den Akker, Stewart, Antoniou, Palmberg, & Jansen, 2014). While traditional therapeutic approaches to suppress binge eating have aimed to suppress episode frequency and severity, the observation of interlocked binge and cue behaviors substantiates the alternative goal of indirectly suppressing binge eating via attenuation of cue reactivity. This approach would broaden the treatment arsenal for binge eating, as medications such as *N*-acetylcysteine (Ramirez-Nino, D'Souza, & Markou, 2013) and varenicline (Franklin et al., 2011) have been shown to suppress cue-induced food- or drug-seeking in preclinical or clinical studies. Non-pharmacological approaches such as transcranial magnetic stimulation (TMS) can also successfully attenuate cue-induced food craving in humans (Van den Eynde et al., 2010). Evidence that the weight loss drug *D*-fenfluramine may suppress food intake via suppression of cue-induced food-seeking further supports the goal to effectively suppress food cue reactivity as a treatment modality in binge eating (Pratt & Ford, 2013).

We hypothesized insula_{ant} activation would alter HFF binge intake and cue reactivity through changes in internal and external cue interpretation. The insula_{ant} is highly implicated in interoceptive processing (e.g., recognition of satiety signals), which is believed to be disrupted in dysregulated eating (Frank et al., 2013). Thus, insula_{ant} activation may restore interoceptive capabilities, evoking greater awareness of satiety. Our findings suggesting that the insula_{ant} is a mediator of hedonic, but not homeostatic, feeding align with a previous study that demonstrated inactivation of the insula_{ant} does not alter food-seeking behavior in food-restricted rats (Cosme, Gutman, & LaLumiere, 2015). Interestingly, studies investigating the role of the mid-insula have demonstrated opposite effects from the present study (i.e., mid-insula *inactivation* suppresses cue-driven food intake and palatable feeding) (Baldo, Spencer, Sadeghian, & Mena, 2015; Livneh et al., 2017), stressing the variability in function of different insula subregions (e.g., posterior regions perceive somatosensation which is not integrated with emotional and cognitive information until reaching the insula_{ant}) (Frank et al., 2013). We suspect that many of the observed effects may occur due to insula_{ant}-mediated activation of efferent neurons that innervate downstream regions such as the hypothalamus and striatum (Noori et al., 2012). For example, the more anterior regions of the insula are known to preferentially innervate the ventromedial hypothalamus over the lateral hypothalamus (Ongur, An, & Price, 1998). Thus, stimulating the insula_{ant} would be expected to promote satiety rather than hunger. Future studies should investigate the neural pathways originating from the insula_{ant} that may be driving the observed effects. Admittedly, the suppression of HFF binge intake seen upon insula_{ant} activation was indeed modest, perhaps due to the limited number of cells exhibiting hM3D expression. However, it is impossible to determine if this effect size would be clinically relevant given the additional motivational factors that drive decision-making in people with obesity that cannot be modeled in preclinical paradigms (e.g., awareness of negative health effects, social stigma, etc.). Thus, it is possible that this seemingly small effect may be just enough to assist a patient who binge eats achieve behavioral modification. In addition, this study only assessed the effects of a singular administration of CNO on binge eating. It is possible that chronic

activation of the insula_{ant} would result in greater effect sizes and should be assessed in future studies.

The insula_{ant} also regulates processing of external cues associated with hedonic eating, perhaps through outflow networks (Frank et al., 2013). Subjects with dysregulated eating demonstrate disrupted functional connectivity of the insula_{ant} with various brain regions (i.e., connectivity is lower in the resting-state but higher after food cue exposure) (Frank et al., 2013; Kullmann et al., 2012). In the present study, insula_{ant} activation occurred prior to cue exposure, suggesting that increasing insula_{ant} activity before cue exposure (as in the resting-state) may be protective in suppressing cue reactivity. Thus, normalization of insula_{ant} function may be necessary to prevent cue-induced food-seeking in individuals who binge eat.

