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Laranci Shanmugarajah

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Under the Direction of Aaron Roseberry, PhD

ABSTRACT

Obesity has become a serious problem in the US. According to the Centers for Disease Control and Prevention, currently ~70% of the US population can be considered overweight or obese. In order to tackle the issue of obesity, it is very important to identify the neural mechanisms that regulate feeding. This will aid us to combat the bigger issue of obesity. The Arcuate nucleus contains two sets of neurons that play an important role in the control of feeding, while the mesolimbic dopamine system plays a major role in most reward based behavior including the reward-related responses to drugs and food. There have been increasing evidence of the melanocortin system interacting with the mesolimbic dopamine system in mediating hedonic feeding. In these studies we tested whether injecting the melanocortin receptor antagonist and agonist, SHU 9119 and MTII, in the ventral tegmental area (VTA) has an effect on reward-based food intake. MTII decreased reward based food intake while SHU9119

affected motivated food intake behavior at a high concentration. Overall, these studies increase our understanding the role of αMSH in the VTA on motivated food reward behavior.

INDEX WORDS: MC3R, MC4R, motivated food intake, VTA, dopamine, αMSH

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Laranci Shanmugarajah

Committee Chair: Aaron Roseberry

Committee: Laura Carruth

Kyle Frantz

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

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1 INTRODUCTION

1.1 Purpose of the Study

Food is necessary for survival. Over historical time, as humans were able to cultivate and grow and store more food than they needed. More types of food have become readily available for many, resulting in an increase in obesity due to an increase in calorie intake. According to the Centers for Disease Control and Prevention, currently ~70% of the US population can be considered overweight or obese. There are many environmental factors that largely contributed to the recent obesity problem. The increased availability of 'tasty' foods high in fat and/or sugar is a possible reason for the increase in obesity rates. There was a scarcity for food in the past, and at that time high fat and sweet tasting food was just meant as a source of high energy. Now in the modern times, with an abundance of food, our bodies still tend to favor the high energy food, resulting in overconsumption and increase in body weight. In a manner similar to animals becoming addicted to drugs, animals prefer certain foods over others. When given a choice between a normal chow and a high fat, high sugar diet, rats tend to eat the high fat, high sugar diet (Jarosz et al., 2007). The body regulates feeding in a complex manner. There are two aspects to the neural control of feeding: homeostatic control and hedonic control. Homeostatic is the basic control of feeding. It is the process of consuming enough food to obtain the energy to maintain a stable weight and other required body functions but not more, hedonic control of food intake refers to the rewarding aspect of food consumption. This involves eating palatable or appetizing foods, beyond the need for energy, because they are 'rewarding' and pleasurable. It is important to increase our understanding of the different neural mechanisms and their interactions that regulate feeding, including the intake of pleasurable high fat and high sugar foods. Only then can we start to combat the issue of obesity.

1.2 Mesolimbic Dopamine system

The mesolimbic dopamine system, consisting of dopamine neurons in the ventral tegmental area (VTA) and their afferent and efferent connections, is primarily associated with regulation of reward processing and drug addiction, but it also plays a role in other processes including the hedonic control of feeding. Dopamine is the essential neurotransmitter in this system; it is involved in numerous behaviors including reward processing, motivated behavior, motor coordination, learning and memory (Wise, 2004). The mesolimbic dopamine system begins in the ventral tegmental area (VTA) of the midbrain and connects to the nucleus accumbens, amygdala, hippocampus and the bed nucleus of the stria terminalis. Dopamine neurons receive inputs from many different brain areas including the lateral hypothalamus, rostra medial tegmental nucleus, ventral pallidum, amygdala, and laterodorsal tegmental nucleus, as well as other regions (Watabe-Uchida et al. 2012).

The mesolimbic dopamine pathway is involved in many different types of behaviors including reward prediction, hedonic behaviors, reinforcement, motivation and incentive salience (DiLeone et al., 2012). Importantly, the mesolimbic dopamine system also plays an important role in the neural control of feeding. For example, extensive depletion of forebrain dopamine has been shown to decrease food intake (DiLeone et al., 2012). It was discovered that the ventromedial striatum was a site in which dopamine depletion altered aspects of food intake. Rats with ventrolateral striatal dopamine depletion had reduced food intake and had decreased rate of feeding (Salamone et al., 1992). In addition to homeostatic feeding, dopamine seems to play a role in regulation of hedonic feeding. Similar to the effects of drugs, food intake increases dopamine release at VTA projection sites such as the ventral striatum (Bassereo and Di Chiara,

1997, 1999; Heffner et al., 1980; Hernanandez and Hoebel 1988; Keugel et al., 2003; Pothos et al. 1995; Wilson et al. 1995). Dopamine and the mesolimbic dopamine system appears to play a larger role in the regulation of hedonic feeding.

1.3 The Melanocortin System

The melanocortin system is a neural circuit that plays an important role in the control of homeostatic feeding. Neurons in the arcuate nucleus of the hypothalamus as well as the brainstem contain peptides associated with the melanocortin system. This system is responsible for the regulation of body weight through the control of food intake as well as energy expenditure and consists of independent sets of neurons in the arcuate nucleus that expresses either neuropeptide Y (NPY) and agouti gene related protein (AgRP) or pro-opiomelanocortin (POMC) (Cone, 2005). NPY has been shown to strongly promote food intake when injected three times/day into paraventricular nucleus resulting in two times more food intake as compared to controls (Stanley et al., 1986). AgRP is co-expressed in the same neurons with NPY and has similar functions. AgRP release increases food intake and decreases energy expenditure. Another set of neurons in the arcuate nucleus of the hypothalamus expresses the POMC gene. POMC consists of a 241 amino acid prohormone that is processed in to multiple different peptides. The proconvertase 1/3 cleaves POMC in to adrenocorticotropic hormone (ACTH) and β-lipotrophin which are further cleaved into α-melanocyte-stimulating hormone (α-MSH) and β-endorphin by proconvertase 2 and carboxypeptidase E (Nillani 2007, Wardlaw, 2011). In the state of hunger, αMSH is decreased while it is increased in the satiated state (Hahn et al. 1998). αMSH has a catabolic effect and works to suppress food intake (Kaska et al., 1998); release of α-MSH results in a decrease of food intake and increase in energy expenditure (Aponte et al, 2011) by acting on melanocortin receptors.

