al., 2010; Rothemund et al., 2007; Stoeckel et al., 2008; Yokum et al., 2014). Although basal differences in neurobehavioral responsivity to food cues likely contribute to weight gain (Demos et al., 2012; Derman and Ferrario, 2018a; Murdaugh et al., 2012; Robinson et al., 2015; Yokum et al., 2014), determining how consumption of sugary, fatty foods may impact these motivational responses to food cues is critical for understanding the mechanisms driving food-seeking and over-eating.

While diet composition has an obvious and direct influence on fat accumulation and metabolism, diets high in fats and sugars also have wide-ranging effects on the function of corticolimbic regions that may render individuals more susceptible to the motivational influence of food cues (Brown et al., 2017; Counotte et al., 2014; Liu et al., 2016). However, only a few preclinical studies have examined how sugar- and fat-rich diets affect motivational responses to Pavlovian cues. One such study found that obesity resulting from consumption of a sugary, fatty "junk-food" diet enhanced willingness to work for a previously learned food CS (i.e., conditioned reinforcement; Robinson et al., 2015). Similarly, another recent study found that consumption of a 60% high-fat diet enhanced instrumental responding in the "incubation of craving" model in outbred rats (Dingess et al., 2017). Together these data suggest that consumption of obesogenic diets may enhance conditioned motivational responses to food CSs. However, whether these effects are related to the predictive validity of a given CS and how these diet manipulations may affect different aspects of cue-triggered motivation are poorly understood. Therefore here, we determined the effects of both, short and- long term "junk-food" consumption on the expression of cue potentiated feeding and conditioned approach.

Cue potentiated feeding is a well-established phenomenon that demonstrates the ability of a learned food-predictive CS to spur on food intake in the face of selective satiation (Reppucci and Petrovich, 2012; Weingarten, 1983). Additionally, the nature of this testing procedure also provides information about conditioned approach to the site of US delivery. Thus, here we examined the impact of "junk-food" consumption on cue-induced consummatory and conditioned appetitive responses. Furthermore, in order to determine whether effects of "junk-food" were specific to Pavlovian motivational processes, we systematically varied the predictive validity of the CS across distinct

training groups by varying the CS-US contingencies between groups. If “junk-food” consumption imparts a change in behavior that is specific to Pavlovian motivation, then effects of “junk-food” should be limited to response elicited by CSs with positive predictive validity, but not by CSs with neutral or negative predictive validity. While the precise neurobiological underpinnings of conditioned approach and cue potentiated feeding are not fully understood, they have been shown to depend upon corticolimbic circuitry and on dopaminergic and glutamatergic transmission in these regions (Blaiss and Janak, 2009; Cole et al., 2015; Keefer and Petrovich, 2017; Ma et al., 2014; Petrovich et al., 2007). Given that consumption of sugar- and fat-rich diets enhance function within these circuits (Baladi et al., 2015; Ferrario et al., 2016; Oginsky et al., 2016; Peng et al., 2015; Robinson et al., 2015), we predicted that “junk-food” consumption would enhance conditioned approach and cue potentiated feeding. Moreover, if this diet manipulation selectively affects Pavlovian motivational processes, then we would expect behavioral differences would manifest only in groups trained with a strong positively predictive CS-US relationship.

2: General materials and methods

2.1: Subjects

Adult male Sprague Dawley rats (total N=139) purchased from Harlan Laboratories (now Envigo) were used. Rats were pair housed and maintained on a reverse light-dark schedule (12/12), where all behavioral experiments were performed during the dark phase. Rats were 70 days old at the start of each experiment. All procedures were approved by The University of Michigan Institutional Animal Care and Use Committee. Additional details for all procedures and housing can be found at: https://sites.google.com/a/umich.edu/ferrario-lab-publicprotocols/.

2.2: Pavlovian conditioning

Prior to training, rats (Experiment 1, n=80; Experiment 2, n=59) were food restricted to 85–90% of their free-feeding bodyweight and maintained at this weight for the duration of training. All training and testing was conducted in standard Med Associate

operant chambers. After reaching their target weight range, rats underwent 2 food cup training sessions (1/day) in which 20 food pellets (US; 45 mg Bioserv #F0021; 0.75 protein, 0.5 fat, 2.36 carbohydrate kCal/g) were delivered into the food cup on a variable time (VT) schedule of 60 s (range, 30″–90″). Next, rats underwent Pavlovian conditioning in 8 daily sessions. Here we describe the general experimental procedures, followed by specific training details about the relationship between CS-US presentations for Experiment 1 and 2 in Sections 3.1 and 3.2, respectively. Each session consisted of four presentations of a 2-minute noise conditioned stimulus (CS) with an average inter-trial-interval (ITI) of 5 min (range 3′–7′). Each session lasted ~28 min and rats received a total of 16 pellet deliveries per session. Food cup entries during the CS and ITI periods were recorded throughout each session.

2.3: Junk-food diet

After initial Pavlovian conditioning, rats were placed on *ad libitum* chow (Test Diet, 5001; 4.5% fat, 23% protein, 48.7% carbohydrates; 4 kcal/g) for 1 day, and then assigned to chow (Chow) or "junk-food" (JF) groups counterbalanced by weight and behavior during Pavlovian conditioning. The "JF" diet is a mash consisting of Chips Ahoy! chocolate chip cookies (260 g), Frito Lays potato chips (80 g), Jif peanut butter (260 g), Nestle Nesquik chocolate powder (260 g), Test Diet, 5001 (400 g) and water (355 ml; 19.6% fat, 14% protein, 58% carbohydrates; 4.5 kcal/g). Bodyweights and food intake (per cage) were monitored for the remainder of the experiment.

2.4: Cue potentiated feeding and conditioned approach testing

Testing for cue potentiated feeding was adapted from existing procedures (Cole et al., 2015; Galarce et al., 2007). Rats were given free access to the same Bioserv food US used in initial training for 1h prior to testing. Next, rats were placed in the operant boxes and food intake was recorded during two distinct 5-minute phases of testing: baseline (BL) and CS presentation (CS test). In each of these phases, a pre-weighed portion (~2–2.5 g) of pellets was placed directly into the food cup without obscuring the infrared beam used to detect food cup entries. During the BL test, there were no CS presentations and rats were free to eat from the food cup during the 5-minute period. The rats were then

briefly removed from the chamber, the remaining pellets were collected, and a fresh pre-weighed portion of pellets was placed in the food cup (~2–2.5 g). Rats were then returned to the chamber for the CS test (5 min). During this test, rats were given two 90 s CS presentations, the first occurred 30 s into testing, and the 2nd occurred 60 s after the first presentation; an additional 30 s of post- CS responding was recorded after the final CS presentation. Our testing procedure was adapted from Galarce et al. (2007) which used a 5-minute test with 2 CS presentations. However, here we shortened the duration of CS to 90 s in order to enhance our ability to observe conditioned approach, while maintaining a 5 min total testing time. After testing, rats were then returned to their home cage and the pellets remaining in the food cup were collected and weighed. Consumption was determined by measuring the difference in weight of the pellets remaining from pellets provided during each phase of testing. In addition, food cup entries were measured throughout.

2.5: Extinction and reinstatement test

During testing borderline effects of JF consumption were found in the Zero Contingency and 50% Positive Contingency groups, which led us to probe this effect further using an extinction/reinstatement protocol. Here, rats underwent 4 days of extinction training; this was identical to their original Pavlovian conditioning, except no USs were delivered. Next, they were tested for reinstatement of conditioned approach to the food cup. In this test, rats received one CS-US pairing at the start of the session (reinstatement of CS-US association), followed by 4 presentations of the CS alone. Food cup entries were recorded throughout extinction and reinstatement testing.

2.6: Progressive ratio testing

Testing under a progressive ratio schedule of reinforcement was used to determine whether JF consumption altered motivation for the US. Rats were trained on a Fixed Ratio-1 (FR1) to press an active lever to earn the same Bioserv pellet US from Pavlovian conditioning (3, 20-min sessions; 1 session/day). Following FR1 training, rats were tested on a progressive ratio (PR) schedule in which the ratio of lever presses required to earn a pellet was progressively increased using the following formula: response ratio [=5e$^{(US}$

$^{delivery \times 0.2)}$]-5 (as in Richardson and Roberts, 1995). The PR test sessions closed after either 30 min passed without any active lever response or the total session length reached 3 h. Lever presses, food cup entries, and the final ratio achieved (i.e., breakpoint) were recorded.

2.7: Statistics

Statistical analyses were performed using GraphPad Prism (Version 7.0c) and included: unpaired and paired t-tests, and one-way and two-way RM ANOVAs. Sidak's and Dunnett's multiple comparisons were used for post-hoc and planned comparisons.

3: Design of individual experiments

3.1: Experiment 1: Effects of limited junk-food consumption

In Experiment 1, four separate groups of rats were trained, each with a distinct CS-US contingency. The purpose here was to determine whether subsequent consumption of a JF diet resulted in effects specific to the initial predictive nature of the CS, or general effects that were not unique to prior learning. Therefore, during Pavlovian conditioning we systematically varied the predictive validity of CS [p(US|CS) - p(US|ITI)] between groups from 100% negative to 100% positive. Specifically, in the 100% Negative Contingency group (n=18), the predictive validity of the CS was set to −1.0, thus US deliveries occurred only during the ITI and never during the CS. In the Zero Contingency group (n=20), the predictive validity of the CS was set to 0.0, where US deliveries occurred with equal probability during both the CS and the ITI period. In the 50% Positive Contingency group (n=20), the predictive validity of the CS was set to +0.5, with US deliveries occurring on half the CS trials and never during the ITI. Finally, for the 100% Positive Contingency group (n=42), the predictive validity of the CS was set to +1.0, such that USs were delivered during every CS trial and never during the ITI. In each of these training groups US deliveries were presented on a VT schedule within the appropriate delivery windows. (see Rescorla, 1968 for discussion of the effects of CS-US contingency on conditioned responding.) Following Pavlovian conditioning, half the rats in each training contingency group were given free access to chow or JF in their home cage (as described above,

Section 2.3) for 14 days before testing. During this period, food intake and bodyweight were recorded at least three times per week. During testing, we found trends for increases in conditioned approach in JF vs. Chow treated rats in the Zero Contingency and 50% Positive Contingency trained groups, which led us to further probe this effect using an extinction reinstatement protocol in these groups as described above (Section 3.4). In addition, potential effects of JF on motivation for the US were evaluated in these same rats using progressive ratio testing (Section 3.5).

3.2. Experiment 2: Effects of prolonged junk-food consumption and junk-food deprivation

For Experiment 2, two groups of rats underwent Pavlovian conditioning: a 100% Negative Contingency group (n=18) and a 100% Positive Contingency group (n=41) as described above (Section 3.1). Briefly, the 100% Negative contingency group only received pellets during the ITI and the 100% Positive Contingency group only received pellets in the presence of the CS. We then assigned rats to one of three diet manipulation groups: *Ad libitum* chow (Chow; 100% negative n=6, 100% Positive n=10), continuous junk-food (JF; 100% negative n=6, 100% Positive n=16) and junk-food deprivation (JF-Dep; 100% negative n=6, 100% Positive n=16) groups. Rats in the continuous JF group (JF) were given *ad libitum* access to junk-food in their home cage for a total of 45 days. Rats in the JF Deprivation group (JF-Dep) received *ad libitum* access to junk-food in the home cage for 30 days before junk-food was removed and replaced by free access to lab chow for 14 additional days. Importantly, groups were counterbalanced for performance during initial acquisition and weight. In addition, JF and JF-Dep groups were counterbalanced for food intake and weight gain across the first 30 days of diet access. Forty-five days following the final day of Pavlovian conditioning session and the start of diet exposure, rats were tested for cue potentiated feeding and conditioned approach as described above (Section 2.4). Thus, for rats in the JF-Dep group, testing occurred 14 days after their last day on the junk-food diet.

4: Results Experiment 1: Effects of CS-US contingency and 14 days of junk-food exposure

4.1: Pavlovian conditioning

For Experiment 1, 80 male Sprague Dawley rats were food restricted and then trained. The average weight prior to food restriction was 297.2 g ± 2.01 g. Fig. 1 shows the rate of food cup entries during Pavlovian conditioning. (Note that food cup responses were taken across the entire session and include responding in the presence of the US.) In the 100% Negative Contingency group (n=18), food cup entries during the ITI increased across sessions relative to entries during the CS− (Fig. 5.1A: two-way RM ANOVA; main effect of phase, $F_{(1,17)}$=13.91, p<0.01; main effect of session, $F_{(7,119)}$=7.26, p<0.01; phase by session interaction $F_{(7,119)}$=4.31, p<0.01), consistent with pellet delivery during the ITI period. In the Zero Contingency group (n=20), food cup entries were slightly, though significantly greater during CS0 presentations than during the ITI, and the magnitude of this effect was fairly stable across training (Fig. 5.1B: two-way RM ANOVA; main effect of phase, $F_{(1,19)}$=120.5, p<0.01; main effect of session, $F_{(7,133)}$=7.26, p<0.01; phase by session interaction, $F_{(7,133)}$=4.8, p<0.01). In the 50% Positive contingency group (n=20) the rate of food cup entries during CS+ presentation increased significantly across sessions compared to the ITI (Fig. 5.1C: two-way RM ANOVA; main effect of phase, $F_{(1,19)}$=399.9, p<0.01; main effect of session, $F_{(7,133)}$=22.15, p<0.01; phase by session interaction, $F_{(7,133)}$=38.8, p<0.01). Likewise, in the 100% Positive contingency group (n=42) food cup entries during the CS+ relative to entries during the ITI increased significantly across training (Fig. 5.1D: two-way RM ANOVA; main effect of phase, $F_{(1,41)}$=582.1, p<0.01; main effect of session, $F_{(7,287)}$=12.15, p<0.01; phase by session interaction, $F_{(7,133)}$=63.7, p<0.01). Although measured in the presence of US deliveries, the progressive increases in the magnitude of responding during the CS period are consistent with the development of conditioned approach to the CS+ in the Positive Contingency groups. In addition to these within-subject assessments, we also compared the rate of food cup entries during the ITI and CS across all the training groups. As expected, the rate of food cup entries during CS presentations varied systematically by training contingency (Fig. 5.1E: two-way RM ANOVA; main effect of group, $F_{(3,96)}$=108.4, p<0.01; main effect of session, $F_{(7,672)}$=16.28, p<0.01). Specifically, the rate of responding

during the CS period was greatest in the 100% Positive Contingency group, whereas the rate in the 50% Positive Contingency group was greater than in the Zero and 100% Negative Contingency groups, and lastly the rate in the Zero Contingency group was greater than in the 100% Negative Contingency group. These group effects became more pronounced across sessions (Fig. 5.1E: two-way RM ANOVA; group x session interaction, $F_{(21,672)}=16.75$, $p<0.01$).

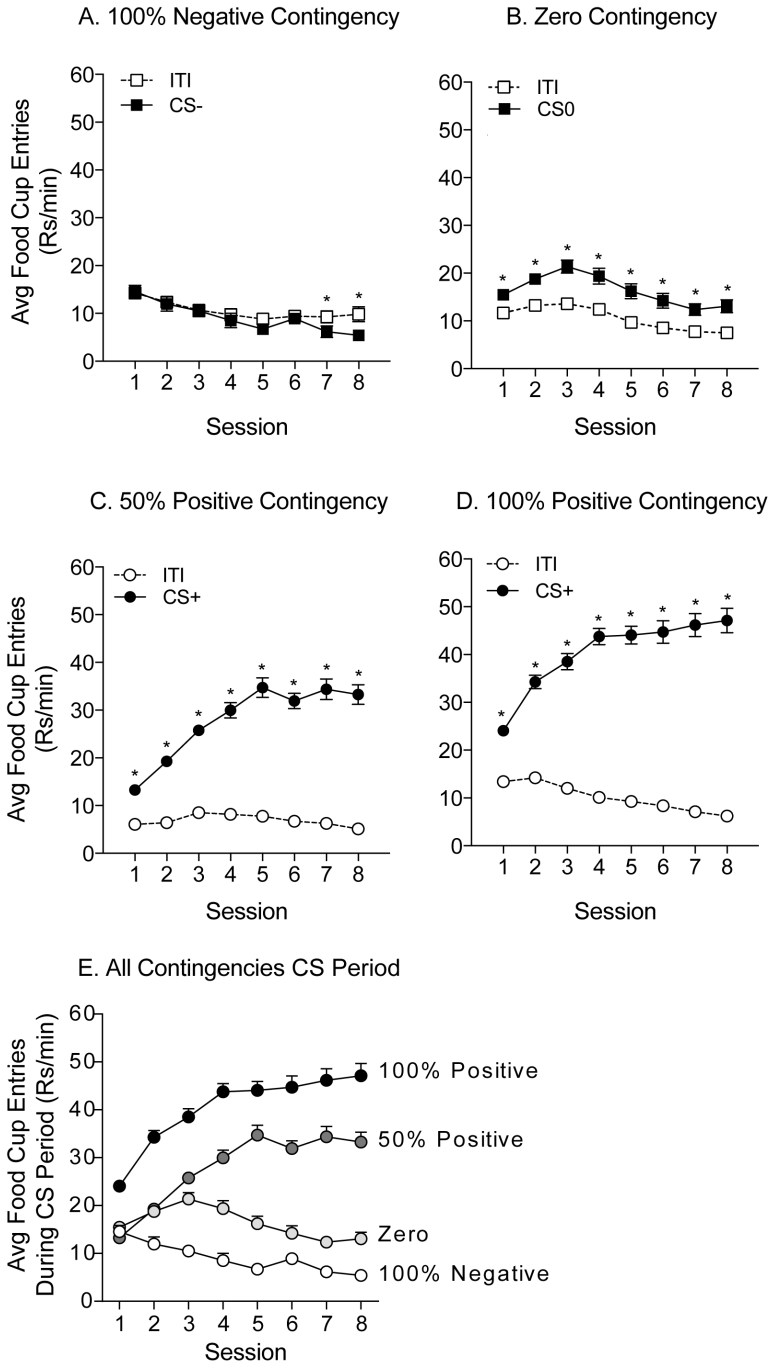

Figure 5.1: Pavlovian conditioning, Experiment 1: Average food cup entries during the ITI and CS presentations across conditioning sessions are shown for each contingency group (±SEM). **A)** 100% Negative Contingency: pellets given during ITI, but never during CS presentations (CS−). By the end of conditioning, entrances into the food cup were greater during the ITI than CS period. **B)** Zero Contingency: pellets given with equal probability during the ITI and the CS period (CS0). Food cup entries were higher during the CS0 throughout conditioning. **C)** 50% Positive Contingency: pellets given on 50% of the CS presentations (CS+) and never during the ITI. The rate of entries during the CS increased across sessions. **D)** % Positive Contingency: pellets given with every CS presentation (CS+) and never during the ITI. The rate of entries during the CS increased across sessions, while entries during the ITI decreased. **E)** Average food cup entries during the CS across conditioning sessions for each contingency group (±SEM). The rate of responding systematically increases as the predictive validity of the CS increases. *=post-hoc comparisons, ITI vs. CS period, $p<0.05$.

The result above, include responding that occurred in the presence of the US, and thus are not a direct measure of conditioned approach per se. Therefore, we also evaluated the latency to approach the food cup following CS onset during the last 4 sessions of Pavlovian conditioning. As expected, the latency to enter the food cup

following CS onset was significantly greater in the 100% Negative Contingency group relative to all other groups, but did not differ between the remaining groups (data not shown: two-way RM ANOVA; main effect of group, $F_{(3,96)}=44.46$, $p<0.01$; main effect of session, $F_{(3,288)}=4.14$, $p<0.01$; significant group by session interaction, $F_{(9,288)}=4.06$, $p<0.01$).

4.2: Post-training junk-food consumption

Following training, rats were relieved from food restriction and split into Chow and JF treatment groups matched for weight and behavior during conditioning (behavioral counterbalancing, data not shown: two-way RM ANOVA; no effects of diet assignment Chow vs. JF groups: 100% Negative Contingency: $p=0.44$; Zero Contingency: $p=0.49$; 50% Positive Contingency: $p=0.75$; 100% Positive Contingency: $p=.24$). Food intake for both Chow and JF groups began relatively high, but then dropped over time (Fig. 5.2: two-way RM ANOVA; main effect of day, $F_{(13,394)}=53.54$, $p<0.01$). This is likely due to a refeeding effect following chronic food restriction during training. In addition, the JF group consumed significantly more calories compared to Chow group across all days (Fig. 5.2: two-way RM ANOVA; main effect of diet, $F_{(1,38)}=45.63$, $p<0.01$; significant day by group interaction, $F_{(13,494)}=6.45$, $p<0.01$).

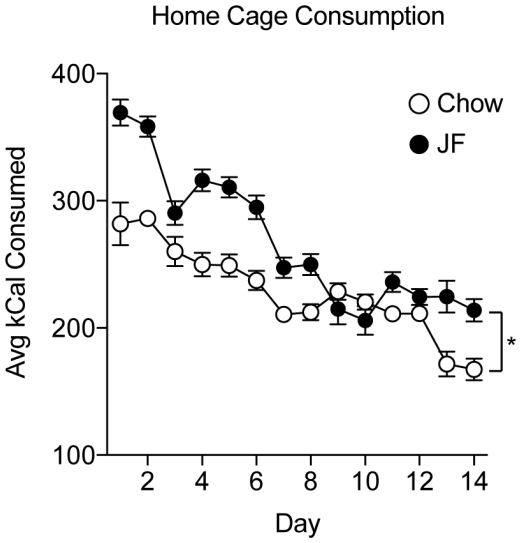

Figure 5.2: Average (±SEM) daily consumption of JF is greater than consumption of Chow during the post-training period of *ad libitum* access. *=p<0.05

4.3: Effects of CS-US contingency and junk-food on cue potentiated feeding and conditioned approach

After 14 days on their respective diets, rats were selectively sated on the US from training (Bioserv pellets) for 1h and then tested for cue potentiated feeding and conditioned approach in the operant chambers. To evaluate the effect of CS presentations on pellet consumption we calculated the proportion of pellets consumed during the CS test relative to the baseline period (CS test/[baseline+CS test]). Cue potentiated feeding differed by training contingency, but did not differ between diet treatment groups (Data not shown: two-way ANOVA; main effect of training, $F_{(3,71)}$=2.91, p=0.04; no effect of diet, p=0.53; no diet by training interaction, p=0.60). Therefore, we collapsed across Chow and JF groups and determined the relationship between initial CS-US contingency and cue potentiated feeding. We found that only the 50% Positive Contingency group exhibited significantly increased pellet consumption in the presence of the CS compared to the 100% Negative Contingency group (Fig. 5.3: one-way ANOVA; main effect of training, $F_{(3,74)}$=2.71, p=0.05; Dunnett's, $q_{(74)}$=2.43, p=0.05). Comparisons here were made back to the 100% Negative Contingency, rather than the Zero Contingency group, because rats trained in the Zero Contingency groups acquired low levels of conditioned approach (see Figs. 5.1B and 5.4B).

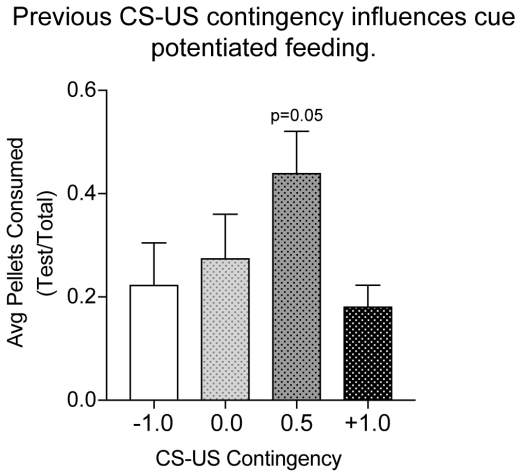

Figure 5.3: Test for cue potentiated feeding: Average proportion of pellet consumption during CS presentations (±SEM). No effects of JF were found, so data are collapsed across JF and Chow groups. Modest cue potentiated feeding was found only in the 50% Positive Contingency group (+0.5) compared to the 100% Negative Contingency group (−1.0).

We next determined whether food cup entries during CS presentation varied as a function of previous training contingency and JF consumption. JF consumption significantly increased conditioned approach relative to Chow groups in rats initially trained with a 50% or 100% Positive CS-US contingency. Specifically, in the 100%

Negative Contingency groups, the rate of food cup entries did not differ between the ITI and the CS, nor was there an effect of diet on this behavior (Fig. 5.4A: two-way RM ANOVA; no effect of phase, p=0.23; no effect of diet, p=0.91; no phase by diet interaction, p=0.20). We also performed planned comparisons between the CS and ITI response rates for each diet treatment alone and neither diet treatment group showed greater responding during the CS vs. ITI (Fig. 5.4A: Sidak's, ITI vs. CS, Chow: p=0.99; JF: p=0.17). In the Zero Contingency groups overall rates of food cup entries did not differ by diet, however the rate of food cup entries was slightly higher during CS presentations than during the ITI (Fig. 5.4B: two-way RM ANOVA; no effect of diet, p=0.39; main effect of phase, $F_{(1,18)}$=13.2, p<0.01; no phase by diet interaction, p=0.61). However, planned comparisons of responding during the ITI vs. CS in each treatment group revealed that this effect was driven primarily by the behavior of the JF treatment group (Fig. 5.4B: Sidak's, ITI vs. CS, Chow: p=0.12; JF: $t_{(18)}$=3.28, p<0.01). Overall, conditioned approach behavior was not dramatically different between JF and Chow treatment groups that previously received 100% Negative or Zero Contingency Pavlovian conditioning.

Behavior differed dramatically in the Positive Contingency groups. Specifically, the 50% Positive Contingency groups showed clear conditioned approach, making significantly more food cup entries during the CS vs. ITI phase of testing (Fig. 5.4C: two-way RM ANOVA; main effect of phase, $F_{(1,18)}$=43.4, p<0.01). In addition, there was a modest effect of diet, such that responding in the JF treated group was elevated compared to the Chow group (Fig. 5.4C: main effect of diet, $F_{(1,18)}$=3.99, p=0.06; no phase by diet interaction, p=0.30). Similarly, the 100% Positive Contingency groups also showed strong conditioned approach (Fig. 5.4D: two-way RM ANOVA; main effect of phase, $F_{(1,26)}$=40.77, p<0.01). Furthermore, this effect was strongly enhanced in JF vs. Chow groups (Fig. 5.4D: two-way RM ANOVA; main effect of diet, $F_{(1,26)}$=19.64, p<0.01; significant phase by diet interaction, $F_{(1,26)}$=5.08, p=0.03). Post hoc analysis of the phase by diet interaction revealed that JF selectively increased entries in the presence of the CS, whereas ITI rates did not differ (Fig. 5.4D: Sidak's, Chow vs. JF, ITI: p=0.25; CS: $t_{(52)}$=4.72, p<0.01). A summary of the relationship between training and diet treatment on conditioned approach is shown in Fig. 5.4E. This illustrates the progressive increase of conditioned approach behavior as a function of CS-US contingency in both groups. In

addition, JF treated rats exhibit stronger rates of responding and this effect is most pronounced with the fully predictive CS (Fig. 5.4E: two-way RM ANOVA; main effect of diet, $F_{(1,72)}$=6.28, p=0.01; main effect of CS-US contingency, $F_{(3,72)}$=6.20, p<0.01; no diet by CS-US interaction p=0.50). Importantly, bodyweights did not differ between Chow and JF groups prior to initial training or on the day of testing, although bodyweights did increase across time, consistent with normal growth (Fig. 5.4F: two-way RM ANOVA; no effect of diet treatment group, p=.48; main effect of time, $F_{(1,78)}$=2096, p<0.01; no diet by time interaction, p=0.52). Thus, effects of JF on conditioned approach described above occurred in the absence of any significant weight differences between Chow and JF groups. In summary, cue potentiated feeding varied by initial Pavlovian training contingencies, but was not strongly affected by diet. In contrast, there was a positive relationship between the expression of conditioned approach and the strength of initial CS-US relationships which was enhanced by JF consumption only in those rats that had received 50% or 100% Positive Contingency training.