In conclusion, this study offers compelling evidence to approach treatment of binge eating in novel ways such as aiming to decrease binge eating indirectly via suppression of food-associated cue reactivity. Furthermore, we have demonstrated that the insula_{ant}, a region amenable to activity modulation via modalities such as TMS, exerts a regulatory role over both HFF binge intake and cue reactivity, thus offering a new therapeutic target in the treatment of dysregulated eating.

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Abbreviations:

ANOVA	analysis of variance
CNO	clozapine <i>N</i> -oxide
DREADD	designer receptors exclusively activated by designer drugs
FR	fixed ratio
HFF	high fat food
insula_{ant}	anterior insula
pcFos	phosphorylated c-Fos
PR	progressive ratio
SF	standard food
VEH	vehicle

Works Cited:

American Psychiatric Association. (2013). Diagnostic and statistical manual of mental disorders : DSM-5 (5th ed.): American Psychiatric Association.

- Anastasio NC, Stutz SJ, Fink LH, Swinford-Jackson SE, Sears RM, DiLeone RJ, ... Cunningham KA (2015). Serotonin (5-HT) 5-HT_{2A} receptor (5-HT_{2AR}):5-HT_{2CR} imbalance in medial prefrontal cortex associates with motor impulsivity. *ACS Chemical Neuroscience*, 6(7), 1248–1258. doi: 10.1021/acscchemneuro.5b00094 [PubMed: 26120876]
- Anastasio NC, Stutz SJ, Fox RG, Sears RM, Emeson RB, DiLeone RJ, ... Cunningham KA (2014). Functional status of the serotonin 5-HT_{2C} receptor (5-HT_{2CR}) drives interlocked phenotypes that precipitate relapse-like behaviors in cocaine dependence. *Neuropsychopharmacology*, 39(2), 370–382. doi:10.1038/npp.2013.199 [PubMed: 23939424]
- Baldo BA, Spencer RC, Sadeghian K, & Mena JD (2015). GABA-mediated inactivation of medial prefrontal and agranular insular cortex in the rat: Contrasting effects on hunger- and palatability-driven feeding. *Neuropsychopharmacology*, 41(4), 960–970. doi:10.1038/npp.2015.222 [PubMed: 26202102]
- Benzon CR, Johnson SB, McCue DL, Li D, Green TA, & Hommel JD (2014). Neuromedin U receptor 2 knockdown in the paraventricular nucleus modifies behavioral responses to obesogenic high-fat food and leads to increased body weight. *Neuroscience*, 258, 270–279. doi:10.1016/j.neuroscience.2013.11.023 [PubMed: 24269937]
- Corwin RL (2006). Bingeing rats: a model of intermittent excessive behavior? *Appetite*, 46(1), 11–15. [PubMed: 16188345]
- Corwin RL, Avena NM, & Boggiano MM (2011). Feeding and reward: perspectives from three rat models of binge eating. *Physiology and Behavior*, 104(1), 87–97. doi:10.1016/j.physbeh.2011.04.041 [PubMed: 21549136]
- Corwin RL, & Wojnicki FH (2006). Binge eating in rats with limited access to vegetable shortening. *Curr.Protoc.Neurosci.*, Chapter 9, Unit9.
- Cosme CV, Gutman AL, & LaLumiere RT (2015). The dorsal agranular insular cortex regulates the cued reinstatement of cocaine-seeking, but not food-seeking, behavior in rats. *Neuropsychopharmacology*, 40(10), 2425–2433. doi:10.1038/npp.2015.92 [PubMed: 25837282]
- Cunningham KA, Fox RG, Anastasio NC, Bubar MJ, Stutz SJ, Moeller FG, ... Rosenzweig-Lipson S (2011). Selective serotonin 5-HT_{2C} receptor activation suppresses the reinforcing efficacy of cocaine and sucrose but differentially affects the incentive-salience value of cocaine-vs. sucrose-associated cues. *Neuropharmacology*, 61(3), 513–523. [PubMed: 21575646]
- Faul F, Erdfelder E, Lang AG, & Buchner A (2007). G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods*, 39(2), 175–191. [PubMed: 17695343]
- Frank S, Kullmann S, & Veit R (2013). Food related processes in the insular cortex. *Frontiers in Human Neuroscience*, 7, 499. doi:10.3389/fnhum.2013.00499 [PubMed: 23986683]
- Franklin T, Wang Z, Suh JJ, Hazan R, Cruz J, Li Y, ... Childress AR (2011). Effects of varenicline on smoking cue-triggered neural and craving responses. *Archives of General Psychiatry*, 68(5), 516–526. [PubMed: 21199958]
- Geliebter A, Benson L, Pantazatos SP, Hirsch J, & Carnell S (2016). Greater anterior cingulate activation and connectivity in response to visual and auditory high-calorie food cues in binge eating: Preliminary findings. *Appetite*, 96, 195–202. doi:10.1016/j.appet.2015.08.009 [PubMed: 26275334]
- Kober H, & Boswell RG (2018). Potential psychological & neural mechanisms in binge eating disorder: Implications for treatment. *Clinical Psychology Review*, 60, 32–44. doi:10.1016/j.cpr.2017.12.004 [PubMed: 29329692]
- Kullmann S, Heni M, Veit R, Ketterer C, Schick F, Haring HU, ... Preissl H (2012). The obese brain: association of body mass index and insulin sensitivity with resting state network functional connectivity. *Human Brain Mapping*, 33(5), 1052–1061. doi:10.1002/hbm.21268 [PubMed: 21520345]
- Livneh Y, Ramesh RN, Burgess CR, Levandowski KM, Madara JC, Fenselau H, ... Andermann ML (2017). Homeostatic circuits selectively gate food cue responses in insular cortex. *Nature*, 546(7660), 611–616. doi:10.1038/nature22375 [PubMed: 28614299]