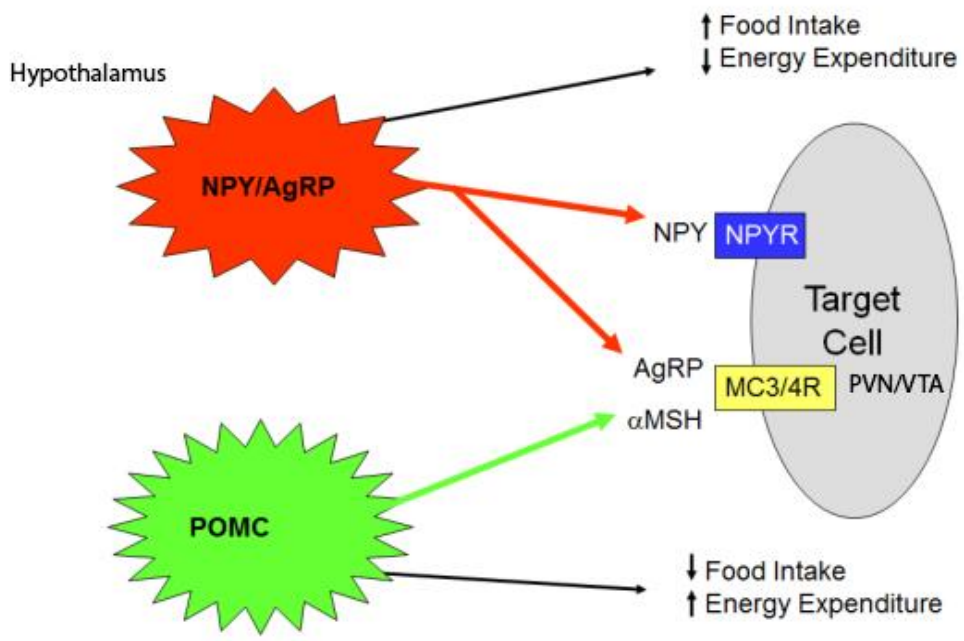

Figure 1: Overview of the melanocortin system. The melanocortin system is comprised of αMSH and AgRP produced in the ARC of the hypothalamus. POMC produces αMSH which decreases feeding and body weight and increases metabolism by acting on MC3/4R, while AgRP released from AgRP neurons increases feeding and body weight by acting as an inverse agonist/antagonist to block MC3/4R activity.

AGRP and α-MSH act on two melanocortin receptors, melanocortin 3 receptor (MC3R) and melanocortin 4 receptor (MC4R) in the central nervous system (CNS) to regulate food intake. The importance of melanocortins became evident with the studies involving the agouti protein, which functions as an antagonist of cutaneous MC1 receptors. MC1Rs are mainly expressed in the hair follicles and reducing MC1 receptors lightens coat color. A spontaneous mouse mutant was identified that ectopically expressed agouti in all tissues, leading to mutants with a yellow coat color and obesity (Cone et al, 1996). When AGRP was cloned, researchers identified that agouti and AGRP act as an antagonist of MC3 and MC4 receptors (Cone et al.,

1996). Thus, αMSH is the endogenous agonist for MC3R and MC4R, while AGRP is an endogenous antagonist of these receptors.

Many melanocortin receptor sites in the CNS receive projections from both agonist expressing POMC neurons, as well as the antagonist expressing AGRP neurons. The melanocortin receptors are located in different areas in the brain. MC3Rs are mainly expressed in the arcuate nucleus, the VTA, and in certain areas in the brain stem, while MC4R are more widely expressed in the brain than the MC3R (Mountjoy et al., 1994). MC4R was found in different areas of the brain such as the cortex, thalamus, hypothalamus, brainstem, and the spinal cord (Mountjoy et al., 1994). Experiments have shown that mice that lack MC4R receptors are very obese, and hyperphagic indicating that signaling MC4R limits food intake (Huszar et al., 1997). MC4R knock outs have significant effects on feeding, energy expenditure and body weight (Butler 2006, Cone 2005, 2006; Huszar et al. 1997). Both MC3R and MC4R has an effect on food intake levels. Therefore, it can be concluded that the melanocortin system plays a role in regulating feeding and body weight.

1.4 Melanocortin system interactions with the mesolimbic system

There is evidence that the melancortin system can act on the mesolimbic dopamine system. Both MC3Rs and MC4Rs are expressed in the VTA (Kishi et al., 2003; Liu et al., 2003; Roselli-Rehfuss et al., 1993) and co-localization of MC3/4R with dopamine neurons has been shown using gfp transgenic mice for MC3R and the MC4R (Lippert et al. 2014).Injections of α-MSH directly into the VTA increased dopamine turnover and increased many dopamine related behaviors such as grooming, rearing, and locomotor activity (Adan et al. 1999; Jansone et al. 2004; Klusa et al. 1999; Lindblom et al. 2002; Sanchez et al. 2001; Torre and Celis 1986, 1988; Yang and Shieh 2005). In addition, some of the effects of intracerebroventricular (icv) injections

of α-MSH and AgRP may be mediated through the dopamine pathways. ICV Injection of MTII, (a MC3/4R agonist) decreased fat preference and selectively decreased fat intake (Samana et al. 2003), which could have been due to changes in the 'rewarding' qualities of these foods. The importance of the melanocortin system having an effect on the dopamine system has been supported through knock out studies. For example, both MC4R -/- mice and rats increased their intake of high fat diets and gained more weight on their high fat diets (Butler et al. 2001; Mul et al., 2012; Srisai et al., 2011).

Since αMSH and AgRP act on the dopamine system, it is possible it mediates reward related behavior as well. There are many studies demonstrating that αMSH and AgRP affect reward related behaviors through the dopaminergic pathways. For example, icv injection of MTII decreased ethanol intake while injection of AgRP increased ethanol intake (Navarro et al., 2005). It has also been shown that melanocortins attenuate acquisition of heroin self-adminstration, hinting that melanocortin may act on the dopamine neurons to affect addiction behavior (Vanree et al., 1981).

Similar to the effects on drug intake, melanocortins can also affect food intake through the mesolimbic dopamine pathway. Studies from the Roseberry lab show that injections of MTII, (a MC3/4R agonist) into the VTA decreases homeostatic feeding by measuring home cage chow intake while SHU 9119, (a MC3/4R antagonist) injected into the VTA increases home cage chow intake (Roseberry, 2013). Another study from this group analyzed whether MTII injections into the VTA affected reward based feeding. Injection of MTII into the VTA decreased consumption of palatable caloric and non-caloric solutions (Yen and Roseberry, 2014). Thus, multiple lines of evidence suggest that αMSH and AgRP regulate multiple dopamine mediated behaviors, including feeding (Fig 2, Roseberry et al, 2015).