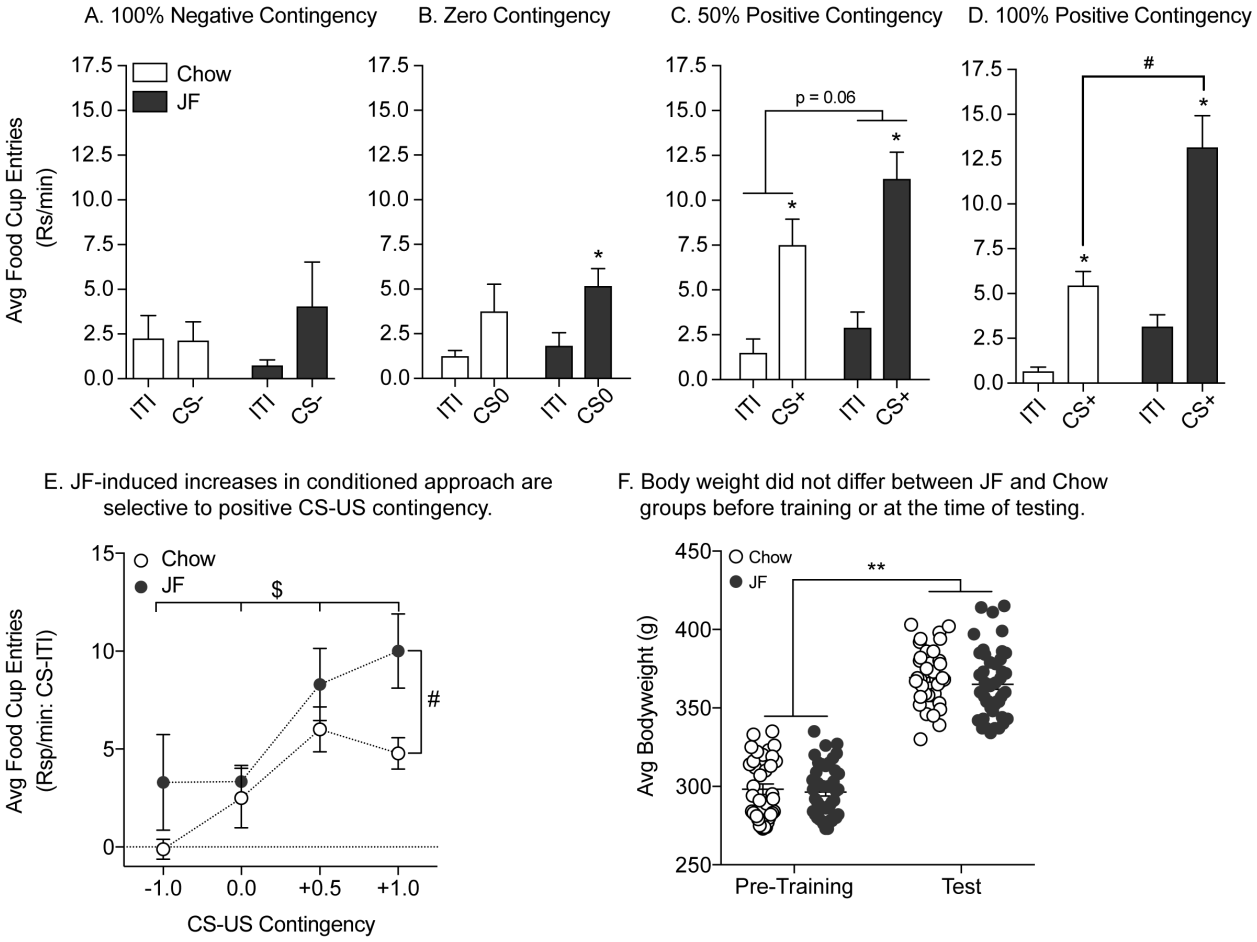

Figure 5.4: Test for cue potentiated feeding and conditioned approach: Average rate of food cup entries (±SEM) during the ITI and CS periods. **A)** In the Negative Contingency groups, rates of food cup entries were similar during the ITI and CS− period, and there was no effect of JF. **B)** In the Zero Contingency groups, CS0 presentation resulted in modest increases in food cup entries relative to the ITI in the JF but not Chow group. **C)** In the 50% Positive Contingency groups, the CS+ elicited conditioned approach in both JF and Chow groups, with significantly greater rates of food cup entries during the CS vs. ITI. In addition, responding was modestly enhanced in the JF vs. Chow group. **D)** In the 100% Positive Contingency groups, the CS+ elicited conditioned approach in both groups, and this effect was enhanced in the JF vs. Chow group. **E)** Summary of conditioned approach in chow and JF groups across CS-US contingency. Conditioned approach varied as a function of CS-US contingency in both groups and JF consumption enhanced conditioned approach to the 100% Positive CS+. **F)** Average body weight (±SEM) for Chow and JF groups. Weight did not differ between groups either prior to initial conditioning, nor at the time of testing. All rats gained significant weight between pre-training and testing. *=planned comparisons, CS vs. ITI, $p<0.05$; #=main effect of diet, $p<0.05$; $=main effect of training; **=main effect of time, $p<0.05$.

4.4: Effects of junk-food on extinction and reinstatement testing

After the testing described above, the same rats from the Zero and 50% Positive Contingency groups then underwent 4 sessions of extinction training followed by reinstatement testing in order to further probe the trends toward an effect of JF on conditioned approach outside of the cue potentiated feeding testing procedure. As expected, in the Zero Contingency groups, food cup entries during the CS and during the ITI were relatively low and were reduced even further by extinction training (Fig. 5.5A: two-way RM ANOVA; Chow group, main effect of session, $F_{(3,21)}$=14.88, p<0.01; main effect of phase, $F_{(1,7)}$=45.7, p<0.01; no session by phase interaction, p=0.12; JF group, main effect session, $F_{(3,33)}$=14.05, p<0.01; main effect of phase, $F_{(1,11)}$=10.53, p<0.01; no session by phase interaction, p=0.10). In addition, behavior between Chow and JF groups was similar across extinction training (Fig. 5.5A: two-way RM ANOVA; ITI: no effect of diet, p=0.55; no diet by session interaction, p=0.67; CS: no effect of diet, p=0.12; no diet by session interaction, p=0.71). In addition, planned comparisons between CS and ITI responding, revealed that by the final session CS responding was not different than ITI for both groups (Sidak's, ITI vs. CS, Chow group: p=0.21; JF group: p=0.72). In the 50% Positive Contingency groups responding to the CS dropped significantly over the four sessions of extinction, and again there were no differences between Chow and JF treatment groups (Fig. 5.5B: two-way RM ANOVA; Chow group, main effect of session, $F_{(3,27)}$=37.46, p<0.01; main effect of phase, $F_{(1,9)}$=37.62, p<0.01; significant phase by session interaction, $F_{(3,27)}$=10.04, p<0.01; JF group, main effect of session, $F_{(3,27)}$=20.69, p<0.01; main effect of phase, $F_{(1,9)}$=171.1, p<0.01; significant phase by session interaction, $F_{(3,27)}$=8.11, p<0.01; Fig. 5.5B: two-way RM ANOVA; ITI, no effect of diet, p=0.70; no diet by session interaction, p=0.36; CS, p=0.68; no diet by session interaction, p=0.77). Importantly, conditioned approach behavior did not fully extinguish in the 50% Positive Contingency groups (Fig. 5.5B: Sidak's, ITI vs. CS, Chow group: $t_{(27)}$=5.43, p<0.01; JF group: $t_{(27)}$=3.75, p<0.01). This was deliberate, as we chose to implement limited extinction training, so as not to completely abolish the CS-US association (see Delamater et al., 2017 for discussion).

Twenty-four hours after the last extinction session, rats were brought back to the operant boxes and given one paired presentation of the CS and US at the start of test

session followed by 4 presentations of the CS alone. As expected, in the Zero Contingency group one CS-US pairing was not sufficient to enhance food cup entries during the CS relative to the ITI, nor did behavior differ between diet treatment groups, although ITI responding did increase following extinction training (Fig. 5.5C: two-way RM ANOVA; main effect of phase, $F_{(3,54)}=4.42$, $p=0.01$; no effect of diet, $p=0.87$; no phase by diet interaction, $p=0.87$). In the 50% Positive Contingency groups, one CS-US pairing was not sufficient to increase conditioned approach behavior above levels reached on the final day of extinction (i.e., to induce reinstatement; Fig. 5.5D: two-way RM ANOVA; main effect of phase, $F_{(3,54)}=19.44$, $p<0.01$; no effect of diet, $p=0.19$; diet by phase interaction, $F_{(3,54)}=2.38$, $p=0.08$). However, planned comparisons examining responding during the ITI vs. CS revealed that during testing conditioned approach was absent in the Chow group, but was maintained in the JF group (Fig. 5.5D: Reinstatement Test: Sidak's CS vs. ITI; Chow Group: $p=.24$; JF Group: $t_{(54)}=4.28$, $p < .01$). Thus, although we did not observe a true reinstatement effect in either group, JF experienced rats nonetheless maintained conditioned approach during the reinstatement test, whereas the Chow group did not. This is consistent with conditioned approach behavior during the cue potentiated feeding test and suggests that JF not only enhances the magnitude, but may also enhance the persistence of conditioned approach.

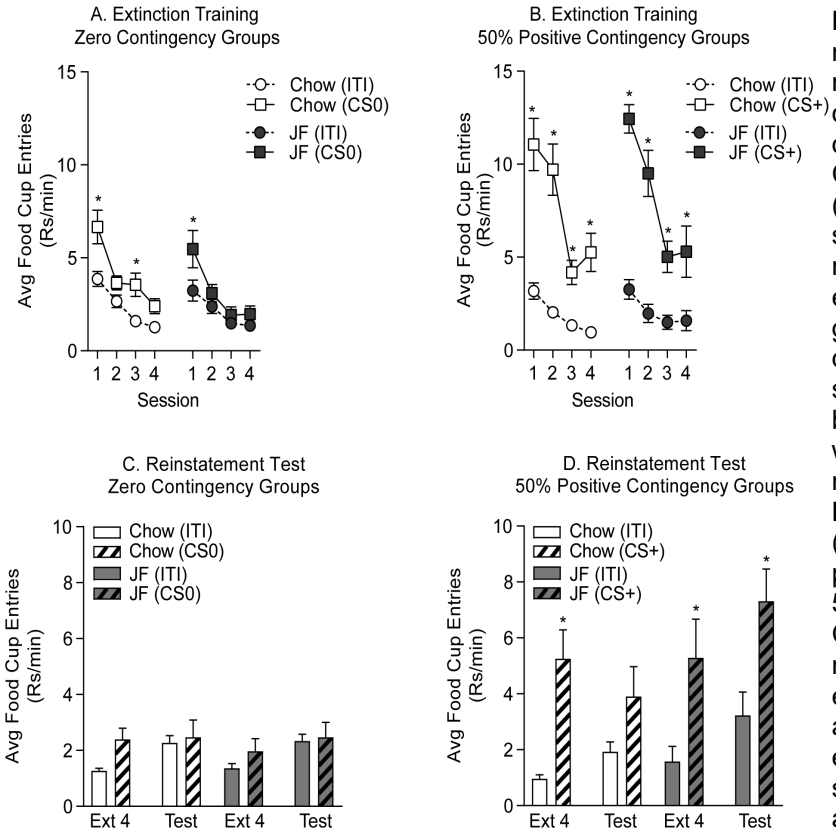

Figure 5.5: Extinction training and reinstatement testing. **A)** Average rate of food cup entries (±SEM) during the ITI and CS0 period during extinction training for Zero Contingency groups. Both Chow (open symbols) and JF (closed symbols) groups decreased responding across the four extinction sessions. In the Chow group, differential responding during the CS0 vs. ITI persisted slightly longer than the JF group, but by the fourth session there were no differences in the rate of responding during the CS and ITI. **B)** Average rate of food cup entries (±SEM) during the ITI and CS+ period during extinction training for 50% Positive Contingency groups. Chow and JF groups significantly reduced the rate of food cup entries during CS+ presentations across extinction. The degree of extinction achieved by the fourth session was similar between Chow and JF groups. **C)** Average rate of food cup entries (±SEM) during the final extinction session and during reinstatement testing (i.e., following one CS-US pairing) in the Zero Contingency groups. Conditioned approach was not observed during reinstatement testing in either the Chow or JF groups with prior zero contingency training. **D)** Average rate of food cup entries (±SEM) during the final extinction session and during reinstatement testing in the 50% Partial Contingency groups. One CS-US pairing did not fully reinstate responding in either Chow or JF groups, although conditioned approach was absent in the Chow group, but maintained in the JF group during reinstatement testing. *=planned comparisons, CS vs. ITI, $p<0.05$.

4.5. Effects of junk-food on instrumental responding for the US

Data above show that JF consumption enhances conditioned responding to food cues, but we had not examined the effect of this diet on motivation for the US itself. Therefore, rats from the 50% Positive Contingency groups were used to examine instrumental responding for the US. Across three sessions of FR1 training, Chow and JF groups increased their rates of active lever responding while decreasing their inactive lever responding (Fig. 5.6A: three-way RM ANOVA; main effect of lever, $F_{(1,108)}=153.9$, $p<0.01$; no effect of session, $p=0.14$; session by lever interaction, $F_{(2,108)}=6.28$, $p<0.01$). Active lever responding in the Chow group was modestly elevated by the end of training compared to the JF group (Fig. 5.6A: three-way RM ANOVA; main effect of diet, $F_{(1,108)}=3.43$, $p=0.07$; main effect of lever, $F_{(1,108)}=153.90$, $p<0.01$; lever by diet interaction,

$F_{(1,108)}$=3.73, p=0.06; no session by diet interaction, p=0.48; no session×lever×diet interaction, p=0.54). Next, rats were shifted to a progressive ratio schedule to determine if JF treatment altered the willingness to work for US deliveries. The Chow group achieved significantly higher breakpoints across three sessions compared to the JF group (Fig. 4B: two-way RM ANOVA; main effect of diet, $F_{(1,18)}$=12.17, p<0.01; no effect of session, p=0.21; no diet×session interaction, p=0.79). In concordance with this result, total active lever responses were greater in the Chow group than in the JF group (Fig. 5.6C: three-way RM ANOVA; main effect of diet, $F_{(1,108)}$=27.17, p<0.01; main effect of lever, $F_{(1,108)}$=87.26, p<0.01; no effect of session, p=0.48; diet by lever interaction, $F_{(1,108)}$=15.57, p<0.01; no diet by session interaction, p=0.81; no session by lever interaction, p=0.45; no session×lever×diet interaction, p=0.97). Moreover, planned comparisons between active v inactive lever responding revealed that, repeated progressive ratio testing in the JF group resulted in a loss of preferential active lever responding on the third test, whereas in the Chow group active lever responding remained significantly higher than inactive responding throughout testing (Fig. 5.6C: Sidak's, active vs. inactive, JF group: p>0.10; Chow group: $t_{(108)}$=4.87, p<0.01). Thus, although incentive motivational responses to the CS were enhanced by JF consumption, the willingness to work for the US itself was reduced. This result is consistent with well-established behavioral and neural dissociations between preparatory and consummatory aspects of feeding (see also discussion, Section 6).

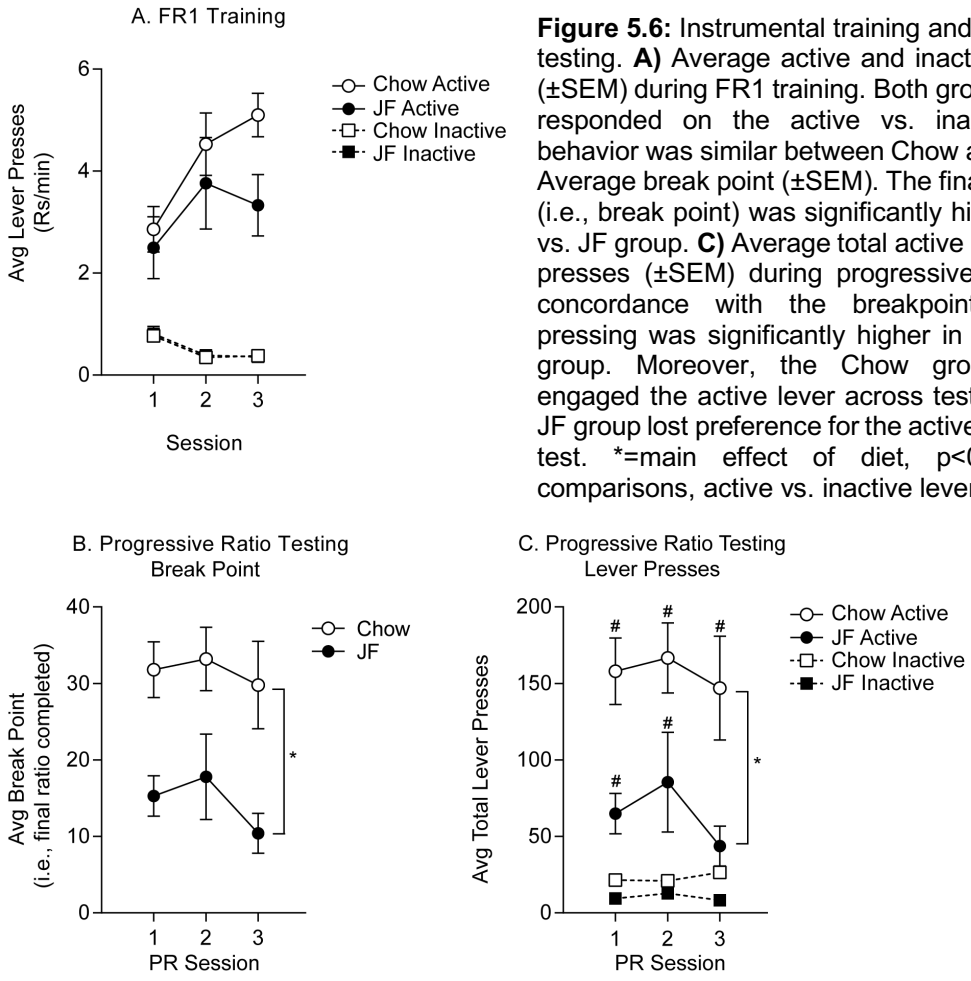

Figure 5.6: Instrumental training and progressive ratio testing. **A)** Average active and inactive lever presses (±SEM) during FR1 training. Both groups preferentially responded on the active vs. inactive lever, and behavior was similar between Chow and JF groups**. B)** Average break point (±SEM). The final ratio completed (i.e., break point) was significantly higher in the Chow vs. JF group. **C)** Average total active and inactive lever presses (±SEM) during progressive ratio testing. In concordance with the breakpoints, active lever pressing was significantly higher in the Chow vs. JF group. Moreover, the Chow group preferentially engaged the active lever across testing, whereas the JF group lost preference for the active lever by the third test. *=main effect of diet, $p<0.05$; #=planned comparisons, active vs. inactive levers, $p<0.05$.

5: Results Experiment 2: Effects of prolonged junk-food exposure, with and without junk-food deprivation

The purpose of this experiment was to determine whether prolonging the JF consumption period, with and without a JF deprivation period, would produce enhancements in cue potentiated feeding and/or conditioned approach.

5.1: Pavlovian conditioning

Rats were trained either in the 100% Negative Contingency (n=18) or the 100% Positive Contingency (n=42) using the same procedure as in Experiment 1 above (average bodyweight: 340 g ± 2.53 g). As expected, in the 100% Negative Contingency group food cup entries were slightly higher during ITI than during the CS− (Fig. 5.7A: two-way RM ANOVA; 100% Negative Contingency group, main effect of phase, $F_{(1,17)}$=41.07, p<0.01; main effect of session, $F_{(7,119)}$=2.24, p=0.04; no phase by session interaction, p=0.13); whereas in the 100% Positive Contingency group, food cup entries were greater during the CS+ than during the ITI, with this difference increasing across conditioning sessions (Fig. 5.7B: two-way RM ANOVA; 100% Positive Contingency group, main effect of phase, $F_{(1,41)}$=821.9, p<0.01; main effect of session, $F_{(7,287)}$=19.7, p<0.01; phase by session interaction, $F_{(7,287)}$=75.51, p<0.01).

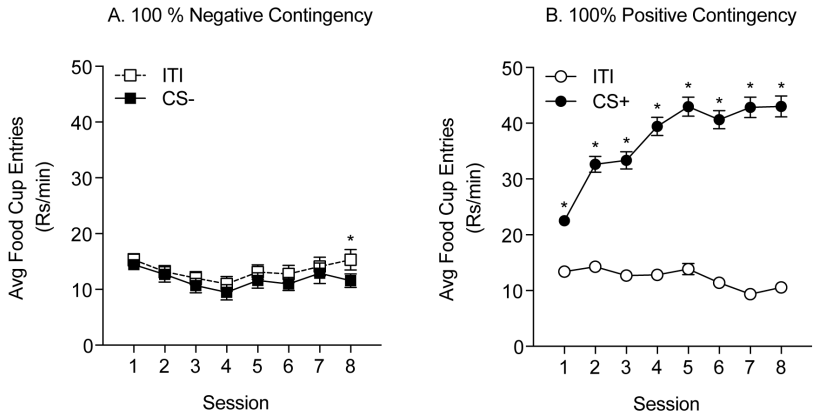

Figure 5.7: Pavlovian conditioning, Experiment 2: Average food cup entries during the ITI and CS presentations across conditioning sessions are shown for each contingency group (±SEM). **A)** 100% Negative Contingency: By the end of training, the rates of food cup entries during the ITI were modestly, though significantly higher than during the CS−. **B)** 100% Positive Contingency: The rate of food cup entries was greater during CS+ vs. ITI presentations, and this difference increased significantly across sessions. *=post-hoc comparisons, ITI vs. CS, p<0.05.

5.2. Post-training junk-food consumption and deprivation

Following initial training, rats were relieved from food restriction and assigned to either Chow (n=15), JF (n=22) or JF-Dep (n=22) diet treatment groups, counterbalanced for behavior during Pavlovian conditioning and weight. (Behavioral counterbalancing of these diet treatment groups, data not shown: two-way RM ANOVA; no effects of diet assignment 100% Negative Contingency group: $p=0.23$; 100% Positive Contingency group: $p=0.73$). Bodyweights in the Chow, JF Dep, and JF groups did not differ at any point throughout study (Fig. 5.8A: two-way RM ANOVA; no effect of diet, $p=0.44$; no diet by time interaction, $p=0.96$). Despite the lack of differences in body weight, daily kCal consumption differed between treatment groups. Specifically, during the first 4 weeks of treatment, caloric intake per cage was significantly higher in both the JF and JD-Dep groups relative to the Chow group, and consumption was similar between JF and JF-Dep groups (Fig. 5.8B: two-way RM ANOVA; main effect of diet treatment, $F_{(6,162)}=192.9$, $p<0.01$; main effect of time, $F_{(2,27)}=11.09$, $p<0.01$; diet by time interaction $F_{(12,162)}=21.31$, $p<0.01$). When rats in the JF-Dep group were put back on a chow only diet, caloric intake dropped dramatically in the first week and was significantly lower in the JD-Dep group compared to both Chow and JF groups (Fig. 5.8B, Week 6: Sidak's: JF-Dep vs. JF, $t_{(189)}=9.97$, $p<0.01$; JF-Dep vs. Chow, $t_{(189)}=2.86$, $p<0.01$). Examination of daily consumption during the JF-deprivation window is shown in Fig. 5.8C. The reduction in caloric intake in the JF-Dep group was immediately apparent after the removal from JF diet and persisted for 4 days (Fig. 5.8C: two-way RM ANOVA; main effect of diet treatment, $F_{(2,27)}=27.72$, $p<0.01$; diet by time interaction $F_{(32,432)}=24.06$, $p<0.01$). After this time, caloric intake between the Chow and JF-Dep groups did not differ (Fig. 5.8C: Day 5, Sidak's: JF-Dep vs, $p<0.31$).

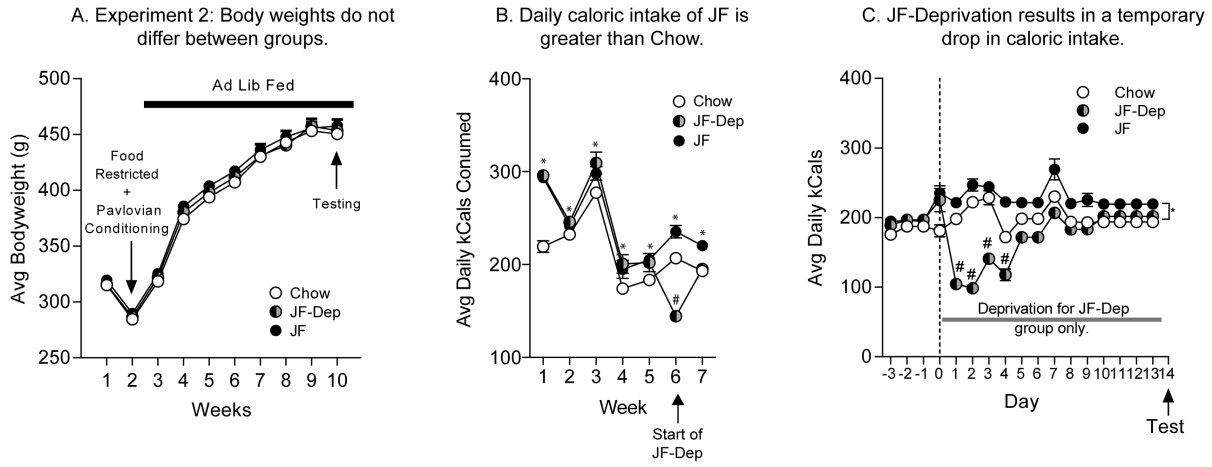

Figure 5.8: Body weight and caloric intake across time. **A)** Average weight (±SEM) prior to and after lifting food restriction. Bodyweights did not differ between the groups at any point in the experiment. **B)** Average daily caloric intake per cage per week (±SEM) across the *ad libitum* feeding period. Food intake started off high and dropped across time in all the groups. This is likely due to a refeeding effect in response to the previous food-restriction period. In addition, caloric intake of JF was greater than that of Chow. JF was available *ad libitum* during weeks 1–5 for both JF and JF-Dep groups. On week 6, JF was removed and replaced by lab chow for rats in the JF-Dep group. Caloric intake of the JF-Dep dropped significantly below both Chow and JF groups in the week following JF deprivation. Chow intake in the JF-Dep then rose to levels comparable to the Chow group the following week. **C)** Average daily caloric intake per cage per day (±SEM). In the JF-Dep group, as soon as JF was replaced by chow (day 0), daily caloric intake dropped dramatically to below that of both JF and Chow groups. This self-imposed suppression of feeding slowly abated over time and returned to levels similar to the Chow group by day 5 of JF deprivation. *=post-hoc comparisons, JF vs. Chow, $p<0.05$; #=post-hoc comparisons, JF-Dep vs. Chow, $p<0.05$.

5.3: Effects of prolonged junk-food, with and without junk-food deprivation, on cue potentiated feeding and conditioned approach

Chow, JF and JF-Dep groups were then tested for cue potentiated feeding and conditioned approach as described above. Briefly, rats were selectively sated on Bioserv pellets for 1 h followed by baseline (BL) test and CS presentation test (CS test) in the operant chambers. Again, we did not find any effect of diet on cue potentiated feeding (Fig. 5.9A: two-way ANOVA; no effect of diet, $p=0.72$; no diet by training interaction, $p=0.78$). However, in this cohort tested 45 days after the final Pavlovian conditioning session, presentation of the CS significantly increased food intake in the 100% Positive Contingency groups (Fig. 5.9A: main effect of training, $F_{(1,54)}=4.00$, $p=0.05$).

Food cup entries during testing are shown in Fig. 5.9. Consistent with results from Experiment 1, in the Negative Contingency groups, the rate of food cup entries did not

differ between the ITI and the CS, nor was there any effects of diet on this behavior (Fig. 5.9B: two-way RM ANOVA; no effect of phase, p=0.12; no effect of diet, p=0.43; no diet by phase interaction, p=0.37). In contrast, in the 100% Positive Contingency groups all groups entered the food cup more during the CS relative to the ITI and this effect interacted with diet (Fig. 5.9C: two-way RM ANOVA; main effect of phase $F_{(1,39)}$=120.9, p<0.01; no effect of diet, p=0.31; phase by diet interaction, $F_{(2,39)}$=3.81, p=0.03). Moreover, post-hoc analysis revealed that food cup entries during the CS were significantly greater in the JF group compared to the Chow group, with no differences in ITI responding (Fig. 5.9C: Sidak's, Chow vs. JF, CS: $t_{(78)}$=2.60, p=0.02; ITI: p=1.00). Fig. 5.9D depicts the number of food cup entries above the ITI elicited by the CS in all groups (i.e., CS-ITI). This illustrates that responding is not altered by diet in the Negative contingency groups, but that JF consumption enhanced conditioned approach in the Positive Contingency groups, even after a period of JF deprivation (Two-tailed t-test: Chow vs. JF-Dep: $t_{(24)}$=1.96, p=0.06).

