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# Appetitive Behavior in the Social Transmission of Food Preference Paradigm Predicts Activation of Orexin-A producing Neurons in a Sex-Dependent Manner

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**Abstract—Orexin-producing cells in the lateral hypothalamic area have been shown to be involved in a wide variety of behavioral and cognitive functions, including the recall of appetitive associations and a variety of social behaviors. Here, we investigated the role of orexin in the acquisition and recall of socially transmitted food preferences in the rat. Rats were euthanized following either acquisition, short-term recall, or long-term recall of a socially transmitted food preference and their brains were processed for orexin-A and c-Fos expression. We found that while there were no significant differences in c-Fos expression between control and experimental subjects at any of the tested timepoints, females displayed significantly more activity in both orexinergic and non-orexinergic cells in the lateral hypothalamus. In the infralimbic cortex, we found that social behavior was significantly predictive of c-Fos expression, with social behaviors related to olfactory exploration appearing to be particularly influential. We additionally found that appetitive behavior was significantly predictive of orexin-A activity in a sex-dependent matter, with the total amount eaten correlating negatively with orexin-A/c-Fos colocalization in male rats but not female rats. These findings suggest a potential sex-specific role for the orexin system in balancing the stimulation of feeding behavior with the sleep/wake cycle.** 

**Key words: orexin/hypocretin, social transmission of food preference, social behavior, appetitive behavior, infralimbic cortex, sex differences.**

## INTRODUCTION

Orexin (also known as hypocretin) producing cells exist in only a small region of the lateral hypothalamic area (LHA), but project to a diverse array of brain regions throughout the central nervous system (Yoshida et al., 2006; Xu et al., 2013). As this extensive network might suggest, the orexin system has been shown to be involved in a wide variety of behaviors including stress responsivity (Eacret et al., 2019; Grafe et al., 2017, 2018), maintenance of sleep and wakefulness (Chemelli et al., 1999; Estabrooke et al., 2001; Mieda et al., 2004; Sakurai, 2007; Sasaki et al., 2011), threat learning (Sears et al., 2013), fear behavior (Chen et al., 2014; Soya et al., 2017), and fear extinction (Johnson et al., 2011; Flores et al., 2014; Sharko et al., 2017; Monfils et al., 2019). In the last decade, emerging research has also implicated orexin in social processes. While the majority of the first studies examining orexin in a social context focused on its role in regulating responses to various forms of social stressors in rodents (Lutter et al., 2008; Weintraub et al., 2010), a broader role of orexin in social behavior was first suggested by a microdialysis study in human seizure patients in which experimenters observed increased orexin-A levels following social interactions (Blouin et al., 2013). Since then, emerging research has implicated orexin in a variety of social behaviors and socially mediated forms of learning including social memory (Yang et al., 2013), play behavior (Reppucci et al., 2020), conditioned social avoidance (Faesel et al., 2021), and general sociability (Abbas et al., 2015; Faesel et al., 2021; though see also Yang et al., 2013).

In addition to their role in social functions, orexinergic neurons have long been known to be involved in the control of food consumption (Sakurai et al., 1998; Haynes et al., 1999, 2000; Lutter et al., 2008) and energy expenditure (Sakurai et al., 1998). Orexin is particularly influential in generating the behavioral response to rewarding, i.e., highly palatable, foods (see Harris and Aston-Jones, 2006 for review). Similarly, orexin seems

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*Abbreviations:* $CS_2$, carbon disulphide; IEG, immediate early gene; IL, infralimbic; LHA, lateral hypothalamic area; STFP, social transmission of food preference.

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to play a role in the acquisition of food-motivated operant behavior (Sharf et al., 2010), taste aversion and non-social taste preference learning (Mediavilla et al., 2011), and in the acquisition (Cole et al., 2015; Keefer et al., 2016) and extinction (Keefer et al., 2016) of cue-food associations. A broader role of orexin in the production and modulation of motivated behaviors has been suggested as a unifying explanation for the how prolific the involvement of orexin in behavior seems to be (Sakurai, 2007, 2014).

Here, we aimed to further disambiguate the potential role of the orexin system in appetitive learning and recall as well as social behavior by examining orexin activity at acquisition and recall of an appetitive-based social learning procedure, the social transmission of food preference (STFP) paradigm. The STFP model involves giving a naïve animal (the *observer*) olfactory access to a conspecific (the *demonstrator*) that has recently eaten a novel food. Rats, along with a number of other rodent species (see Monfils and Agee, 2019 for review), will subsequently display a preference for consuming the novel food item that said conspecific had recently eaten (Galef and Wigmore, 1983; Strupp and Levitsky, 1984; see Galef, 2012 for review). STFP is a frequently used behavioral model for studying the neurobiological mechanisms underlying a number of cognitive functions including social (Agee et al., under review) and olfactory learning (Sánchez-Andrade et al., 2005), as well as the acquisition and storage of long-term memories (Ross and Eichenbaum, 2006; Smith et al., 2007; Lesburguères et al., 2011). The STFP behavioral paradigm differs from other forms of appetitive learning not only in the social aspect, but in that the transmission of these preferences is mediated by the semiochemical carbon disulfide ($CS_2$) that is present in breath exiting the nasal cavity (Galef et al., 1988). Given the aforementioned activity of orexin neurons during acquisition and recall of cue-food associations, it could be expected that pairing $CS_2$ exposure with a novel scent or re-exposing rats to a novel scent that was previously $CS_2$ paired (i.e., demonstrated) might result in increased orexin activity.

In the present study, male and female observer rats that had undergone STFP acquisition, short-term STFP recall (recall immediately post-acquisition), or long-term STFP recall (recall 48 h post-acquisition) were euthanized and perfused. We then double-stained their fixated brain tissue for both orexin-A producing cells and the immediate early gene (IEG) c-Fos (a marker of neural activation). In addition to examining orexinergic cell and c-Fos colocalization in the LHA, we examined c-Fos activity in non-orexin LHA cells and c-Fos activity in the infralimbic (IL) cortex. The IL was selected due to past research indicating its involvement in STFP recall (Smith et al., 2007), as well as due to research indicating a large number of outgoing projections from the IL to LHA orexin neurons, (Yoshida et al., 2006) and the presence of orexin receptors in the IL (Marcus et al., 2001), which suggests reciprocal connections between the IL and LHA orexin neurons. To determine whether these regions played a role in the acquisition or recall of socially transmitted food preferences, experimental animals were compared to controls that had gone through analogous behavioral procedures without having acquired or recalled a STFP. Additionally, c-Fos expression in these regions was further related to pre-fixation behavior using a series of linear models to determine whether appetitive or social behavior was predictive of activation in any of these areas.

## EXPERIMENTAL PROCEDURES

### Apparatus and diets

We prepared two novel diets by mixing a 100 g of powdered 5LL2 Purina rodent chow with either 1 g of McCormick ground cinnamon (Diet Cin) or 2 g Hershey cocoa powder (Diet Co). Control rats and, in most cases, their demonstrators ate plain powdered 5LL2 Purina rodent chow that all animals had been habituated to during the food restriction period (Diet Plain). Choice tests (described in detail below) and demonstrator/control eating took place in standard rat cages (26.7 cm × 48.3 cm × 20.3 cm) while the interaction phase took place in a large plastic bin (50.5 c m × 37.4 cm × 37.5 cm) with woodchip bedding covering the bottom. Bedding in the bin was refreshed between groups and bins were sanitized with an ammonia-based cleaner at the end of each day. Rat cages used for choice tests and consumption were freshly sanitized and new cages were swapped in for each subject. Food during the choice tests and consumption tests was presented in a hanging food cup constructed from 12-gauge steel utility wire and 4 oz. (118.3 ml) hexagonal glass jars. Between uses, food cups were washed thoroughly with soap and hot water and then sanitized with a 70% alcohol solution. All behavioral procedures were performed under red light during the rats' dark cycle.

### Procedures

*Food restriction.* Food restriction began 5 days before rats went through the acquisition phase of the social transmission of food preference task, with the total duration of food restriction lasting from 5 days to 7 days based on experimental condition. Food hoppers containing the rats' normal feed – pelleted Purina 5LL2 chow – were removed from all home cages at the beginning of the food restriction period and were kept out for the duration of the experiment. Rats were given 1 hour of *ad libitum* access to a hanging food cup containing the plain powdered version of the 5LL2 chow every 23 hours from the start of food restriction.

*Social transmission of food preference task.* (See Fig. 1 for graphical depiction) Rats were housed in dyads with one rat in each cage assigned to the demonstrator condition and one rat assigned to the observer condition. In the first phase of the social transmission of food preference task, demonstrator assigned rats were removed from their home cages and transferred to a fresh cage on the opposite side of the colony room with a hanging food cup containing 30 g of

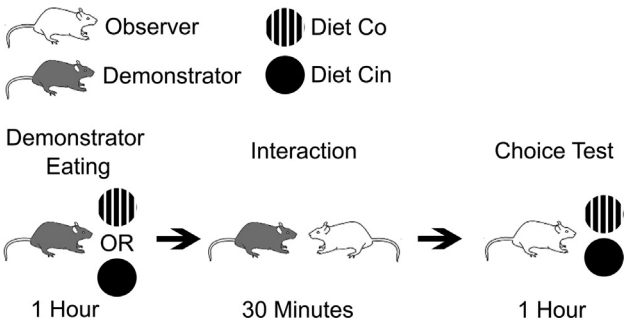


**Fig. 1.** Basic social transmission of food preference procedure. This figure displays the standard design for transmission of a food preference and verification of transmission.

the diet they were demonstrating. Demonstrator rats paired with observers assigned to most control conditions, with the exception of the short-term memory controls (see explanation in next section), were given Diet Plain, while Demonstrators paired with observers in the other conditions were given either Diet Cin or Diet Co. Novel diet assignments were given such that every other demonstrator in each condition was given Diet Cin. Demonstrators were allowed 1 hour to eat before being moved along with their paired observer (transported in a separate cage) to a room down the hall containing the bins for the interaction phase of the experiment to take place. The contents of the demonstrator's food cups were weighed and recorded to ensure that each demonstrator had eaten. Each demonstrator and their observer were allowed to interact for 30 min before they were returned to their home cages or, in the case of observer animals in the short-term recall conditions, to the cages containing food for their choice test/control Diet Plain consumption. The full interaction period for every group was recorded by handheld digital cameras mounted over the interaction box and was later scored for social behavior.