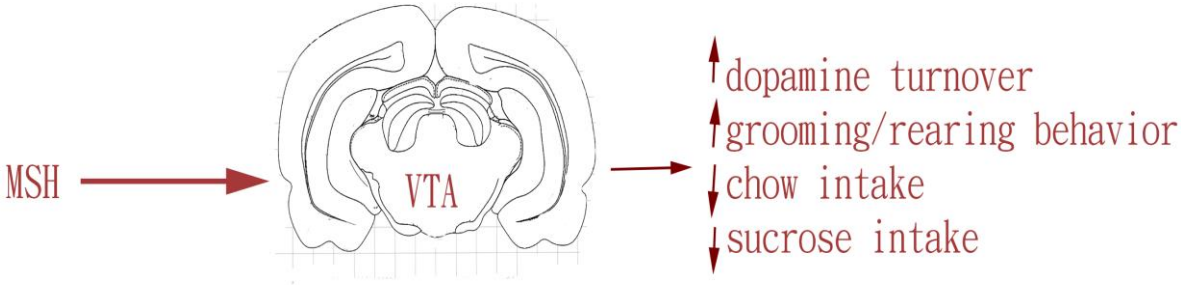

Figure 2: Effect of MSH in the mesolimbic dopamine system. αMSH act in the VTA to regulate dopamine dependent behaviors. Injection of αMSH into the VTA increases dopamine turnover, and grooming and rearing behavior, and decreases chow and sucrose intake

Since dopamine neurons in the VTA are involved in addictive behavior and evidence shows that melanocortins may act in the VTA, it is possible that the melanocortins act in the VTA to affect reward based food behavior. In the studies proposed here, we tested the hypothesis that injection of the melanocortin receptors agonist and antagonist, MTII and SHU9119 directly in to the VTA alters reward-related food intake through the melanocortin receptors signaling in the VTA. We predict that MC3/MC4R agonist will decrease the acquisition of palatable sucrose pellets as well as decrease the amount rats are willing to work for food in food self-administration assays, while the MC3/4 antagonist will increase the acquisition of sucrose pellets and increase the amount that rats are willing to work for food. We will test our hypothesis through self-administration tests.

2 Materials and Methods

2.1 Animals

Male Sprague Dawley rats (Harlan Laboratories, Madison, WI, USA), about 1.5 – 2 months old, were used for these experiments. The rats weighed between 250 to 300 g at the start of the experiments. All protocols and procedures were approved by the Institutional Animal Care and Use Committee at Georgia State University and conformed to the NIH Guide for the Care and Use of Laboratory Animals. Rats were housed standard polycarbonate cages with ad libitum food and water access, unless otherwise noted.

2.2 Implantation of indwelling cannula

Rats were anesthetized with isoflurane, and stainless steel guide cannulas were bilaterally inserted in to the brain targeting the VTA using standard flat skull techniques. The coordinates used to target the VTA were: 5.4 mm posterior to the bregma, 2.2 mm lateral to the midline and 7.5 mm ventral to the skull surface at a 12^{o} angle to the midline. The cannulas were anchored to the skull using skull screws and dental cement. Cannulae were placed 1.0 mm above the target injection site, with the injectors extending 1.0 mm beyond the tip of the cannulae. After the surgery, stainless steel stylets were placed in the cannulae to prevent any occlusion. Ibuprofen was given to the rats in their drinking water 2 days prior to the surgery and then for 4 days after the surgeries to aid with the pain and stress pre and post-surgery. The rats were allowed two

weeks to recover from the surgeries, during that time their food consumption returned to pre-surgery levels before training and testing.

2.3 Microinjections

Rats were gently restrained by hand with a hand towel, stylets removed, and stainless steel injectors (33ga) extending 1.0mm beyond the tip of the cannula inserted bilaterally. The injections (0.2 µl) were performed over the course of 1 minute using a Hamilton Syringe connected to a microinfusion pump at the rate of ~0.2 µl/min. After the injections, the injectors were left in place for another minute to allow for diffusion and to limit any backflow up the cannulae. The stylets were replaced and the rats were returned to the home cage for 30 minutes before starting the operant behavior tests. The rats were acclimated to the injection procedure once prior to the test injections by inserting the injectors without injecting any substances.

2.4 Drugs

The rats received injections of MTII, (MC3/4R agonist), SHU9119 (MC3/4R antagonist), or saline. MTII and SHU9119 were dissolved in sterile saline. The saline vehicle was used as the control injection. The rats received a maximum of five injections spaced 2-4 days apart to allow for full recovery from the previous treatment. During the intervening non-test days the rats continued with the self-administration procedures. All injections were performed in the early to middle of the light phase. The injections were counterbalanced; on each test day half of the cohort received one treatment while the other half would receive a different treatment.

2.5 Measurements of food intake and food restriction

Daily food intake was measured as described below. Food was given *ad libitum* during the post-surgery recovery period for about 2 weeks. Rats were fasted for 24-hours prior to the start of the first fixed ratio one (FR1) session. They were then food restricted to 80% of their baseline daily food intake until they completed two food self-administration sessions obtaining more than 10 pellets with time out of 5 seconds. Afterwards, the rats were given chow *ad libitum* in their home cage for the rest of the experiment. Food was measured daily at the same time each day throughout the experiment. For the measurement of food intake, rats were given a set, pre-weighed amount of food, which was then weighed 24-hours later. Additional food was added prior to returning the food to the cage to ensure that the rats were exposed to the same amount of food daily.

2.6 Operant conditioning for food self-adminstration

Operant conditioning chambers enclosed in a sound-attenuating, ventilated environmental cubicles (Med Associates, Inc.) were used for this experiments. Each chamber had a metal bar floor, with two retractable levers with a round white stimulus light above each. In the middle of the levers is the food receptacle which delivers a 20mg sucrose pellet (Bio-Serv, Frenchtown, NJ) with presses on the active lever. Each reinforced response lit a cue light directly above the active lever, which stayed on for 2s. Presses on the inactive lever were recorded but had no scheduled consequences.

Fixed ratio schedule

After the stereotaxic surgeries, rats were allowed two weeks for recovery before initiating the operant conditioning. Two different cohorts of rats were used for this experiment. One cohort

(n=12) was used for the injections of MT11 while another cohort (n=8) was used for the SHU9119 injections. Rats were initially trained in the food self-administration procedures using a fixed ratio-1 (FR1) schedule of reinforcement. At the onset of the fixed ratio (FR) training rats were food deprived for the first 24 hours and then food restricted to 80% of their diet for approximately one week until they obtained more than 10 pellets per session with time out of 5 seconds. The rats were trained once every day under a FR1 ratio, where a single active lever press resulted in a sucrose pellet. Sessions ran for 30 minutes or until the rats obtained 50 pellets after a successful active lever press, no more sucrose pellets could be obtained during a specified time out period. The time out period started with 5 seconds, and after two complete sessions with rats obtaining more than 10 pellets with time out 5 seconds, the time out was increased to 10, and then 20 seconds. The rats continued on the FR1 ratio with 20 seconds timeout until they reached a stable amount of active lever presses that were more than 10 and did not vary more than 15% for 2 consecutive days (Sharf et al. 2004). At this time, testing with the different drug doses was initiated.