In sum, in rats tested 45 days after initial training (Exp. 2), a food CS with 100% predictive validity elicited cue potentiated feeding, whereas this potentiation effect was absent when testing was conducted 14 days after initial training (Exp. 1). Furthermore, results from Experiment 2 replicated JF induced enhancements in conditioned approach observed in Experiment 1. Additionally, the inclusion of the JF-Dep group extended these findings by demonstrating that enhancements in incentive motivation persisted after JF consumption has ceased.

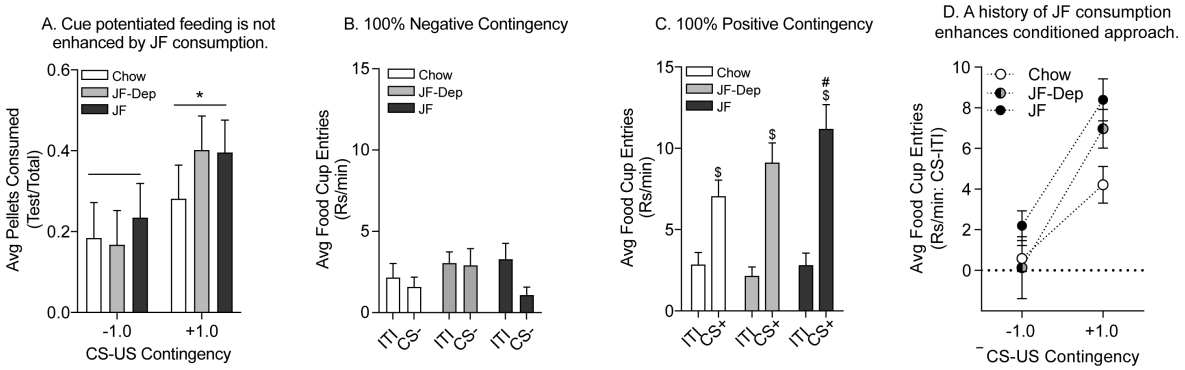

Figure 5.9: Test for cue potentiated feeding and conditioned approach, Experiment 2. **A)** Average proportion of pellet consumption during CS presentation (±SEM). In this cohort, rats in the 100% Positive Contingency groups showed cue potentiated feeding compared to 100% Negative Contingency groups. There was no significant effect of diet on this behavior. B–D) Average rate of food cup entries (±SEM) during the ITI and CS presentations. **B)** There were no effects of CS or diet manipulation on conditioned approach in the 100% Negative Contingency groups. **C)** All groups with prior 100% Positive Contingency training showed conditioned approach, with significantly greater magnitude of food cup entries during the CS+ vs. ITI. In addition, the rate of CS+ evoked food cup entries were significantly greater in the JF vs. Chow group. In the JF-Deprivation group, this effect of JF was modest, but not completely absent. **D)** Summary of conditioned approach in Chow, JF-Dep and JF groups across CS-US contingency (CS-ITI). In the 100% Negative contingency trained groups, CS evoked increases in the rate of food-cup entries did not differ across diet treatment groups (−1.0). In the 100% Positive Contingency trained groups (+1.0), JF enhanced conditioned responding, and this effect of JF was reduced, but not completely absent in the JF-Deprivation group. *=main effect of diet, $p<0.05$; $=main effect of phase, CS vs. ITI; #=post-hoc comparisons of CS+, Chow vs. JF, $p<0.05$.

6: Discussion

6.1: Overview

In the current study, we determined how limited and prolonged JF consumption alter cue potentiated feeding and conditioned approach in response to a previously established food paired CS. In addition, to establish the degree to which these behaviors and JF effects are influenced by the predictive validity of the CS, we included groups trained with distinct CS-US contingencies ranging from −1.0 to +1.0. We found that both cue potentiated feeding and conditioned approach varied as a function of CS-US contingency, with greater potentiation of feeding and conditioned approach induced by CSs that are more predictive of US delivery. Moreover, we found that both limited (14 day) and prolonged (45 day) JF consumption enhanced conditioned approach, but not cue potentiated feeding. Furthermore, this effect of JF on behavior was dependent on the previously established CS-US contingency. That is, consumption of JF following training

enhanced approach to the food cup only in response to CSs with previously positive predictive validity. This selective effect of JF on incentive motivation was further supported by maintenance of conditioned responding in the JF vs. Chow group following extinction and reinstatement testing. Interestingly, these JF induced increases in incentive motivational responses to the CS occurred alongside reduced motivation for the US itself assessed by progressive ratio testing. Together, these data show that JF consumption in the absence of overt obesity selectively enhances incentive motivational responses to previously established food CSs, without dramatically altering consumption induced by these same CSs. In addition, they extend our understanding of how CS-US contingency influences cue potentiated feeding.

6.2: Effects JF consumption and CS-US contingency on cue potentiated feeding

During initial Pavlovian conditioning, we varied the CS-US contingency from −1.0 to +1.0. We then formed Chow and JF groups counterbalanced by weight and initial performance during conditioning. Rats were tested for cue potentiated feeding and conditioned approach after either 14 (Exp. 1) or 45 (Exp. 2) days of free access to chow or JF, or 30 days of JF followed by 14 days of JF-deprivation (Exp. 2). We did not find any effect of JF on cue potentiated feeding, either when JF was given continuously for 14 or 45 days, or following JF-deprivation. The lack of an effect of JF cannot be explained by differences in the amount of food consumed during selective satiation, as JF and Chow groups ate similar amounts during this period. Moreover, pellet consumption during the 5-minute baseline test period, which served as the comparison for determining the cue potentiated feeding effect, was similar between all groups. Thus, the data indicate that all rats ate to a level of similar satiety prior to CS presentation.

To our knowledge, this is the first study to examine the effect of CS-US contingency (i.e., the predictive validity of the CS) on cue potentiated feeding. We found cue potentiated feeding in rats trained with a partially predictive CS (Exp 1), a fully predictive CS (Exp 2), but not to neutral CS (Exp 1). These data are important because they establish that increased consumption in response to CS presentation is not an all or none phenomenon, but is related to the nature of the relationship between the CS and US. In addition, in rats tested 45 days after initial training (Exp. 2), a food CS with 100%

predictive validity elicited cue potentiated feeding, whereas this potentiation effect was absent when testing was conducted 14 days after initial training (Exp. 1). Although speculative, this suggests that the expression of cue potentiated feeding may increase as a function of time since initial Pavlovian conditioning. This phenomenon is reminiscent of "incubation of craving" effects reported for both food and drug reinforcers, in which "cue-triggered reward- seeking" intensifies as a function of time since the last instrumental self-administration session (Lu et al., 2004). However, additional studies directly examining the effect of time off on cue potentiated feeding are needed, as comparisons between day 14 and 45 testing are limited here as these were conducted in completely separate experiments.

The absence of a cue potentiation of feeding effect in the 100% Positive Contingency groups found in Experiment 1 was unexpected. However, it is worth noting that although there is a significant amount of variation in the procedures used to study cue potentiated feeding, most have implemented a within-subjects design that inherently incorporates discrimination learning (e.g., Galarce et al., 2007; Holland et al., 2002). Thus, in previous studies the same rats were trained and tested with a CS+ and a CS-, rather than the between-subjects design used here. Therefore, it is possible that conditioning using a within-subject CS+/CS− discrimination, rather than the between-subjects approach used here, may have better engaged sensory specific learning about the CS+ which in turn may have enhanced the consummatory drive acquired by the CS+ (for discussion of enhanced discrimination learning see Delamater, 1998; Delamater, 2012). This raises interesting questions about the impact of initial learning on the expression of cue potentiated feeding that should be addressed in future.

In Experiment 1, the 50%, but not 100% predictive CS resulted in modest cue potentiated feeding effect (Fig. 5.2). This may have been due to the amplification of incentive salience induced by uncertainty of the partially predictive CS. Indeed, uncertainty enhances specific aspects of incentive salience of Pavlovian conditioned stimuli (Anselme et al., 2013; Boakes, 1977). While the process mediating the attribution of incentive motivational properties to CSs may be distinct from the mechanism driving cue potentiated feeding, it is possible that they share a common sensitivity to uncertainty. That is to say, uncertainty may also enhance the degree to which a CS is imbued with

consummatory drive in a manner that is similar to its effects on incentive motivation. When considering the role of uncertainty in consummatory behaviors, such as foraging uncertainty has been consistently shown to potentiate feeding behaviors (Anselme et al., 2017; Forkman, 1993). Thus, uncertainty inherent in the 50% Positive Contingency training conditions may have amplified the cue potentiated feeding effect by sensitizing the consummatory drive of the CS.

6.3: Effects of JF consumption and CS-US contingency on conditioned approach

In addition to examining cue potentiated feeding, we also determined how JF and the CS-US contingency affected conditioned approach to the food cup during this same test session (food cup entries during the CS vs. the ITI). In two separate sets of experiments we found that JF consumption enhanced conditioned approach. Specifically, when JF was given continuously for 14 days the magnitude of conditioned approach was significantly increased relative to Chow groups (Fig. 5.1F). In addition, this effect of JF scaled according to previously established CS-US contingencies such that CSs with stronger predicative validity elicited a greater magnitude of conditioned approach in JF groups (Fig. 5.1B-E). Thus, JF did not simply enhance general responding to stimuli; rather JF consumption selectively increased incentive motivation for CSs tightly linked to US delivery. That is, the relative predictive value of a given CS interacted with JF consumption to impact the magnitude of subsequent cue-triggered motivational responses. This suggests that JF consumption may result in amplification of the incentive value of CSs that are more consistently paired with palatable food. Of course, whether the enhancing effect of JF is limited to food CSs that predict palatable foods or may extend to all food CSs generally is an outstanding question that will need further investigation.

6.4: Dissociable effects of JF on conditioned approach vs. cue potentiated feeding

One intriguing aspect of the current results is that although JF amplified conditioned approach in all groups trained on a positive contingency (Exp 1 and 2), CS+ presentation did not enhance cue potentiated feeding in JF vs. Chow treated rats. In the testing procedure, the food pellets are present inside the food cup throughout testing. Thus, although rats in the JF groups were entering the food cup more frequently in

response to the CS+ than rats in the Chow groups, this failed to translate into enhanced consumption. While direct comparisons between conditioned approach and cue potentiated feeding have not been conducted, lesion studies have demonstrated that cue potentiated feeding requires an intact central nucleus of the amygdala, whereas conditioned approach relies on the function of the basolateral amygdala (Holland and Gallagher, 2003; Holland et al., 2002; Parkinson et al., 2000b). Thus, the dissociable effects of JF on conditioned approach vs. cue potentiated feeding may suggest differential effects of JF on the function of these sub-regions.

Another interesting feature of the data presented here is that rates of instrumental responding and break points for the US itself were reduced in JF vs. Chow groups (Fig. 5.6). Thus, in the same rats the food pellet US itself was less desired while motivational responses to the CS+ were enhanced by JF. It is tempting to speculate that this dissociation of JF effects on appetitive and consummatory behavior may be similar to established dissociations between "wanting" and "liking" following psychostimulant-induced sensitization (Castro et al., 2015; Robinson et al., 2016). However, it is important to note that we did not explicitly measure hedonic responses to food consumption here, and changes in the willingness to work for the US do not necessarily reflect changes in "liking" (Pecina et al., 2003). However, using the same junk-food diet used here, we previously found that that prolonged "junk-food" consumption (30 days) diminished positive orofacial hedonic responses to liquid sucrose regardless of obesity, but enhanced incentive motivation (Robinson et al., 2015). Thus, the reduced break points alongside enhanced conditioned approach following junk-food consumption found here are consistent with this previous result. Though speculative, this is also consistent with results in obese humans, where hedonic and neural responses to food itself are blunted while motivational and neural responses to food cues are enhanced (see Small, 2009 for review).

Following conditioned approach and cue potentiated feeding measures, the Zero Contingency and 50% Positive Contingency groups were tested for reinstatement following extinction training. Rats were given 4 days of extinction training followed by one CS-US pairing. Approach to the food cup during CS presentation and the ITI was then determined in the absence of food (i.e., reinstatement testing). Although neither group

showed a true reinstatement effect, rats in the JF group continued to exhibit robust conditioned approach during testing, whereas conditioned approach was absent in the Chow group (Fig. 5.5D). Taken with data above, this provides further support for the idea that JF consumption amplifies the incentive motivational properties of CSs, even when the CS-US association has been undermined by extinction training (Delamater et al., 2017).

6.5: Effects of JF vs. JF-deprivation

In Experiment 2, we included a group that was given 30 days of continuous access to JF and then underwent 14 days of JF deprivation before testing (i.e., JF replaced with *ad libitum* chow). The purpose of including this group was to determine how long-lasting the impacts of JF consumption were. Conditioned approach in the 100% Positive Contingency groups was greater in the JF-Dep group compared to the Chow group (Fig. 7A). Furthermore, the magnitude of approach was similar between JF-Dep and JF groups. Thus, prolonged JF consumption had long-lasting effects on incentive motivation, even withstanding a 14-day deprivation period. This is an important observation because it shows that once the incentive motivational properties of a CS have been amplified by JF consumption, voluntary reductions in caloric intake (Fig. 5.8C) and elimination of palatable food consumption are insufficient to reverse this process. This finding may have important clinical relevance, as an individual's diet history may be an important factor to consider when establishing treatment plans for weight loss. Of course, it is also possible that longer JF deprivation may be able to reverse this effect; this will be determined in future studies.

6.6: Relationship between junk-food effects and obesity

In the current study, rats given JF did not differ significantly in weight from Chow groups. This is not surprising given that the diet used is relatively low in fat content (~20%), and that diet exposure was relatively limited (45 days at maximum), compared to regimens usually used to induce obesity or metabolic dysfunction (typically 40–60% fat for 6–8 weeks). In addition, the distribution of weight was similar between Chow and JF groups (Figs. 5.4F and 5.8A). Although we did not measure adiposity directly, these data

suggest that JF induced increases in incentive motivation may not require profound weight gain or metabolic dysregulation. Indeed, this is consistent with the development of obesity, as over-consumption necessarily precedes weight gain. Of course, these effects may be further potentiated in the obese state, and/ or in individuals that are susceptible to weight gain (see Ferrario et al., 2016 for discussion). Consistent with a role of individual susceptibility, we recently found that obesity-prone rats show stronger Pavlovian-to-instrumental transfer (PIT) than obesity-resistant rats prior to obesity (Ferrario, 2018a) in the absence of obesity; that is, they are more sensitive to the incentive motivational impact of a food CS. Thus, it will be worthwhile in future to determine how effects of JF described here may interact with individual difference in susceptibility to obesity.

6.7: Additional considerations and conclusions

The JF diet used here contains several different kinds of fats and sugars, as well as salt. Thus, we do not know which component or combination of components may be more important in the observed behavioral effects. However, given the current food environment and wide range of dietary fats, sugars, etc. consumed by people, we think this diet manipulation should provide results that are generalizable across species. Of course, understanding how, for example, one particular type of fat affects similar behaviors would also be informative in future.

In regard to underlying neural mechanisms, there is a limited understanding of the neural substrates of conditioned approach behavior. However, this behavior relies on activity within mesolimbic systems, as well as on the integrity of the central nucleus of the amygdala (Cardinal et al., 2002; Parkinson et al., 2000a; Parkinson et al., 2000b). Furthermore, activity within these circuits can be profoundly altered by consumption of sugary, fatty, diets. For example, consumption of the same JF diet used here enhances acute amphetamine- induced locomotor activity compared to chow fed controls, regardless of degree of weight gain (Robinson et al., 2015). Psychostimulant induced locomotion is a well-established, though general, indicator of mesolimbic reactivity (Robinson et al., 1985; Vezina, 2004), and locomotor sensitization induced by repeated psychostimulant exposure enhances incentive motivational response to food CSs (Taylor

and Horger, 1999; Wyvell and Berridge, 2000; Wyvell and Berridge, 2001). Thus, increases in amphetamine-induced locomotor activity following JF consumption are consistent with enhanced responsivity of mesolimbic circuits. This is also consistent with effects of both sugar, and high fat on responsivity to direct acting dopamine receptor agonists (e.g., McGuire et al., 2011) increases in dopamine uptake (Fordahl and Jones, 2017), and enhanced striatal excitatory transmission (Dingess et al., 2017; Oginsky et al., 2016; Tukey et al., 2013).

In sum, the data presented above add to our understanding of the psychological and behavioral alterations induced by consumption of sugary, fatty diets, and expand on previous studies examining the processes underlying cue-potentiated feeding. The persistent and selective enhancement of incentive motivation in the face of reduced motivation for the food itself by JF suggest that interventions to prevent weight gain must include targeting neurobehavioral responses to food CSs, as well as food itself. Supplementary data to this article can be found online at https://doi.org/10.1016/j.physbeh.2018.03.012.

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Chapter 6: The Role of CamKII Basolateral Amygdala Neurons in Sensory Specific PIT and Pavlovian Outcome Devaluation Effect

Abstract:

Pavlovian stimuli not only acquire the ability to elicit conditioned approach responses, but can also develop the ability to influence instrumental behaviors, a phenomenon known as Pavlovian-to-instrumental transfer (PIT). Importantly, PIT can emerge through a Sensory Specific process that depends on the basolateral amygdala (BLA; Sensory Specific PIT) or via a general affective process (Gen PIT) independent of the BLA. Lesion and inactivation studies were the first to identify the role of the BLA in SS PIT, however to date little is known regarding the BLA cell populations mediating this effect. Therefore, here we sought to determine the role of glutamatergic BLA neurons in SS PIT. Prior to training, rats underwent surgeries to deliver either an inhibitory DREADD virus or a control virus into the BLA; these viral vectors contained a CamKIIa promotor thereby limiting viral expression to glutamatergic neurons. Rats were then trained and tested for both SS and Gen PIT. Administration of the DREADD activating ligand, CNO, selectively blocked SS PIT in DREADD infected, but not control rats. In contrast, CNO blocked the expression of General PIT in both groups. These data demonstrate that CamKII BLA neurons mediate SS PIT and independently that CNO acting via a nonspecific mechanism can block General PIT likely through a shift in interoceptive state. We went on to demonstrate that inactivation of CamKII BLA neurons did not block outcome devaluation effects on Pavlovian conditioned approach, indicating that CamKII BLA neurons do not mediate the recall of stimulus-outcome associations. Therefore, the role of CamKII BLA neurons in SS PIT appears to be mediating recall of the appropriate instrumental behavior associated with the memory of the outcome elicited by the Pavlovian stimulus.

1: Introduction:

During Pavlovian appetitive conditioning, repeated pairing of a conditioned stimulus (CS) with a rewarding outcome results in the formation of an association between the stimulus and outcome (S-O). The formation of this association can be inferred through the manifestation of complex behaviors including the emergence of anticipatory conditioned responding (CR) directed toward the site of expected reward delivery (e.g., food cup approach). The nature of the S-O representation is composed of multiple distinct elemental associations between the CS and various experiential features of the outcome including the outcome's sensory properties and the hedonic and emotional effects of consuming the outcome (Delamater, 2012a, b; Konorski, 1967). Importantly, these complex S-O associations can acquire the capacity to spontaneously modulate the expression of learned instrumental behaviors supported by response-outcome associations (R-O). This phenomenon, known as Pavlovian-to-instrumental transfer (PIT), is thought to play a role in a wide range of naturally occurring behaviors in both human and non-human animals, and may also contribute to the development of motivational disorders such as addiction and obesity (Boutelle and Bouton, 2015; Bouton, 2011; Derman and Ferrario, 2018; Watson et al., 2018).

In the laboratory setting, appetitive PIT can be measured by determining how the presentation of a previously established CS augments established instrumental responding for the same or similar outcome predicted by the CS. Since its first demonstration by Walker (1942), PIT has been studied using a variety of paradigms, most notably, Single Outcome PIT (SO PIT), General PIT (Gen PIT), and Sensory Specific PIT (SS PIT). While not initially recognized by researchers as distinct, it is now established these variants of PIT are each psychologically and neuronally dissociable. SO PIT is observed when presentation of a learned CS excites instrumental responding for the same outcome predicted by the CS; critically in this procedure only one CS-US and one response-outcome association is trained (first demonstrated by Walker, 1942). Gen PIT in contrast, is observed when CS presentation non-selectively excites instrumental responding for any outcome within the same general motivational properties or modality (i.e., ingestive, sexual, fear, aggression etc.), independent of the specific outcome associated with the instrumental response (first demonstrated by Balleine, 1994). Finally,

SS PIT is classically observed when presentation of a CS selectively excites instrumental responding for the same outcome predicted by that CS as compared to instrumental responding for a different outcome (first demonstrated by Colwill and Motzkin, 1994).

Of the three established forms of PIT, Gen and SS PIT are most strongly dissociable *psychologically and neurobiologically*. Contemporary psychological frameworks distinguishing Gen and SS PIT take inspiration from Konorski's (1967) theories of motivation which distinguish between sensory specific and general affective mechanisms of behavioral control. Sensory specific associations carry information about the distinct sensory components of an experience, independent of affective influences; for instance, the flavor, viscosity, and temperature of an imbibed liquid, but not satisfaction, or relaxation that could result from its ingestion. Here, subsequent behaviors are influenced via activation of memories of these specific sensory properties (i.e., sensory specific memories). In contrast, general affective associations contain information about the emotional content of an experience; for instance, the feeling of comfort associated with consuming a warm beverage on a cold day. Via the general affective mechanism, behaviors are controlled by memories of the emotional experience. Of course, a given experience can have both sensory specific and affective properties. However, distinguishing between affective and sensory specific processes, as can be achieved experimentally by the expression of Gen and SS PIT has important and broad reaching implications for the study of motivated behaviors and associated mental illnesses. For instances, this sets forth the possibility that aberrations in motivational systems underlie disorders such as obesity and addiction may arise via alterations in one or both of these independent processes and may even come to interact in complex ways as motivational disorders manifest. Thus, our onus to elucidate the psychological and neuronal boundaries and overlap between these mechanisms of behavioral control is of utmost importance.

Early research attempting to identify the major subnuclei of the network mediating PIT were initially hampered by the absence of a clear framework distinguishing Gen PIT, and SS PIT. Although it was clear that activity within the amygdala and the nucleus accumbens (NAc) were critical for the expression of PIT (Blundell et al., 2001; Corbit et al., 2001; Hall et al., 2001), determining which specific subnuclei were critical produced

seemingly contradictory results. For instance, basolateral amygdala (BLA) lesions were found to block SO PIT, but to have no effect on SS PIT, while NAc core lesions blocked SO PIT, but had no effect on SS PIT, and yet NAc Shell lesions blocked SS PIT, but did not prevent the expression of SO PIT (Blundell et al., 2001; Corbit et al., 2001; Hall et al., 2001). In an effort to clarify the discrepant findings with respect to the role of the amygdala in PIT, in 2005, Balleine and colleagues designed an approach to measure Gen and SS PIT in the same subject and evaluated the effects of BLA or central nucleus (CN) lesions on both SS and Gen PIT (Corbit and Balleine, 2005). In this procedure, two distinct response-outcome (R1-O1, R2-O2) and three distinct CS-US associations (CS1-O1, CS2-O2, CS3-O3) were trained. Importantly, the instrumental actions shared common outcomes with CS1 and CS2, but not with CS3. With this design, SS PIT could be measured by contrasting the effects of CS1 and CS2 on instrumental responding, whereas presentation of CS3 enabled evaluation of Gen PIT - all within the same subject. This study provided the first evidence that the ability for a CS to modulate expression of instrumental behaviors can arise via at least two distinct non-overlapping neural mechanisms. Specifically, they found that lesions of the BLA blocked SS PIT, but not Gen PIT, whereas lesions of the CN blocked Gen PIT, but not SS PIT. Thus, not only were these forms of PIT separable on a behavioral level, but they were also neuronally dissociable. Following this breakthrough, lesion and disconnection inactivation studies revealed that SS PIT depended on connections between the BLA and the NAc Shell, whereas Gen PIT was by the CN and the NAc Core (Corbit and Balleine, 2011; Shiflett and Balleine, 2010). However, to this date, the explicit circuitry and the cell populations of the amygdalo-striatal pathway that mediate PIT has yet to be explicitly identified.

Anatomical studies have established that there are direct glutamatergic connections from the BLA to the NAc Shell, suggesting this glutamatergic BLA efferent as a strong candidate for the putative cell population mediating SS PIT (Groenewegen et al., 1999; Sah et al., 2003; Shinonaga et al., 1994). However, anatomical studies have yet to reveal a direct connection (glutamatergic or GABAergic) between the CN and the NAc Core, suggesting that an indirect, rather than direct pathway between these sites mediates Gen PIT. Thus, this pathway likely involves an intermediate nucleus, with brain stem nuclei such as the Ventral Tegmental Area (VTA) or the Raphe Nucleus (RN), given

that these nuclei receive CN afferents and send glutamatergic efferents to the NAc Core (Brog et al., 1993; Chang et al., 2011; Sah et al., 2003; Taylor et al., 2014). In the current study, we sought to refine the understanding of the circuitry involved in the expression of SS PIT by selective inhibition of glutamatergic neurons within the BLA during PIT testing. This was accomplished using viral-mediated expression of an inhibitory G_i coupled Designer Receptors Exclusively Activated by Designer Drugs (DREADDS; hM4Di). To selectively target our hM4Di expression to glutamatergic neurons we utilized CamKII promotor which limits expression to CamKII expressing cells. CamKII is a protein kinase whose expression has been shown to be largely restricted to excitatory neurons (Jones et al., 1994). Using an adapted version of the procedure pioneered by Balleine and collogues (2005) that enables testing for both SS and Gen PIT, we tested whether DREADD mediated inhibition of CamKII BLA neurons via systemic injections of CNO would attenuate SS PIT, but not Gen PIT. In addition, we tested the effects of CamKII BLA inhibition on the expression of Pavlovian outcome devaluation effects. The goal here was to determine if inhibition of CamKII BLA would alter the ability to recall a current representation of the CS-O relationship, which has implications for how to interpret PIT effects. This is based on the idea that SS PIT arises when presentation of a CS activates a sensory specific memory of the predicted outcome (CS-O), which in turn activates the instrumental R-O associative memory, thereby selectively invigorating the distinct motor response of that instrumental association (i.e., S-O-R accounts of SS PIT; Alarcon and Bonardi, 2016; Alarcon et al., 2018; de Wit and Dickinson, 2009). Thus, a loss of SS PIT can theoretically occur via disruption of the S-O association (i.e., an inability to recognize the S) or via disruption of the R-O (i.e., an inability to link a given response to a specific outcome). If inactivation of CamKII BLA neurons disrupts the S-O aspect of PIT, then this manipulation should block the expression of devaluation. On the other hand, if CamKII BLA neurons mediate activation of the O-R branch, then we would not expect to see a loss of Pavlovian devaluation effects.

2: Materials and methods:

2.1: Subjects

Adult Sprague Dawley rats (total N=56; male, n=28; female, n=28) purchased from Envigo (Haslett, MI) were used for the study presented here. Rats were housed in groups of two or three and maintained on a reverse light-dark schedule (12/12). All behavioral experiments were performed during the dark phase. Rats were 65 days old at the start of each experiment. All procedures were approved by The University of Michigan Institutional Animal Care and Use Committee. Additional details for all procedures and housing can be found at: https://sites.google.com/a/umich.edu/ferrario-lab-public-protocols/. All behavioral training was conducted in red light conditions.

2.2: Viral vectors and drugs

Two CamKII dependent viral vectors were used in this study a DREADD vector, AAV(2/10) CamKII-hM4Di-mCherry (titer, $3.83x10^{13}$ vgc/ml) and a control vector, AAV(2/10) CamKII-GFP (titer, $1x10^{13}$ vgc/ml) . The DREADD used here is a Gi coupled receptor which is activated by Clozapine-N-oxide (CNO). The control vector expressed the fluorophore, GFP. The plasmids for these viral vectors were purchased from Addgene, having been deposited by Bryan Roth (pAAV-CaMKIIa-EGFP: Addgene plasmid # 50469 ; http://n2t.net/addgene:50469 ; RRID:Addgene_50469; pAAV-CaMKIIa-hM4D(Gi)-mCherry: Addgene plasmid # 50477 ; http://n2t.net/addgene:50477 ; RRID:Addgene_50477). These plasmids were then used to produce virus by Caroline Bass (University at Buffalo) using a combination serotype of AAV2 and AAV10.