- McCue DL, Kasper JM, & Hommel JD (2017). Regulation of motivation for food by neuromedin U in the paraventricular nucleus and the dorsal raphe nucleus. *International Journal of Obesity* (2005), 41(1), 120–128. doi:10.1038/ijo.2016.178 [PubMed: 27748746]
- Naqvi NH, Gaznick N, Tranel D, & Bechara A (2014). The insula: a critical neural substrate for craving and drug seeking under conflict and risk. *Annals of the New York Academy of Sciences*, 1316, 53–70. doi:10.1111/nyas.12415 [PubMed: 24690001]
- Nathanson JL, Yanagawa Y, Obata K, & Callaway EM (2009). Preferential labeling of inhibitory and excitatory cortical neurons by endogenous tropism of adeno-associated virus and lentivirus vectors. *Neuroscience*, 161(2), 441–450. doi:10.1016/j.neuroscience.2009.03.032 [PubMed: 19318117]
- Ng L, & Davis C (2013). Cravings and food consumption in binge eating disorder. *Eat Behav*, 14(4), 472–475. doi:10.1016/j.eatbeh.2013.08.011 [PubMed: 24183139]
- Noori HR, Spanagel R, & Hansson AC (2012). Neurocircuitry for modeling drug effects. *Addiction Biology*, 17(5), 827–864. doi:10.1111/j.1369-1600.2012.00485.x [PubMed: 22978651]
- Ongur D, An X, & Price JL (1998). Prefrontal cortical projections to the hypothalamus in macaque monkeys. *J Comp Neurol*, 401(4), 480–505. [PubMed: 9826274]
- Paxinos G, & Watson C (2005). *The Rat Brain in Stereotaxic Coordinates* (5th ed.). Burlington, MA: Elsevier Academic Press.
- Pratt WE, & Ford RT (2013). Systemic treatment with D-fenfluramine, but not sibutramine, blocks cue-induced reinstatement of food-seeking behavior in the rat. *Neuroscience Letters*, 556, 232–237. doi:10.1016/j.neulet.2013.10.030 [PubMed: 24157852]
- Price AE, Anastasio NC, Stutz SJ, Hommel JD, & Cunningham KA (2018). Serotonin 5-HT_{2C} receptor activation suppresses binge intake and the reinforcing and motivational properties of high-fat food. *Frontiers in Pharmacology*, 9(821). doi:10.3389/fphar.2018.00821
- Ramirez-Nino AM, D'Souza MS, & Markou A (2013). N-acetylcysteine decreased nicotine self-administration and cue-induced reinstatement of nicotine seeking in rats: comparison with the effects of N-acetylcysteine on food responding and food seeking. *Psychopharmacology*, 225(2), 473–482. doi:10.1007/s00213-012-2837-3 [PubMed: 22903390]
- Richardson NR, & Roberts DC (1996). Progressive ratio schedules in drug self-administration studies in rats: a method to evaluate reinforcing efficacy. *Journal of Neuroscience Methods*, 66(1), 1–11. [PubMed: 8794935]
- Schienze A, Schafer A, Hermann A, & Vaitl D (2009). Binge-eating disorder: reward sensitivity and brain activation to images of food. *Biological Psychiatry*, 65(8), 654–661. doi:10.1016/j.biopsych.2008.09.028 [PubMed: 18996508]
- van den Akker K, Stewart K, Antoniou EE, Palmberg A, & Jansen A (2014). Food cue reactivity, obesity, and impulsivity: Are they associated? *Curr Addict Rep*, 1, 301–308.
- Van den Eynde F, Claudino AM, Mogg A, Horrell L, Stahl D, Ribeiro W, ... Schmidt U (2010). Repetitive transcranial magnetic stimulation reduces cue-induced food craving in bulimic disorders. *Biological Psychiatry*, 67(8), 793–795. doi:10.1016/j.biopsych.2009.11.02 [PubMed: 20060105]