Progressive ratio schedule

Another cohort of rats (n=6) were used for progressive ratio test. Rats undergoing progressive ratio testing were trained using the same FR1 schedule described above until their responding was stable. Once their responding in fixed ratio was stable, the rats were moved to progressive ratio schedule of reinforcement. In the progressive ratio schedule the number of responses required to obtain a sucrose pellet increased with each successive reward. The progression was derived from the equation: response ratio= $(5 \times e^{(.2 \times \text{infusion no.})} - 5)$, which yields response ratio of 1,2,4,6,9,12,15,20,25, etc. (Richardson and Roberts, 1996). The sessions lasted until a period of 30 minutes without obtaining a sucrose pellet had passed, or for a maximum of

4 hours total. The break point, the last ratio where the rats no longer continued to press the lever, was measured. Testing in the progressive ratio schedule began when the number of pellets earned did not vary by more than 15% on two consecutive days (Sharf at al. 2004).

2.7 Histological confirmation of injector location

At the end of all experiments, the injection site was confirmed by using Nissl stain. At the end of the studies the rats were injected with 0.2 µl of the fluorescent Nissl stain, NeuroTrace, 24hours prior to fixation of their brain. The rats were perfused transcardiac with cold saline followed by 4% paraformaldehyde. The brains were then removed and post fixed with 4% paraformaldehyde at 4 ^{o}C overnight. They were then stored in saline until sectioning. Coronal sections of 100 µm thickness were cut through the extent of the VTA with a vibrating blade microtome. The sections were then mounted to slides, stained with cresyl violet, and the location of the cannula was confirmed with light microscopy.

2.8 Data Analysis

The independent variables for these experiments were the different doses of injections (MTII and SHU9119). The dependent variable were the number of pellets earned, pellets consumed, total active lever press, inactive lever presses, and the 24 hour home cage food intake. All of the data are presented as means ± SEM. Data were plotted using Igor Pro. (Wavemetrics, Inc.) and statistical analysis was performed using SigmaPlot (v1 1.0, Systat Software, Inc.). One way repeated measures ANOVA and Tukey tests were used for post-hoc for all statistical comparisons. All analysis were performed with a significance level of $p<0.05$ set a priori.

3 Results

Previous evidence demonstrated that the MC3/4R agonist, MTII, and the MC3/4R antagonist, SHU9119, affects food intake when injected directly into the VTA. This is true for both normal chow intake (Roseberry, 2013) and the intake of appetizing sucrose and saccharin solutions (Roseberry and Yen, 2013). In the present studies, we tested whether injection of MTII and SHU9119 directly into the VTA also affected hedonic feeding by examining their effects on the self-administration of palatable sucrose food pellets.

We initially tested whether injection of the MC3/4R agonist, MTII, into the VTA affected food self-administration using a fixed ratio-1 schedule, where each press of the active lever during the active period resulted in the delivery of a sucrose pellet. MTII dose dependently decreased the number of pellets earned (Fig 3a; $F(3,33)=26.297$ $p<0.001$) and had a similar effect on the consumption of the pellets obtained by the active lever presses (Fig 3b; $F(3,33)=20.677$ $p<0.001$) Total active lever presses (the number of active lever presses during both the active and the time out periods) were also significantly reduced by MTII (Fig 3c; $F(3,33)=27.118$ $p<0.001$). We also measured 24 hour home cage chow intake during these experiments, as a control for the effects of MTII. Similar to the results from previous studies (Roseberry 2013), intra-VTA MTII injection also dose-dependently decreased 24-hour home-cage chow intake (Fig 3d; $F(3,33)=27.825$ $p<0.001$). These effects appeared to be specific to food-directed behavior and were not a result of general inactivity, as inactive lever presses and total activity (measured by total beam breaks in the chambers) were not affected by MTII injection (Table 1).

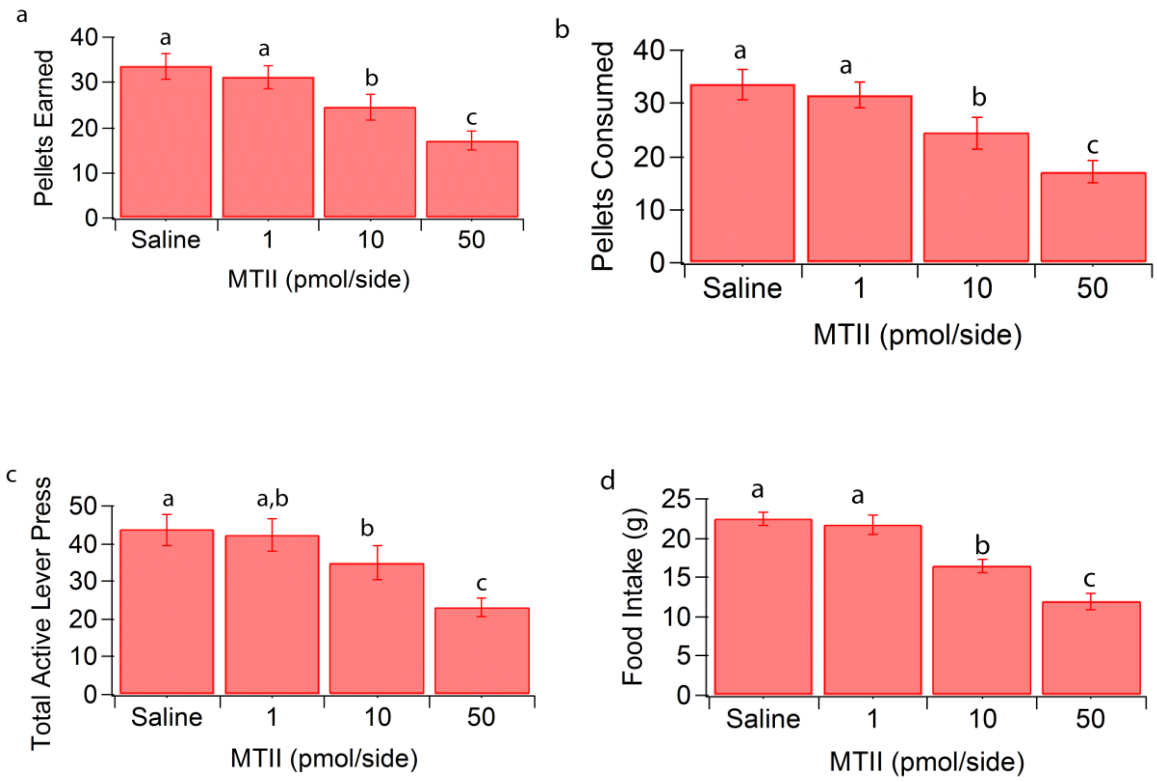

Figure 3: Injection of MTII into the VTA decreased FR1 sucrose self-administration. A. MTII effect on total sucrose pellets earned. B. MTII effect on total sucrose pellet consumed. C. MTII effect on total active lever presses. D: MTII effect on 24h home cage chow intake. Bars with different letters are significantly different from each other ($p<0.05$), n= 12

[MTII]/side	Inactive Lever	Total Beam Break
Saline	4.4± 1.0	887± 173
1 pmol	4.5± 0.9	968± 181
10 pmol	3.1± 0.9	819± 76
50 pmol	2.7± 0.8	965± 143

Table 1: Effect of MTII injected directly into the VTA on inactive lever presses and total beam breaks during FR1 food self-administration experiments.