*Choice test.* (See Fig. 1 for graphical depiction) The choice test was conducted similarly to the first phase of the STFP task with the exception that (1) the rats undergoing the test were the observers from our short-term and long-term memory conditions and (2) rats were provided with two separate hanging food cups on opposite ends of a fresh cage containing 30 g of Diet Co and 30 g of Diet Cin, respectively. Rats in the control short-term and long-term memory conditions were provided only with food cups containing Diet Plain. All observers were given 1 hour to eat the food provided to them before they were removed and returned to their home cages. Food cups from all cages were then emptied and the content were weighed. For rats that had undergone a choice test, the amount of Diet Co and Diet Cin eaten were weighed separately and then used to calculate the percent of total eaten for each diet type using the following equations: % Eaten Diet Co = $D_{Co}$/($D_{Co}$ + $D_{Cin}$) * 100; % Eaten Diet Cin = $D_{Cin}$/($D_{Co}$ + $D_{Cin}$) * 100 where $D_{Diet}$ = total amount in grams eaten of a given diet.

## Animals

Subjects were male ($n$ = 60) and female ($n$ = 48) Sprague-Dawley rats ordered between 9 and 10 weeks of age and obtained from Envigo (Houston, TX, USA). Rats were housed in same-sex dyads with their demonstrator for the duration of the experiment. Twelve rats of each sex were reused as demonstrators throughout the experiment, so observers that arrived after the first cohort of each sex were rehoused with a demonstrator a day or two after arrival. Rats were allowed at least 5 days to habituate to the colony before food restriction began. Subjects were kept on 12 h reversed light/dark cycle (lights off at 8 am) from arrival to experiment end. All parts of this experiment were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Experimental Animals and were approved for use by The University of Texas at Austin Animal Care and Use Committee.

## Overview of condition assignments

One rat in each dyad – the rat that would be acquiring the STFP – was assigned to the observer condition while the other rat was assigned to be their paired demonstrator. Observers were then assigned to one of 6 STFP acquisition/recall conditions (3 experimental, 3 control). Descriptions of the treatment of animals in each condition are summarized in text below and graphically in Fig. 2 (**Note:** Descriptions of basic behavioral procedures can be found above):

Acquisition Experimental (ACQ): Rats in the ACQ condition underwent the standard STFP acquisition procedure with a demonstrator that had eaten a Diet Cin or Diet Co. Observers were then returned to their home cage while their demonstrator was rehoused. Observers were euthanized and perfused 1 hour after being returned to their home cage without ever undergoing a choice test. This delay was instituted for this group and all subsequent groups to maximize c-Fos protein expression in the brain (Müller et al., 1984; Barros et al., 2015).

Acquisition Control (ACQ-CTRL): Rats in the ACQ-CTRL condition were treated identically to rats in the ACQ condition, with the exception that the demonstrator that they interacted with had eaten Diet Plain, which all rats had been extensively exposed to, prior to their interaction.

Short-term Memory Experimental (STM): Rats in the STM condition underwent standard STFP acquisition with a demonstrator that had eaten Diet Cin or Diet Co. Immediately following STFP, observers underwent the standard choice test procedure while their demonstrator was rehoused in a new home cage. Following the choice test, observers were moved back to their original home cage for 1 hour before they were euthanized and perfused.

Short-term Memory Control (STM-CTRL): Rats in the STM-CTRL condition were treated identically to rats in the STM condition with the exception that they were allowed 1 hour access to Diet Plain rather than undergoing the choice test. Notably, this does mean

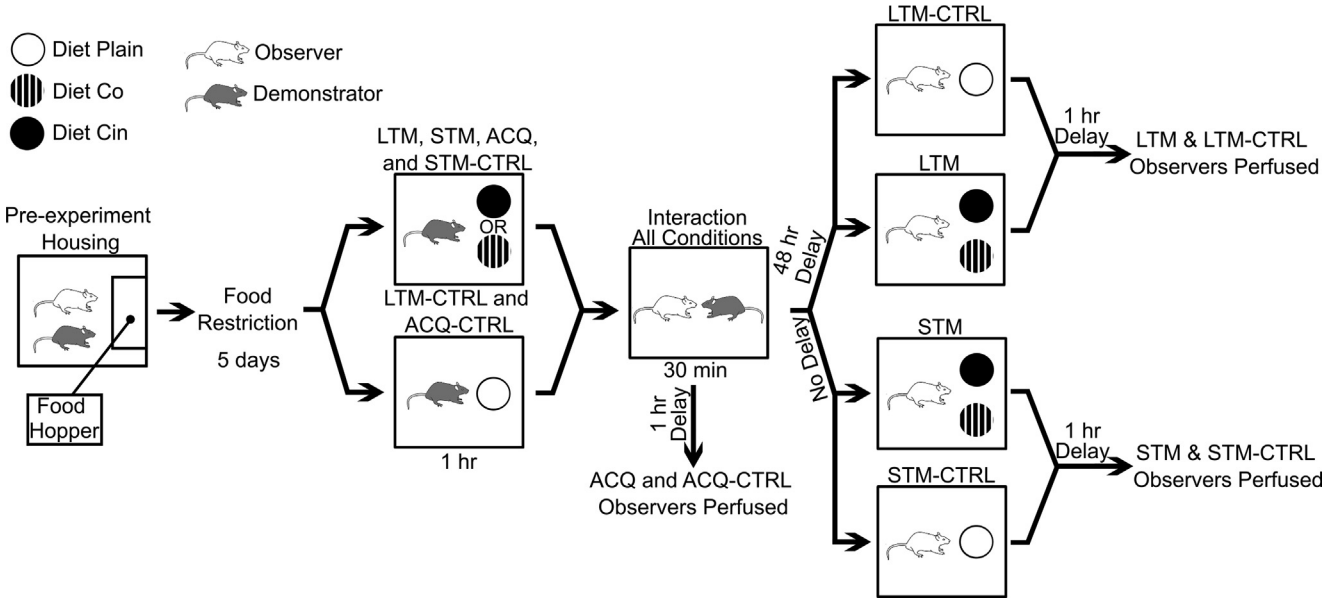


**Fig. 2.** Overview of experimental design. This figure outlines the behavioral experience and endpoints for rats in all six conditions: acquisition (ACQ), acquisition control (ACQ-CTRL), short-term memory (STM), short-term memory control (STM-CTRL), long-term memory (LTM), and long-term memory control (LTM-CTRL).

that observers in this condition interacted with a demonstrator that had consumed a novel diet. This was to ensure that any differences in c-Fos activity that was observed between the rats in the STM condition and rats in the STM-CTRL condition were the result of STFP recall and not lingering c-Fos expression from the acquisition period.

Long-term Memory Experimental (LTM): Rats in the LTM condition went through the standard STFP acquisition procedure with a demonstrator that had eaten Diet Cin or Diet Co. Following STFP acquisition, the demonstrator was rehoused with another demonstrator, and the observer was returned to their home cage alone and left undisturbed with the exception of the feeding procedures described in the *Food Restriction* section above. After a 48-hour delay, observers went through the standard choice test procedure. After this, observers were returned to their home cage and left undisturbed for an hour before they were euthanized and perfused.

Long-term Memory Control (LTM-CTRL): Rats in the LTM-CTRL condition were treated identically to rats in the LTM condition with the following exceptions: [1] during the STFP acquisition period they interacted with a demonstrator that had eaten Diet Plain and [2] rather than completing a choice test just prior to euthanasia they were allowed 1 h access to Diet Plain.

## Behavioral scoring

Footage of the social interaction period of the social transmission of food preference task was recorded on tripod-mounted handheld cameras and scored for observer social behavior for all rats in the ACQ, ACQ-CTRL, STM, and STM-CTRL groups. As our interest was primarily in examining the potential acute role of social behavior on c-Fos expression in orexin expressing cells, this footage was not scored for rats in the LTM and LTM-CTRL groups. The following observer social behaviors were scored as described: (1) face sniffing, scored for when observers were sniffing at or around the face of their demonstrator without making direct snout-to-snout contact; (2) nose contact, scored for when observers and demonstrators were making physical snout-to-snout contact; (3) body contact, scored for when observers and demonstrators were huddled together or otherwise making physical contact that did not fall under any of the other scoring categories; (4) paw contact, scored for when observers were making physical contact with the demonstrator with just their paw. In the case that demonstrators were making paw contact to the observer, this was scored as body contact; (5) body sniffing, scored for when observers were actively sniffing at the body area of their demonstrators; (6) grooming, scored for when observers were actively grooming their demonstrator (e.g., cleaning their demonstrators fur by nibbling at/licking the area being groomed or genital grooming); (7) play behavior, scored for when observers were initiating nape contact or responding to nape contact initiated play (i.e., displaying play as described in Pellis and Pellis, 1991, Pellis et al., 1993). Only one type of social behavior – whichever best described the behavior of the observer – was scored for at a time during the interaction period. As such, the total amount of time that each observer spent in social contact with their demonstrator during the interaction period could be calculated by summing together the time engaged in each kind of social behavior. Scored separately from observer social behavior was demonstrator-initiated grooming, which occurred when the demonstrator engaged in grooming behavior towards the observer as described.

## Tissue analysis

*Brain preparation.* All observer rats were euthanized with a pentobarbital and phenytoin solution injected peritoneally (Euthasol; Virbac Animal Health) 1 h following the end of behavioral procedures. Following injection, full unconsciousness was confirmed by toe pinch and rats were transcardially flushed with PBS and then perfused using 4% paraformaldehyde (PFA) phosphate buffered solution. Brains were then extracted and submerged in the 4% PFA solution for 48 hours to allow for post-fixation before being transferred to a solution of 30% sucrose in phosphate buffered saline (PBS) for cryoprotection. Once brains had sunk to the bottom of the sucrose solution (typically ~48 h after submersion), they were flash frozen in powdered dry ice before being transferred to a −80 °C freezer for storage until sectioning. Brains were sectioned coronally from about +3.7 from bregma to −5.6 from bregma on a sliding microtome at a thickness of 40 μm and stored in four series. Brain sections were stored in a PBS and a 0.2% sodium azide in PBS solution at 10 °C until immunohistochemical processing could occur.