CNO was used to test the behavioral effects of inactivating CamKII BLA neurons. CNO solution was prepared by dissolving CNO powder in 100% dimethyl sulfoxide (DMSO) and then diluting this solution with sterile saline (0.9%) to a final concentration of 5mg/mL CNO and 5% DMSO. CNO was administered at a 5mg/kg (i.p.) dose for all studies. The vehicle solution for these injections was 5% DMSO. CNO was provided by the NIDA drug supply program.

Lithium chloride (LiCl) was used for outcome devaluation. Lithium chloride was dissolved directly in sterile saline (0.9%) for a 0.3M concentration (dose, 63.6 mg/kg) and saline was used as the vehicle solution for these injections (i.p.).

2.3: Stereotaxic surgery

Rats were allowed to acclimate to the vivarium for 7 days before surgeries were performed. Stereotaxic surgery was performed to deliver viral vectors into the BLA. Rats were infected with either the CamKII-hM4Di-mCherry DREADD vector (n=35) or the CamKII-GFP control vector (n=21). Stereotaxic surgeries were conducted as previously described (Derman & Ferrario, 2016). Anesthesia was induced with 5% isoflurane and maintained with 1.5-5% isoflurane. For analgesia, rats were subcutaneously administered Carprofen (5mg/kg; Rimadyl) pre- and postoperatively (24 hours later). Bilateral injections of virus were made at AP, -2.28mm, ML, $\pm$ 5.00mm from bregma, and DV, -7.2 from dura. A volume of 0.5ul of virus was injected at a rate of 1ul/min using a microliter syringe (Hamilton, 800 series, Model 85; 26 gauges) attached to a motorized pump (Harvard, Pump 11 Elite Nanomite). Before each injection, the syringe was tested to ensure proper flow and the needle was lowered to -0.5 DV below the intended DV coordinate. The needle was left in place for 5 min, then raised to the DV target site and the injection was initiated. The injection lasted for 30 sec and the needle was left in place for an additional 9.5 min and was then slowly withdrawn. Rats were left to recover for 7-10 days before food-restriction and training described below.

2.4 Instrumental and Pavlovian conditioning

The training details were identical to those outlined in Chapter 3 (section 2) and were adapted from Corbit and Balleine (2005; 2011). Briefly, rats were food restricted 85-90% of their *ad libitum* weights and maintained at this weight throughout the remainder of the study. Rats were then food cup trained with three distinctly flavored 45 mg pellets (Bioserv: Unflavored #F0021; Banana #F0059; Chocolate #F0299).

Table 6.1 illustrated the experimental design of instrumental training, Pavlovian conditioning, and PIT testing. During instrumental training, rats were trained to acquire two distinct R-O associations, where pressing on two distinct levers was reinforces with

two distinct outcomes (Lever1-O1 and Lever2-O2). Rats were initially trained on a continual reinforcement schedule until reaching the acquisition criterion of earning 50 consecutive pellets in under 40 minutes. Then rats were shifted to variable interval (VI) schedules of reinforcement which were made leaner across sessions, culminating in a VI60" schedule (VI10", VI30", VI45", and VI60"). Each lever was trained once a day and in isolation from the other lever (details for instrumental training are outlined in Chapter 3, section 2.5). In the next phase rats underwent Pavlovian conditioning with three distinct conditioned stimulus (CS) associations: CS1-O1, CS2-O2, and CS3-O3. (details for Pavlovian conditioning are outlined in Chapter 3, section 2.6).

2.5: Pavlovian-to-instrumental transfer testing

Rats were given an instrumental "reminder" session one day before each PIT test. To determine the effect of DREADD mediated inhibition of CamKII BLA neurons on PIT, rats were administered either vehicle or CNO (5mg/kg). Injections were conducted in the home cage and 20 min later rats were transported to and placed into operant chambers for testing. Testing was identical to the procedures outline in Chapter 3 (section 2.7). Briefly, both levers were available for the entire 44 min duration of testing, but pellet delivery was omitted. After a 10 min instrumental extinction phase, each CS was presented three times in a quasi-random order using a fixed 2 min inter-trial-interval (ITI). Lever responses and food cup entries were recorded throughout and sessions were video recorded. Rats were tested once under each treatment condition (Vehicle, CNO), with the order of treatment assignments counterbalanced across rats.

2.6: Conditioned taste aversion training

To test the effects of CamKII BLA neuronal inactivation on the expression of Pavlovian outcome devaluation, a subset of rats first (n=29; GFP, n=10; hM4Di, n=19) underwent conditioned taste aversion (CTA) training to devalue one of the three outcomes from

Instrumental Training	Pavlovian Conditioning	PIT Testing
R1-O1	CS1-O1	CS1: R1 v R2
R2-O2	CS2-O2	CS2: R1 v R2
	CS3-O3	CS3: R1 v R2

Table 6.1: Experimental design of training and PIT testing.

Pavlovian training (procedure adapted from Derman et al., 2018). Outcome devaluation was achieved by pairing post-ingestive injections of LiCl to induce temporary illness. Rats were placed into individual locomotor chambers each outfitted with a metal tube feeder filled with a pre-weighed amount of one type of flavored food pellet (~20 g, ~445 pellets) and left to freely consume for 20 min (Fig. 6.4A depicts an illustration of these training chambers). Rats were then removed from these chambers and immediately injected with either saline (control sessions) or LiCl (63.6mg/kg, i.p.; devaluation sessions) and placed back in their home cages in the absence of any food. Unconsumed pellets were weighed and recorded to determine the amount consumed in each session. If notable spillage had occurred this too was weighed and accounted for. To prevent any carryover of taste aversion to their standard lab chow, after returning to their home cages rats were fed no earlier than 2 hrs. post injection. CTA training was conducted in 5, 3 session cycles. Each cycle consisted of one devaluation session and two control sessions. Outcome devaluation assignments were counterbalanced across rats within each group.

2.7: Devaluation testing

To test the effect on DREADD mediated inhibition of CamKII BLA neurons on the expression of Pavlovian devaluation, rats were injected with vehicle or CNO 20 min prior to devaluation testing. Test sessions lasted for 36 min and were conducted under extinction conditions. Each CS was presented 3 times, in a quasi-random order, separated by a fixed 2 min ITI. Food cup entries were recorded throughout and each test session was video recorded. Rats were tested once under each treatment condition, where the order of treatment assignments was counterbalanced based on consumption rats in the final cycle of CTA training.

2.8: Choice consumption testing for taste aversion

To test the effect on DREADD mediated inhibition of CamKII BLA neurons on the expression of conditioned taste aversion rats were given Vehicle or CNO 20 min prior to testing. Each test consisted of a 20 min session in which rats were given *ad libitum* access to all three outcomes from Pavlovian and CTA training. Testing was conducted in the same chambers as CTA training was performed; each chamber was outfitted with 3

feeder tubes each filled with a pre-weighed amount of one of the outcomes from training (~10.2g; 227 pellets). Once testing was completed, returned to their home cages and the remaining pellets were weighed and recorded. Rats were tested under both treatment conditions where the order of treatment assignments was counterbalanced based on consumption levels in the final cycle of CTA training.

2.9: Histology, fluorescent immunochemistry, imaging and microscopy

For brain extraction, rats were injected with a fatal dose of pentobarbital and intracardially perfused. and fixed with 4% paraformaldehyde (PFA; w/v). The brain was then extracted and placed into a room temperature 50/50 cocktail of 4% PFA and 30% sucrose (w/v) after saturating (~24hrs), brains were transferred into a 30% sucrose solution till saturation and then sectioned. Brains were sectioned coronally at 60μm using a cryostat (Leica) and sections were stored in cryoprotectant (50% 0.1M Phosphate Buffer; 30% Ethylene Glycol; 30% Sucrose) at -20°C till processed with immunohistochemistry (IHC). Free-floating IHC was performed to evaluate viral expression, the specificity of viral expression to CamKII neurons. Brain slices were washed 12 times with 1X Phosphate Buffered Saline (PBS; 10 min/wash), then blocked (5% Normal Goat Serum; 0.04% Triton-X; 95% 1X PBS) for 1.5 hrs. Tissue was then incubated with a primary antibody in blocking solution overnight (15-20 hrs.). Next the tissue was washed 5 times in 1X PBS (5 min/wash) and then incubated in with a secondary antibody in blocking solution for 1.25-1.5 hrs., after which it was washed again 5 times in 1X PBS (5 min/wash) and then mounted onto Superfrost Plus microscope slides (Fisherbrand) coverslipped with Prolong Gold +DAPI mounting medium (Invitrogen, P36931).

All IHC was conducted at room temperature using a standard orbital shaker (Tallboys). All primary and secondary antibodies were incubated at a 1:2K concentration. The primary antibodies used to amplify signals were as follows: mCherry: Rb anti-RFP (Rockland, 600-406-379); GFP: Rb anti-GFP (Invitorgen A6455); CamKII: Ms anti-cFos (Abcam, AB22609); and Parvalbum: Rb anti-Parvalbumin (Abcam, AB11427). The secondary antibodies used were as follows: Alexa Fluor 555 Goat anti-Rabbit (Invitrogen,

A32732); Alexa Fluor 555 Goat anti-Mouse (Invitrogen, 32727); DyLight 488 Goat anti-Rabbit (Invitrogen, 35553); Alexa Fluor 488 Goat anti-Mouse (Invitrogen, A32723).

Coverslipped slides were visualized using an upright epifluorescence manual system microscope (Olympus, BX43) with an XM10 camera; images were taken at 2x, 10x, and 20x (cellSens). Assessment of viral expression location was performed visually, using standard anatomical landmarks to identify the BLA (Paxinos and Watson, 2007). For DREADD infected rats, only data from subjects with bilateral hM4Di-mCherry expression localized to the BLA were included for analysis (20 included, 15 rejected).

3. Experimental design and statistical analysis

All behavioral experiments were designed for within subject comparisons. To control for unintended effects of viral infection and potential off target effects of CNO, a CamKII-GFP control group was included in all testing. Data was processed and organized with Microsoft Excel (Version 16.16.16) and statistical analyses were performed using the GraphPad statistical software suite Prism (Version 8.0.2). Data was assessed using, student's t-tests, one-way ANOVAs, repeated measures ANOVAs (RM ANOVAs) and Holmes-Sidak post-tests for planned and post-hoc multiple comparisons. Instrumental and Pavlovian behavioral data were analyzed as response rates per minute or per 10 sec and, when relevant, as a change from pre-CS rates (60 sec and 10sec prior, respectively). For instrumental responding during VI training, the rate of responding under each VI schedule was averaged for analysis. For Pavlovian training, PIT testing, and Pavlovian devaluation testing the data were averaged across trials within a session, with the expectation of lever responding in the instrumental extinction phase at the start of each PIT test. For this phase, data were analyzed in 60" bins.

For each behavioral measure, exclusion criteria were established to ensure that analyses were limited to individuals who expressed the behavior being tested (e.g., SS or Gen PIT). All inclusion criteria were applied based on performance under vehicle conditions. Our goal in this study was to identify the role of CamKII BLA neurons in PIT and the expression of outcome devaluation effects on conditioned approach. As such, we set inclusion criteria to limit our analysis to subjects that exhibited the expectant behaviors under vehicle conditions. The inclusion criterion for expression of SS PIT was that the CS

elicited greater lever responding on the lever whose outcome was the same versus the lever whose outcome was different than that predicted by the CS in presentation (Same>Diff; as defined by Colwill and Motzkin, 1994; Delamater and Holland, 2008). For Gen PIT the inclusion criterion was that CS elicited lever responding had to be greater than pre-CS lever responding (CS>Pre; averaged across levers). The inclusion criterion for rats exhibiting devaluation effects was that CS elicited food cup approach was greater during presentation of the non-devalued CSs versus the devalued CS (N-Dev>Dev; averaged across N-Dev CSs). For post-devaluation testing, the inclusion criterion for post Devaluation testing and was that CS evoked food cup approach rates were greater to the non-devalued CSs than to the devalued CS. Finally, inclusion criteria for CTA testing was that consumption of the non-devalued outcome was greater than the devalued outcome.

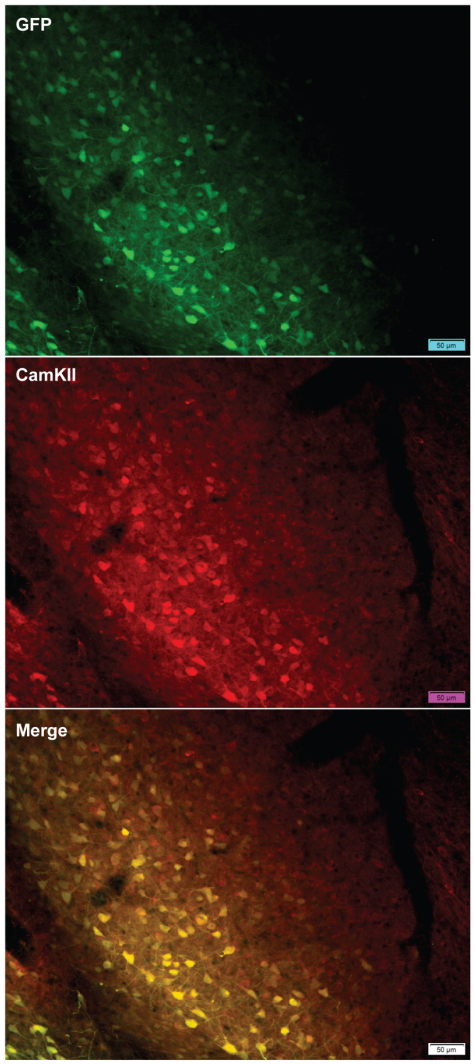

Figure 6.1: Evaluation of specificity of viral expression to CamKII cells infected with AAV(2/10) CamKII-GFP in the BLA. **A)** GFP labeled CamKII BLA neurons. **B)** CamKII labeled neurons in the BLA neurons. **C)** Channel s merged, yellow cells illustrate the overlap in labeling and demonstrate the highly specific expression of GFP to CamKII positive cells.

4: Results

4.1: Histology

Only data from CamKII-hM4Di rats with bilateral on target BLA infection were included in analyses. Exemplar images of bilateral infections are depicted in Fig 6.3D for CamKII-hM4Di and Fig 6.4E for CamKII-GFP. Images were taken of sections processed for IHC to amplify the reporter fluorophores used to assess infection sites. Among rats infected with CamKII-hM4Di, 20 had bilateral on target infection sites, 9 had bilateral infections that were off target, and 6 had unilateral infection. Only CamKII-hM4Di rats with bilateral on target infection sites were included in analyses (N=20). Among rats

infected with the control CamKII-GFP, 10 were bilateral on target, 4 were bilateral, but off target, 5 had unilateral, and 2 showed no sign of infection. We did not observe notable behavioral differences between these infection conditions within this control group, thus data was included from all rats.

IHC was performed to qualitatively confirm that viral expression was limited to putative glutamatergic neurons (i.e., positive for CamKII and negative for parvalbumin). An exemplar image of this overlap is shown in Fig. 6.1. As a negative control we assessed the overlap between virally infected cells and parvalbumin cells (as a marker of inhibitory interneurons). We found nearly exclusive overlap between infected cells and cells positive for CamKII labeling, and virtually no overlap of infected cells and parvalbumin labeling.

4.2: Instrumental training

Rats were first trained to press one lever to receive one flavored outcome (i.e., food pellet) and another lever to receive a different flavored outcome on a continual reinforcement schedule in separate sessions (Fig. 6.2A; Lever 1-O1 and Lever 2-O2). Rats were trained to an acquisition criterion of earning 50 consecutive pellet deliveries before moving on to a VI schedule (see also methods). The mean time to acquire this task was 24.6 min (±SEM: 3.7) and did not differ between levers within either group (Data not shown, Paired t-test, Lever 1 versus Lever 2; CamKII-GFP: $p=0.60$; CamKII-hM4Di: $p=0.14$). Next, rats were transitioned to a variable interval (VI) schedule of reinforcement that was made leaner across sessions to encourage higher rates of responding. As expected, the rate of lever pressing increased as a function of VI schedule in each group (Fig. 6.2B. CamKII-GFP: Mixed-effects analysis: main effect of VI schedule: $F_{(3,48)}=58.37$, $p<0.01$; CamKII-hM4Di: Two-way RM ANOVA: $F_{(3,51)}=51.31$, $p<0.01$). Inversely, the number of outcomes earned decreased as a function of VI schedule in each group (Fig. 6.2C. CamKII-GFP: Mixed-effects analysis: main effect of VI schedule: $F_{(3,48)}=163.7$, $p<0.01$; CamKII-hM4Di; Two-way RM ANOVA: $F_{(3,51)}=437.6$, $p<0.01$).

4.3: Pavlovian conditioning

Rats were next conditioned to associate 3 distinct CS-O pairs (Fig. 6.2D). In this procedure, outcomes were never delivered within the first 10 sec of CS presentation thus

providing a window during which true conditioned anticipatory responding could be measured across training. Fig. 6.2E depicts the temporal structure of the CS-O contingencies implemented here. Anticipatory conditioned food cup approach rapidly increased across the first 3 sessions and then plateaued to asymptotic levels for the remaining sessions in both groups (Fig. 6.2F. Two-way RM ANOVA: CamKII-GFP: main effect of session: $F_{(8,128)}=9.43$, $p<0.01$; CamKII-hM4Di; Two-way RM ANOVA, $F_{(8,136)}=5.99$, $p<0.01$). As an additional measure of conditioning, we assessed the latency to enter the food cup following CS onset and offset (ITI) across training. In both groups, the latency to enter the food cup following CS onset decreased across training (most notably between sessions 1 and 2), whereas the latency to enter following ITI onset increased across training (Fig. 6.2G. Two-way RM ANOVA: CamKII-GFP: main effect of session: CS: $F_{(8,128)}=5.08$, $p<0.01$; ITI: $F_{(8,128)}=2.25$, $p=0.03$; CamKII-hM4Di: main effect of session: CS: $F_{(8,136)}=9.92$, $p<0.01$; ITI: $F_{(8,136)}=8.33$, $p<0.01$), indicating acquisition of an expectancy of reward following CS onset.

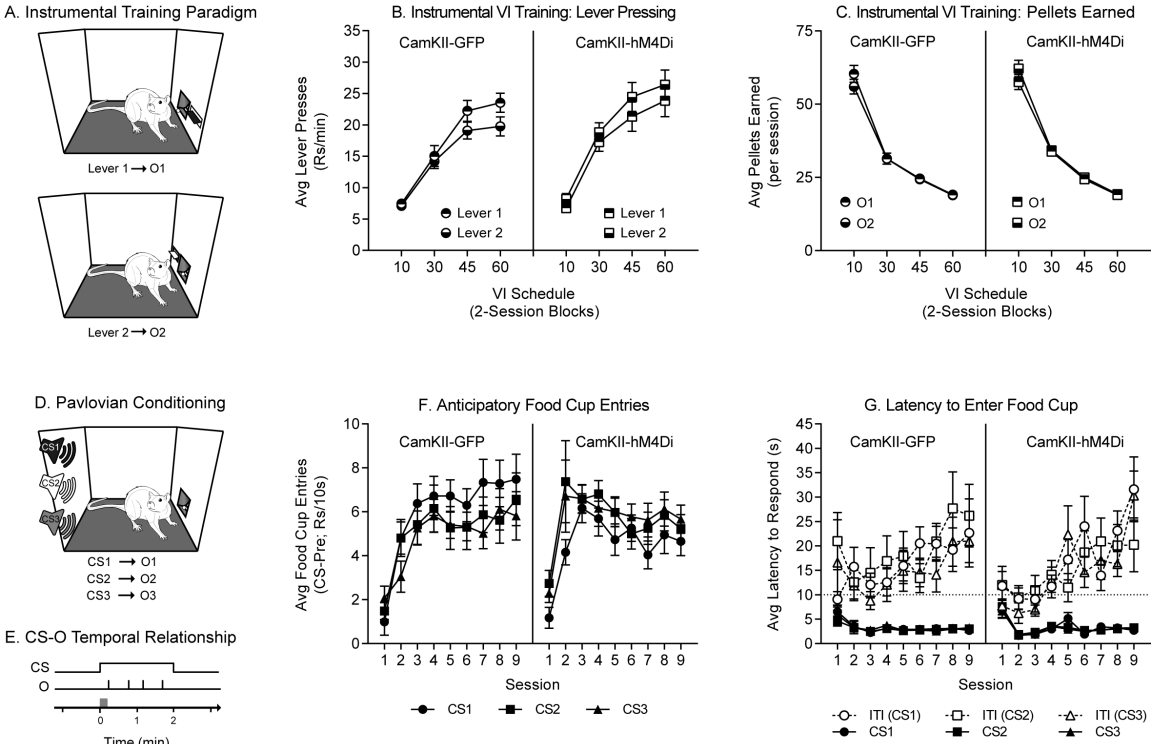

Figure 6.2: Instrumental and Pavlovian conditioning. **A)** Schematic of instrumental training, where rats learn two independent R-O associations. **B)** Lever pressing on both levers increased across instrumental VI training as the schedule of reinforcement thinned. **C)** The number of pellets earned in VI instrumental training decreased as the schedules of reinforcement grew. **D)** Schematic of Pavlovian conditioning, where rats were conditioned to associate three independent CS-O associations. **E)** Schematic of the temporal relationship between the CS and the paired outcomes. Each CS was presented for two minutes and four pellets were delivered randomly after the first 10 seconds following CS onset. The grey box denotes over the time line illustrates the first 10 seconds of the CS during which pellets were never delivered. **F)** Anticipatory food cup entries increased within the first three sessions and then stabilized for the remaining sessions. Entries were similar across CSs. **G)** The latency to enter the food cup following CS onset was rapid and stable across sessions. Latencies were similar between CSs. In contrast, latencies to enter the food cup following CS offset slowed dramatically across training. (All data are shown as averages ±SEM, unless otherwise noted**).**

4.4: CNO selectively blocks expression of sensory specific PIT in DREADD infected rats

Next, rats were tested for PIT following injections of either vehicle or CNO (within subject, treatment order counter balanced). The timeline for injections and testing is illustrated in Fig. 6.3A. PIT testing begins with a 10 min instrumental extinction phase, followed by intermittent presentation of each CS (3 trials/CS). SS PIT is observed when presentation of the sensory specific CSs (CS1, CS2) elicits greater responding on the lever that previously generated the same outcome predicted by that CS versus the lever that generated a different outcome. Gen PIT is observed when presentation of the general CS (CS3), which does not share a common outcome with either lever, elicits an increase in responding on either lever above pre-CS levels (see schematic Fig. 6.3C). Analysis of the effects of CNO on SS PIT were conducted separately, given that not all rats who showed SS PIT also showed Gen PIT under Vehicle conditions (CamKII-GP: 12/21; CamKII-hM4Di: 14/20). The data from PIT testing are shown in Fig. 6.3; data from CamKII-GFP controls are shown in panels F-I, and data from the CamKII-hM4Di group is shown in panels J-M.

In CamKII-GFP control rats, administration of CNO did not disrupt lever responding during the first 10 min of instrumental extinction and as expected response rates dropped steadily across the 10 min phase (Fig. 6.3F. Mixed-effects analysis: no effect of drug: $p=0.62$; main effect of time, $F_{(9,135)}=15.25$, $p<0.01$; no drug x lever interaction, $p=0.25$). To determine whether CNO altered SS PIT, planned comparisons were made between CS elicited lever responses made on the lever that shared the 'Same' outcome as the CS in presentation versus lever responses made on the other lever that previously produced a 'Different' outcome. In CamKII-GFP rats, CNO administration did not disrupt SS PIT. Following both Vehicle and CNO injections, rats showed comparable SS PIT behavior, preferentially responding on the lever that shared the Same outcome as the CS in presentation (Fig. 6.3G. Two-way RM ANOVA: main effect transfer, $F_{(1, 12)}=12.45$, $p<0.01$; no effect of drug, $p=0.37$; no drug x transfer interaction, 0.95; Holm-Sidak's multiple comparisons test, Same versus Different: Vehicle: $t_{(12)}=2.55$, $p=0.05$; CNO: $t_{(12)}=2.46$, $p=0.05$). Similarly, the SS PIT magnitude (Same[-]Diff), or the sensory specificity of the transfer effect, was similar between Vehicle and CNO treatments (Fig. 6.3H. Paired t-test, $p=0.94$). In addition to assessing the effect of CNO on SS PIT, we also evaluated its

effects on conditioned approach during SS PIT trials (i.e., CS1 and CS2 trials). Rats showed robust conditioned approach during these trials and this did not differ following Vehicle or CNO treatment (Fig. 6.3I. Two-way RM ANOVA: no effect of drug, p=0.81; main effect phase, $F_{(1, 12)}$=36.97, p<0.01; no drug x phase interaction, p=0.94; Holm-Sidak's multiple comparisons test pre-CS versus CS: Vehicle: $t_{(12)}$=4.8, p<0.01; CNO: $t_{(12)}$=4.9, p<0.01). Collectively, these data demonstrate that in CamKII-GFP control rats, CNO does not disrupt 1) lever responding generally, 2) the expression of SS PIT, nor 3) conditioned approach.

In DREADD expressing rats, CNO administration did not affect responding during the instrumental extinction phase (Fig. 6.3J. Three-way RM ANOVA: no main effect of drug: p=0.24; main effect of time, $F_{(9,144)}$=31.00, p<0.01; no drug by lever interaction, p=0.44). In contrast to controls, SS PIT was selectively disrupted by CNO administration (Fig. 6.3K-L). Specifically, following Vehicle injection CS elicited lever pressing was greater on the lever that shared same outcome as the CS in presentation versus the lever with different outcome, whereas this preference was lost following CNO injection (Fig. 6.3K. Two-way RM ANOVA: main effect transfer, $F_{(1, 13)}$=10.13, p<0.01; no effect of drug, p=0.29; drug x transfer interaction $F_{(1,13)}$=3.79, p=0.07; Holm-Sidak's multiple comparisons test: Vehicle: $t_{(13)}$=3.91, p<0.01; CNO: $t_{(13)}$=1.16, p=0.27). This effect is also apparent when we directly compared the magnitude of SS PIT between Vehicle and CNO treatments (Fig. 6.3L. Paired Two-tailed, $t_{(13)}$=1.96, p=0.07). In contrast to the effects of CNO on SS PIT in the CamKII-hM4Di infected rats, conditioned food cup approach to the SS CSs was fully intact following CNO administration (Fig. 6.3M. Two-way RM ANOVA: main effect phase, $F_{(1, 13)}$=47.80, p<0.01; no effect of drug, p=0.52; no drug x phase interaction, p=0.44; Holm-Sidak's multiple comparisons test pre-CS versus CS: Vehicle: $t_{(13)}$=6.05, p<0.01; CNO: $t_{(13)}$=4.92, p<0.01). In summary, in contrast to the CamKII-GFP control group, administration of CNO selectively disrupted SS PIT in DREADD expressing rats, without altering conditioned food cup approach.