We next tested whether injection of the MC3/4R antagonist, SHU9119, could also affect food self-administration when injected into the VTA using the FR1 schedule. Injection of 100 pmol SHU9119 significantly increased the total number of sucrose pellets earned, while there was a non-significant trend for increases with the other doses as well (Fig 4a). SHU9119 had a similar effect on the consumption of the pellets eaten; the 100 pmol injection increased pellet consumption, while the other doses showed a trend toward increased intake (Fig 4b). The injections of SHU9119 did not exert significant effects on the total active lever presses, but as with the pellets earned and eaten, there appeared to be a non-significant trend toward increased lever pressing with increasing doses of SHU9119 (Fig 4c). When the 24-hour home cage chow was measured, SHU9119 significantly increased home-chow intake (Fig 4d; $F(5,82)= 9.569$ $p<.001$.), as has been observed previously (Roseberry, 2013). As with MTII, there were no changes in total beam breaks or inactive lever presses after SHU9119 injections (Table 2).

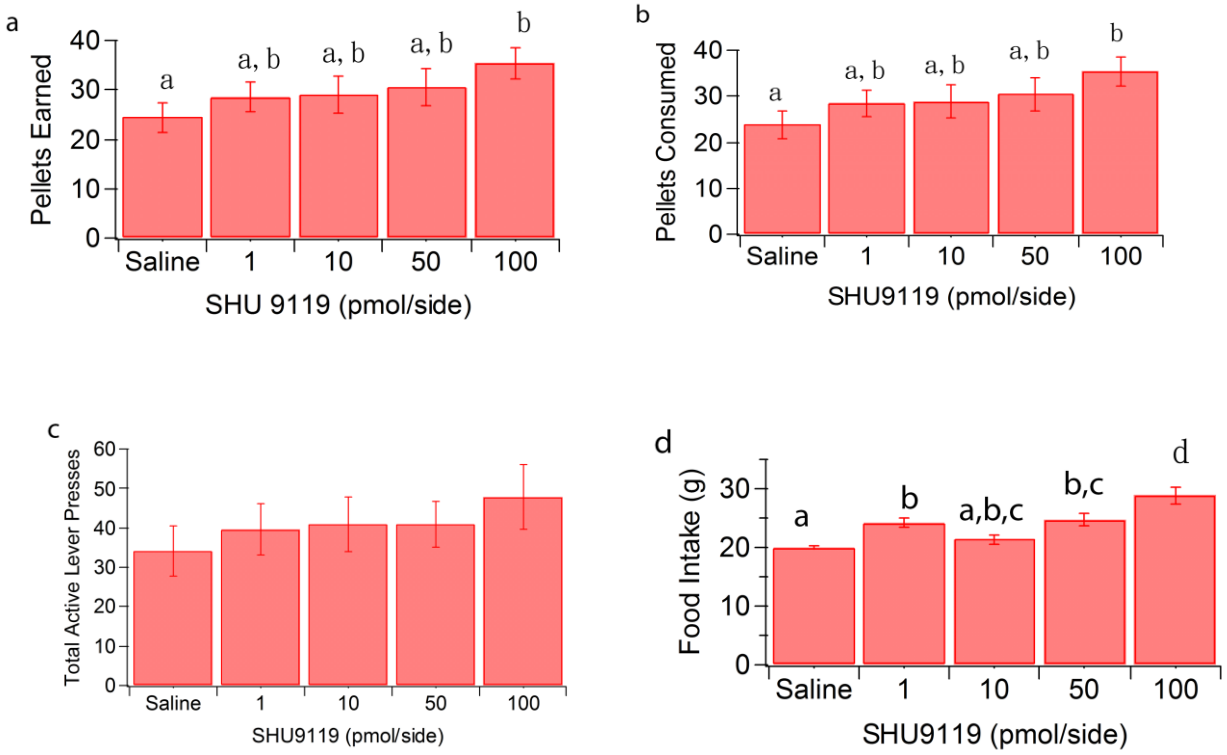

Figure 4: Injection of SHU9119 into the VTA increased FR1 sucrose self-administration. A: SHU9119effect on total sucrose pellets earned. B. SHU9119 effect on total sucrose pellet consumed. C. SHU9119 effect on total active lever presses. D: SHU9119 effect on 24h home cage chow intake. Bars with different letters are significantly different from each other ($p<0.05$), n= 8

[SHU 9119]/side	Inactive Lever	Total Beam Break
Saline	2.8± 1.1	583± 133
1 pmol	1.0± 0.4	542± 87
10 pmol	2.5± 0.8	560± 135
50 pmol	2.0± 0.6	549± 120
100 pmol	1.9± 0.4	703± 146

Table 2: Effect of SHU 9119 injected directly into the VTA on inactive lever presses and total beam breaks during the fixed ratio food self-administration experiments.

We next tested the effects of MTII and SHU9119 in the VTA by examining their ability to affect the motivation of rats to work for appetizing sucrose pellets in progressive ratio (PR) self-administration assays. Injection of 50 pmol of MTII significantly decreased session time, total active lever presses, and the number of sucrose pellets earned (fig 5a; $F(2,10)=6.255$ $p=0.017$, fig 5b; $F(2,10)=4.506$ $p=0.040$, fig c; $F(2,10)=7.282$ $p<0.001$), and the 10 pmol dose caused a small but non-significant decrease in each of these measures. The break point ratio was also decreased by MTII injection (Figure 6d). (Fig 6d). In contrast, injection of SHU9119 did not have an effect in these experiments (Fig 6). As expected, 24-hr home cage chow intake was significantly decreased by MTII in these experiments (fig 5e; $F(2,10)=48.515$ $p<0.001$), but SHU9119 did not have an effect(fig 6e). The inactive lever presses were not affected by the injections of either MTII or SHU9119 during the progressive ratio experiment (Table 3).