*Immunohistochemistry.* For each rat, a full series was dual stained for c-Fos protein expression and for orexin-A producing cells. Free floating sections were PBS washed following removal from storage before being incubated for an hour in a 1% detergent (Tween20; Sigma) and PBS solution. Sections were then PBS washed and transferred to the primary orexin antibody solution (1:1000 Anti-Orexin-A in Mouse; R&D Systems) and incubated overnight at 10 °C. Sections were subsequently washed and transferred to the secondary orexin antibody solution (1:1000 Donkey Anti-Mouse 568 (red); Alexa Fluor; Abcam) and incubated for 1 hour before being washed and blocked in 2% normal goat serum for 1 hour. Following blocking, the tissue was transferred immediately to the primary c-Fos antibody solution (1:1000 Anti-c-Fos in Rabbit; Immunostar) and incubated for 2 days at 10 °C. Finally, sections were washed and transferred to the secondary c-Fos antibody solution (1:1500 Goat Anti-Rabbit 488 (green); Alexa Fluor; Abcam) and incubated overnight at 10 °C before being mounted and coverslipped with a fluorescence mounting medium (Prolong Gold Antifade Mounting medium; Thermofisher). Slides were kept in a dark fridge at 10 °C until imaging.

*Imaging.* All imaging was completed using an Axio Scope A1 microscope (Zeiss; Thornwood, NY, USA). Regions of interest were identified in accordance with Paxinos & Watson (2006) under a 10× objective and imaged at 20× (actual magnification 200×). Images were taken of the lateral hypothalamus (~Bregma −2.3 to −3.14) and infralimbic cortex (Bregma +3.52 to +2.2). All viable sections and areas containing orexin cells in the lateral hypothalamic area (LHA) were imaged under the fluorescent wavelengths for the secondary antibodies used for c-Fos and for orexin cells, while the infralimbic cortex (IL) was only imaged for c-Fos. Orexin and c-Fos images from the LHA were merged automatically using a custom macro in the ImageJ software with FIJI (NIH, Bethesda, MD) (see Fig. 5A for example image). Image files were coded to blind experimenters to all details and counts were then taken using the ImageJ cell counting tool. While only raw c-Fos counts were taken for the IL, in he LHA c-Fos counts, orexin cell counts, and counts of orexin cells displaying c-Fos activity were all taken. To gauge non-orexin-A LHA activity the total c-Fos counts were subtracted out from the counts for c-Fos expressing orexin cells. Counts were averaged across all images for each subject and used for analysis. A minimum of 6 viable images/region was required for a rat to be included in the statistical analysis of a given area.

## Statistical analyses

All statistical analyses and graphs were completed/produced using the R and Rstudio software. Where necessary, data/residual normality and variance were assessed using Shapiro and Levene tests, respectively. If testing assumptions were not met, data were log transformed in order to bring them in line with testing assumptions. If standard transformations did not suffice to bring data in line with assumptions, a series of Kruskal–Wallis tests were run as a nonparametric alternative. Effect sizes were calculated using the appropriate eta-squared value for the statistical test used. Post-hoc pairwise comparisons were made using Tukey's HSD formula, but otherwise no adjustments were made for multiple comparisons.

*Formation of predictive models of c-Fos activation.* To further explore the relationship between social behavior, appetitive behavior, and c-Fos activation across the various regions/cell types that we had examined, we constructed a series of predictive mixed-effect models. The following behaviors/subject characteristics were using as predictor variables: Total grams eaten, time since colony lights were turned off prior to start of behavioral testing, all forms of social contact scored for during the social interaction period (see **Behavioral Scoring** section) with the exception of body contact (removed due to covariance with other predictors), total time spent in social contact, timepoint (long-term memory, short-term memory, or acquisition), condition type (control or experimental), pre-perfusion STFP acquisition (ACQ, STM, and STM-CTRL conditions), and sex. All continuous predictor variables were standardized by taking the *z*-score prior to model building. Data from subjects that had been tested at the acquisition timepoint were assessed separately from subjects tested at the long-term memory timepoint due to differences in the behavioral measures available. The data from rats tested at the short-term memory timepoint were included with both of the aforementioned datasets as data for both social behavior and appetitive behavior was available and potentially relevant to their c-Fos expression.

Predictors were also assessed independently for polynomial relationships with the dependent variable and for any significant interactions between predictors. If

any were detected, they were added to the list of potential variables for the final model. Additionally, interaction terms were included for any continuous predictor variables that were known to be affected by the level of included factor terms. Variable selection was completed using the best-subsets method for linear regression with the max number of predictors set to 6 to ensure there were at least five subjects per predictor in all cases. BIC, adjusted $R^2$, and Mallows's CP were checked for the best models at all predictor levels and the final model was selected based on combined consideration of all three. In order to reduce model prediction bias resulting from the inclusion of all subjects, leave-one-out cross validation (LOOCV) was performed to obtain less biased predictions for each point. The $R^2$ value for each model was calculated using the mean-squared error (MSE) obtained from the differences between the LOOCV predicted value of the dependent variable and the actual value ($MSE_{LOOCV}$) and the MSE obtained from the null regression ($MSE_{Null}$) using the standard formula:

$$R^2 = (MSE_{Null} - MSE_{LOOCV})/MSE_{Null}$$

As our analysis found that sex was a highly predictive variable in all models, LOOCV was also run on a model containing sex alone to ascertain that the other predictors were contributing. Finally, the relative contribution of individual predictors was roughly estimated by calculating the proportionate reduction of error (PRE) produced by the full model compared to the model without the variable of interest. This was calculated by using the residual sum of squares (RSS) achieved through LOOCV for each model in the following equation:

$PRE = 1 - RSS_{Full\ Model}/RSS_{Reduced\ Model}$ (see Judd et al., 2011).

*Predictive model validation.* The use of best subsets variable selection along with our small sample size and the large number of candidate predictors remained a possible source of inflation (both for our $R^2$ values and for the probability of obtaining a significant *p*-value), particularly for our social models. In order to provide a better gauge of how likely our findings would be to occur at random given the techniques that were used, we formed dummy datasets by randomly resampling the dependent variable in each dataset without replacement. Using this new dependent variable-scrambled dataset, we selected a new model using best subsets selection under the same parameters described in the last section, with the exception that the number of variables was selected based on which model had the highest adjusted $R^2$ value. The *p*-value of the new model was recorded and an $R^2$ value for the model was calculated using LOOCV as described in the previous section. This was repeated over 10,000 iterations for all the datasets we had found to be significant in order to form a rough probability distribution of *p*-values and $R^2$ values for each. The probability of obtaining the reported values for each model was calculated by finding the percent of the total values falling above ($R^2$ values) or below (*p*-values) the obtained values on the distribution for a given model. To further assess how applicable our results were between timepoints, new models were formed using the selected predictors for each area/model type using just the acquisition timepoint data, the STM timepoint data, or the LTM timepoint data. These new models were then used to try and predict the dependent variable values of the other timepoint (e.g., acquisition model to predict STM) and an $R^2$ value and rough *p*-value were obtained by regressing the predicted values against the actual values.

## RESULTS

### Behavioral results

*STFP social interaction results.* A two-way factorial ANOVA (type II) was run to gauge the effect of sex and experimental condition (i.e., ACQ, ACQ-CTRL, STM, or STM-CTRL in this case) on the amount of time the observers spent engaging in each social behavior. ANOVAs were also run for the total amount of time observers spent in social contact and the amount of time their demonstrator spent grooming them. Our ANOVAs found no effect of condition or sex on the social behaviors of face sniffing, play behavior, paw contact, grooming, and body sniffing (all $p > 0.1$). While no overall significant effect of condition was detected on any of the other kinds of social behavior (all $p > 0.1$), a significant overall effect of sex was found on nose contact ($F_{1,43} = 4.876$, $p = 0.033$, $\eta^2 = 0.098$), body contact ($F_{1,43} = 12.41$, $p = 0.001$, $\eta^2 = 0.202$), demonstrator grooming ($H_1 = 8.885$, $p = 0.003$, $\eta^2_H = 0.161$) (Fig. 3A), and overall social contact ($F_{1,43} = 14.47$, $p < 0.001$, $\eta^2 = 0.201$) (Fig. 3B) with males engaging more in all cases. Additionally, a significant interaction was found between sex and condition in overall social contact ($F_{3,43} = 2.85$, $p = 0.048$, $\eta^2 = 0.19$). A Tukey HSD post-hoc test confirmed the overall effect of sex for social contact ($p < 0.001$) but found no significant within sex differences between the different conditions (data not shown).

*Choice test/diet plain consumption results.* Preference for the demonstrated flavor was assessed using one-sample t-tests which tested the percent of total eaten that was the demonstrated flavor for the LTM and STM groups (separately) against a population mean of 50. Our results found that while rats in the STM groups showed a robust preference for the demonstrated flavor ($t_{13} = 4.898$, $p = 0.000291$, $\eta^2 = 0.649$), rats in the LTM group did not show a significantly higher preference for the demonstrated flavor ($t_{13} = 0.814$, $p = 0.43$, $\eta^2 = 0.049$) (see Fig. 4A) indicating that while our STFP behavioral model was sufficient for transmission, some learning degradation did occur over time. A two-way ANOVA (type II) was run for rats in the LTM, LTM-CTRL, STM, & STM-CTRL on the total