Figure 1.

Schematic representation of the experimental procedures completed in Cohort 1 (Top), 2 (Middle), and 3 (Bottom). Numbers below the timelines represent day of the experiment.

HFF, high fat food; **SF**, standard food; **FR**, fixed ratio; **PR**, progressive ratio.

Figure 2.

High fat food (HFF) binge intake and cue reactivity are positively correlated. Cohort 1 rats underwent sequential HFF binge intake and cue reactivity assessments. Pearson's correlation revealed a positive correlation between HFF binge intake and cue reactivity ($r=0.8585$, $p<0.05$; Pearson's correlation).

Figure 3.

hM3D is accurately localized within the insula_{ant}. Representative images of accurate hM3D expression in the insula_{ant} determined by visualization of mCherry using a DFP-1 Dual Fluorescent Protein Flashlight (**Figure 3A**) or fluorescent microscopy using a 5X objective for a final magnification of 50X (**Figure 3B**). Histologically verified hM3D placements of included rats from Cohort 2 (n=9; **Figure 3C, left**) and Cohort 3 (n=7; **Figure 3C, right**) using templates from Paxinos and Watson 2005 (Paxinos & Watson, 2005) to depict the central point of expression (insula_{ant} depicted in gray).

Figure 4.

Insula_{ant} activation induces phosphorylated c-Fos (pcFos) expression. Administration of clozapine *N*-oxide (CNO; 2 mg/kg, i.p.) 90 minutes prior to insula_{ant} dissection demonstrated a significantly higher level of pcFos protein expression normalized to β -actin compared to vehicle (VEH; 1 mL/kg, i.p.) administration (**Figure 4A**). Composite data are represented as mean $\pm$ standard error of the mean (SEM). * $p < 0.05$ vs. VEH (Student's *t*-test). Immunohistochemical qualitative analyses using a 20X objective for a final magnification of 200X indicated greater evidence of pcFos expression in rats treated with CNO (2 mg/kg, i.p.) 90 minutes prior to transcardial perfusion compared to VEH (1 mL/kg, i.p.) administration (**Figure 4B**). Yellow arrows indicate select cells exhibiting both pcFos and mCherry expression while white arrows indicate select cells exhibiting pcFos expression but not mCherry expression.