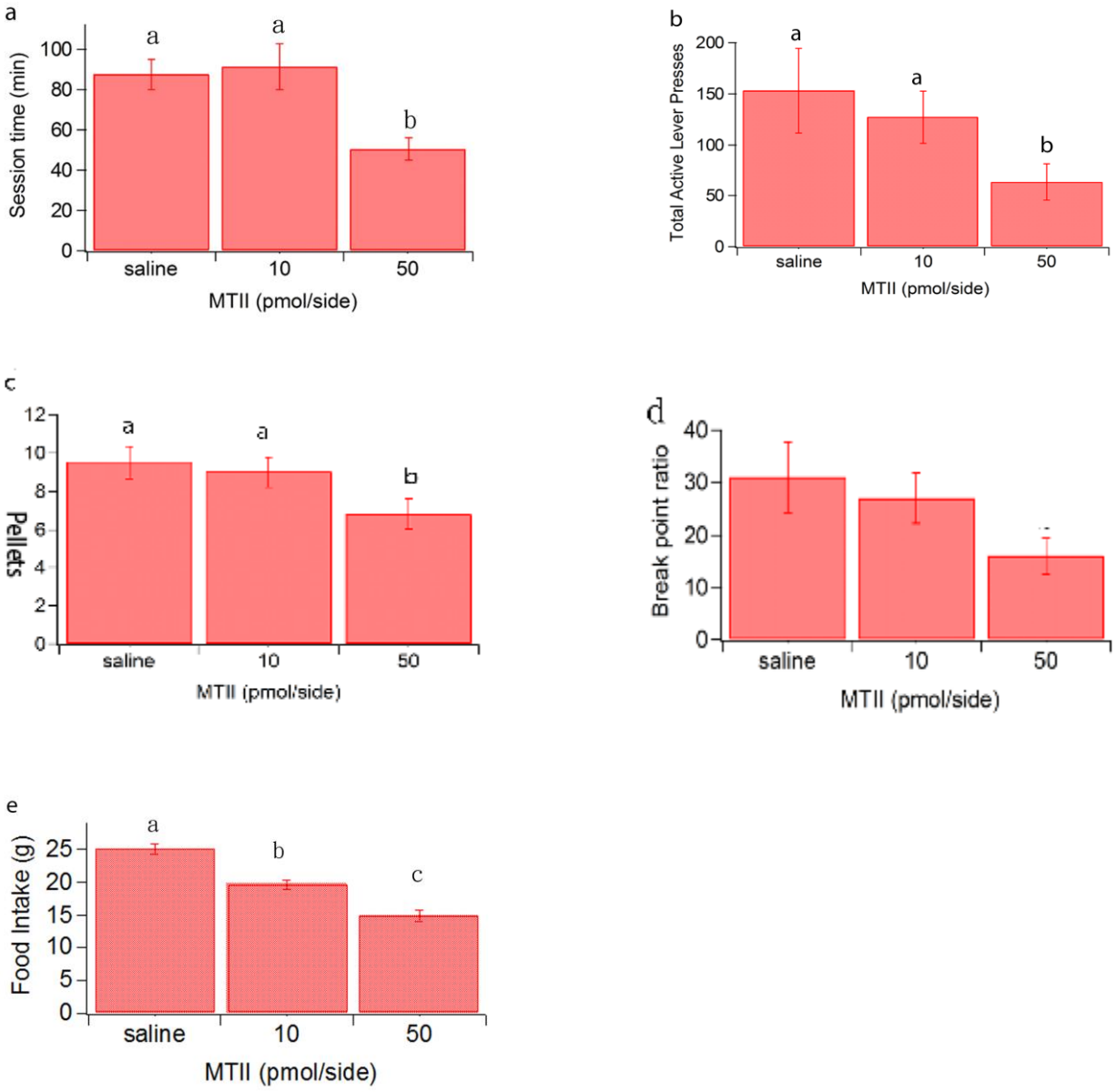

Figure 5: Effects of injection of MTII into the VTA on food self-administration using a PR schedule. A: Session time with injection of MTII. B: Total active lever presses with the injection of MTII. C: Effect of MTII on the number of rewards. D: Break point ratio with injection of MTII E: Effects of MTII on 24-hr home cage chow intake Bars with different letters are significantly different from each other ($p<.05$), n= 6