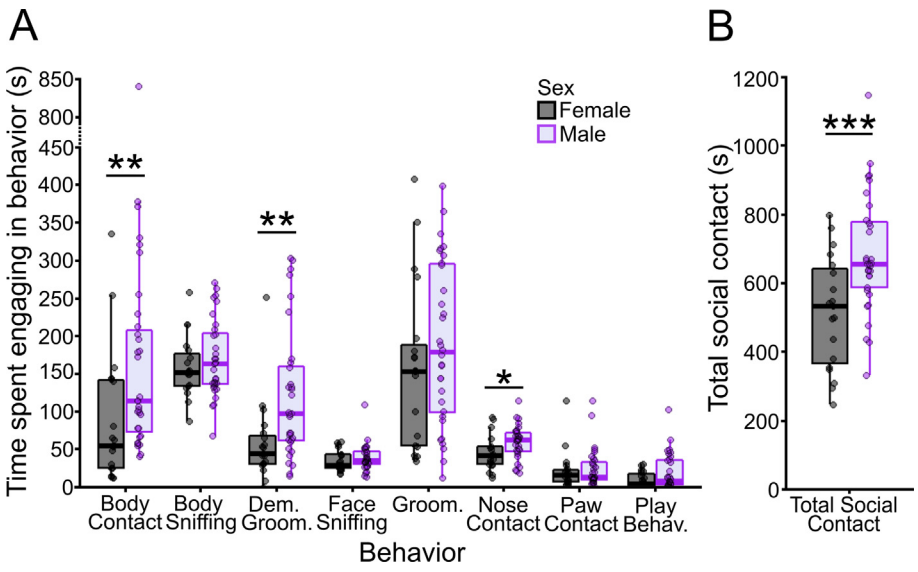


**Fig. 3.** Social behavior during STFP acquisition. The above graphs outline the average (±s.e.m) amount of time male ($n$ = 32) and female ($n$ = 19) rats spent engaging in **(A)** each discrete form of social behavior and **(B)** the total time observers spent interacting socially with their demonstrator during STFP transmission. Overall, males were significantly more social than females. (Abbreviations: Demonstrator Grooming [Dem. Groom.], Grooming [Groom.], Play Behavior [Play Behav.]). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

grams eaten with sex and experimental condition as the independent variables. A significant effect was found for sex ($F_{1,47}$ = 14.78, $p$ < 0.001, $\eta^2$ = 0.15) and condition ($F_{3,47}$ = 9.38, $p$ < 0.0001, $\eta^2$ = 0.286). Additionally, a significant interaction between the two was detected ($F_{3,47}$ = 2.81, $p$ = 0.049, $\eta^2$ = 0.086). A post-hoc Tukey HSD confirmed a significant effect of sex ($p$ < 0.001) and found a significant differences between the amount eaten by rats in the LTM-CTRL group and rats in the STM ($p$ < 0.0001), STM-CTRL ($p$ = 0.004), and LTM ($p$ = 0.048) conditions. The effect of experimental condition appeared to be driven entirely by the males, as post-hoc testing found no significant differences between conditions when comparing between only females (all $p$ > 0.1) (See Fig. 4B).

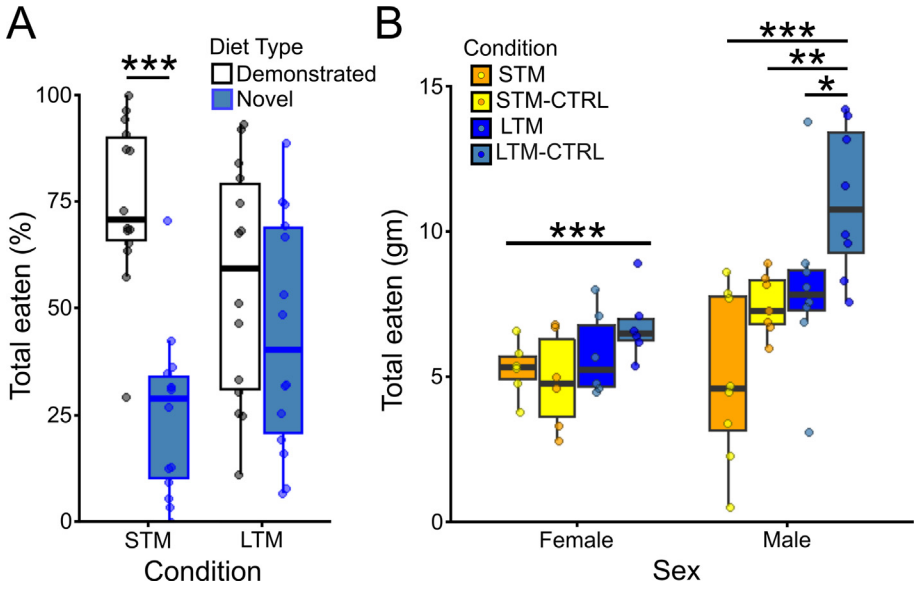


**Fig. 4.** Choice test results and between-group comparison of total amount eaten. **(A)** Rats in the STM condition ($n$ = 14), but not rats in the LTM condition ($n$ = 14), displayed a strong preference for consuming the demonstrated flavor at the choice test as compared to the 50% baseline. **(B)** During the choice task (STM and LTM conditions) or Diet Plain consumption (STM-CTRL [$n$ = 13] and LTM-CTRL [$n$ = 14] conditions), male rats ($n$ = 31) overall ate more than female rats ($n$ = 24). Within the male group, LTM-CTRL rats ate more than their STM, STM-CTRL, and LTM counterparts. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

## Immunohistochemical analysis results

*Condition and sex comparisons.* Two-way factorial ANOVAs (type II) were run to determine whether there was an effect of sex or timepoint (acquisition, short-term recall, or long-term recall) on c-Fos expression in orexin neurons, non-orexin LHA expression, and expression in the IL. A significant effect of sex ($F_{1,68}$ = 32.654, $p$ < 0.0001, $\eta^2$ = 0.312) (Fig. 5C) was detected, but no significant effect of timepoint ($F_{2,68}$ = 1.28, $p$ = 0.286, $\eta^2$ = 0.024) and no significant interaction between sex and timepoint ($F_{2,68}$ = 0.725, $p$ = 0.488, $\eta^2$ = 0.014) were found in orexin cells displaying c-Fos activation (Fig. 5B). In the infralimbic cortex, a significant effect of timepoint ($F_{2,67}$ = 5.88, $p$ = 0.004, $\eta^2$ = 0.141) was detected, with post-hoc testing finding a significant difference between IL c-Fos expression at the acquisition and long-term memory timepoints ($p$ = 0.006) and a significant difference between rats at the short-term memory and long-term memory timepoints ($p$ = 0.025) (see Fig. 6A). No effect of sex ($F_{1,67}$ = 1.98, $p$ = 0.16, $\eta^2$ = 0.024) (Fig. 6B) and no interaction between sex and timepoint ($F_{2,67}$ = 1.518, $p$ = 0.227, $\eta^2$ = 0.036) were detected. Finally, in non-orexin LHA cells a significant effect of sex on c-Fos activity was detected ($F_{1,68}$ = 20.87, $p$ < 0.0001, $\eta^2$ = 0.22), with females showing more c-Fos activity than males (see Fig. 6C). No significant effect of timepoint ($F_{2,68}$ = 1.55, $p$ = 0.22, $\eta^2$ = 0.032) or sex/timepoint

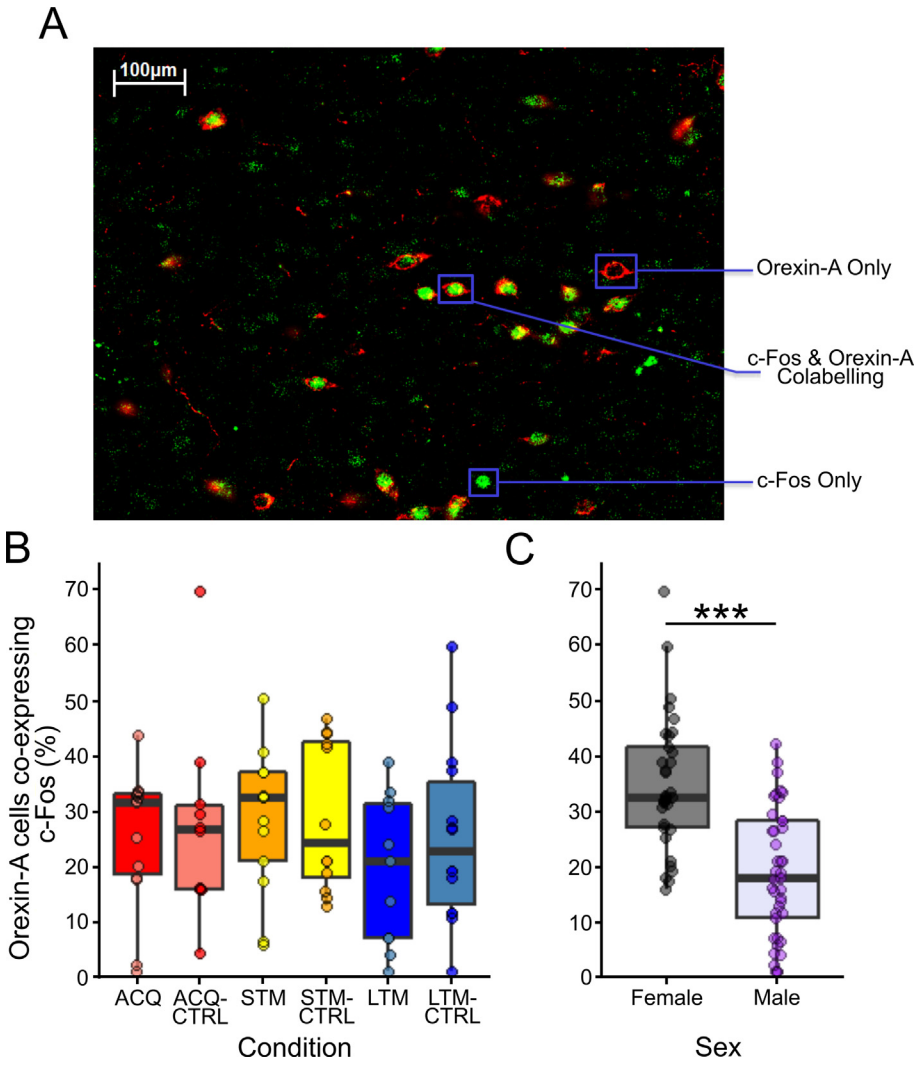


**Fig. 5.** Orexin/c-Fos co-localization findings. **(A)** A representative picture of our orexin-A (red) and c-Fos (green) labelling. **(B)** No significant difference was detected between control and experimental rats at any timepoint or between rats run at the acquisition timepoint (ACQ: $n = 14$, ACQ-CTRL: $n = 10$), short-term memory timepoint (STM: $n = 13$, STM-CTRL: $n = 12$), and rats at the long-term memory timepoint (LTM: $n = 11$, LTM-CTRL: $n = 14$). **(C)** Female rats ($n = 33$) displayed overall higher levels of orexin/c-Fos co-localization than did male rats ($n = 41$). $^{***}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

interaction ($F_{2,68} = 1.65$, $p = 0.2$, $\eta^2 = 0.035$) was detected (data not shown). To test for differences between control and experimental groups at each timepoint, c-Fos activity at each examined area/cell type was divided by the average expression in the control group for each timepoint. As there was a clear effect of sex in every area/cell type examined, control averages were taken separately for each sex and timepoint combination and the percent of control activation for experimental groups was calculated using only the same-sex averages. The resulting percentages were tested using a one-sample t-test against a μ of 100 (i.e., the value indicating no difference from control). No significant differences between experimental and control conditions were found at any of the timepoints for any of the examined areas (all $p > 0.05$).