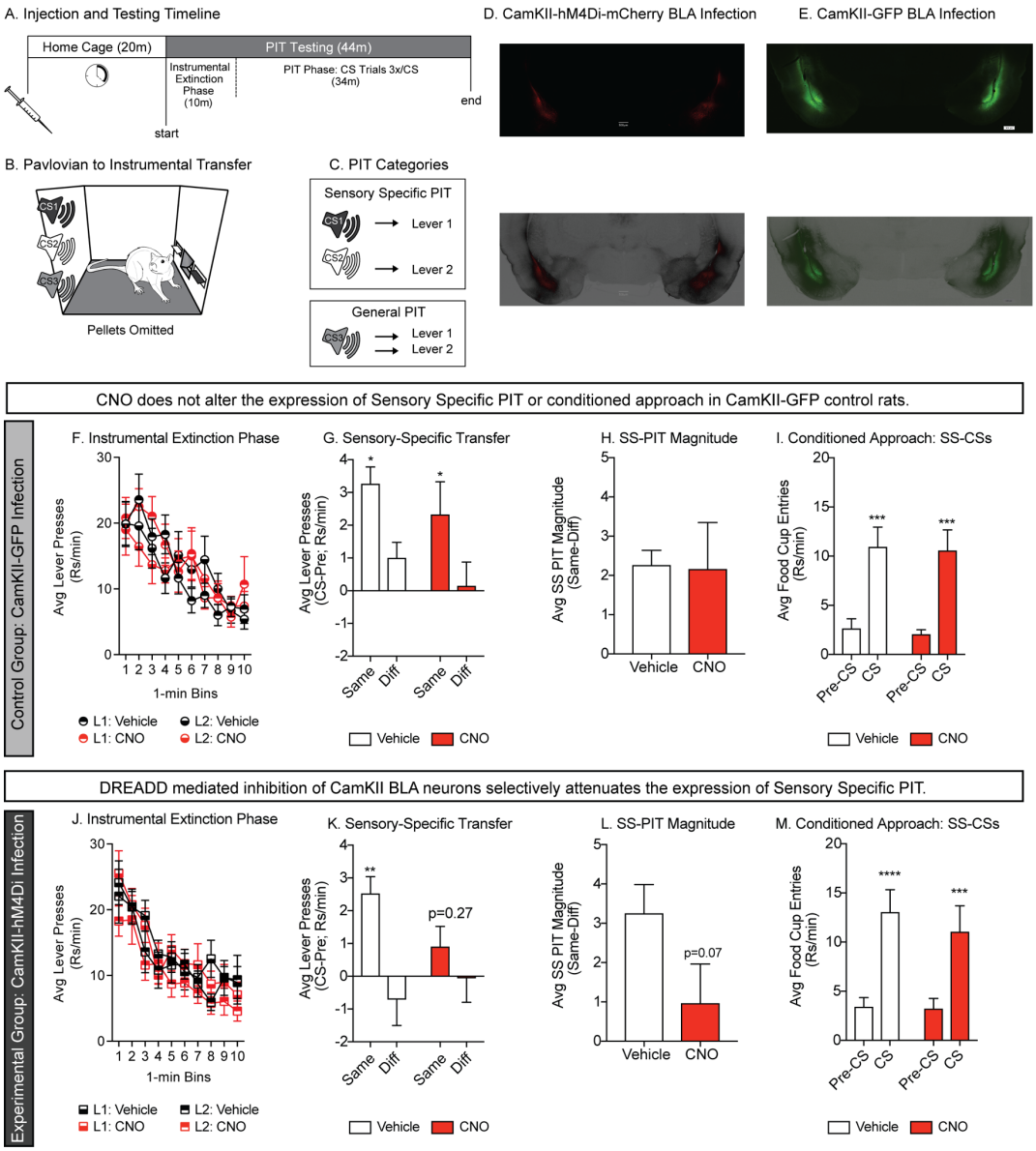

Figure 6.3: Effects of inactivation of CamKII BLA neurons on PIT. **A)** Injection timeline for testing. Rats were injected in the home-cage and then tested 20 min later. **B)** Schematic of PIT testing, rats were given access to both levers under extinction conditions and CSs were presented intermittently after an initial 10 min lever extinction period. **C)** Categories of PIT tested here, SS and Gen PIT. **D)** Exemplar of BLA CamKII-hM4Di-mCherry infections. **E)** Exemplar of BLA CamKII-GFP infections. **F)** Lever pressing in GFP infected rats decreased across the first 10 minutes of testing, prior to CS presentation and was similar following Vehicle and CNO injections. **G)** SS PIT in GFP infected rats was unaffected by CNO administration, as is evident by greater lever pressing on the lever previously generating the Same versus the Different outcome than predicted by the CSs, following both Vehicle and CNO injections. **H)** In GFP infected rats, the magnitude of SS PIT was similar following Vehicle and CNO injections. **I)** In GFP infected rats, conditioned approach was similar following Vehicle and CNO injections. **J)** Lever pressing in DRREADD infected rats decreased across the first 10 minutes of testing, prior to CS presentation and was similar following Vehicle and CNO injections. **K)** In DREADD infected rats, CNO disrupted SS PIT demonstrated by the loss of preferential responding on the Same lever following CNO injections. **L)** In DREADD infected rats, SS PIT magnitude was diminished by CNO administration. M) In DREADD infected rats, conditioned approach was unaffected by CNO administration. (All data are shown as averages ±SEM, unless otherwise noted; *=p<0.05).

4.5: Effect of CNO on General PIT

Analysis of the effects of CNO on General PIT were conducted separately, given that not all rats who showed Sensory Specific PIT also showed General PIT under Vehicle conditions (CamKII-GFP: 13/21; CamKII-hM4Di: 12/20). General PIT is observed when presentation of the General CS (CS3) evokes an increase in lever responding above pre-CS rates. Administration of CNO disrupted this transfer effect in both control and experimental groups (Fig. 6.4).

In CamKII-GFP controls following Vehicle injection, presentation of the General CS elicited a robust increase in lever responding, which was absent following CNO administration (Fig. 6.4A. Two-way RM ANOVA: main effect phase, $F_{(1, 12)}$=19.38, p<0.01; phase x drug interaction, $F_{(1, 12)}$ = 8.66, p=0.01; Holm-Sidak's multiple comparisons test: Vehicle: $t_{(12)}$=5.32, p<0.01; CNO: $t_{(12)}$=1.15, p=0.27). A similar effect was observed in our DREADD infected rats (Fig. 6.4B. Two-way RM ANOVA: main effect phase, $F_{(1, 11)}$=26.55, p<0.01; phase x drug interaction, $F_{(1, 11)}$=5.867, p=0.03; Holm-Sidak's multiple comparisons test: Vehicle: $t_{(11)}$=4.99, p<0.01; CNO: $t_{(12)}$=1.57, p=0.15). Comparison of the PIT magnitude between Vehicle and CNO conditions further illustrates this effect; CNO reduced the magnitude of General transfer in both experimental and control groups (Fig. 6.4C. Paired t-test, $t_{(12)}$=2.76, p=0.02; Fig. 3D. Paired t-test, $t_{(11)}$=2.47, p=0.03).

We also evaluated the effect of CNO on conditioned food cup approach in response to presentations of the General CS. Food cup entries were significantly increased above pre-CS response rates following both Vehicle and CNO injection in both the experimental and control groups (Fig. 6.4E. Two-way RM ANOVA: main effect phase, $F_{(1, 12)}$=24.94, p<0.01; no effect of drug, p=0.18; no phase x drug interaction, p=0.11; Holm-Sidak's multiple comparisons test: Vehicle: $t_{(12)}$=5.80, p<0.01; CNO: $t_{(12)}$=3.36, p=0.01; Fig. 6.3F. Two-way RM ANOVA: main effect phase, $F_{(1, 11)}$=60.07, p<0.01; no effect of drug, p=0.94; no phase x drug interaction, p=0.56; Holm-Sidak's multiple comparisons test: Vehicle: $t_{(11)}$=5.47, p<0.01; CNO: $t_{(11)}$=6.31, p<0.01). Collectively these data reveal that independent of DREADD expression, CNO exerts a robust depressive effect on General PIT, without strongly affecting conditioned food cup approach elicited by the General CS.

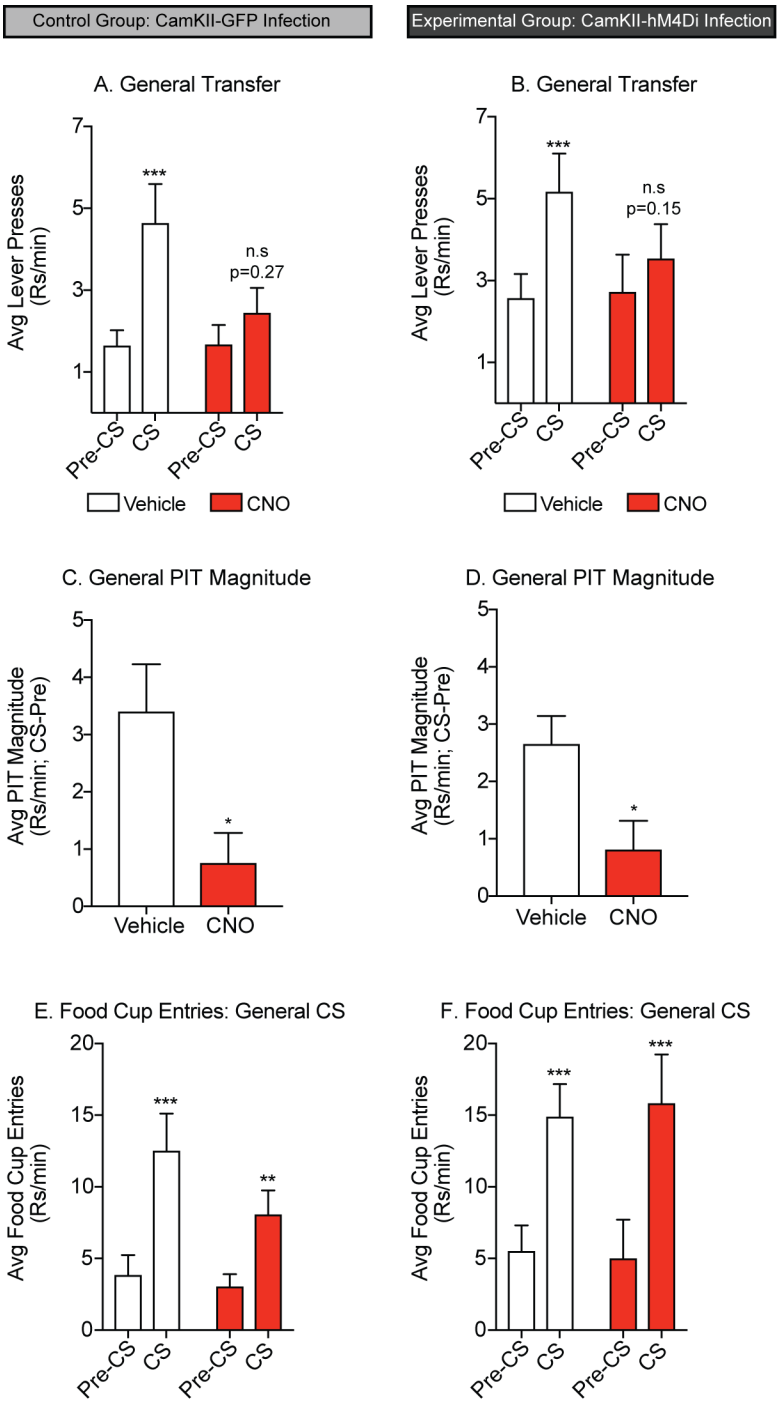

Figure 6.4: Nonspecific effects of CNO on Gen PIT, but not conditioned approach. **A)** In GFP infected control rats, CNO disrupted the expression of Gen PIT. **B)** Similarly, in DREADD infected rats CNO blocked the expression of Gen PIT. **C)** In GFP infected control rats General PIT magnitude is greatly diminished by CNO. **D)** In DREADD infected rats CNO reduced the magnitude of Gen PIT. **E)** In GFP infected control rats, conditioned food cup approach to the Gen-CS was unaffected by CNO administration. **F)** In DREADD infected rats CNO administration did not alter conditioned approach to the Gen-CS. (All data are shown as averages ±SEM, unless otherwise noted; *=p<0.05).

4.6: Conditioned taste aversion training

Following PIT testing, a subset of rats underwent conditioned taste aversion (CTA) conditioning. The purpose here was to devalue one of the outcomes from Pavlovian conditioning in order to subsequently assess the effects of inhibition of CamKII BLA neurons on the expression of Pavlovian devaluation effects. For each subject, one of the three outcomes from training was devalued by pairings with post-ingestive injections of LiCl to induce illness, whereas the other two outcomes were left non-devalued and were paired instead with post- ingestive saline injections. Two CTA training conditions were used here: for some rats the devalued outcome was the outcome associated with the Gen CS (GFP, n=4; hM4Di, n=3), whereas for the remaining rats, the devalued outcome was one of the outcomes paired with a SS CS (GFP, n=6; hM4Di, n=9).

Data from each CTA condition (Gen versus SS outcomes devalued) were first examined to determine if the associative nature of the devalued outcome (Dev Gen-O versus Dev SS-O) affected CTA acquisition. Post-ingestive pairings of the General outcome with LiCl injections (Gen-O: LiCl) significantly suppressed consumption of these pellets across training, whereas consumption of the non-devalued SS outcomes (SS-O: Sal) paired with saline injections did not decrease across training across training (Fig. 6.5B. Two-way RM ANOVA: cycle x outcome interaction, CamKII-GFP: $F_{(8,\ 24)}$=4.63, p<0.01; CamKII-hM4Di: $F_{(8,\ 16)}$=6.16, p<0.01; Holm-Sidak's multiple comparisons test: Cycle 5, SS-O1: Sal v Gen-O: LiCl: CamKII-GFP: $t_{(12)}$=5.39, p<0.01; CamKII-Hm4Di: $t_{(16)}$=8.45, p<0.01; SS-O2: Sal v Gen-O: LiCl CamKII-GFP : $t_{(12)}$=5.56, p<0.01; CamKII-Hm4Di: $t_{(16)}$=7.67, p<0.01). The same pattern was observed when the devalued outcome was one of the SS-Os and notably, there was no difference in the consumption between the non-devalued General outcome (Gen-O: Sal) and the non-devalued SS outcome (SS-O: Sal) (Fig. 6.5C. Two-way RM ANOVA: cycle x outcome interaction, CamKII-GFP: $F_{(8,\ 31)}$=14.6, p<0.01; CamKII-hM4Di: $F_{(8,\ 64)}$=10.99, p<0.01; Holm-Sidak's multiple comparisons test: Cycle 5, Gen-O: Sal v SS-O: LiCl: CamKII-GFP: $t_{(39)}$=6.09, p<0.01; CamKII-Hm4Di: $t_{(64)}$=8.61, p<0.01; SS-O: Sal v SS-O: LiCl: CamKII-GFP : $t_{(39)}$=7.69, p<0.01; CamKII-Hm4Di: $t_{(64)}$=8.32, p<0.01). Collectively, the emergence of conditioned taste aversion was evident by the reduction in consumption of the LiCl paired outcomes and was present whether the devalued outcome was the Gen-O or an SS-O.

4.7: Effect of CNO and CamKII BLA inactivation on expression of outcome devaluation effects

After CTA training, rats were tested for expression of Pavlovian outcome devaluation effects following Vehicle or CNO injections (within subject, treatment order counterbalanced). The purpose of this testing was to determine if inactivation of BLA CamKII neurons disrupted the ability for the CS to call up a current representation of the outcome. Testing was performed in the operant chambers under extinction conditions (3 trials/CS). Outcome devaluation is observed when presentation of a CS whose outcome has undergone devaluation (Dev CS), evokes significantly less food cup approach than presentations of the CS whose outcome was never devalued (ND CS). This behavior depends, in part, on the ability for the rat to utilize the newly updates value status of the outcome to appropriately guide conditioned responding.

Among rats for whom the devalued outcome was associated with the Gen CS, we did not observe reliable devaluation effects on conditioned approach in either CamKII and GFP groups. Of 8 total rats who underwent this CTA training, all but 2 of them showed reduced rate of conditioned food cup approach to presentations of the devalued CS. Thus, analysis of the nonspecific or DREADD mediated effects of CNO on this behavior was not possible in this training group.

In contrast, among rats for whom the devalued outcome was associated with the one of the SS-Os majority of the rats expressed outcome devaluation effects following Vehicle injections (Total, 10/15; CamKII GFP, 4/6; CamKII-hM4Di, 6/9). Here, presentation of the Dev-CS evoked fewer food cup entries than presentation of the N-Dev-CSs (Fig. 6.5E. Two-way RM ANOVA: CamKII-GFP: main effect of CS, $F_{(1, 3)}=6.54$, $p=0.08$; CamKII-hM4Di: $F_{(1, 5)}=5.05$, $p=0.08$). Importantly, administration of CNO did not attenuate expression of this devaluation effect (Fig. 6.5E. Two-way RM ANOVA: CamKII-GFP: no effect drug, $p=0.36$; CamKII-hM4Di: $p=0.76$). Due to the above alpha p-values here and given that we did not observe notable group or drug effects here, we further tested the veracity of this trending data by performing a 3-way ANOVA with both viral expression groups and both drug conditions. This test confirmed expression of outcome devaluation effects both groups following both Vehicle and CNO injection (Fig. 6.5F. Three-way RM ANOVA: main effect of CS, $F_{(1, 8)}=11.78$, $p<0.01$; no effect of drug, $p=0.33$;

no drug x group x CS interaction, p=0.93). In sum, devaluation effects were not disrupted by CNO administration via either a non-specific or DREADD mediated mechanism among rats for whom SS-O had been devalued. This indicates that inactivation of CamKII BLA neurons does not disrupt the ability to call up a representation of the outcome, which implies that disruption of SS PIT via in DREAD infected rats arises via an inability for this recall to then access the correct instrumental response associated with that same outcome.

Within the SS-O devaluation condition, the two non-devalued outcomes each had a unique training history (Gen-O versus SS-O), where only one shared a similar training history with the SS-O. Specifically, the Gen-O was only trained in a CS-O association, whereas the SS-O was trained in both an instrumental R-O and Pavlovian CS-O association. Thus, of these two non-devalued outcomes, the ND SS-O had more training features in common with the Dev SS-O, than the ND Gen-O. We therefore chose to analyze responding to these non-devalued CSs separately in order to determine whether devaluation of the SS-CSs carried over to the other non-devalued SS-CS, or non-devalued Gen-CS. First, we determined whether the pattern of responses to these CSs (ND Gen-CS, ND SS-CS, and Dev SS-CS) differed between Vehicle and CNO conditions and found no effect of drug in either group (Supp. Fig. 3. Three-way RM ANOVA, no effect of drug, p=0.34; no drug x group interaction, p=0.55). We then determined whether conditioned approach differed across the CSs, and found a significant difference in responding to each CS (Data not shown. Three-way RM ANOVA, main effect of CS, $F_{(1, 8)}$=11.78, p<0.01; no CS by group interaction, p=0.53; no CS x drug interaction, p=0.76). For ease of illustration and due to the lack of an effect of drug, viral infection group or interaction of these factors with the CSs, we collapsed across drug and viral infection conditions. Figure 6.5F shows these collapsed data where the difference in conditioned approach across the CSs is apparent (One-way RM ANOVA, main effect of CS $F_{(1.44, 12.94)}$=5.46, p=0.02). Here we see a strong devaluation effect between conditioned approach elicited by the ND Gen-CS and the Dev SS-CS (Fig. 6.5F, Holm-Sidak's multiple comparisons test: $t_{(9)}$=3.00, p=0.03). In contrast a weaker devaluation effect was observed between the ND SS-CS and the Dev SS-CS (Fig. 6.5F, Holm-Sidak's multiple comparisons test: $t_{(9)}$=2.10, p=0.07). These data demonstrate carryover devaluation

effects between the SS-CSs suggesting that the shared associative properties of these outcomes render them similar enough to support these carryover effects.

Collectively, the results here show no substantial effect of CNO on expression of outcome devaluation effects, either via a non-specific mechanism or a DREADD mediated effect. Furthermore, additional analysis of these data reveals that expression of outcome devaluation is impacted by the commonality of training histories between non-devalued and devalued outcomes.

4.8: Effect of CNO on the expression of conditioned taste aversion in a choice consumption test

During CTA training, outcomes were presented in isolation to allow rats to acquire the new post-ingestive effects of a given outcome. Testing the rats in a 3-choice test session enables confirmation of the taste aversion, and allows us to ask about any carryover effects of devaluation between the outcomes that might otherwise be masked by a forced choice test. Therefore, rats were next tested in a 3-choice consumption test following injections of Vehicle or CNO to evaluate the efficacy of aversion training, to test for carryover effects and to determine whether CNO effected this behavior. Data were analyzed separately for rats in the two different CTA training conditions (Dev Gen-O training versus Dev SS-O training).

In the group for whom the devalued outcome was the Gen-O, testing revealed a robust aversion to the devalued outcome. Consumption of both non-devalued SS-Os was significantly higher than consumption of the devalued Gen-O (Fig. 6.4H. Three-way RM ANOVA, main effect of outcome, $F_{(2,\ 10)}=39.38$, $p<0.01$; Holm-Sidak's multiple comparisons test: ND SS-O1 versus Dev Gen-O, , $t_{(10)=}6.86$, $p<0.01$; ND O2 versus Dev Gen-O, , $t_{(10)=}8.31$, $p<0.01$). Moreover, consumption was similar between the non-devalued outcomes (Holm-Sidak's multiple comparisons test: ND SS-O1 versus ND SS-O2, p=0.18). Finally, this taste aversion did not differ between CamKII-GFP and CamKII-hM4Di groups, and there was no difference in this effect following Vehicle or CNO injections (Fig. 6.4H. Three-way RM ANOVA, no main effect of group, p=0.41; no main effect of drug, p=0.23; no drug x group x outcome interaction, p=0.80). Thus, the absence of devaluation effects on CS elicited approach, are not due to a failure to acquire

conditioned taste aversion as demonstrated by the results of the choice test and their consumption during CTA training (Fig. 6.4A).

The pattern of consumption was somewhat different in the groups for whom the devalued outcome was one of the SS-Os. First, as expected, we observed a strong aversion to the devalued SS-O; rats barely consumed the devalued outcome and consumed substantially more of both non-devalued outcomes (Fig. 6.4I. Three-way RM ANOVA, main effect of outcome, $F_{(2,\ 24)}$=61.77, p<0.01; Holm-Sidak's multiple comparisons test: ND Gen-O versus Dev SS-O, $t_{(24)}$=11.10, p<0.01; ND SS-O versus Dev SS-O, $t_{(24)}$=6.07, p<0.01). However, similar to the effects during devaluation testing (Fig 6.4E), we observed substantial carryover of CTA from the devalued SS-O to the non-devalued SS-O. This was evident by the observation that consumption of the non-devalued SS-O was substantially less than consumption of the non-devalued Gen-O (Fig 6.4I; Holm-Sidak's multiple comparisons test: ND SS-O versus ND Gen-O, $t_{(24)}$=5.03, p<0.01). Importantly, these effects were not different between CamKII-GFP and CamKII-hM4Di groups and CNO did not alter this effect (Fig. 6.4I; Three-way RM ANOVA, no main effect of group, p=0.28; no main effect of drug, p=0.24; no drug x group x outcome interaction, p=0.67). It is worth reiterating that the flavors of the assigned outcomes (banana, chocolate, or unflavored pellets) were counterbalanced across rats and groups, precluding the interpretation that similarities between the intrinsic sensory properties of the SS-Os may account for this effect. Collectively, these data demonstrated that CNO does not attenuate the expression of conditioned taste aversion either through a non-specific effect or via a DREADD mediated effect. Furthermore, the similarity of training history between given outcomes renders them sensitive to carryover effects of taste aversion.

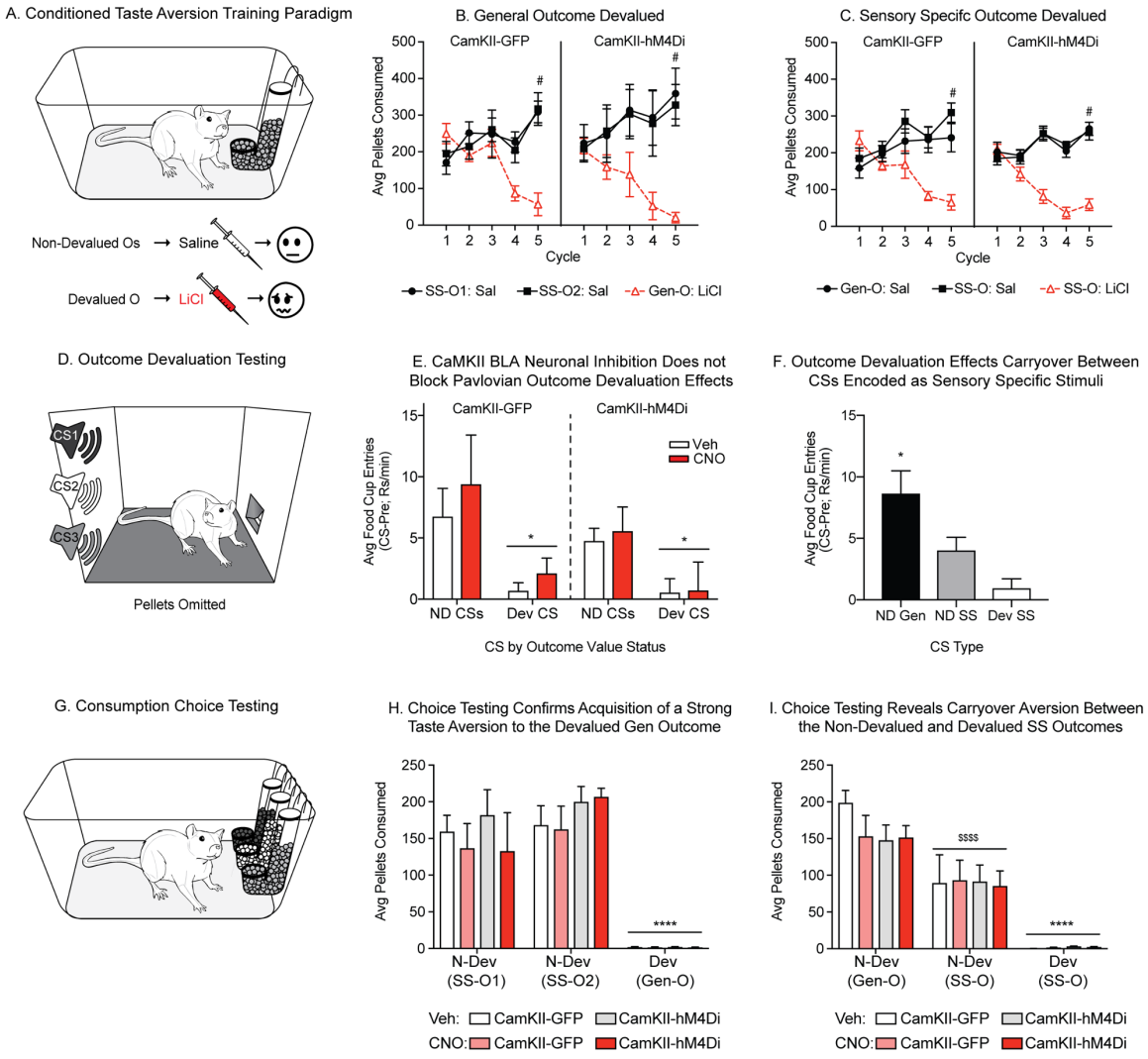

Figure 6.5: Conditioned taste aversion training, devaluation testing, and consumption choice test. **A)** Schematic of taste aversion training where the devalued outcome was paired with post-ingestive injections of LiCl and the non-devalued outcomes were paired with saline injections. **B)** Conditioned taste aversion emerged in both GFP control and DREADD infected rats, when the LiCl paired outcome was the General outcome. **C)** Similarly, taste aversion emerges in both GFP control and DREADD infected rats when the LiCl paired outcome was one of the Sensory Specific outcomes. **D)** Schematic of the test for effects of outcome devaluation on conditioned approach. **E)** Outcome devaluation effects were observed in rats for whom the devalued outcome one of the Sensory Specific outcomes. This was apparent by the reduction in conditioned approach to the presentations of the CS associated with the devalued versus the non-devalued outcomes. These effects were not altered by CNO injections in either GFP control or DREADD infected rats. **F)** Collapsing across drug and viral groups reveals a carryover devaluation effect between the devalued SS CS and the non-devalued SS CS. This is apparent by the relative similarity in approach suppression between the devalued and non-devalued SS CSs, but the apparent lack of suppression in approach during the non-devalued General CS. **G)** Schematic of the consumption choice test, were rats were given free access to all three outcomes in a single test session. **H)** Strong conditioned aversion to the devalued General outcome with no carryover effects to the non-devalued Sensory Specific outcomes. No differences were observed between viral groups CNO did not alter these effects. **I)** In contrast, the conditioned taste aversion is seen to the devalued Sensory Specific outcomes with substantial carryover effects the non-devalued Sensory Specific outcome. This is apparent by the strongest preference for the General non-devalued outcome over the Sensory Specific outcome. (All data are shown as averages ±SEM, unless otherwise noted; *,#,$=p<0.05)

5: Discussion

Pavlovian conditioned stimuli often acquire the ability to control instrumental behaviors, a phenomenon known as Pavlovian to Instrumental transfer (Walker, 1942). This phenomenon is thought to play an important role in a wide range of naturalistic behaviors and in the development of disordered appetitive behaviors underlying conditions like addiction and obesity (Boutelle and Bouton, 2015; Bouton, 2011; Derman and Ferrario, 2018; Watson et al., 2018). Moreover, in recent years, the ecological validity of PIT has been made increasingly apparent as the number of PIT studies in humans have grown (Colagiuri and Lovibond, 2015; De Tommaso et al., 2018; Hogarth et al., 2018; Jeffs and Duka, 2017; Nadler et al., 2011; Prevost et al., 2012; Seabrooke et al., 2018a; Seabrooke et al., 2018b; Watson et al., 2014). Furthermore, some of these studies in humans have begun to provide support for the implication of PIT in appetitive disorders, such as obesity and internet gaming disorders (colloquially, gaming addiction; Lehner et al., 2017; Vogel et al., 2018). Thus, the importance of expanding our current understanding of the neural basis for this phenomenon is highly pertinent.