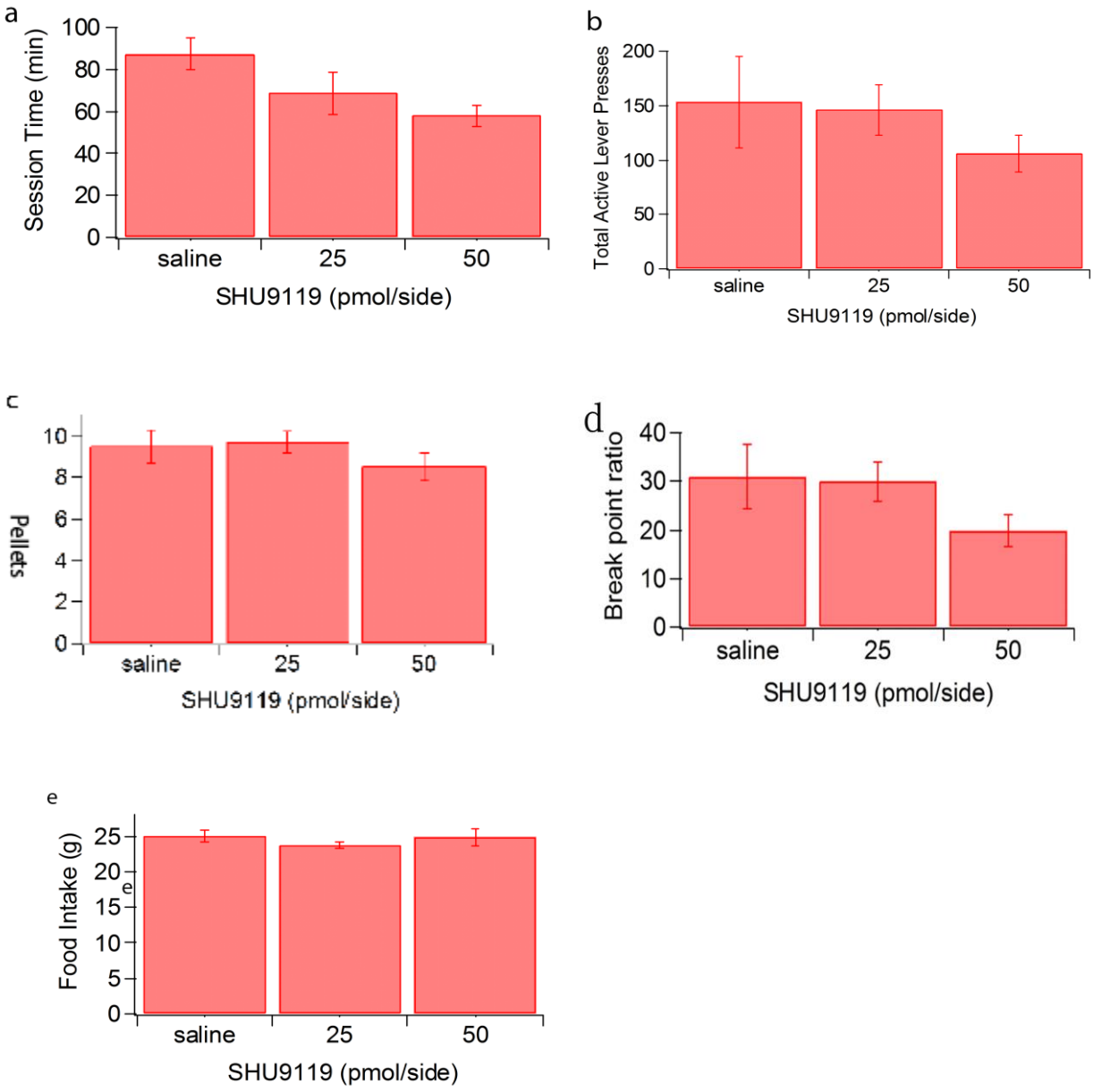

Figure 6: Effects of injection of SHU9119 into the VTA on food self-administration using a PR schedule. A: Session time with injection of SHU9119. B: Total active lever presses with the injection of SHU9119. C: Effect of SHU9119 on the number of rewards. D: Break point ratio with injection of SHU 9119 E: Effects of SHU9119 on 24-hr home cage chow intake Bars with different letters are significantly different from each other ($p<.05$), n= 6

Injection	Inactive Lever Presses
Saline	4.5± 1.2
10 pmol MTII	6± 2.9
50 pmol MTII	2.2± 1.1
25 pmol SHU9119	5± 1.7
50 pmol SHU9119	5± 2.8

Table 3: Effect of MTII and SHU9119 on inactive lever presses during the progressive ratio self-administration

For all experiments, the injection sites were verified at the end of the experiment. The location of the injections and sample images of two injections are shown in Figure 7.

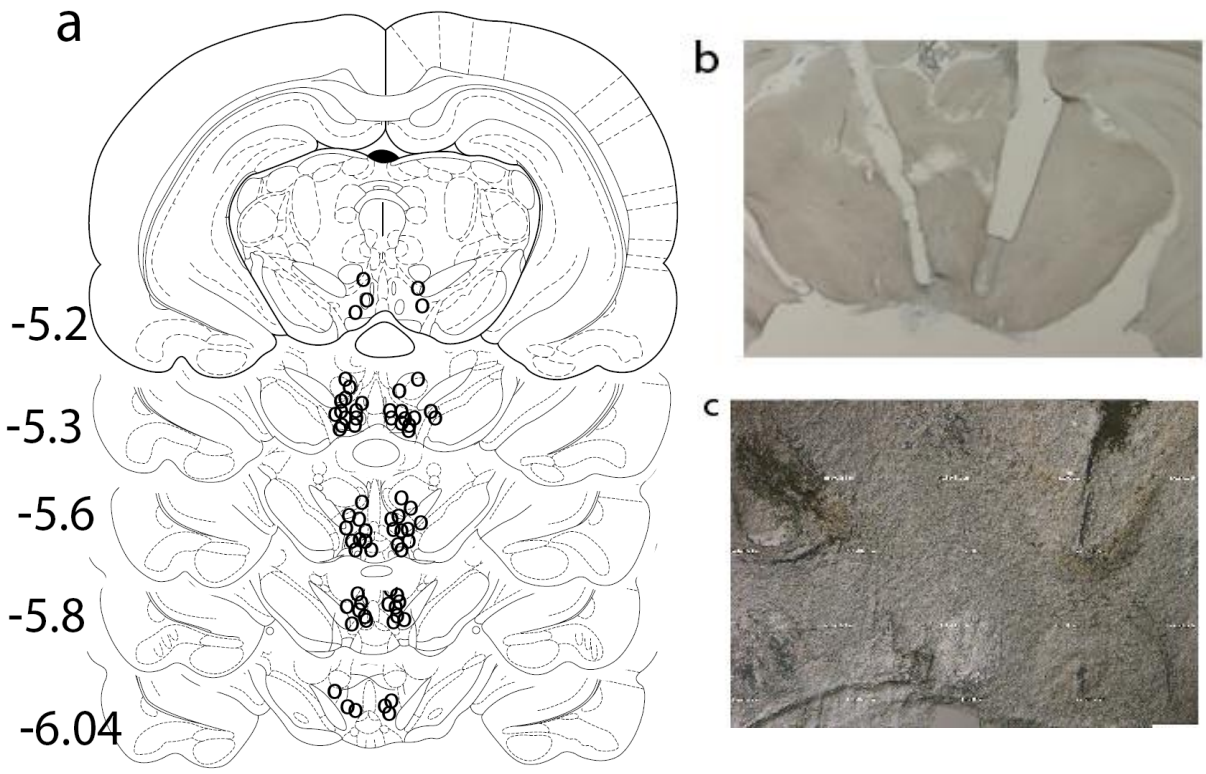

Figure 7: Verification on the cannula placements. A: coronal sections of the rat brains demonstrating injector location, which are indicated by *circles* on panels taken from a rat brain atlas (Paxinos and Watson 1998). Numbers next to each section indicate the location of the section relative to bregma. B: sample image of a brain section showing the injector location. C: sample brightfield

4 Discussion

In these studies, we examined the impact of microinjections of MC3/4R antagonists and agonists directly into the VTA on reward-related food intake. Injection of the MC3/4R agonist, MTII, dose-dependently decreased the amount of lever presses and sucrose pellets earned in the fixed ratio schedule of reinforcement. This effect on the food self-administration behavior suggests that MTII may play a role not only in homeostatic food control but also hedonic feeding. The decrease in the number the number of pellets earned and consumed with the injections of MTII, shows that the agonist effectively reduced the animals' reward-related responding. In this set of experiments, injections of MTII did not affect locomotor activity, measured by photocell beam breaks in the operant conditioning chambers, nor did it affect inactive lever pressing These control measures suggest that the decrease in obtaining the sucrose pellets earned and consumed was not a result of lack of movement but rather was directly related to food reward.

In order to measure how the animals' motivation to obtain their reward, progressive ratio experiments were performed. The progressive ratio schedule examined the incentive motivation for the sucrose pellets. Injections of 50 pmol of MTII significantly decreased session time, total active lever presses and total number of rewards obtained suggesting a decrease in motivation. The 10 pmol MTII dose injected into the VTA may have had an effect on self-administration (total lever presses and number of pellets earned), but this effect was not statistically significant. Injection of 10 pmol did significantly decrease 24hr food intake however. This suggests that the influence of MTII on feeding may depend on the type of food, whereby more αMSH/MTII is required to decrease intake of highly appetizing foods compared to normal chow, which supports a previous study (Roseberry and Yen, 2013). The sample size for the progressive ratio schedule

experiment was very small (n=6), which may have limited our ability to observe significant differences, especially with the lower dose of MTII, which has a much smaller effect. Thus, this experiment should be repeated in order to have a greater sample size. Overall, our results suggest that activation of MC3/4 receptors in the VTA can decrease hedonic feeding.