*Regression analyses.* We ran a series of linear regressions (random effect models) to determine whether a relationship existed between our primary behavior measures (total grams eaten, total social contact, and percent of total eaten that was the demonstrated food) and c-Fos activity across the brain, as well as to determine whether there was a relationship in c-Fos activity between regions. A significant negative relationship was detected between total grams eaten and c-Fos expression in orexin stained cells ($F_{1,48} = 15.38$, $p < 0.001$, $R^2_{adjusted} = 0.23$, $r = -0.493$) (Fig. 7A) and in the infralimbic cortex ($F_{1,46} = 6.011$, $p = 0.018$, $R^2_{adjusted} = 0.09$, $r = -0.34$) (Fig. 7C), and a borderline significant relationship was found in the LHA ($F_{1,48} = 3.49$, $p = 0.068$, $R^2_{adjusted} = 0.05$, $r = -0.26$) (Fig. 7B). A significant relationship between social contact and c-Fos expression in the IL was detected ($F_{1,42} = 4.27$, $p = 0.045$, $R^2 = 0.07$, $r = 0.303$) (Fig. 7F), while only a borderline significant relationship was found between social contact and orexin c-Fos expression ($F_{1,42} = 3.325$, $p = 0.075$, $R^2_{adjusted} = 0.05$, $r = -0.271$) (Fig. 7D). No significant relationship between was found between non-orexin LHA c-Fos expression and social contact ($F_{1,42} = 2.6$, $p = 0.11$, $R^2_{adjusted} = 0.036$, $r = -0.24$) (Fig. 7E). In contrast, there was no relationship between the percent of total eaten that was the demonstrated food and c-Fos expression in any of the examined areas (all $p > 0.4$). As post-hoc data imaging suggested that sex might serve as a mediating factor in the relationship between grams eaten and orexin activity, female and male data were separated out and a second series of regressions were run on the separated datasets. We found that while no relationship between grams eaten and c-Fos expressing orexin cells existed in the female data alone ($F_{1,19} < 0.01$, $p = 0.95$, $R^2_{adjusted} < 0$, $r = 0.02$) a moderate negative correlative relationship was present in the male animals ($F_{1,27} = 7.657$, $p = 0.01$, $R^2_{adjusted} = 0.192$, $r = -0.47$) (see Fig. 8).

Initial visual assessment of the data when examining c-Fos activation across brain regions suggested non-linear relationships. As such, regressions were run with both

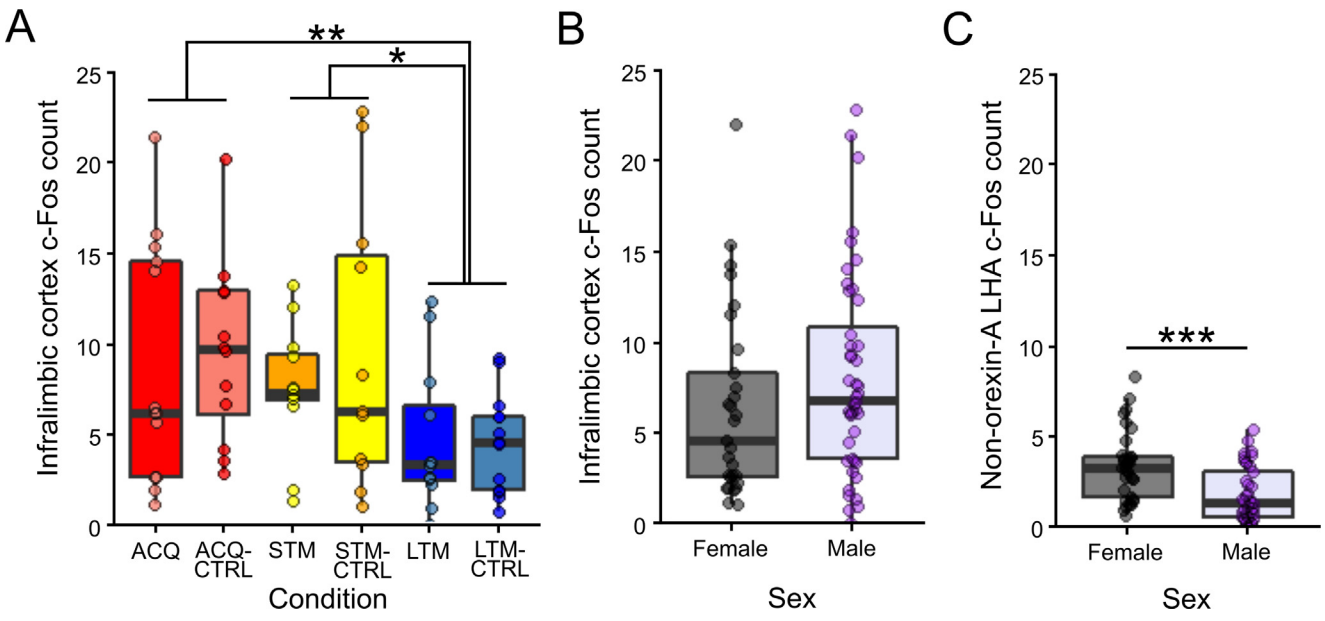


**Fig. 6.** Infralimbic and non-orexin LHA c-Fos Results. In the infralimbic cortex, **(A)** rats sacrificed at the long-term memory timepoint (LTM: $n = 12$, LTM-CTRL: $n = 13$) showed significantly less c-Fos expression than rats sacrificed at the acquisition timepoint (ACQ: $n = 13$, ACQ-CTRL: $n = 12$) or short-term memory timepoint (STM: $n = 12$, STM-CTRL: $n = 11$). **(B)** While there was no significant difference in c-Fos activity in the infralimbic cortex between females ($n = 29$) and males ($n = 44$), **(C)** a significant effect of sex was detected in non-orexin c-Fos expression in the lateral hypothalamic area (females: $n = 33$, males: $n = 41$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

the first and second order term of their predictor and analyses had to be run with every dependent/independent variable combination (e.g., with y = IL counts and x = LHA counts as well as y = LHA counts and x = IL counts). The first and second order terms for LHA c-Fos counts were significantly predictive of both IL c-Fos expression ($F_{2,62} = 10.57$, $p_1 = 0.0002$, $p_2 = 0.022$, $R^2_{adjusted} = 0.23$) (Fig. 9A), and orexin c-Fos expression ($F_{2,71} = 23.04$, $p_1 < 0.00001$, $p_2 = 0.016$, $R^2_{adjusted} = 0.377$) (Fig. 9B). Only the first order term of IL c-Fos expression was significantly predictive of expression in the LHA ($F_{2,62} = 8.549$, $p_1 = 0.0002$, $p_2 = 0.37$, $R^2_{adjusted} = 0.191$) (Fig. 9C) and of c-Fos expression in orexin cells ($F_{2,62} = 8.965$, $p_1 = 0.0003$, $p_2 = 0.076$, $R^2_{adjusted} = 0.199$) (Fig. 9D). Finally, the first and second order terms for orexin c-Fos \expression were significantly predictive for activity in the IL ($F_{2,62} = 8.68$, $p_1 = 0.01$, $p_2 = 0.0024$, $R^2_{adjusted} = 0.194$) (Fig. 9E) and for activity in non-orexin LHA cells ($F_{2,71} = 29.98$, $p_1 < 0.00001$, $p_2 = 0.018$, $R^2_{adjusted} = 0.44$) (Fig. 9F). Given the large number of outgoing connections from the infralimbic cortex to orexinergic neurons in the LHA (Yoshida et al., 2006) we were particularly interested in how the different behavioral procedures might influence the relationship between infralimbic activity and orexin c-Fos expression. As such, we ran another series of linear regression analyses with data separated out by timepoint. Surprisingly, while these analyses found a significant first order predictive relationship between IL activity and orexin activation in the LTM/LTM-CTRL timepoint subjects ($F_{2,21} = 4.28$, $p_1 = 0.0254$, $p_2 = 0.11$, $R^2_{adjusted} = 0.22$), no relationship was present in our STM/STM-CTRL timepoint ($F_{2,18} = 1.248$, $p_1 = 0.154$, $p_2 = 0.601$, $R^2_{adjusted} = 0.024$) or ACQ/ACQ-CTRL timepoint subjects ($F_{2,17} = 1.426$, $p_1 = 0.147$, $p_2 = 0.472$, $R^2_{adjusted} = 0.043$) (data not shown).

*Predictive modeling results.* (An overview of model findings can be found in Table 1).

The Social Model of the percent of orexin cells expressing c-Fos, constructed using data from rats in the ACQ, ACQ-CTRL, STM and STM-CTRL groups, was found to be overall significant ($F_{5,38} = 7.458$, $p < 0.0001$, $R^2 = 0.338$). Best subsets selected the six variable model containing the predictors minutes since colony light's off ($t_{43} = 2.945$, $p = 0.0055$, PRE = 0.136), body sniffing ($t_{43} = -1.69$, $p = 0.099$, PRE = 0.002), play behavior ($t_{43} = 1.593$, $p = 0.119$, PRE < 0), the sex variable ($t_{43} = -5.12$, $p < 0.0001$, PRE = 0.353), and the sex and paw contact interaction term ($t_{43} = 1.813$, $p = 0.078$, PRE = 0.051). Sex alone was not as predictive of c-Fos activation, confirming contributions of the other predictors ($R^2 = 0.223$). The Appetitive Model for orexin, using data from rats in the LTM, LTM-CTRL, STM, and STM-CTRL conditions, was similarly successful ($F_{3,46} = 21.24$, $p < 0.0001$, $R^2 = 0.512$) and included sex ($t_{49} = -6.53$, $p < 0.0001$, PRE = 0.458), minutes since colony lights off ($t_{49} = 3.224$, $p = 0.0023$, PRE = 0.153), and the interaction term between sex and grams eaten ($t_{49} = -2.647$, $p = 0.011$, PRE = 0.103). LOOCV with sex alone was not as accurate, again confirming the contributions of behavioral measures ($R^2 = 0.357$).