Since its discovery by Walker (1942), psychology research has developed a rich understanding of the associative and motivational mechanisms governing PIT. Importantly, work in the recent two decades has led to the understanding that the process by which CSs come to influence instrumental behaviors seems to arise via two distinct psychological processes, a sensory specific process versus general affective process (Corbit and Balleine, 2005). SS PIT is canonically observed when presentation of one CS preferentially spurs one instrumental response associated with the same unique outcome as that predicted by the CS, over other instrumental response associated with different outcomes (Collwill and Motzkin, 1994). Gen PIT, in contrast, is observed when presentation of a CS non-specifically augments instrumental responses within the same broader motivational system, without any outcome driven specificity in this responding (Balleine, 1994).

These two forms of PIT have been shown to be neuroanatomically and psychologically independent. Both forms of PIT depend on the VTA, but are controlled by distinct sub-regions of downstream nuclei in the amygdala and NAc (Corbit and Balleine, 2005; Corbit and Balleine, 2011; Corbit et al., 2007). Whereas SS PIT depends on the

BLA and the NAc Shell, Gen PIT, in contrast, is mediated by the CN and the NAc Core. Progress has been made in attempts to identify receptors within these nuclei that mediate these distinct phenomena (Laurent et al., 2012; Lichtenberg and Wassum, 2017; Malvaez et al., 2015). These studies have provided important insights as to the receptors mediating PIT, but leave open the question of what cell populations within these nuclei are primarily responsible for these behaviors. Here we attempt to answer this question by focusing on the role of CamKII neurons within the BLA in the expression SS- versus Gen PIT.

Rats were infected with an hM4Di DREADD under the control of the CamKII promotor, thereby restricting expression to CamKII BLA neurons, putative glutamatergic neurons (Jones et al., 1994). Importantly we included a control group that were infected with a virus that expresses GFP under the CamKII promotor, in order to control for viral infection and for potential off target effects of CNO (Gomez et al., 2018), the DREADD activating agonist. Consistent with previous lesion and inactivation studies implicating the BLA in SS PIT, we found that reduction of activity in CamKII BLA cells via CNO administration blocked the expression of SS PIT in DREADD infected rats, but not in GFP-expressing controls (Corbit and Balleine, 2005; Shiflett and Balleine, 2010). Furthermore, CNO administration did not block the expression of Pavlovian devaluation effects, suggesting that the inhibition of excitatory BLA neurons was not the result of an inability for the recall of the outcomes associated with the CSs, but instead a disruption of the ability to use that information to guide appropriate instrumental responding (i.e., S-O-R accounts for SS PIT; Alarcon and Bonardi, 2016; Alarcon et al., 2018; de Wit and Dickinson, 2009). In addition, we found that administration of CNO blocked Gen PIT in both DREADD-expressing and GFP-expressing control groups. This result suggests that CNO administration itself may alter affective experience, upon which Gen PIT relies (Manvich et al., 2018). Finally, we tested whether inhibition of CamKII BLA neurons would alter the expression of conditioned taste aversion and found this behavior intact.

5.1 Effect of DREADD mediated inhibition of CamKII BLA neurons on sensory specific PIT

Our results demonstrate that inactivation of CamKII BLA neurons at the time of testing selectively blocks the expression of SS PIT. These results are consistent with the ability of non-selective BLA lesions to block the expression of this behavior (Corbit and Balleine, 2005). These data expand upon our understanding of the neuronal circuitry of this effect by identifying putative glutamatergic, CamKII-expressing BLA output neurons as a critical cell population mediating SS PIT within the BLA. Of course, this does not rule out a potential contribution by other populations within the BLA that influence these output neurons. For example, recent work in other brain regions has pointed to the importance of acetycholinergic receptor in the expression of PIT, suggesting that BLA cholinergic interneurons may play a key role too (Collins et al., 2016; Ostlund et al., 2017). Nevertheless, given that there are direct glutamatergic connections between the BLA and NAc shell (Groenewegen et al., 1999; Sah et al., 2003; Shinonaga et al., 1994), and that both nuclei are required for the expression of SS PIT (Corbit and Balleine, 2005; Corbit and Balleine, 2011; Shiflett and Balleine, 2010), our results provide further support for this BLA-NAc circuitry in the expression of SS PIT.

An outstanding question, is which target nuclei of these CamKII BLA efferents mediate the expression of SS PIT? Of the major efferents of the BLA (Sah et al., 2003), projections to the pre-frontal cortex (PFC) and to the striatum are the most likely target nuclei serving a role in SS PIT. Indeed, a recent study by Lichtenberg et al., (2017) demonstrated that projections from the BLA to the orbital frontal cortex (OFC) are critical for SS PIT. In this study, optogenetic inhibition of BLA efferent terminals within the OFC blocked expression of SS PIT and post-synaptic excitatory transmission in the OFC. This identifies another important leg of the amygdalar circuitry mediating PIT.

5.2: Non-specific effects of CNO on General PIT

Our study was carefully designed to assess any off-target behavioral effects of CNO administration. CNO did not alter basal levels of instrumental responding or have any notable impact on Pavlovian conditioned food cup approach. This is important because it demonstrates that our DREADD mediated disruption of SS PIT is a specific

effect of reducing activity in CamKII BLA cells and not the result of some off-target effects on instrumental responding generally or recall of the Pavlovian associations. However, CNO administration abolished Gen PIT in both our control group and our DREADD-expressing experimental group, thereby indicating that this effect was independent of DREADD expression. This may be due to differences in sensitivity of Gen PIT vs SS PIT to internal state.

One of the interesting psychological distinctions between SS and Gen PIT is that, SS PIT is not sensitive to shifts in motivational states, whereas Gen PIT is, at least in some cases, altered by motivational shifts. Specifically, testing in a satiated versus hungry state abolishes Gen PIT, but does not alter the sensory specificity of SS PIT (Corbit et al., 2007; though see Watson et al., 2014). Similar results have been observed with thirst (Balleine, 1994; De Tommaso et al., 2018). Motivational shifts in hunger and thirst states are notably distinct from drug induced state shifts, but considering the labile nature of this type of transfer, the loss of Gen PIT we observed following CNO administration may be the result of a shift in interoceptive state. Indeed, a recent study in mice and rats demonstrated that CNO can, in fact, produce a change in interoceptive state detectable in rats. In this study, rats were first trained to use the interoceptive state associated with Saline versus Clozapine (1.25mg/kg) as a discriminative stimulus to guide lever responding, such that one lever (e.g., left) was reinforced under Saline conditions, whereas the other lever, (e.g., right) was reinforced under Clozapine conditions. Once these behaviors were acquired, rats and mice were challenged with different doses of CNO and lever preference was tested under extinction conditions. Clozapine was used as the comparator drug here, because recent work has shown that CNO can be back metabolized into Clozapine which is a psychoactive antipsychotic drug (Gomez et al., 2017; Manvich et al., 2018). During testing, rats injected with CNO demonstrated a preference for responding on the lever reinforced during Clozapine training. This finding illustrates that CNO can produce an interoceptive state that is distinguishable by rats from the interoceptive state of Saline. Therefore, our finding that CNO blocked Gen PIT regardless of DREADD expression suggests that this effect may have arisen from a drug-induced shift in interoceptive state.

To our knowledge, no previous studies have directly tested whether drug induced shifts in interoceptive state have a similar effect on Gen PIT as shifts in thirst and hunger. However, given the affectable nature of Gen PIT, that we observed a loss of Gen PIT following CNO administration, and that CNO can produce an interoceptive state, it is likely that Gen PIT may be shifted by a wider range of states and interoceptive stimuli than previously identified. This also opens the possibility of using drug-induced state shifts to alter undesired cue-triggered motivation that arises via a Gen PIT and identifies Gen PIT an alternative paradigm for studying the interoceptive properties of drugs.

On a more practical note, however, these results also demonstrate a potential challenge for researchers attempting to use CNO and DREADDs to explore the neuronal basis for general affective motivation. Identification of a truly inert DREADD ligand has proven challenging, thus far. One potentially viable alternative is Compound 21, which activates hM3Dq and is not back metabolize into Clozapine (Chen et al., 2015). However, its efficacy at the hM4Di DREADD has yet to be tested, and full characterization of off-target behavioral effects remains to be completed. Yet, this may be a promising new tool for DREADD research.

5.3: CamKII BLA neurons are not required for expression of Pavlovian outcome devaluation effects

Following PIT testing, a subset of rats underwent conditioned taste aversion and were subsequently tested for expression of Pavlovian outcome devaluation effects after CNO or Vehicle injections. The purpose of this experiment was to determine whether DREADD mediated attenuation of SS PIT was the result of an inability for the CS to call up a current representation of the outcome, one of the primary mechanisms by which SS PIT is thought to be mediated (i.e., S-O-R accounts for SS PIT; Alarcon and Bonardi, 2016; Alarcon et al., 2018; de Wit and Dickinson, 2009). CNO administration did not alter expression of Pavlovian outcome devaluation effects on conditioned approach (Fig. 6.5E). This suggest that the loss of SS PIT via inhibition of BLA CamKII cells does not arise from the animal's inability to call up the distinct sensory properties of the CS-O association, but instead likely entails the inability for the memory of the outcome to access the appropriate motor networks mediating the instrumental transfer effect. In other words,

assuming an S-O-R framework of PIT, BLA CamKII neurons appear to be critical for the ability of the outcome memory to activate the appropriate instrumental memory.

It is worth noting that evidence for the necessity of the BLA in the expression of Pavlovian outcome devaluation effects is mixed. Some studies have supported its role (Baxter et al., 2000; Johnson et al., 2009; Lichtenberg et al., 2017) and yet others, including ours here, show that disruption of BLA does not alter the expression of Pavlovian devaluation effects (Blundell et al., 2003; Pickens et al., 2003; Wellman et al., 2005). However, among these studies, only two have looked at manipulations of the BLA at the time of testing, as we did here. And while here too the results are seemingly inconsistent, the discrepant results in these cases maybe more readily explained by differences in the depth of devaluation and in sensory specificity of the experiment. Specifically, Lichtenberg et al (2017) found that inhibition of BLA to OFC efferents at the time of testing blocked Pavlovian outcome devaluation effects in rats using a 1-hour pre-feeding satiety induced devaluation procedure. In contrast, Wellman et al., (2005) found that muscimol induced BLA inactivation at the time of testing did not disrupt satiety induced Pavlovian outcome devaluation effects in monkeys. Potential species differences aside, a key difference here, is that the devaluation manipulation conducted by Wellman et al., and that used here produced very robust devaluation. In their study, monkeys had *ad libitum* access to the outcome and testing was only performed once each subject had stopped consuming the food. In our current study, we used LiCl to devalue the outcome, this produces a very robust devaluation, and like Wellman et al., (2005) we saw no effect of BLA manipulations at the time of testing on outcome Pavlovian outcome devaluation effects. Therefore, the apparent discrepancies in determining the necessity of the BLA for expression of Pavlovian outcome devaluation effects may be explained by the degree of devaluation, with sufficiently strong devaluation reducing the role of BLA activity in the expression of this behavior. Another potentially important distinction is that the rats in Lichtenberg's study were trained with two CS-O associations, whereas our study used 3 CS-O pairs. Thus, it is possible that a richer associative training experience may result in less reliance on the BLA for outcome mediated effects.

5.4: CamKII BLA neurons are not required for expression of conditioned taste aversion

As a final test to examine whether CamKII BLA neurons mediate the ability to distinguish the sensory properties of the outcomes and to evaluate the acquisition of CTA, rats were given a choice consumption test following CNO or Vehicle injections. During taste aversion training the outcome exposure sessions were done in isolation in order to effectively establish the appropriate post-ingestive associations with a given outcome. In the choice test for taste aversion the rats were presented with a choice to consume all three outcomes. Here, rats exhibited robust avoidance of the devalued outcome (previously paired LiCl) following vehicle and CNO injections in all rats. This finding confirmed that CamKII BLA neurons do not disrupt sensory processing of the outcome nor the ability to recall the recently updated post-ingestive effects of these outcomes. These results are consistent with previous lesion and inactivation studies demonstrating that expression of CTA occurs independent of the BLA (Johnson et al., 2009; Pickens et al., 2003; West et al., 2012).

5.5: Outcomes trained under conditions that promote sensory specific encoding versus those general affective encoding exhibit enhanced sensitivity to carry over effects of outcome devaluation

One interesting and unexpected behavioral finding in this study that is separate from the question of the role of BLA in PIT, was that rats showed “carryover” effects between outcomes trained under conditions promoting sensory specific encoding versus general affective encoding. Specifically, we found that when CTA was performed using one of the outcomes from the CS-Os used to promote SS PIT (e.g., O1 or O2), Pavlovian devaluation effects were seen to both the devalued SS-CS, and to the non-devalued SS-CS, but not to the non-devalued Gen-CS (Fig. 4F). This carryover effect while mild, was notable. Furthermore, a conceptually similar but stronger carryover effect was observed during the taste aversion choice testing (Fig. 4I). During choice testing, rats vastly preferred the non-devalued Gen-O versus the non-devalued SS-O. Critically, the flavors had been carefully counterbalanced and therefore similarity in sensory perception of outcomes alone cannot account for this effect.

These data suggest that CTA can produce aversions not just to stimuli directly associated with the devalued outcomes, but also to other stimuli (outcomes and CSs) that share the same associative modalities (i.e., SS versus Gen). In this PIT paradigm, the outcomes that support SS PIT are each independently associated with a Pavlovian CS (CS-O) and an instrumental response R-O association. In this way the outcome is embedded in a wider S-O-R network. In contrast, the outcome that supports Gen PIT is only experiences within a Pavlovian CS-O association, but not an instrumental R-O association. In this way the Gen-O exists in a smaller S-O network. The SS-Os share a common associative structure, which is distinct from that of the Gen-O, which render the outcomes trained under conditions promoting SS encoding vulnerable to effects observed. If outcomes trained within the same associative history are more vulnerable to carryover effects of CTA training, then we would expect a similar effect between a devalued Gen-O and a non-devalued Gen-O, where a non-devalued SS in this paradigm should be immune to carryover. This hypothesis has yet to be tested. Nevertheless, the finding here of carryover between a devalued SS-O and a non-devalued SS-O, but not a Gen-O, provides further support for the idea that independent encoding processes exist for sensory specific associative structures and general affective associative structures (as canonically proposed by Konorski, 1967), and presumably dissociable neural mechanisms underlaying these processes.

5.6: General summary

In sum, the data presented in this study refine our understanding of the neural circuits involved in the expression of PIT by showing that putative glutamatergic neurons within the BLA are critical for the expression of SS PIT. In addition, effects of CNO alone on Gen PIT suggest that drug induced interoceptive cues alone may also affect the expression of this behavior in a manner similar to shifts in interoceptive states related to hunger and thirst drives, though future studies need to test this idea more directly. This also speaks to potential confounds of using CNO to explore the neural mechanism of affective motivation. Finally, our control studies using CTA further support the specific role of BLA in SS PIT, and also provide additional new insights into the independence of associations encoded via sensory specific versus general affective processes. Future

studies will need to identify the critical afferent sites for BLA CamKII neurons in the expression of PIT. In addition, the distinct cell populations and circuitry mediating Gen PIT still remains to be elucidated.

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Chapter 7: Discussion

The studies presented in this dissertation explore the psychological and neurobiological components of Pavlovian motivation, particularly within the context of obesity-vulnerability. Overall, the data here support the idea that heightened sensitivity to the motivational influence of food associated Pavlovian stimuli is a pre-existing trait associated with obesity susceptibility. In addition, these data identify Nucleus Accumbens (NAc) Core calcium permeable AMPA receptors (CP-AMPARs) as a common neuronal mechanism mediating the expression of Pavlovian-to-instrumental transfer (PIT; a classic measure of Pavlovian motivation) in obesity vulnerable selectively bred and outbred populations. These data expand our understanding of the psychological and neurobiological processes contributing to heightened Pavlovian motivation associated with obesity-vulnerability.

Below, I begin by discussing behavioral differences in selectively bred obesity-prone and obesity-resistant rats and in outbred rats identified as obesity susceptible. I then discuss how consumption of a palatable diet potentiates certain aspects of incentive motivation, independent of individual susceptibility to obesity. This is followed by a discussion of the neurobiological factors mediating cue-triggered food-seeking (i.e., PIT). Then in the final section, I explore the overlap and distinguishing features underlying addiction and obesity within the context of the role of NAc Core CP-AMPAR in mediating incentive motivation.

1.0 Behavioral differences in selectively bred obesity-prone and obesity-resistant rats

Data presented in this dissertation demonstrate that instrumental and Pavlovian incentive processes are enhanced in obesity-prone rats relative to obesity-resistant rats. However, these differences were not apparent under all training and testing conditions. Therefore, I begin by discussing some experimental factors that may influence the degree to which these intrinsic differences manifest in instrumental (Section 1.1), Pavlovian

(Section 1.2), and PIT (Section 1.3) behaviors. In addition, I will introduce some additional data in the discussion here, to help clarify some of the results presented in the data chapters 2-6. This will help identify factors that influence the manifestation of heighted Pavlovian motivational processes and inform both variability in the data and provide valuable ecologically relevant insights regarding the interaction between extrinsic and intrinsic factors mediating these behaviors.

1.1: Instrumental behaviors

Goal directed behaviors such as food-seeking often entail instrumental behaviors. Incentivized instrumental behaviors are mediated by response-outcome (R-O) associations that develop when specific actions reliably produce a biologically significant outcome (Thorndike, 1898; Tolman, 1938). Across several experiments, we observed that obesity-prone rats exhibit enhanced instrumental responding for food under specific training conditions. This enhanced responding emerged only when instrumental training included multiple outcomes and rats were trained without an inert response manipulandum (i.e., inactive lever). When trained with a simple instrumental discrimination task, where pressing on an active lever was reinforced, but pressing on a simultaneously present inactive lever was never reinforced, no consistent differences between obesity-prone and -resistant rats were observed. This apparent absence of a reliable group difference was found across multiple independent replications (Fig 2.2B; Fig 7.1; details of these replications are outlined in Appendix A). In contrast, when rats were instead trained with two independent lever R-O associations, obesity-prone rats exhibited elevated responding which emerged across training (Fig 3.4B). A

Across two independant replications, lever pressing was similar between obesity-prone and -resistant rats when trained on a simple instumental discrimination task.

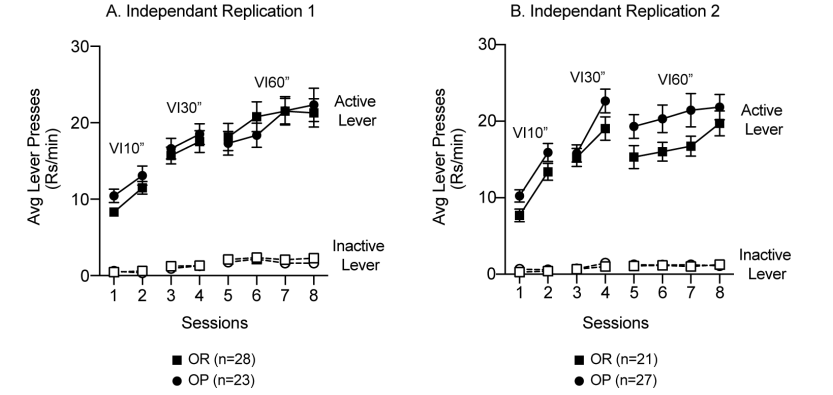

Figure 7.1: Lever pressing on a simple instrumental discrimination task was similar between obesity-prone and -resistant rats across two independent replications. (All data are shown as averages ±SEM.)

similar effect was observed in an independent control study designed to test for intrinsic flavor preferences across three different outcomes. In this control study, rats learned to press on a single lever to earn three uniquely flavored pellets, where in a given session only one flavor was available and no inactive lever was present. We found that obesity-prone rats responded significantly more for pellets than obesity-resistant rats (Fig 7.2; Appendix B). These findings suggest that richer instrumental training conditions (i.e., variety of available outcomes) reveal enhanced instrumental incentive motivation in obesity-prone rats. Moreover, the elimination of the inactive lever may have also promoted this enhanced instrumental incentive motivation by preventing any dampening effects exerted by the engagement of inhibitory learning processes required for response inhibition on the inactive manipulandum.

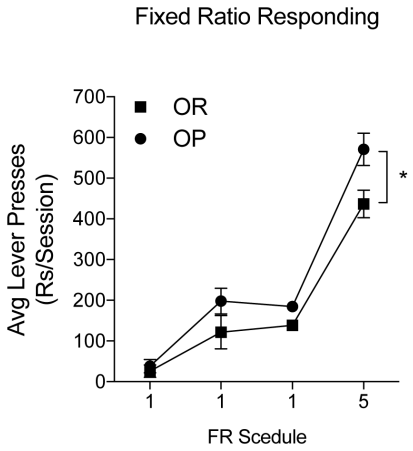

Figure 7.2: Obesity-prone rats exhibited greater lever pressing than obesity-resistant rats, when trained to press one lever to earn multiple outcomes on FR reinforcements schedules. (All data are shown as averages ±SEM; *=p>0.05.)

Interestingly, while obesity-prone rats responded more strongly under richer training conditions, this effect was not sustained under instrumental extinction conditions (Figure 3.5A). This has an important implication regarding the nature of the associative structure mediating this effect. Specifically, this finding demonstrates that enhanced instrumental responding in obesity-prone rats is mediated by an explicit R-O association, rather than a habitual stimulus-response (S-R) association. If an S-R association drove this effect, then the presence of the lever would ballistically evoke a press response, devoid of any mental representation of the outcome. Behaviors mediated by S-R mechanisms can be identified via their insensitivity to changes in the value of their associated outcomes or by changing the reinforcement schedule (Adams, 1982; Dickinson, 1998; Dickinson and Weiskrantz, 1985). Consequently, the finding that when pressing on the lever that was no longer reinforced, obesity-prone rats quickly adapted and any evidence of group differences disappeared, demonstrates that the previously observed enhanced instrumental behavior was driven by an incentive, R-O, process. This also implies that the outcomes themselves exert greater motivational influence in obesity-

prone rats. This is consistent with our finding (discussed above) that as we increase the variety of outcomes available in training, enhanced lever responding begins to emerge in obesity-prone versus -resistant rats.

In sum, when rats were trained under sparse reinforcement conditions, instrumental responding in obesity-prone rats did not differ from obesity-resistant rats. However, training in relatively rich conditions enhanced instrumental responding in obesity-prone versus obesity-resistant rats. These data reveal that selective breeding for obesity susceptibility indirectly selects for enhanced instrumental incentive motivation.

1.2: Pavlovian conditioned behaviors

Pavlovian associations and their resultant behaviors comprise an important category of goal directed behaviors that play a critical role in food-seeking. Pavlovian behaviors are mediated by stimulus-outcome (S-O) associations that form between a conditioned stimulus (CS) and its biologically meaningful outcome. Throughout several experiments, obesity-prone rats displayed stronger Pavlovian incentive motivation than obesity-resistant rats. Yet these differences were only reliably apparent under specific conditions.

When rats were trained with a simple Pavlovian discrimination task (CS+/CS-) using long duration CSs (120 sec), obesity-prone rats displayed stronger conditioned approach than obesity-resistant rats, an effect that was made more reliable with increased training (Fig 7.3; Appendix C). With limited training this effect was not always apparent, which led me to conduct additional studies to determine if increasing the amount of conditioning would stabilize this effect. Sure enough, increasing the duration of conditioning reliably elevated conditioned anticipatory response rates in obesity-prone rats, relative to obesity-resistant rats (Fig 7.3A). Anticipatory responding is measured in the absence of pellets (during the first 10 sec of the CS) and is therefore a pure measure of conditioned responding. However, observation of responding during the remainder of the CS presentation when pellets are intermittently delivered provides highly ecologically relevant measure of incentive behavior. Consider that in real world settings, food CSs and food consumption are typically experienced concurrently. Thus, it is of particular interest that obesity-prone rats showed reliably enhanced food cup approach during the

CS in the presence of pellet delivery earlier in training than was observed with anticipatory responding (Fig 7.3B; Appendix C). Lastly, observation of conditioned approach under extinction conditions provides information about the robustness of the incentive motivational properties of a CS. Here, we found that conditioned responding under extinction was greatly enhanced in obesity-prone rats after 16 sessions, whereas this effect was variable at earlier time points (Fig 7.4; Appendix C). Thus, when trained with this relatively lean Pavlovian discrimination procedure (16 pellet-CS pairings/60-minute/day), prolonged training is required in order to reliably reveal enhanced Pavlovian incentive motivation in obesity-prone rats (>12 days).

CS+ food cup approach is greater in obesity-prone vs obesity-resistant rats with extended conditioning.

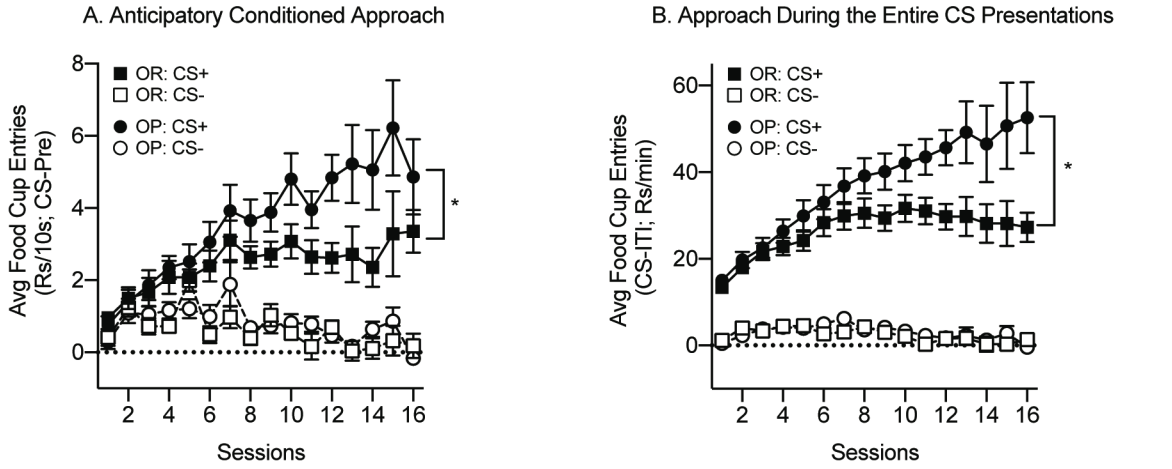

Figure 7.3: With extended Pavlovian conditioning obesity-prone rats exhibited stronger CS+ associated food cup approach than obesity-resistant rats. **A)** As training progressed, obesity-prone rats express greater anticipatory food cup approach in the first 10 seconds of the CS+ prior to pellet delivery, than obesity-resistant rats. **B)** Similarly, food cup approach across the entire CS+ during which pellets are variably delivered, became increasingly stronger in obesity-prone versus obesity-resistant rats, with extended conditioning. (All data are shown as averages ±SEM; *=p<0.05.)

Consistent with the instrumental behavior discussed above, training under a richer paradigm reveled enhanced Pavlovian incentive motivation after fewer training sessions. When trained in a Pavlovian sensory discrimination task, where three distinct associations (CS1-O1; CS-O2; CS3-O3) were established using long duration CSs (120 sec), obesity-prone rats showed much stronger conditioned approach compared to obesity-resistant rats when tested under extinction conditions after only 9 days of conditioning (Figure

3.5D). Interestingly this enhanced conditioned responding was not apparent during training, though there was a trend toward enhanced approach in the presence of reward delivery (F3.4F). Taken together with the response pattern seen in the Pavlovian discrimination task, perhaps the development of differences in anticipatory responding requires more extensive training when a long duration CSs are used. Nevertheless, the data from testing reveal that conditioning under this relatively richer paradigm (48 pellet-CS pairings/90 minutes/day) supports the expression of enhanced Pavlovian incentive motivation in obesity-prone rats, with less training than was observed in the leaner Pavlovian discrimination paradigm.