In these studies, the effects of SHU9119 were much smaller than the effects of MTII. Although there was a clear increasing trend of pellets earned and consumed following SHU9119 injection in the fixed ratio testing, only the highest dose tested significantly increased the number of pellets earned and eaten. A similar trend was observed for total lever presses but there were no significant changes in the lever presses with the injection of SHU during the fixed ratio testing. In contrast however SHU9119 still had an effect on homeostatic feeding in this study as was observed in previous studies (Roseberry, 2013). During the progressive ratio experiment, SHU9119 did not have any effect on the food self-administration, and it also did not change the 24hr chow intake. One potential explanation for these results is that they could be the result of a problem with the SHU9119 solution used in this study (e.g. improper preparation or degradation of SHU9119 before testing). Another possible interpretation of these studies is that SHU only acts in the VTA to affect homeostatic feeding but not reward-related food intake. Injection of SHU9119 did not have an effect on self-administration, but it did have an effect on the 24hr home cage chow intake in the FR1 schedule. This suggests that SHU9119 may act by different mechanisms to affect homeostatic food intake and reward related food intake in the VTA. Alternatively, there was a trend for increased responding in the fixed ratio experiments, which may mean that SHU9119 can play a role in more that homeostatic feeding. Combined with the low sample size and the inability of SHU9119 to affect either PR responding or home cage chow intake in the progressive ratio experiment, it is possible that SHU9119 does affect reward-related

feeding, and that these experiments were too limited to observe any potential effects. Thus, these experiments need to be repeated with more animals to allow for a better examination of whether SHU9119 affects food self-administration. The 24-hr food intake for the progressive ratio cohort was greater than the 24-hr food intake for the cohorts for the fixed ratio. Since the rats were consuming more pellets in general, perhaps they already had more activation of AgRP that the injection of AgRP did not have an increasing effect. Future studies will be required to test whether the baseline home cage chow intake of the animals influenced the ability of SHU9119 to act in the VTA to affect homeostatic and hedonic feeding however.

These experiments were completed during the light cycle, since according to the previous studies done by our lab, SHU9119 in the VTA only had an effect in the light cycle (Roseberry, 2013). During the light cycle when the rats are not normally eating, injection of SHU9119 to the VTA caused them to increase their food consumption, but its effect was muted following injection at the onset of the dark cycle when the animals were already eating at a maximal level. A previous study has reported that the firing rate of AgRP neurons are significantly elevated during the dark cycle compared to the light cycle (Krashes et al. 2013). Near the dark phase, more AgRP is present to block melanocortin receptors, allowing the animals to consume majority of their food intake at the dark phase. Due to a "ceiling" effect, adding more antagonist (SHU9119) at a time where a maximum amount of AgRP already present, will not have an effect. Therefore in order to see an effect of SHU9119, it needs to be injected at a time when there isn't a lot naturally released in the brain during the light cycle. This was taken in to consideration when performing these experiments. The action of AgRP suggests that αMSH mainly act during the light phase to suppress food intake.

There were no differences between the number of pellets earned and the consumption of the sucrose pellets. If the rats pressed the lever to obtain a sucrose pellet, they ate the pellet unless they dropped the pellet through the cage wire floor. This indicates that the rats were pressing the lever to obtain and consume the sucrose pellets, and that these pellets served as a functional reinforcer. The number of inactive lever presses was also measured, and there was not any change in the number of presses for inactive lever presses, suggesting that the rats effectively associated the active lever presses with sucrose pellets so the antagonists and the agonists strictly had an effect on the motivation to obtain a sucrose pellet and not on the action of pressing the lever. In the beginning of training the rats pressed the inactive lever more but as they learned to associate the active lever with the sucrose pellet, the inactive lever decreased dramatically or in some cases nonexistent. This supports that the effect of the MTII and SHU9119 is mostly specific for food, since there were not any changes in the inactive lever press or beam breaks.

There are different types of melanocortin (MC1R,MC2R,MC3R, and MC4R) in the brain, and MTII and SHU9119 can act on two different receptor subtypes, MC3R and MC4R. AgRP and αMSH from the arcuate nucleus can both bind to these receptors to either promote or inhibit food related behavior. In the VTA, MC3R and MC4R are both present but it is unknown whether either receptor subtype plays an equal role on mediating this effect. Although both receptors are expressed in the VTA, there is evidence that there are more MC3Rs in the VTA as compared to MC4Rs (Lippert et al, 2014). This suggests that MC3Rs may play a bigger role in the VTA in mediating motivated food intake behavior. Further studies should be conducted to determine which receptors in the VTA mediate these effects, and whether different receptors regulate homeostatic and hedonic feeding.

For this research we used young adult male rats. Our studies focused on male rats to be consistent with previous studies from our lab (Roseberry, 2013; Yen and Roseberry, 2013). Previous evidence indicates that there may be sex differences in response to αMSH in dopamine systems, however, depletion of MC3 receptors (whole body knockout) increased dopamine levels and decreased sucrose intake and preference in female but not male mice (Lippert et al, 2014). The increased dopamine levels seemed to be dependent on female reproductive hormones since this increase was reversed by ovariectomy and were not observed in pre-pubertal females (Lippert et al, 2014). Lippert hypothesized that estrogen and melanocortin receptor signaling may interact together and that the loss of MC3R drives an increase in estrogen sensitivity that primes the dopaminergic system to remain elevated in adults (Lippert et al, 2014) causing an increased sucrose preference in female. This raises the question as to whether there may be differences between sexes of the rats in the responses to αMSH and AgRP in the VTA. Thus, further research should examine sex differences between the effects of melanocortins acting on dopamine pathways.

In summary, we have shown that injection of MTII directly into the VTA decreases motivational food intake, by decreasing the pellets earned and consumed in the fixed ratio schedule as well as decreasing the number of rewards at the highest MTII dose during the progressive ratio schedule. Injection of SHU9119 appeared to increase fixed ratio self-administration, by increasing the number of pellets earned and consumed at the highest dose, but its effect on progressive ratio is unclear. Further studies need to be completed to determine the role of SHU9119 on reward-related food intake. Some next steps that can be taken from our results, may be to look at which receptor (MC3R *vs* MC4R) mediates these effects. This can be looked at through different approaches such as receptor knockouts, knockdown with shRNA or

using CRISPR. We can also run further experiments to determine the role of AgRP and its different effect on high fat diets versus high sucrose diet, since there have been mixed results on the effect of AgRP and our experiments did not show any effect on the progressive ratio tests. Another future direction may be to look at the sex differences in the interaction on the melanocortin system with the mesolimbic dopamine system and its effect on food intake. Overall, our results further supports that αMSH can act on the dopamine neurons in the VTA to control homeostatic food intake as well as motivational food reward intake.

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