The social model of non-orexin LHA c-Fos expression was also significant ($F_{5,38} = 4.177$, $p = 0.004$, $R^2 = 0.106$) with sex ($t_{42} = -3.41$, $p = 0.0016$, PRE = 0.183), demonstrator grooming ($t_{42} = 2.769$, $p = 0.0087$, PRE = 0.062), nose contact ($t_{42} = -2.566$, $p = 0.014$, PRE = 0.114), grooming ($t_{42} = 2.412$, $p = 0.021$, PRE = 0.092), and the interaction between sex and nose contact ($t_{42} = 1.894$, $p = 0.066$,

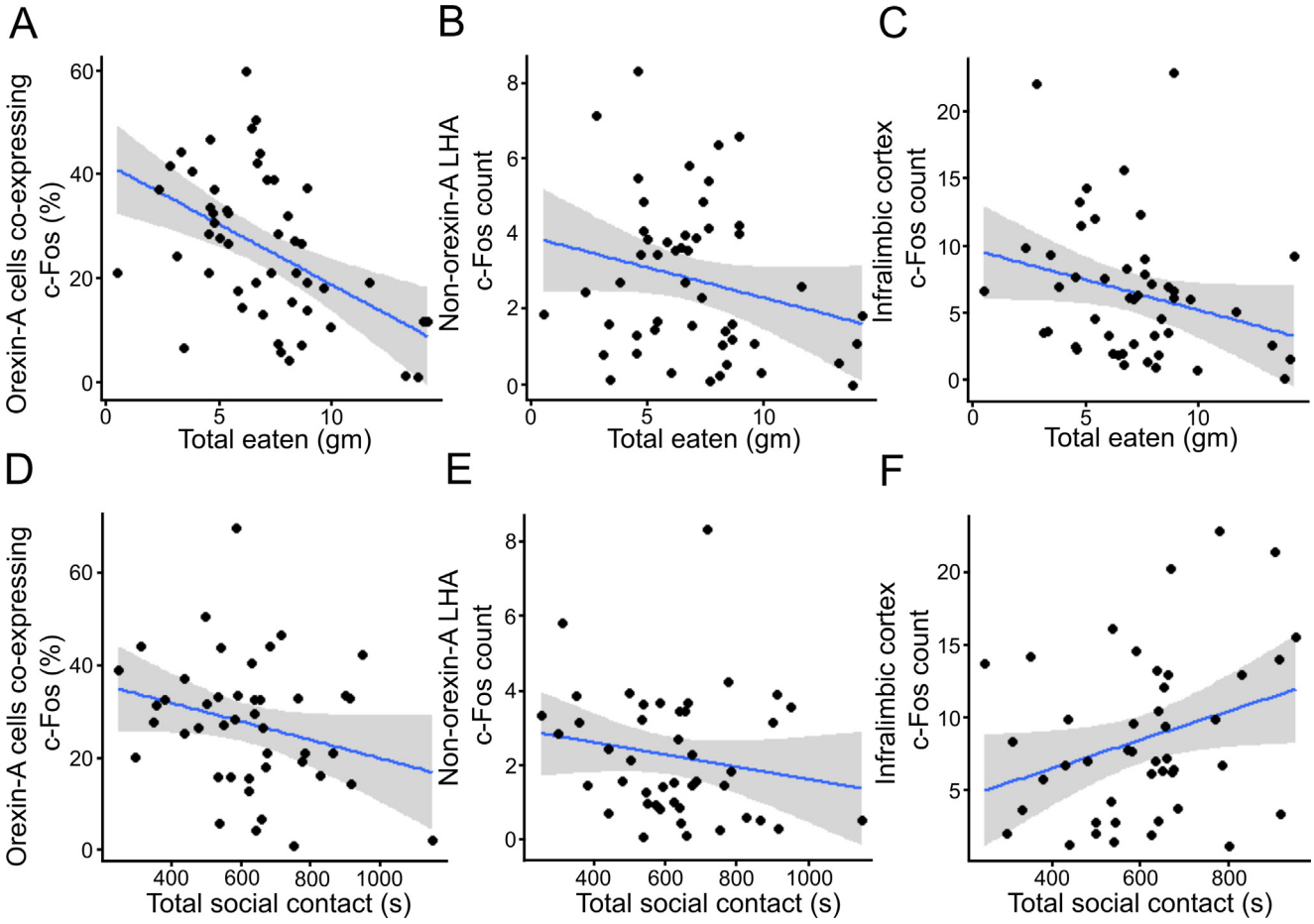


**Fig. 7.** Correlative relationships between primary behavioral measures and c-Fos expression. The above graphs display the correlative relationship between the percent of orexin cells co-expressing c-Fos and **(A)** grams eaten ($n = 50$; $p < 0.001$, $r = -0.493$) and **(D)** total social contact ($n = 44$; $p = 0.075$, $r = -0.26$), the number of non-orexin c-Fos counts in the LHA and **(B)** grams eaten ($n = 50$; $p = 0.068$, $r = -0.26$) and **(E)** total social contact ($n = 44$; $p = 0.114$, $r = -0.241$), and the average c-Fos count in the IL cortex and **(C)** total grams eaten ($n = 48$; $p = 0.018$, $r = -0.34$) and **(F)** total social contact ($n = 44$; $p = 0.045$, $r = 0.304$).

PRE = 0.043) being included in the model. LOOCV run with sex alone as a predictor was slightly less accurate in predicting the data ($R^2 = 0.053$), though the predictive value of the full model was minimal itself. Furthermore, separately calculating the LOOCV $R^2$ value for the acquisition timepoint subjects and short-term memory timepoint subjects found that the model was only accurate for predicting non-orexin LHA activity in the short-term memory group ($R^2_{STM} = 0.378$, $R^2_{ACQ} < 0$). The Appetitive Model for non-orexin LHA c-Fos expression selected for only the sex variable in the initial best subsets analysis, indicating no additional predictive value for any of the other predictors.

The Social Model for infralimbic c-Fos activation was found to be significant ($F_{6,37} = 5.868$, $p = 0.00022$, $R^2 = 0.299$) and predictive beyond what was found for sex alone ($R^2 = 0.024$), with included variables being whether the rat has acquired a STFP ($t_{43} = -3.38$, $p = 0.0392$, PRE = 0.05), sex ($t_{43} = 4.294$, $p = 0.007$, PRE = 0.151), the interaction between total social contact and sex ($t_{43} = 4.732$, $p = 0.00013$, PRE = 0.284), nose contact ($t_{43} = -1.915$, $p = 0.016$, PRE = 0.127), face sniffing ($t_{43} = 2.135$, $p = 0.024$, PRE = 0.081), and body sniffing ($t_{43} = -2.302$, $p = 0.0015$, PRE = 0.21). The Appetitive Model for IL c-Fos found that the best predictive model contained only the timepoint variable, indicating no predictive value of any of the continuous variables.

*Model validation results.* (Note: Model Validation results are not displayed in a table)

For our orexin activity findings, there was a low probability of obtaining the results of the social model ($p_{probability} = 0.0123$, $R^2_{probability} = 0.0098$), and values rivaling the $p$ and $R^2$ values obtained for our eating model did not occur even once in the 10,000 iterations ($p_{probability} < 0.0001$, $R^2_{probability} < 0.0001$). The values obtained for our social model of infralimbic activity also suggested that the connection between our predictor variables and outcome variable was valid ($p_{probability} = 0.0189$, $R^2_{probability} = 0.0108$). In contrast, the results of our social model of non-orexin LHA activity were found to be fairly likely to occur even if there were truly no connection between the predictors and the outcome variables ($p_{probability} = 0.192$, $R^2_{probability} = 0.192$).

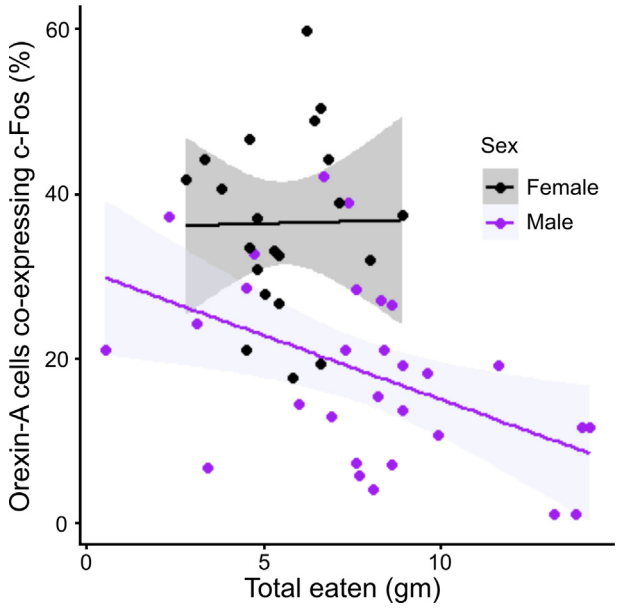


**Fig. 8.** Sex differences in the relationship between appetitive behavior and c-Fos expression in orexin cells. When split by sex, we found that the negative correlation that was detected between grams eaten and orexin activity was present in male rats ($n$ = 31; $p$ = 0.01, $r$ = −0.47) but not female rats ($n$ = 24; $p$ = 0.948, $r$ = 0.015). Shaded areas represent 95% confidence intervals for the regression lines.

In our social orexin model, we found that our STM model was highly predictive of our acquisition data ($p$ = 0.0029, $R^2_{adjusted}$ = 0.334) and our acquisition model was highly predictive of our STM data ($p$ < 0.0001, $R^2_{adjusted}$ = 0.581). Similarly, our appetitive LTM timepoint orexin model was highly effective at predicting STM timepoint data ($p$ = 0.00016, $R^2_{adjusted}$ = 0.447) and vice versa ($p$ < 0.0001, $R^2_{adjusted}$ = 0.558). The infralimbic social model also effectively predicted IL c-Fos expression between timepoints (Acquisition model: $p$ = 0.0058, $R^2_{adjusted}$ = 0.302; STM model: $p$ = 0.0054, $R^2_{adjusted}$ = 0.282). In contrast, and in line with its failure of our previous validation test, our social non-orexin LHA activity model was not uniformly effective at predicting between timepoints (STM model: $p$ = 0.108, $R^2$ = 0.08; Acquisition model: $p$ = 0.0004, $R^2$ = 0.453).