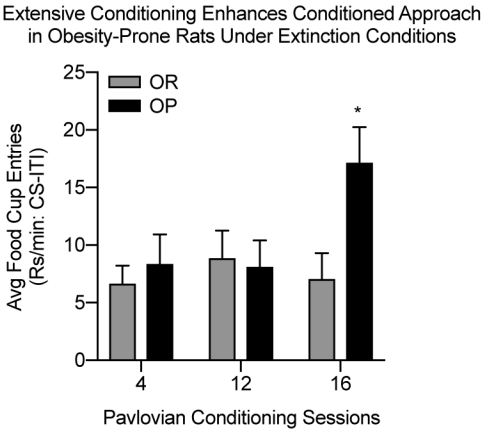

Figure 7.4: Conditioned food cup approach to the CS+ under extinction conditions was greater in obesity-prone versus obesity-resistant rats only after 16 conditioning sessions. (All data are shown as averages ±SEM; *=p<0.05).

In addition to training rats with long duration CSs (like those used during conditioning prior to PIT), I also evaluated conditioned responding using short duration CSs (Appendix D). Classically, long duration CSs do not typically support high levels of conditioned responding, whereas short CSs do (Silva and Timberlake, 1997). Consequently, when trained with a 10 second CS that terminated just prior to pellet delivery, we observed dramatically stronger conditioned responding in obesity-prone versus obesity-resistant rats, and this effect emerged much earlier in training than when rats were conditioned with long duration CSs (Fig 7.5).

Another factor that can influence the expression of Pavlovian incentive motivation is hunger state. For studies in Chapters 2 and 3, rats were food restricted throughout training and testing. In the abovementioned experiment utilizing a short duration CS, I also compared whether hunger state influenced condition responding by including groups that were either food restricted or fed *ad libitum* during training and testing. I found that food restriction amplified acquisition of conditioned responding in both groups, and that group differences emerged more rapidly under these conditions (Fig 7.5). However, by the end of training, obesity-prone rats showed stronger conditioned approach than

obesity-resistant rats, regardless of whether they were trained satiated or hungry. These data revealed the conditioning rats with short CSs and in a state of hunger accelerated the manifestation of enhanced Pavlovian incentive motivation in obesity-prone rats. Interestingly, while the food restriction increased early rates of conditioned approach in both groups, by the end of training, approach rates were similar between hunger states within each group. However, in obesity-prone rats food restriction sustained enhanced conditioned responding over *ad libitum* fed rats for longer than in obesity resistant-rats (Fig 7.5C-D). These data suggest that hunger drive during training does not change the final motivational salience with which a CS is imbued, but intrinsic susceptibility to obesity does and hunger more strongly influences responding in obesity-prone rats.

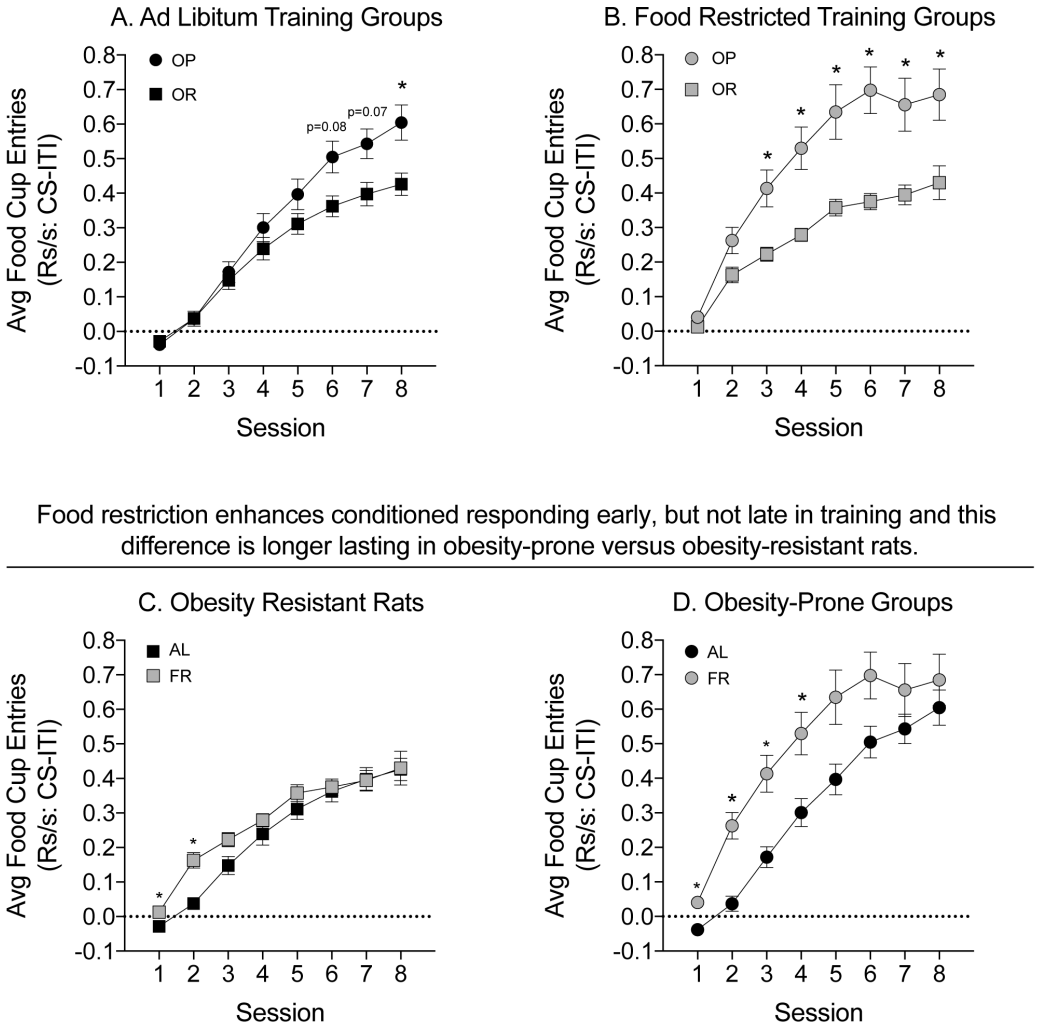

Figure 7.5: Conditioned with a 10 second CS more readily reveals enhanced conditioned food cup approach in obesity-prone versus obesity-resistant rats. **A)** Conditioned approach was initially similar between obesity-prone (OP) and obesity-resistant (OR) rats trained under *ad libitum* conditions, but obesity-prone rats ultimately reached higher rates of responding. **B)** In contrast, when trained under food restriction, obesity-prone rats readily exhibited stronger conditioned approach than obesity-resistant rats very early in training (Session 3). **C)** Comparing the effects of food restriction in obesity-resistant rats, reveals a modest and transient increase conditioned approach in food restricted (FR) versus *ad libitum* (AL) obesity-resistant rats**. D)** In contrast, in obesity-prone rats food restriction enhanced conditioned food cup responding for longer, but by the end of training approach rates were similar between food ad libitum and food-restricted obesity-prone rats. (All data are shown as averages ±SEM; *=p<0.05.)

Collectively these studies examining the expression of Pavlovian motivation in obesity-prone and -resistant rats demonstrate that under three distinct conditioning paradigms obesity-prone rats exhibit robust enhancements in conditioned approach relative to obesity-resistant rats. Furthermore, this effect is systematically enhanced as the richness of training increases and speed at which these differences emerge is accelerated by training in a hungry versus food restricted state.

1.3: Pavlovian to instrumental transfer effects

Modulatory effects comprise another essential mechanism influencing goal directed behaviors (Bouton, 2007). These effects arise when a stimulus develops the ability to modulate expression of a behavior that is directly elicited by an independent association. A classic modulatory effect is the phenomenon of PIT (Walker, 1942). Here a Pavlovian stimulus exerts motivational influence over an instrumental behavior supported by an R-O association. Consistent with the enhancements in incentive Pavlovian and instrumental motivation that obesity-prone rats exhibit discussed above, we also found enhancements in the expression of PIT.

My initial studies examined whether single outcome PIT (SO PIT) differed between obesity-prone and obesity-resistant rats. Although obesity-prone rats displayed stronger SO PIT than obesity-resistant rats in three separate experiments (Fig 2.3, 2.6, 2.7, and 7.6; Appendix E), this effect was not always apparent in other cohorts (Fig 7.7; Appendix C). The variability in the enhanced expression of SO PIT in obesity-prone rats is likely the result of the spartan properties of the training procedures employed to capture SO PIT. For this variant of PIT, rats were trained with instrumental and Pavlovian discrimination tasks discussed above; both are lean protocols that did not readily support enhanced instrumental or Pavlovian incentive motivation in obesity-prone rats. Consequently, it is likely that the sparsity of these training procedures

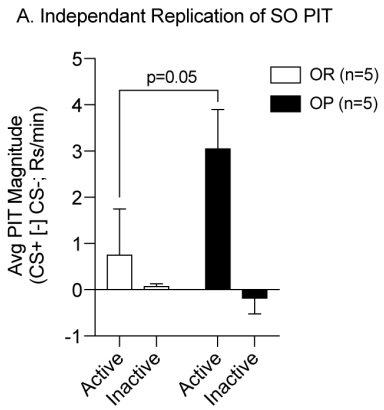

Figure 7.6: In an independent replication, obesity-prone rats exhibit moderately greater SO PIT than obesity-resistant rats. (All data are shown as averages ±SEM.)

prevented the CS+ from reliably developing enhanced modulatory functionality in obesity-prone rats.

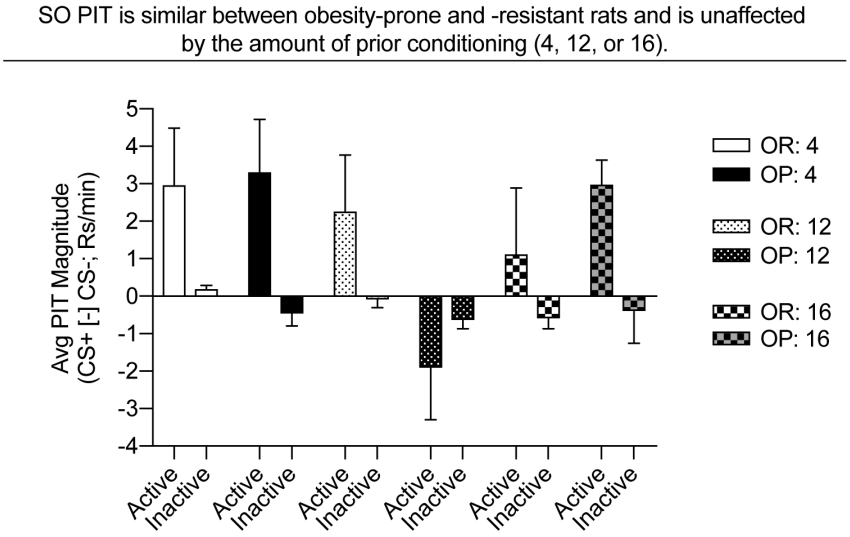

Figure 7.7: In an independent study examining how the amount of Pavlovian conditioning impacted SO PIT expression, no group differences between obesity-prone and obesity-resistant rats were found. (All data are shown as averages ±SEM.)

PIT effects can emerge via a sensory specific process and separately via a general affective process (SS PIT and General PIT, respectively; Corbit and Balleine, 2005). SO PIT does not fall discretely into either of these categories, and shares features of both SS and General PIT. Thus, it's possible that differences in SO PIT between obesity-prone and -resistant groups are driven more strongly by either SS or General PIT processes, and if so the variance in the magnitude of SO PIT may also be attributable to the ambiguity of the underlying transfer mechanism. When I implemented a procedure that allowed me to measure SS and General PIT within the same subjects (Chapter 3), we found that differences between obesity-prone and -resistant groups were most substantially driven by General PIT (observed in both Different and CS3 transfer; Fig 3.5C). Thus, in obesity-prone rats CSs acquire an enhanced capacity to influence instrumental responding via a general affective process. The implications of this behavioral difference for neurobiological differences between obesity-prone and obesity-resistant rats is discussed further in section 2.0 below.

Although rats also showed SS PIT, whether this form of transfer is also enhanced in obesity-prone rats is not totally clear from these results. We observed considerable transfer to the Different lever in both groups. Given that sensory specificity of transfer is typically determined by the comparing differential impact of CSs on the Same versus Different levers, the strong general transfer to the Different lever interfered with our ability to confidently determine the magnitude of sensory specific transfer as classically defined (see Chapter 3 for additional discussion).

Section 1.4: Summary of enhanced incentive motivation in selectively bred obesity-prone rats

Overall, my studies examining incentive motivation reveal that selectively-bred obesity-prone rats exhibit enhanced incentive motivation for food and enhanced motivational control of Pavlovian CSs over instrumental food-seeking. Specifically, we demonstrated instrumental, Pavlovian, and PIT behaviors are all enhanced in obesity-prone rats relative to obesity-resistant rats. We also demonstrated that these effects were most reliable under rich training conditions and when explicit inhibitory associations were absent during training. Furthermore, richer training conditions also supported stronger General PIT, illustrating that the enhanced ability for CSs to modulate instrumental responding in obesity-prone rats is mediated by an affective process. Critically, all of these data were gathered in lean animals trained and tested while food restricted. Consequently, these data provide substantial support for the hypothesis that enhanced incentive sensitivity precedes weight gain in vulnerable individuals.

It is interesting to consider that the obesity epidemic in humans has emerged as our environments have grown richer in both the caloric content, amount and variety of foods available. Furthermore, consider that while BMI and body weight are highly heritable the emergence of the obesity epidemic is a recent phenomenon. Therefore, it would seem that our replete environments may have enabled the phenotypic manifestation of this heritable, vulnerability to obesity. The data presented here provide support for this by demonstrating that under spartan training conditions, differences in incentive motivation for food are less apparent between selectively bred obesity-prone and -resistant rats. Whereas when training conditions are made richer in both the amount

and quantity of food available within these associations, obesity-prone rats reliably exhibit enhanced food-seeking behaviors.

However, one major missing link in the data presented here is whether these training experiences would lead to enhanced weight gain in obesity-prone rats in a more naturalistic setting. This could be answered by conducting an experiment wherein all food is exclusively available through instrumental and Pavlovian associations within the home-cage environment. If a causal relationship exists between enhanced incentive sensitivity for food and weight gain, then systematically increasing the number of associations available in the environment across experimental groups should selectively enhance self-administration and weight gain in obesity-prone rats. Inclusion of yoked control groups receiving an equal number of unpaired stimulus and outcome presentations with access to one R-O relationship would be essential. If the number of associations plays a critical role in the manifestation of obesity-vulnerability, then yoked controlled obesity-prone rats should not self-administer nearly as many outcomes and consequently gain less weight.

2.0: Obesity susceptibility in outbred rats is associated with enhanced General PIT

The data from selectively bred obesity-prone rats provides support for the heritability of enhanced incentive motivation for food, which is consistent with the heritability of body phenotypes, such as BMI and weight (Hur et al., 2008; Maes et al., 1997). However, selective breeding may concentrate this incentive sensitivity beyond that observed in outbred populations, thus determining what this sensitivity looks like in the absence of artificial selection process is important. Moreover, selective breeding carries with it the risk of inadvertent selection of traits that are unrelated to obesity that might seem intuitively linked to obesity, but are in fact unconnected. Therefore, determining whether natural variance[1] in susceptibility to obesity is also associated with enhanced incentive motivation for food provides a means to corroborate the reliability and validity of the effects observed in selectively bred rats. To address this, in Chapter 3 we trained and tested outbred Sprague Dawley rats for PIT and then gave them unrestricted access to a moderately fatty junk-food diet that allows us to evaluate individual differences in degree of weight gain. We then evaluated whether the degree of diet induced weight gain

[1] Variance arising independent of artificial selection processes and assortative mating processes.

correlated with behavior during initial training and testing. Given that the primary goal of this dissertation was to examine Pavlovian motivational processes, studies in outbred rats focused on PIT. Though it is worth noting that no positive correlations with weight and instrumental or conditioned approach was observed (discussed in more detail below).

Using procedures that allow us to distinguish SS PIT from General PIT, we found a strong positive correlation between bodyweight after 5 weeks on JF and the magnitude of General PIT prior to any weight gain (Fig 3.3E-G). This correlation was observed via two independent measures of General PIT: transfer generated by CS3 presentations and in transfer to the Different lever during CS1 and CS2 presentations. These data are consistent with our observation that obesity-prone rats show stronger General PIT, and provide additional evidence of stronger affective Pavlovian motivational control in obesity susceptible populations.

Section 3: Commonalities and differences in incentive motivational processes between selectively bred obesity-prone and outbred obesity susceptible individuals

Instrumental responding and Pavlovian conditioned approach were enhanced in selectively bred-obesity-prone, but not in outbred obesity susceptible rats. This discrepancy suggests that selective breeding for obesity potentiates incentive sensitivity in susceptible individuals. It is also worth noting that while we found no correlation with JF induced weight gain and conditioned approach, Robinson et al., (2015) found that conditioned responding was greater in outbred rats that subsequently gained more weight on a JF diet than less, using a categorical analysis. A critical difference between these studies, is that we used long duration CSs (necessary for supporting PIT effects), whereas Robinson et al (2015) used a short CS. As noted above short duration CSs more robustly support conditioned responses and manifest group difference between obesity-prone and -resistant rats more readily than long CSs. Thus, it is likely that had we used shorter CSs in outbred studies here we would have seen a positive correlation between conditioned approach and JF induced weight gain. Nevertheless obesity-prone rats exhibited stronger conditioned approach to these long duration CSs under parallel

conditions where outbred susceptible rats did not, thereby demonstrating that selective breeding heighted Pavlovian incentive sensitivity.

The capacity of Pavlovian stimuli to modulate instrumental responding (i.e., PIT) via affective mechanism was enhanced in selectively bred obesity-prone rats and was correlated with obesity susceptibility in outbred rats. However, obesity susceptibility was not reliably associated with enhancements in other forms of PIT. In particular, while in some studies SO PIT was enhanced in selectively bred obesity-prone rats, this effect was not always reliable and in outbred rats SO PIT was not correlated with diet induced weight gain. However as discussed above, the mechanisms mediating SO PIT effects do not discretely align with either SS or General PIT processes, and likely represent a chimera of the two processes. This could account for the variability in enhanced expression in obesity-prone rats and the lack of a correlation between obesity susceptibility and SO PIT in outbred rats. In contrast, General PIT, but not SS PIT, was commonly associated with obesity vulnerability in both selectively bred obesity-prone rats and in outbred obesity susceptible rats. In addition to these behavioral findings there are some mechanistic differences mediating Pavlovian incentive motivation and PIT effects between selectively bred and outbred obesity vulnerable individuals; this is discussed below.

In summary, behavioral data reveal that selective breeding for obesity susceptibly amplifies direct incentive motivational control of instrumental and Pavlovian responding. Whereas the ability for Pavlovian CSs to modulate instrumental responding via an affective PIT process is similarly enhanced in both selectively bred and outbred obesity susceptible individuals. The finding that PIT is a more sensitive at detecting variance in motivational sensitivity in outbred populations than conditioned approach measures is not surprising. It is consistent with previous studies demonstrating that PIT is an acutely sensitive measure of the integrity of S-O associations following Pavlovian extinction than conditioned approach (Delamater, 1996; Delamater et al., 2017). Considering the acute sensitivity of PIT, these data demonstrate that an intrinsic trait accompanying obesity susceptibility is enhanced affective motivational control of Pavlovian stimuli over instrumental behaviors.

4.0: Effects of diet on Pavlovian incentive motivation in outbred rats.

Thus far we have discussed the role that individual differences play in incentive Pavlovian motivational processes, however reward experiences themselves can amplify these processes in a manner that may or may not interact with individual variation. Therefore, we also sought to determine whether unrestricted consumption of the JF diet could amplify Pavlovian incentive motivation to a previously established Pavlovian association. To achieve this, outbred rats were initially conditioned to associated a single long duration CS (120 sec) with food delivery and then subsequently placed on an unrestricted diet of JF or standard lab chow. Following this *ad libitum* period rats were tested for conditioned approach and CS potentiated feeding to determine if JF consumption altered Pavlovian incentive processes (Chapter 5). We found that food cup approach elicited by CS presentation (conditioned approach) was enhanced after 14 or 45 days of JF consumption, compared to rats given unrestricted access to Chow (Fig 5.4 and 5.9). Moreover, this effect depended on the predictive validity of the previously trained CS. Thus, JF consumption didn't indiscriminately enhance CS elicited food cup approach, but instead amplified the existing Pavlovian incentive motivation originally established in training. Furthermore, this effect was robust and not easily undermined as reinstatement of conditioned approach following mild extinction was stronger in rats fed a JF diet (Fig 5.5).

Interestingly, while Pavlovian conditioned approach was enhanced by JF consumption, instrumental incentive motivation for these training pellets was markedly reduced in rats fed JF versus chow (Fig 5.6). This demonstrates that JF diet reduced the reinforcing properties of the training pellets, and illustrates a dissociation between Pavlovian motivation and instrumental incentive motivation. Along with this dissociation, we also found that CS potentiated feeding of these training pellets was not different between JF and chow fed rats (Chapter 5, section 4.3; Fig 5.9). Together these findings demonstrate a three-way dissociation between the impact of JF on CS evoked approach, CS potentiated feeding and instrumental responding. Critically, all of these behaviors were measured in the presence of training pellets. Thus, despite the presence of the training pellets with a now reduced reinforcing capacity, conditioned approach was still enhanced in JF treated rats.

These data reveal that palatable diets influence Pavlovian behaviors differently from instrumental and consummatory behaviors. This split is reminiscent of the distinction proposed by Konorski (1967), between preparatory and consummatory motivational processes. Where approach behaviors are classified under preparatory behaviors and instrumental responding and ingestive behaviors are classed as consummatory behaviors. In this framework consumption of JF amplified the preparatory aspects of this Pavlovian CS, while depressing the consummatory behaviors supported by its associated outcome.

Similarly, the split in the effects of JF here are also reminiscent of the distinction made by the incentive sensitization theory between desire for and enjoyment of a given outcome ("wanting" vs "liking"; Berridge et al., 2009). If we consider conditioned approach as a measure of "wanting" and consumption as a proxy for "liking", then JF seemed to sensitize the "wanting", but not the "liking" of the training pellets. One caveat here is that instrumental responding is typically thought to providing a measure of "wanting", but our data here show a distinction between "wanting" as measured by conditioned approach and "wanting" as measured by instrumental responding. However, this disparity might be explained by considering that the Pavlovian S-O association was formed prior to JF exposure and in a state of food restriction, whereas the instrumental R-O association was acquired after JF exposure and in a satiated state. Therefore, the saliency of the memory of the outcome from the S-O association if preserved, would be distinct from the saliency of its current value. Considering this, it seems plausible that JF consumption amplified "wanting" of a preserved representation of the outcome, whereas it suppressed "wanting" for the current representation of the outcome. Thus, the data here might represent an added dissociation between "wanting" driven by a salient memory of an outcome and "wanting" driven by the current representation of that outcome. This added distinction intuitively fits with the colloquial description of "chasing the dragon" that many drug-addicts experience. However, in this case experience with a highly palatable diet may have produced a similar effect with food-seeking.

In regard to the relationship between these effects of JF and individual susceptibility, in this same study, JF-induced weight gain did not correlate with behavior during initial pre-JF conditioning or during post JF testing. Moreover, in this study weight

gain between chow and JF rats did not differ, which is a bit of a curiosity considering the breadth of weight range here (Fig 5.4F and 5.8A). Nevertheless, the absence of a correlation between weight gain and conditioned approach in testing illustrates that the effects of JF on approach cannot be explained by differences in obesity susceptibility. This does not rule out the possibility that the JF induced amplification of conditioned responding may interact with obesity susceptibility under different conditions. But within the confines of the current data, JF diet alone amplified conditioned approach. Collectively these data suggest that consumption of a palatable diet amplifies the motivational capacity of a previously established Pavlovian CS independently of obesity susceptibility. However, some caveats to this conclusion are that weight gain across the post-training *ad libitum* period was similar between chow and JF fed rats, suggesting a homogenous population which may have obscured our ability to observe any correlations.

5.0: Mechanisms underlying differences in incentive motivation in obesity-susceptible populations.

The studies discussed so far have focused on identifying behavioral traits associated with obesity vulnerability. Not only do these provide valuable insights with potential clinical translational relevance, but they also help hone in on brain nuclei that may differ between obesity susceptible and resistant individuals. Notably, the Nucleus Accumbens (NAc) plays a critical role in the expression of PIT (Corbit and Balleine, 2011; Hall et al., 2001), consequently my studies examining the neural mechanisms contributing to differences in PIT between prone and resistant individuals, focused on this nucleus.

5.1: The role of NAc CP-AMPARs and PIT in selectively bred obesity-prone rats

As discussed in Chapter 2, the NAc plays a role in food-seeking behaviors and activity within the NAc elicited by food cues has been linked to enhanced susceptibility to weight accumulation in humans (Demos et al., 2012; Yokum et al., 2014). The NAc receives dopaminergic and glutamatergic afferents that interact to drive goal directed behaviors (Di Ciano et al., 2001; Faure et al., 2008). AMPA receptors mediate glutamatergic transmission and fall into two categories 1) calcium impermeable GluA2 containing receptors 2) calcium permeable GluA2 lacking receptors (Wolf, 2010). NAc

calcium permeable AMPARs (CP-AMPARs) have been implicated in the “incubation” of craving for drugs induced by forced abstinence (Ferrario et al., 2010; Scheyer et al., 2016). It has been suggested that NAc CP-AMPAR upregulation is a unique feature of this form of drug craving and thereby plays a key role in addiction (Wolf, 2016). However, challenging this idea, Oginsky et al., (2016), demonstrated that NAc CP-AMPARs were upregulated following brief period of unrestricted consumption of JF in obesity-prone rats. Moreover, this upregulation was observed only 1 day following removal from this diet. This illustrates two critical points: 1) experience with palatable food (as opposed to drugs of abuse) can drive upregulation of NAc CP-AMPARs, and 2) this effect is rapid and does not require extended abstinence. This surprising finding suggested that NAc Core CP-AMPARs are not strictly tied to aberrations associated to drug intake, but may instead be associated with more naturalistic experiences, particularly in populations exhibiting heightened incentive sensitivity. In support of the notion that NAc CP-AMPARs are also associated with experience with natural rewards, Dingess et al., (2017) found that in rats trained to self-administer chow pellets, NAc CP-AMPARs were upregulated after 30 days of time away from this task, paralleling the forced abstinence induced upregulations found for drugs of abuse (Conrad et al., 2008; Ferrario et al., 2011a; Loweth et al., 2014; Scheyer et al., 2016). Jointly, these studies lead us to the idea that perhaps NAc CP-AMPARs played an important role in incentive motivation processes more generally and particularly in selectively bred obesity-prone rats that exhibit heightened incentive sensitivity for food.

First, we found that following instrumental and Pavlovian conditioning, cell surface expression of NAc CP-AMPARs was elevated in obesity-prone rats relative to untrained rats, whereas this effect was not seen in obesity-resistant rats (Fig 2.5). To determine the functional role of these receptors in PIT, we infused the selective CP-AMPAR antagonists, NASPM, into the NAc Core immediately prior to SO PIT testing. NASPM infusions attenuated the expression of SO PIT in obesity-prone, but not obesity-resistant rats (Fig 2.6 and 2.7), an effect that was replicated in a subsequent independent study, these data are shown here in Fig 7.8 (details in Appendix E). Interestingly, conditioned approach was not affected by NASPM infusions (Fig 2.6 and 2.7). Jointly, these data demonstrated that NAc CP-AMPARs mediate the ability for Pavlovian stimuli to modulate instrumental

responding in obesity-prone, but not obesity-resistant rats. Thus, selective breeding for obesity vulnerability carries with it a change in the underlying neuronal mechanisms mediating the ability for Pavlovian stimuli to motivationally influence instrumental responding for food.