Figure 5.

Insula_{ant} activation suppresses high fat food (HFF) binge intake. Cohort 2 rats underwent standard food (SF) and HFF intake testing under either a freely-fed or food-restricted condition after vehicle (VEH) or clozapine *N*-oxide (CNO; 2 mg/kg) administration. A two-way repeated measures ANOVA followed by Bonferroni's multiple comparisons test revealed insula_{ant} activation suppressed HFF (i.e., binge eating; $p<0.05$), but not SF, intake in freely-fed rats (**Figure 5A**). Insula_{ant} activation did not alter SF or HFF intake in food-restricted rats (**Figure 5B**). There was no effect of 2 mg/kg CNO in surgically naïve rats on HFF binge intake (**Figure 5C**). Composite data are represented as mean $\pm$ SEM. * $p<0.05$ vs. HFF VEH (Bonferroni's multiple comparisons test).

Figure 6.

Insula_{ant} activation suppresses high fat food (HFF) cue reactivity. The role of insula_{ant} activation on HFF cue reactivity was assessed in Cohort 3 after vehicle (VEH) or clozapine *N*-oxide (CNO; 2 mg/kg) administration. An unpaired Student's *t*-test revealed insula_{ant} activation suppressed previously active lever presses ($p < 0.05$; **Figure 6A**), but not inactive lever presses (**Figure 6B**). A time course of lever presses in rats with hM3D expression is shown in **Figure 6C**. There was no effect of 2 mg/kg CNO on previously active lever presses (**Figure 6D**) or inactive lever presses (**Figure 6E**) in rats from Cohort 3 that demonstrated no evidence of hM3D expression. A time course of lever presses for rats without hM3D expression is shown in **Figure 6F**. Composite data are represented as mean $\pm$ SEM. * $p < 0.05$ vs. VEH (unpaired Student's *t*-test).

Figure 7.

Insula_{ant} activation does not suppress HFF-reward efficacy. The role of insula_{ant} activation on FR responding for HFF pellets was assessed in Cohort 3 after vehicle (VEH) or clozapine *N*-oxide (CNO; 2 mg/kg) administration. A paired Student's *t*-test revealed insula_{ant} activation did not alter active lever presses (**Figure 7A**) or inactive lever presses (**Figure 7B**). There was also no effect of 2 mg/kg CNO on active lever presses (**Figure 7C**) or inactive lever presses (**Figure 7D**) in rats from Cohort 3 that demonstrated no evidence of hM3D expression. Composite data are represented as mean $\pm$ SEM. **p* < 0.05 vs. VEH (paired Student's *t*-test).

Figure 8.

Insula_{ant} activation does not suppress motivation for HFF. The role of insula_{ant} activation on PR responding for HFF pellets was assessed in Cohort 3 after vehicle (VEH) or clozapine *N*-oxide (CNO; 2 mg/kg) administration. A paired Student's *t*-test revealed insula_{ant} activation did not active lever presses (**Figure 8A**), inactive lever presses (**Figure 8B**), or breakpoint (**Figure 8C**). There was also no effect of 2 mg/kg CNO on active lever presses (**Figure 8D**), inactive lever presses (**Figure 8E**), or breakpoint (**Figure 8F**) in rats from Cohort 3 that demonstrated no evidence of hM3D expression. Composite data are represented as mean $\pm$ SEM. * $p < 0.05$ vs. VEH (paired Student's *t*-test).