## DISCUSSION

The primary goal of our study was to determine whether activity in orexin neurons or related structures was induced during the acquisition or recall of a socially transmitted food preference. While our experiment did not support a primary role of orexin in either the acquisition or recall of a socially transmitted food preference specifically, our analysis of social and appetitive behavior during the associated pre-perfusion behavioral procedures uncovered a strong, sex-mediated relationship between appetitive behavior and orexin activity. Notably, and in accordance with past research (Estabrooke et al., 2001), we also noted a positive correlation between the hours since the start of our rat's dark cycle and the percent of orexin cells that were displaying activity. Additionally, our results suggest a relationship between activity in the infralimbic cortex and orexinergic activity in the LHA, which is supported by findings that the infralimbic cortex projects extensively to orexin neurons in the LHA and receives reciprocal projections back (Yoshida et al., 2006). In the following subsections we will go over our findings in regard to the role of the investigated areas in STFP acquisition and recall, appetitive behavior, social behavior, and sex differences detected in our findings.

### Involvement of investigated regions in the acquisition and recall of socially transmitted food preferences

Regarding our primary research question of whether orexinergic neurons play a role in the acquisition and recall of socially transmitted food preferences, our results suggest that such a relationship may not exist. While our findings do point towards a role of orexinergic neurons in the regulation of the primary behaviors associated with the expression of a STFP (food consumption), no statistically significant differences were detected between any of our experimental and control groups at any of the examined timepoints. It remains possible that there is some orexin involvement in the acquisition of a socially transmitted food preference that we were unable to detect with our sample size. Future research examining orexin activity at the acquisition or short-term recall timepoints could very well produce results that counter what we have found here. Additionally, it remains possible that orexin-B signaling specifically might play a role in STFP acquisition/recall.

In regard to the other examined areas, while the lack of difference in c-Fos expression between experimental and control animals in non-orexin LHA neurons was not necessarily unexpected, our lack of results in the infralimbic cortex runs counter to past research findings examining STFP associated c-Fos activity in this region. Unlike in Smith et al. (2007), who utilized near-identical behavioral procedures to ours, our LTM animals failed to show a significant increase in c-Fos expression in the infralimbic cortex over their controls. While this is potentially due to strain differences (Long Evans in Smith et al. vs. Sprague-Dawleys in our experiment), it is notable that Ross and Eichenbaum (2006) also did not find significantly elevated c-Fos levels at the 48-h recall timepoint for STFP in Long Evans, though their data did trend strongly in that direction. Though sex was somewhat controlled for by using only same-sex controls to calculate experimental rats' difference from baseline, it remains possible that there are sex differences in the involvement of the infralimbic cortex in STFP recall which may have resulted in our group differences being obscured. Finally, it is also notable that despite our STM timepoint rats clearly displaying STFP acquisition at testing – indicating that our STFP transmission procedure was sufficient for learning to occur – our LTM rats did not display a significant preference for the demonstrated flavor (though they did trend in that direction). Many stud-

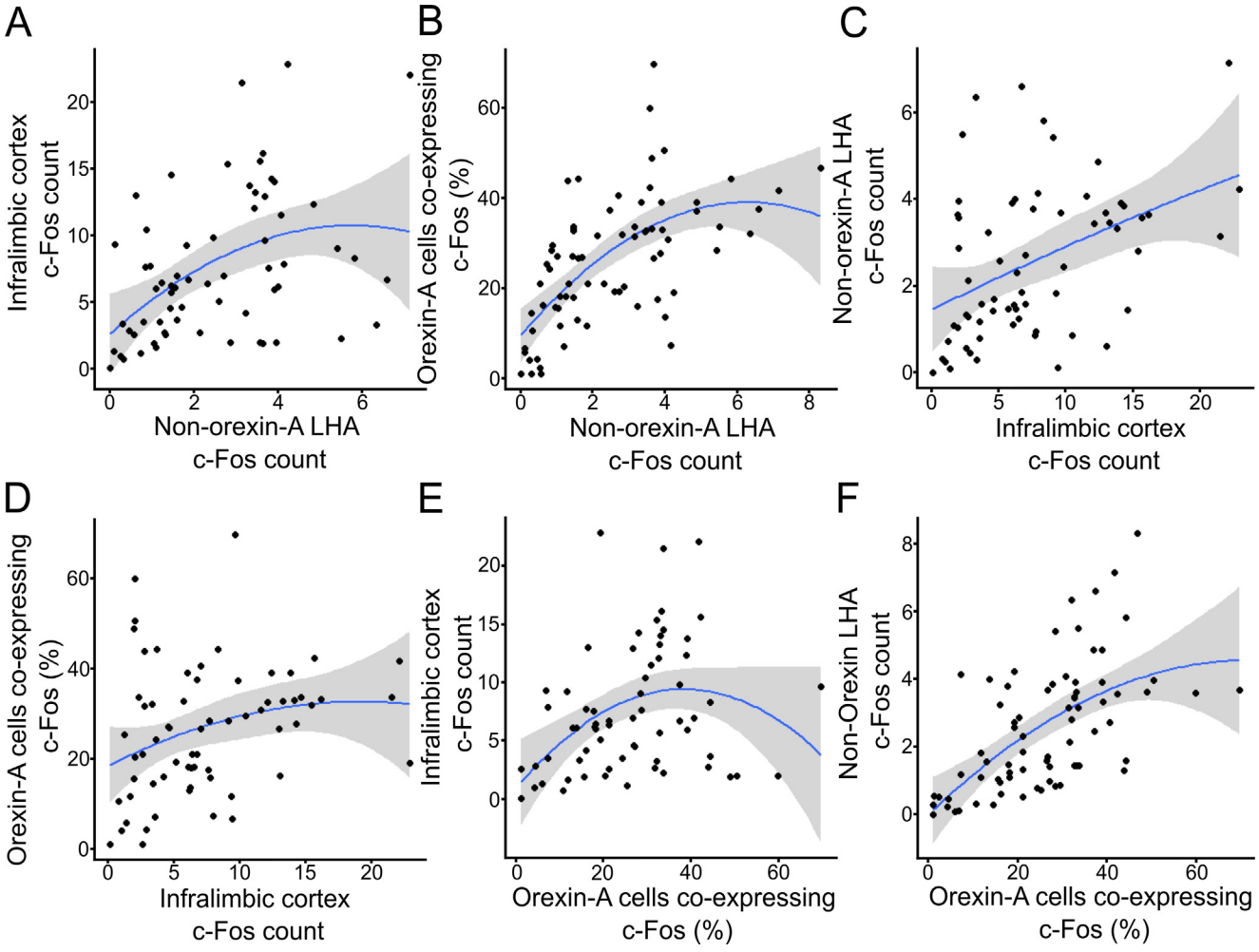


**Fig. 9.** Results of between area regression analyses of c-Fos expression. The above graphs summarize the statistical results of regression run on the second-degree polynomial of predictors (IL included: $n = 65$, otherwise: $n = 74$). c-Fos expression in non-orexin-A LHA cells was found to be predictive of **(A)** c-Fos expression in the IL ($p_{1st\ order} = 0.0002$, $p_{2nd\ order} = 0.022$, $R^2_{adjusted} = 0.23$) and **(B)** c-Fos expression in orexin-A cells ($p_{1st\ order} < 0.0001$, $p_{2nd\ order} = 0.016$, $R^2_{adjusted} = 0.377$). Similarly, IL cortex c-Fos counts were predictive of **(C)** c-Fos counts in non-orexin-A LHA cells ($p_{1st\ order} = 0.0002$, $p_{2nd\ order} = 0.37$, $R^2_{adjusted} = 0.191$) and **(D)** c-Fos counts in orexin-A positive LHA cells ($p_{1st\ order} = 0.0003$, $p_{2nd\ order} = 0.077$, $R^2_{adjusted} = 0.199$). Finally, c-Fos activity in orexin-A producing LHA cells was predictive of **(E)** c-Fos activity in the IL cortex ($p_{1st\ order} = 0.01$, $p_{2nd\ order} = 0.0024$, $R^2_{adjusted} = 0.194$) and **(F)** c-Fos activity in non-orexin-A LHA cells ($p_{1st\ order} < 0.0001$, $p_{2nd\ order} = 0.018$, $R^2_{adjusted} = 0.44$).

ies have demonstrated that rats can display an STFP more than a week after acquisition (Ross and Eichenbaum 2006; Smith et al., 2007; Lesburguères et al., 2011) making this apparent failure to retrieve somewhat puzzling. As the use of Sprague-Dawley strain rats in STFP research is relatively uncommon, it is possible that this effect is strain specific.