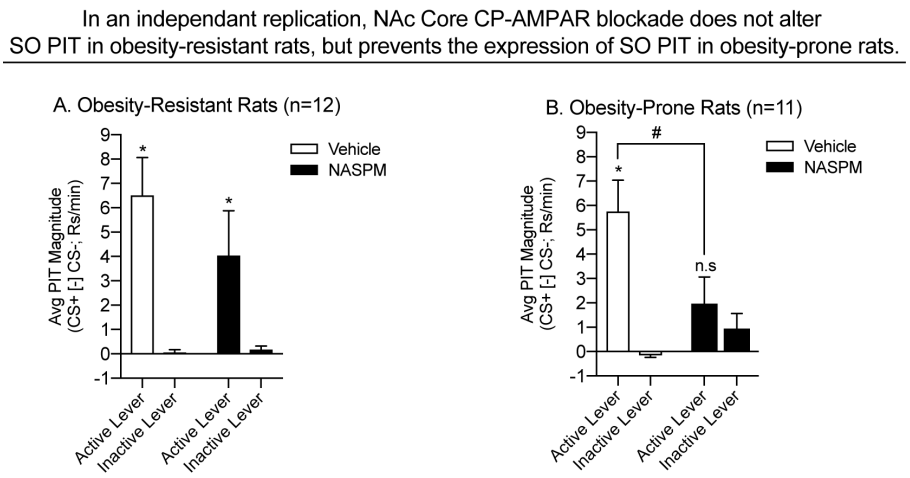

Figure 7.8: An independent replication of the selective effects of NAc Core CP-AMPAR blockade on SO PIT in obesity-prone, but not obesity-resistant rats. **A)** In obesity-resistant rats, infusion of NASPM into the NAc Core did not alter the expression of SO PIT significantly. **B)** In contrast, in obesity-prone rats, infusion of NASPM blocked the expression of SO-PIT as is apparent by the reduction in transfer on the active lever between Vehicle and NASPM conditions and in the loss of differential transfer effects to the active versus the inactive lever. (All data are shown as averages ±SEM. *,#= $p<0.05$)

These data open numerous questions that remain to be addressed. For instance, which aspect of experience during training drove CP-AMPAR upregulation in obesity-prone rats? It is possible that CP-AMPARs emerge with cumulative associative experiences including both acquisition of instrumental learning, or the acquisition of Pavlovian learning? Instead, perhaps only one of these processes promotes CP-AMPAR surface expression, or even more simply that merely consuming the pellets used in training drive this effect. The finding by Ognisky et al., (2016) that consumption of JF increases NAc Core CP-AMPARs in obesity-prone rats, suggests that experience with rewarding food alone can drive this effect. Yet the highly specific role of CP-AMPARs in SO PIT might also suggest that this upregulation is specific to synaptic plasticity associated with the R-O and or S-O associations acquired in training. These possibilities are all open to exploration and would provide important insights as to the behavioral driving force of this upregulation.

Relatedly, the precise molecular mechanism driving this CP-AMPAR up-regulation remains unknown. It is possible that upregulation arises via increased synthesis of GluA1 relative to GluA2 stochastically resulting in a greater abundance of GluA2 lacking

receptors available for trafficking to the surface (Boudreau et al., 2012; Conrad et al., 2008). However, in our data, while training increased surface GluA1 it concomitantly reduced intracellular GluA1, pointing to the more likely possibility that trafficking upregulation of CP-AMPARs was due to preferential trafficking of GluA1 containing AMPARs to the surface. Trafficking of GluA1 to peri-synaptic sites in the cell surface is mediated by phosphorylation of the 845-serine residue via PKA (Oh et al., 2006; Roche et al., 1996; Wolf, 2010). At the surface, transmembrane AMPA receptor regulatory proteins (TARPs) associated with AMPARs to stabilize them in the membrane. In particular γ-4 associates with GluA1 containing AMPARs in peri-synaptic sites of the NAc (Ferrario et al., 2011b). Another TARP, γ-7 has been shown to preferentially associates with GluA1 and mediates retention of CP-AMPARs in synaptic sites of the cerebellum (Bats et al., 2013; Studniarczyk et al., 2013). Thus, perhaps the enhancements in surface CP-AMPAR in obesity-prone rats depends on enhanced activity or expression of γ -4 or γ -7 TARPs. However, whether γ-7 serves a similar role in CP-AMPAR synaptic retention in the NAc is an open question. Experiments exploring these possibilities would provide valuable insights to the basic processes mediating CP-AMPAR upregulation generally and more specifically expand our understanding of molecular underpinnings of incentive motivational processes.

5.2: The role of NAc CP-AMPARs and PIT in outbred obesity susceptible rats

The data taken from selectively bred rats suggest NAc CP-AMPARs mediate PIT in obesity-prone, but not obesity-resistant rats. This raises the question as to whether similar mechanisms would be observed in obesity susceptible individuals in outbred populations, or alternatively whether this is a unique product of selective breeding. In Chapter, 4 I examined this by testing outbred rats for SO PIT under NAc Core CP-AMPAR blockade or vehicle conditions. Following testing rats were place on an unrestricted JF diet to induce weight gain and then correlational analyses were performed between post-JF weight and pre-JF PIT testing data. These data revealed that the degree to which CP-AMPAR blockade by NASPM reduced SO PIT was strongly positively correlated with weight (Fig 4.4C). However, weight did not correlate with the magnitude of PIT under vehicle conditions (Fig 4.4A). This is not entirely surprising considering the extensive

discussion above about how SO PIT may be driven by different mechanisms (sensory specific vs affective) across individuals. Nevertheless, taken together with the data from selectively bred rats, the mediational role of NAc Core CP-AMPARs in PIT is a common neuronal mechanism associated with obesity susceptibility.

5.3: Commonalities and differences in the role of NAc Core CP-AMPARs in Pavlovian behaviors in selectively bred and outbred rats

Our data from studies in selectively bred and outbred rats reveal that NAc Core CP-AMPARs mediate the ability for Pavlovian stimuli to modulate instrumental behaviors in obesity susceptible individuals. However, one point of divergence between outbred and selectively bred rats, was that in outbred rats NAc CP-AMPAR blockade attenuated conditioned approach, but this effect was not seen in obesity-prone rats. Moreover, while NASPM attenuated conditioned approach in most outbred rats, the magnitude of this blockade effect was positively correlated with weight, suggesting that CP-AMPARs more strongly mediated Pavlovian conditioned approach in obesity susceptible individuals. Also of note, while in outbred rats NASPM reduced conditioned approach, it did not entirely eliminate the behavior or the discriminatory CS+/CS- responding. This, suggests that NAc Core CP-AMPARs only play a partial role of in this behavior in outbred rats. This latter point suggests that conditioned approach is also supported by either other receptors in the NAc Core or by other brain regions. This might help explain why NAc Core CP-AMPAR blockade did not attenuate conditioned approach in obesity-prone rats. It is possible that other nuclei and or other receptors in the NAc Core were better able to compensate for the loss of NAc Core CP-AMPAR function. This is consistent with the idea that under sufficiently rich training conditions selectively bred obesity-prone rats show stronger conditioned approach, suggesting that the underlying circuitry mediating approach is stronger in these rats. This may in turn render them less sensitive to the effects of NAc Core CP-AMPAR blockade because the wider circuitry mediating conditioned approach can better compensate for this perturbation. Thus, selective breeding for obesity susceptibility seems to have bolstered the wider circuitry mediating conditioned approach.

Considering that selectively bred and outbred obesity susceptible rats share a common neural mechanism for SO PIT and that both populations exhibit enhanced General PIT, this raises the question as to whether NAc Core CP-AMPARs also mediate General PIT. This seems especially likely, considering that SO and General PIT shares some overlapping circuitry and both depend on the NAc Core (Corbit and Balleine, 2011; Hall et al., 2001). Yet, this is an outstanding question that remains to be answered.

Relatedly, an interesting unresolved challenge regarding General PIT is the identification of the direct circuitry driving this effect. Specifically, while both the central nucleus of the amygdala (CN) and the NAc Core are critical for General PIT (Corbit and Balleine, 2005; Corbit and Balleine, 2011), there is no direct connection between these nuclei (Sah et al., 2003). Therefore, at least one intermediary nucleus must serve as a connection hub between these structures. Moreover, the role of CP-AMPARs in SO PIT implicates glutamatergic transmission in the NAc as essential. Thus, if SO PIT and General PIT are governed by similar neural processes in obesity susceptible individuals, then it is likely that the intermediary connection between the CN and NAc Core is glutamatergic. The identity of this intermediary nucleus is not clear; however, two candidate nuclei are the ventral tegmental area (VTA) and the Raphe Nucleus (RN). While the VTA is primarily composed of dopaminergic neurons, and the RN of serotonergic neurons, each of these nuclei send glutamatergic projections to the NAc Core and both are innervated by the CN (Brog et al., 1993; Chang et al., 2011; Sah et al., 2003; Taylor et al., 2014). Along with the NAc Core and CN, the VTA has also been implicated in General PIT (Corbit et al., 2007), yet whether a circuit between these three structures mediates Generate PIT remains open to study.

In contrast, the role the serotonergic RN in PIT has not been examined directly. However, systemic depletion of tryptophan (the serotonin precursor) in humans has been shown to block aversive, but not appetitive PIT (Geurts et al., 2013). This suggests that the serotonergic RN may play a role in at least some forms of PIT. This potential role of the RN and more specifically whether a CN-RN-NAc Core circuit exists and whether it mediates General PIT needs to be examined. One additional consideration in these proposed lines of research is that utilization of DREADD techniques to explore this would be challenging, considering my finding in Chapter 6, that CNO (the DREADD activating

ligand) abolishes General PIT regardless of DREADD expression, likely via a shift in interoceptive state. Therefore, optogenetic tools might be better suited for these proposed studies.

Lastly, our data here also open the question as to what mechanisms control SO PIT in obesity-resistant and outbred rats not susceptible to obesity. In obesity-resistant rats, expression of SO PIT was often weaker than obesity-prone rats and sometimes absent (Fig 2.7 and 7.6A). Moreover NAc CP-AMPARs were not increased by training and nor did blockade of NAc Core CP-AMPARs disrupt SO PIT in obesity-resistant rats. There are several potential explanations for this lack of an effect by NAc Core CP-AMPAR blockade. One tempting explanation is that perhaps in obesity-resistant rats SO PIT is primarily mediated by a sensory specific process, in which case expression would be controlled by the NAc Shell as opposed to the Core. If this is the case then SO PIT expression in obesity-resistant rats may be more readily disrupted by NAc Shell manipulations. Studies have shown that connection between the BLA and NAc Shell mediate SS PIT (Shiflett and Balleine, 2010) and in Chapter 6, I demonstrated that SS PIT relies on the CamKII BLA neurons. Thus, we might also expect that manipulation of glutamatergic transmission between the BLA and NAc Shell might disrupt SO PIT in obesity-resistant rats, if in fact this behavior is mediated by a sensory specific process.

Another possibility is that other receptors within the NAc Core may mediated SO PIT in obesity-resistant rats. We attempted to address this by administering CNQX which theoretically should act as a general AMPAR antagonist, and yet found no effect on SO PIT. However as noted in Chapter 2, the ability for CNQX to block activity at AMPARs is influenced by both the concentration of glutamate at the synapse and by the type auxiliary proteins associated with the AMPARs (Kawai, 1991; Kott et al., 2009; Maclean and Bowie, 2011; Menuz et al., 2007). Thus, it is possible that the endogenous glutamate concentrations may have precluded the antagonists of AMPARs here, though given the high binding affinity of CNQX this possibility seems less plausible. On the other hand, it seems quite plausible that the auxiliary proteins associated with the AMPARs within the NAc Core synapse may have prevented CNQX from effectively blocking AMPAR transmission at these synapses. Therefore, perhaps infusion of a selective non-competitive AMPAR antagonist, such as Perampanel, may better determine the

contribution of NAc Core AMPARs generally in the expression of SO PIT among obesity-resistant rats (Rogawski, 2011; Rogawski and Hanada, 2013).

6: A broader role for NAc CP-AMARs in enhanced incentive motivation for food and drug

Studies identifying the role of NAc Core CP-AMPARs in the incubation of drug craving proposed that these receptors serve a unique and defining role in drug craving and addiction (Conrad et al., 2008; Loweth et al., 2014; Ma et al., 2014; Scheyer et al., 2016; Wolf, 2016). However, a few studies have challenged this by demonstrating that experience with food reward can trigger NAc upregulation in CP-AMPARs (Dingess et al., 2017; Oginsky et al., 2016). My biochemical data presented in Chapter 2, provide yet another demonstration that naturalistic food related experience can drive upregulation in NAc CP-AMPARs. However, my NASPM data go one step farther in demonstrating that NAc Core CP-AMPARs mediate Pavlovian incentive motivational processes for natural rewards. These data collectively challenge the idea that NAc CP-AMPARs are uniquely involved in addiction processes.

However, my finding that the role of NAc CP-AMPAR in the expression of PIT was specific to obesity susceptible individuals suggests that these NAc CP-AMPARs might be uniquely tied to enhanced incentive motivation. With this in mind, let us consider the incubation of drug craving studies. In these studies, NAc CP-AMPARs are not simply associated with drug intake, but rather are specifically upregulated across a forced abstinence period. Importantly, during this same period incentive motivation for drug increases and selective blockade of NAc CP-AMPARs blocks the expression of this enhanced motivation by bringing post-abstinence response levels down to those observed after only one day of withdrawal. Similarly, the increases in NAc CP-AMPARs found in chow self-administering rats following 30 days off-task was paralleled by enhanced lever pressing for chow at this same time point (Dingess et al., 2017). Thus, here too CP-AMPAR upregulation parallels increased incentive motivation. A common feature across all these studies and my results is that NAc CP-AMPARs are associated with heightened incentive motivation. For the incubation of craving studies for drug and chow, heighted incentive motivation was induced by force abstinence or time off-task, respectively, whereas in my study this heighted incentive motivation was linked with

intrinsic traits associated with obesity vulnerability. Therefore, CP-AMPARs may more broadly represent enhanced incentive motivation for both drug and natural reward, such as food.

One notable distinction worth addressing between my studies here and those using the incubation of craving model, is that training and testing procedures used in the latter do not distinguish between instrumental and Pavlovian incentive motivation. For incubation of craving experiments, rats learn to lever press for concomitant presentation of a CS and an intravenous infusion of cocaine (i.e., the reward, R). Once the outcome has been delivered, presses on the lever are not reinforced during a brief timeout. This training is likely to promote at least two unique associative structures: R-S-O and S-O. Then in testing, rats are asked to lever press under drug extinction conditions, where pressing results in presentation of the CS alone. Responding in the first phase would not be expected to be especially high considering that lever was not reinforced in the absence of CS delivery during training. In the second phase, responding may be driven by either expectation of the outcome or by the properties or reinforcement imbued in the CS. This poses a challenge for interpreting the incentive mechanisms mediating lever responding in this phase. In contrast, PIT, cleanly distinguishes between instrumental and Pavlovian incentive motivation. During training these R-O and S-O associations are acquired in isolation and during testing food cup approach and lever pressing in the presence and absence of the CS. This provides three distinct readouts, instrumental incentive motivation (lever pressing alone), Pavlovian incentive motivation (conditioned approach), and the ability for Pavlovian stimuli to modulate ongoing instrumental behavior (PIT). Thus, in contrast to the incubation of craving studies, our data pinpoint the role of NAc CP-AMPARs specifically to Pavlovian motivational processes. In sum, CP-AMPARs in the NAc Core play an important role in the expression of heightened incentive motivation and this does not appear to be unique to drugs of abuse.

7: Summary and future direction:

In summary, the behavioral data presented here demonstrate that individual susceptibility to obesity is associated with heightened sensitivity to affective motivational control by Pavlovian stimuli. Furthermore, selective breeding enhances both instrumental

and Pavlovian incentive motivation which is especially apparent under rich training experiences. These data help identify psychological traits associated with obesity vulnerability that may mediate expression of this vulnerability by triggering enhanced food-seeking. Independently, I also showed that consumption of a palatable JF diet enhances Pavlovian incentive motivation and that did not interact with obesity susceptibility. This finding illustrates that rewarding experiences with food may also trigger enhanced food-seeking. Collectively, these data identify both intrinsic and extrinsic factors that mediate enhanced food-seeking and likely contribute to the obesity epidemic. These findings also reveal a few potential avenues for obesity-prevention and treatment. First, the finding that richness of training potentiates Pavlovian incentive motivation, suggests that individuals with known obesity susceptibility may be benefited by reducing the variety of food options available in their home environment. Second, given the underlying mechanism of enhanced cue-triggered food-seeking in obesity susceptible individuals is an affective process, and that this form of motivational control is especially sensitive to hunger, it would seem that maintaining a stable sate of general satiation may prevent the expression of heightened food-seeking in these individuals. Of course, satiation would have to be maintained in a calorically conscientious manner. Lastly, the finding that chronic consumption of a palatable diet potentiated Pavlovian motivation across the weight spectrum suggests that limiting daily consumption of highly palatable food might help prevent this effect. So, while both intrinsic susceptibility to obesity and experience with highly palatable foods independently potentiate Pavlovian approach, awareness of these specific factors should help us design prevention and treatment plans to contain heighted food-seeking.

One unanswered question worth considering here is how consumption of a palatable diet interacts with intrinsic incentive sensitivity in selectively bred obesity-prone rats. Rather than merely being a point of scientific curiosity, this has actual ecological relevance given that humans exhibit assortative mating practices according to body types (Ajslev et al., 2012; Dawson et al., 2013; Hur, 2003; Jacobson et al., 2007). Although in outbred rats we did not find a relationship between weight and JF induced enhancements in conditioned approach, what this looks like in obesity-prone rats is unknown. We might expect that intrinsic incentive sensitivity would result in even stronger effects in these rats.

Furthermore, JF consumption in obesity-prone rats increases NAc Core CP-AMPARs, however, we also showed that these receptors do not critically mediate conditioned approach in these rats. This does not rule out the possibility that CP-AMPARs are enhanced in the wider network mediating conditioned approach or that JF consumption produces other changes essential for expression of this behavior. Understanding how intrinsic incentive sensitivity interacts with rewarding food experiences is an essential next step for understanding how obesity arises and may be maintained.

Our neurobiological studies here have identified the dependence of motivational control of Pavlovian stimuli over instrumental food-seeking on NAc Core CP-AMPARs as an intrinsic mechanism tied to obesity-vulnerability. These data tie together studies in outbred rats, selectively bred obesity-prone rats and even shed light on the possible mechanisms mediating enhanced NAc activity found in humans susceptible to weight gain (e.g., Demos et al., 2012). Furthermore, this finding establishes a central role for NAc Core CP-AMPARs in Pavlovian motivation, by specifically linking individual susceptibility to obesity and enhanced Pavlovian motivation. Whether CP-AMPARs serve a similar role in other nuclei critical for the expression of incentive motivation, such as the amygdala, is an open question.

Relatedly, whether the propensity for synaptic trafficking of CP-AMPARs exists as a spectrum across individuals and experiences has yet to be systematically illustrated. Perhaps highly sensitive individuals possess the molecular mechanisms/machinery that more readily facilitates CP-AMPAR synaptic incorporation, but that given a sufficiently salient experience this effect would be observed across all individuals. This would seem likely, given that incubation of craving associated increases in NAc CP-AMPARs for both food and drug have been shown in outbred populations, where no specific traits had been selected for (Conrad et al., 2008; Dingess et al., 2017; Loweth et al., 2014; Scheyer et al., 2016). In further support of this, our data in outbred rats suggests that the role of CP-AMPARs in mediating Pavlovian motivation exists on a continuous spectrum that is tied to bodyweight. Systematic demonstration of this spectrum can theoretically be achieved by carefully titrating the saliency of given experience and then measuring either the functional contribution of CP-AMPARs to related behaviors in vivo or to glutamatergic transmission ex vivo. Whether or not a true spectrum of CP-AMPAR synaptic inclusions

readiness exists, out data clearly demonstrate that intrinsic susceptibility to obesity is associated the mediation role of CP-AMPARs in Pavlovian motivation.

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Appendices

Appendix A: Independent Replications of Instrumental Discrimination Tasks in Obesity-Prone and Obesity-Resistant Rats

In two independent studies, male obesity-prone (replication 1, n=23; replication 2, n=27) and obesity-resistant rats (replication 1, n=28; replication 2, n=21) were trained on a simple instrumental discrimination task as detailed in Chapter 2, Section 2.2. Responding in both replications increased with training (Figure 7.1: Two-way RM ANOVA; main effect of session, Figure 7.1A: $F_{(7,343)}=67.22$, $p<0.01$; Figure 7.1B:$F_{(7,322)}=39.37$, $p<0.01$) and did not differ significantly between groups (Figure 7.1: Two-way RM ANOVA; no effect of group, Figure 7.1A: $p=0.93$; Figure 7.1B: $p=0.07$; no session by group interactions, Figure 7.1A: $p=0.31$; Figure 7.1B: $p=0.10$).

Appendix B: Fixed Ratio Responding for Three Distinct Pellets is Stronger in Obesity-Prone Versus Obesity-Resistant Rats

Male obesity-prone (n=4) and obesity-resistant rats (n=4) were trained to press one lever to earn three flavored 45 mg pellets (Bioserv: Unflavored #F0021; Banana #F0059; Chocolate #F0299). Sessions lasted 20 minutes and one flavor of pellets was delivered per session on a fixed ratio (FR) schedule. For the first three sessions, an FR1 schedule was used and then rats were shifted to and FR5 schedule in the final session. Responding for the different flavors did not differ in either group (Data not shown, Two-way RM ANOVA; no effect of flavor, OP: $p=0.08$; OR: $p=0.13$), therefore, for ease of analysis responding across pellets was collapsed. Lever pressing increased over training and was significantly higher in obesity-prone versus obesity-resistant rats (Figure 7.2 Two-way RM ANOVA; main effect of session, $F_{(2.00, 12.01)}=131.6$, $p<0.01$; main effect of group, $F_{(1, 6)}=7.27$, $p=0.03$; no session by group interaction, $p=0.13$).

Appendix C: Extensive Conditioning on a Simple Pavlovian Discrimination Task Enhances Conditioned Approach in Obesity-Prone Rats Relative to Obesity-Resistant Rats

Following instrumental training rats from Appendix A (Independent Replication 1), were conditioned in a simple Pavlovian discrimination task as described in Chapter 2, Section 2.2. In this experiment, obesity-prone and obesity-resistant rats were trained for 4, 12, or 16 sessions (Groups 4: OP, n=8; OR, n=7; Group 12: OP, n=14; OR, n=12; Group 16: OP, n=9; n=7). For ease of representation and to increase power of analysis, these data were analyzed with training groups collapsed using a mixed effects analyses. These analyses reveal that conditioned anticipatory approach to CS+ presentation increased across training and was greater in obesity prone rats (Figure 7.3A: Mixed-effects model: main effect of session, $F_{(5.20,187.4)}=131.6$, $p<0.01$; main effect of group, $F_{(1,55)}=6.04$, $p=0.02$). In contrast, anticipatory approach to CS- presentation decreased with training and did not differ between groups (Figure 7.3A: Mixed-effects model: main effect of session, $F_{(7.367, 265.7)}=3.88$, $p<0.01$; no effect of group, $p=0.44$). Similarly, food cup approach across the entire CS+ increased with training and was stronger in obesity-prone rats (Figure 7.3B: Mixed-effects model: main effect of session, $F_{(2.470, 89.07)}=35.98$, $p<0.01$; main effect of group, $F_{(1,55)}=6.32$, $p=0.02$; session by group interaction, $F_{(15, 541)}=3.57$, $p<0.01$). In contrast approach during the CS- decreased across sessions and did not differ between group (Figure 7.3B: Mixed-effects model: main effect of session, $F_{(7.510, 270.9)}=4.90$, $p<0.01$; no effect of group, $p=0.80$).

Following conditioning, rats were then tested for PIT under extinction conditions as described in Chapter 2, Section 2.2. During this testing we measured both conditioned approach and PIT. Conditioned approach during the CS+ was greater in obesity-prone rats conditioned with 16 sessions than obesity-resistant rats, but did not differ in the groups trained in 4 and 12 conditioning sessions (Figure 7.4: Mixed-effects model: main effect of group, $F_{(1,52)}$=5.10, p=0.03; planned comparison OP v OR: Group 4, p=0.86; Group 12, p=0.86; Group 16, p=0.01). In contrast, PIT was similar between obesity-prone and obesity-resistant groups independent of the amount of prior conditioning (Figure 7.5 Two-way RM ANOVA: main effect lever, $F_{(1,43)}$=9.70, p<0.01; no effect of group, p=0.15; no lever by group interaction, p=0.22).

Appendix D: When Trained with a Short Duration CS, Obesity-Prone Rats Exhibit Enhanced Conditioned Approach Relative to Obesity-Resistant Rats and this Effect is Enhanced by Food Restriction

Adult male obesity-prone and obesity-resistant (OP, n=51; OR, n=75) rats underwent simple Pavlovian conditioning with a short duration CS. Rats were split into groups trained *ad libitum* (AL; OP, n=28; OR, n=34) or trained food restricted (FR; OP, n=34; OR, n=40). FR rats were restricted to 85-90% of their *ad libitum* weights and maintained at this weight throughout the remainder of the study. AL rats were allowed unrestricted access to consumption of chow in their home-cages. Once reaching the target weight range, rats were initially trained in one session to retrieve food pellets (Bioserv: Unflavored #F0021) as described in Chapter 3, Section 2.4. Next rats were conditioned to associate a 10-second white noise with delivery of two pellets at its offset. Pavlovian conditioning was conducted in eight, 30 min sessions. Each session consisted of 25 CS trials that were separated by a 60-sec (range, 30”-90”) variable inter-trial interval (ITI). For the data reported here, rats were excluded from all behavioral analysis if they did not acquire conditioned anticipatory food cup approach to CS presentations. The criteria for this behavior was that food cup entries had to be increased above pre-CS entry levels during the final two sessions of conditioning (excluded: OR-AL, n=1).

First, we compared conditioned approach between obesity-prone and obesity-resistant rats within each training condition. Among groups trained under *ad libitum* conditions, conditioned approach increased over training and toward the end of training

approach grew stronger in obesity-prone versus obesity-resistant rats (Figure 7.5A: Two-way RM ANOVA: main effect of session, $F_{(3.35,197.5)}$=161.8, p<0.01; main effect of group, $F_{(1,59)}$=4.96, p=0.03; session by group interaction, $F_{(7,413)}$=4.96, p<0.01). In groups trained under food restriction, approach increased across training, but approach was stronger in obesity-prone rats and this group effect was evident early in training (Figure 7.5B: Two-way RM ANOVA: main effect of session, $F_{(7,420)}$=79.47, p<0.01; main effect of group, $F_{(1,60)}$=24.07, p<0.01; session by group interaction, $F_{(7,420)}$=5.57, p<0.01). Next, we compared the effects of food restricted within each strain. Among obesity-resistant rats, conditioned responding did not significantly differ between training conditions (Figure 7.5C: Two-way RM ANOVA: no effect of training conditions, p=0.11). In contrast, in obesity-prone rats, food-restriction enhanced conditioned approach early in training, but by the end of training approach was similar between training conditions (Figure 7.5D: Two-way RM ANOVA: main effect of training condition, $F_{(1,48)}$=10.17, p<0.01; session by training condition interaction, $F_{(7,336)}$=2.46, p=0.02).

Appendix E: In an Independent Replication, NAc Core CP-AMPAR Blockade Abolishes SO PIT in Obesity-Prone, but N ot Obesity-Resistant Rats

In an independent study evaluating male obesity-prone (n=14) and obesity-resistant (n=12) rats, were trained, implanted with cannulae, and tested in the manner described in Chapter 2, Section 2.2 and 2.4. One distinction between the procedures used in the original study (Chapter 2) and those used here, was that rats in the current study received Vehicle and NASPM infusions, but never received CNQX. During testing, 3 obesity-prone rats had blocked cannulae and therefore data from these rats was excluded from analysis. Histological analysis as described in Chapter 2, revealed all cannulae placements to be on target and without extensive tissue damage, therefore no additional exclusions were made. Obesity-resistant rats exhibited SO PIT following both Vehicle and NASPM infusions and NASPM did not diminish lever responding (Figure 7.8A: Two-way RM ANOVA: main effect of lever, $F_{(1,11)}=12.35$, $p<0.01$; no effect of infusion, $p=0.14$; no lever by infusion interaction, $p=0.11$; Planned comparison: active vs inactive, Vehicle, $t_{(11)}=6.22$, $p<0.01$; NASPM, $t_{(11)}=3.73$, $p<0.01$). In contrast, in obesity-prone rats, SO PIT was observed only following Vehicle infusions, whereas NASPM infusions blocked SO PIT, most notably by diminishing transfer to the active lever (Figure 7.8B: Two-way RM ANOVA: main effect of lever, $F_{(1,10)}=20.89$, $p<0.01$; no effect of infusion, $p=0.20$; significant lever by infusion interaction, $F_{(1,10)}=7.43$, $p=0.02$; Planned comparison: active vs inactive, Vehicle, $t_{(10)}=4.67$, $p<0.01$; NASPM, $p=0.80$; Post hoc comparison: Vehicle vs NASPM, active lever, $t_{(10)}=3.01$, $p=0.02$; inactive lever, $p=0.41$)