### Involvement of orexin and investigated regions in appetitive behavior

Unexpectedly, the results of this study indicted a negative correlative relationship between the amount of food eaten and the percent of orexin-A producing cells that were co-expressed c-Fos. This finding seems counter to the results of numerous studies that have found that central or intracerebroventricular (ICV) administration of orexin-A generally results in increases in food intake (Sakurai et al., 1998; Edwards et al., 1999; Haynes et al., 1999; Ida et al., 1999; Borgland et al., 2009), while orexin depletion or blockage results in hypophagia (Haynes et al., 2000; Yamada et al., 2000; Niimi et al., 2001; Borgland et al., 2009). Both these results would suggest that we should be observing increases in consumption with more orexin activity. One potential explanation for our results may be the time of day in which our experiment was run. Most of our terminal behavioral procedures began within one to five hours after the start of our rat's dark cycle, with only $n = 8$ of our 48 male rats, and none of our females, being run at a later time. Past research examining the effects of orexin-A on feeding behavior throughout the day have indicated that continuous ICV infusion of orexin-A results in increased feeding during the light cycle and decreased feeding during the dark cycle (Haynes et al., 1999). While at the time these researchers reasonably hypothesized that this decrease in intake during the dark cycle was the result of countermeasures by the orexin system to compensate for hyperphagia during the light cycle, our results suggest that increased orexin-A activity during the early dark cycle

**Table 1.** Predictive modeling results

| | *F*/*t*-value | *p*-Value | PRE | $R^2$ | $R^2_{\text{Sex Only}}$ |
|---|---|---|---|---|---|
| **Orexin-A c-Fos – Social Model** | $F_{5,38} = 7.458$ | < 0.0001 *** | | 0.338 | 0.223 |
| Time Since Lights Off | $t_{43} = 2.945$ | 0.0055 ** | 0.136 | | |
| Sex | $t_{43} = -5.12$ | < 0.0001 *** | 0.353 | | |
| Body Sniffing | $t_{43} = -1.69$ | 0.099 | 0.002 | | |
| Play Behavior | $t_{43} = 1.593$ | 0.119 | < 0 | | |
| Sex × Paw Contact | $t_{43} = 1.813$ | 0.078 | 0.051 | | |
| **Orexin c-Fos – Appetitive Model** | $F_{3,46} = 21.24$ | < 0.0001 *** | | 0.512 | 0.357 |
| Sex | $t_{49} = -6.53$ | < 0.0001 *** | 0.458 | | |
| Time Since Lights Off | $t_{49} = 3.224$ | 0.0023 ** | 0.153 | | |
| Sex × Total Grams Eaten | $t_{49} = -2.647$ | 0.011 * | 0.103 | | |
| **Non-Orexin-A LHA c-Fos – Social Model** | $F_{4,38} = 4.177$ | 0.004 ** | | 0.106 | 0.053 |
| Sex | $t_{42} = -3.41$ | 0.016** | 0.183 | | |
| Demonstrator Grooming | $t_{42} = 2.769$ | 0.0087 ** | 0.062 | | |
| Grooming | $t_{42} = 2.412$ | 0.021 * | 0.092 | | |
| Nose Contact | $t_{42} = -2.566$ | 0.014 * | 0.11 | | |
| Sex × Nose Contact | $t_{42} = 1.894$ | 0.066 | 0.043 | | |
| **Infralimbic c-Fos – Social Model** | $F_{6,37} = 5.868$ | 0.00022 *** | | 0.299 | 0.024 |
| STFP Experience | $t_{43} = -3.38$ | 0.0392 * | 0.05 | | |
| Sex | $t_{43} = 4.294$ | 0.007 ** | 0.151 | | |
| Nose Contact | $t_{43} = -1.915$ | 0.016 * | 0.127 | | |
| Face Sniffing | $t_{43} = 2.135$ | 0.024 * | 0.081 | | |
| Body Sniffing | $t_{43} = -2.302$ | 0.0015 ** | 0.21 | | |
| Sex × Total Social Contact | $t_{43} = 4.732$ | 0.00013 *** | 0.284 | | |

**Notes:** Results for the IL appetitive model and the non-orexin LHA appetitive model are not included due to overall model non-significance and full predictive dependence on the sex variable, respectively.

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

might function to actively suppress food intake. Somewhat in support of this, it has been found that targeted deletion of orexin-producing cells results in increases in food intake in mice (González et al., 2016). That said, this increase occurred specifically in the late dark cycle (6–9 h following the beginning of the dark cycle) during what was normally a period of decreased feeding for mice.

Alternatively, it is possible that the decrease in orexin activity we observed in rats that had consumed more food was the result of decreased orexin firing in response to the act of eating. This interpretation is supported by the findings of González et al. (2016), who found via live orexin cell recordings in mice that while food presentation resulted in a brief sharp up-regulation of orexin cell activity followed by rapid downregulation to below baseline during eating. Cessation of eating behavior resulted orexin cells being rapidly reactivated and returning to baseline firing. Additionally, González et al. found that sustained eating resulted in sustained down-regulation of activity. This might account for the negative correlation we observed between orexin-A producing cell activation and the total amount eaten at testing, as we would expect rats that ate more to have longer bouts of eating and, therefore, longer periods of sustained downregulation in orexin activity. It is also possible that increased satiation might result in longer term downregulation of orexin activity, with rats that had eaten more ultimately having less orexin activity. In line with this, González et al. and past researchers (Yamanaka et al., 2003; Burdakov et al., 2005) have also demonstrated that orexin firing is inhibited in the presence of concentrations of glucose in the 5–30 mM range. Another potential explanation is that, rather than directly reducing feeding behavior, increased orexin-A activity indirectly decreases feeding by promoting increased locomotion. In line with this, stimulating activity in certain subsets of orexin-A cells leads to increases in locomotor activity (Kosse et al., 2017; Karnani et al., 2020).

This negative correlation between orexin activity and food consumption was also only observed in our male rats, suggesting that this potential inhibitory effect of orexin might be a sex-specific effect. Indeed, there are a number of studies that implicate sex differences in orexin-mediated feeding behavior (Funabashi et al., 2009; Fukushima et al., 2015; Buczek et al., 2020; Freeman et al., 2021). These sex differences may be partially mediated by the higher baseline orexin cell activity and orexin production in females (Taheri et al., 1999; Jöhren et al., 2002; Grafe et al., 2017, 2019) and the greater expression of the orexin receptor $OX_2R$ in females (Loewen et al., 2017). Sex differences in the role of the orexin system in mediating appetitive behavior might be of clinical relevance given women's higher incidence of eating disorders (Hudson et al., 2007; Merikangas et al., 2010), the higher incidence of obesity in adult women (Sung et al., 2019), and sex differences in the efficacy of certain weight-loss treatments (Lovejoy and Sainsbury, 2009). Our findings

do provide evidence that our understanding of the orexin system in appetitive behavior – which has been based primarily on research using exclusively male subjects – is likely not fully applicable to the female half of the population. Future research aimed at disambiguating how the orexin system mediates consummatory behavior in females is still necessary for a full understanding of how this system operates.

### Involvement of orexin and investigated regions in social behavior

Our findings indicated a possible correlative relationship between social behavior and orexin activity, though our models would suggest that this relationship, if it exists, is weak. While a number of social behaviors were selected for in our final predictive model of orexin activity based on social behavior, analysis of the predictive contribution of social behaviors revealed that most added no or negligible predictive value to the model. The only possible exception to this would be the amount of paw contact made during interaction, though this effect was specific to male rats and fairly small. It should be noted that it is possible that the accuracy of our predictions was decreased somewhat by the bundling of data from rats that underwent the short-term memory behavioral procedures along with rats that had underwent acquisition procedures. As the social interaction period for rats in the STM and STM-CTRL conditions ended 2 h before rats were sacrificed and perfused, we would expect some amount of degradation – though not complete loss – of the c-Fos produced by initial socialization (Müller et al., 1984; Barros et al., 2015). Similarly, while our social model of non-orexin-A activity in the LHA was found to be initially significant, the model did not survive our validation tests.

Social activity was more definitively found to predict for c-Fos expression in the infralimbic cortex, with olfactory-based behavior appearing to be one of the major contributors to our ability to predict activity. Models constructed using data from only the acquisition timepoint were effective for predicting c-Fos activity in rats tested at the short-term recall timepoint and vice versa, again indicating a surprising retention of socially induced c-Fos at the more remote short-term memory sacrifice timepoint. Though the infralimbic cortex has not been a brain region that has received extensive scrutiny for its role in social behavior in rodent research, there have been some studies that have drawn a connection (Shah and Treit, 2003; Minami et al., 2017; Huang et al., 2020). The findings of this research tend to point towards a role of the infralimbic cortex in mediating social disengagement. While our results also suggest that the infralimbic cortex may play a role in mediating general social interaction and/or social exploration, social engagement did not overall negatively correlate with increases in IL activity as might have been expected based on this past research. Furthermore, our findings also suggest a potential role of sex in mediating the relationship between IL activity and social behavior, as is indicated by our social model's selection of the sex and total social contact duration interaction term.

### Sex differences

While sex differences relevant to the already discussed topics has been covered in the relevant sections, we also found a number of generalized sex effects. Our results indicated that a higher percentage of orexin-A producing cells were found to express c-Fos in the brains of female rats as compared to male rats, and this effect seemed stable across behavioral treatments and tested timepoints. This finding is in line with past research which has suggested higher baseline prepro-orexin expression (Jöhren et al., 2002; Grafe et al., 2017), orexin-A/c-Fos co-localization (Grafe et al., 2017), orexin cell spine density (Grafe et al., 2019) and lateral and posterior hypothalamic immunoreactivity to orexin-A (Taheri et al., 1999) in female rats over male rats. This replication of results provides further evidence that the orexin system likely serves as an important mediator of sex differences in behavior and brain signaling. Similarly, non-orexin-A producing LHA cells also showed increased c-Fos expression in females over males. This latter effect could possibly still be representing differences in orexin activity, as orexin-B producing cells remained unstained in our sample and would have been included in this population. Research explicitly examining sex differences in orexin-B activity would help to clarify this.

Overall, our findings do not support a role of orexin-A activity in the acquisition or recall of a socially transmitted food preference. In regard to generalized social behavior, activity in the infralimbic cortex was more closely correlated to social measures while a connection between orexin-A activity and sociability was less clear. In contrast, our findings did strongly support a relationship between orexin-A activity and appetitive behavior. Furthermore, our results suggest that counter to past findings, increased orexin-A activity was not associated with increased food intake, and, in fact, correlated negatively with food intake. Finally, our finding that the negative correlative relationship between the amount of food eaten prior to perfusion and orexin-A activity is sex-mediated may indicate a differing role of the orexin system in processing satiation cues in females as compared to males. Future research more directly aimed at dissociating how orexin cells respond to food/satiety cues in females as compared to males (e.g., via direct recording of orexin cell activity in female and male animals) would be hugely beneficial in determining the mechanisms behind these observed sex differences.

## DECLARATIONS

**Competing interests:** The authors have declared that no conflict of interest exists.

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**Author's Contributions:** LAA designed the experiment, gathered and analyzed data, processed and imaged tissue samples, and drafted the manuscript. VN assisted with tissue imaging and CAM completed most tissue sectioning. MHM and HJL contributed to the experimental design and approved the final manuscript.

**Availability of data and material:** Raw data files are available in The Monfils Lab repository, housed in the Texas Data Repository in Dataverse (https://dataverse.tdl.org/dataverse/MonfilsFearMemoryLab). All other materials are available by request to the authors.

**Code availability:** Code is available alongside data and materials in the Monfils Lab repository (see above).

**Ethics approval:** All parts of this experiment were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Experimental Animals and were approved for use by The University of Texas at Austin Animal Care and Use Committee.

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