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# The Role of Lateral Septal Opioid and $GABA_A$ Receptors in Feeding Behavior

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by

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ABSTRACT OF THE DISSERTATION

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by

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Morphine, a μ opioid agonist, elicits eating in rats when injected into multiple brain regions related to feeding and reward including the ventral tegmental area (VTA), nucleus accumbens shell and multiple regions of the hypothalamus (Castro & Berridge, 2014). It produces a particularly large feeding effect when injected into the lateral septum (LS) (Stanley et al., 1988), an area previously linked to several motivational and affective behaviors. The LS has connections to cortical and subcortical regions associated with motivation, and emotion, which makes it a potentially important integrative site for control and modulation of feeding-related behaviors. In this dissertation, I sought to establish receptor specificity, site-specificity and behavioral specificity of opioid-stimulation induced feeding in the LS.

I replicated the robust feeding effects found by Stanley et al. (1988) at a lower morphine dose of 5 μg and found that this effect was reliable across days. I found that naloxone (a competitive opioid receptor antagonist) attenuated the feeding effect of morphine without changing baseline food intake, suggesting that the elicited feeding is

likely specific to opioid receptors. I found that both the mu specific receptor agonist DAMGO and the GABA agonist muscimol increased feeding behavior when injected into the lateral septum. The effects of morphine were blocked at high doses by the mu specific receptor antagonist CTAP, suggesting that both mu opioid and GABA receptors may play a similar role in the modulation of feeding behaviors by the lateral septum.

Although there are many sites in the brain in which stimulation of opioid receptors might stimulate feeding there are differences in feeding response to mu opioid and GABA agonists within the lateral septum. Specifically, muscimol was effective in the ventral and rostral lateral septum, while opioids are more effective in the medial septum, even more than the lateral septum. This interesting finding could reflect interactions of opioid and GABAergic receptors or receptor distribution within the septum. It may mean that the septal control of feeding and motivation involves multiple mechanisms in multiple regions.

The Role of Lateral Septal Opioid and $GABA_A$ Receptors in Feeding Behavior

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## Chapter 1: Background and Significance

Obesity poses multiple substantial health risks including an increased incidence of heart disease, stroke, high blood pressure, diabetes, respiratory problems, sleep apnea, and certain cancers. In the United States obesity is a widespread problem, as an estimated 39.6% of adults are obese (Hales et al., 2017). Therefore, understanding the neural mechanisms underlying feeding and reward is important as it could shed light on how to better treat obesity and other eating disorders, such as binge eating, anorexia, or bulimia.

Classically, feeding behavior has been viewed as a homeostatic function and the study of feeding behavior has largely focused on peripheral hormones and hypothalamic control of feeding responsible for maintaining energy balance. Indeed, the release and detection of peripheral hormones are one of the many ways the body responds to a fed or fasted state. For example, major classes of neurons in the arcuate nucleus of the hypothalamus detect blood-borne signals such as leptin, a fat-derived hormone that inhibits hunger following a meal, and ghrelin, a stomach-derived hormone that can induce hunger (Saper et al., 2002). These arcuate nucleus neurons then transmit the appropriate feeding stimulatory or satiety messages to the paraventricular nucleus, lateral hypothalamus (LH), and multiple extra-hypothalamic sites (Anand & Brobeck, 1951; Minor et al., 2009). However, if eating was solely controlled by homeostatic mechanisms then the United States would not have an obesity problem. People, as well as rats, will overeat highly palatable foods enriched with fat and sugar even if they have consumed enough calories to meet their metabolic needs. Bodyweight homeostasis is

sub-served by mechanisms like peripheral hormones, and hypothalamic nuclei have primary and complex roles in feeding control; however, the strong motivation to eat highly palatable foods even after metabolic needs have been satisfied and the consequent obesity has led to an increased interest in non-homeostatic mechanisms.

The hypothalamus and the LH are well established as having key roles in the control of feeding. Electrolytic lesions of the LH decrease food intake and electrical stimulation of the LH increases food intake (Anand & Brobeck, 1951). Stimulation of LH glutamatergic receptors or suppression of LH GABA receptors increases feeding even in satiated animals, while inhibition of LH neurons decreases feeding and leads to significant loss of body weight (Stanley et al., 1996, 2011; Turenius et al., 2009; Urstadt & Stanley, 2015). In addition, the sight or taste of food changes the firing rates in a subset of neurons in the LH in hungry but not satiated animals (Mora et al., 1976). While the LH is primarily considered as important in homeostatic mechanisms of feeding, the LH also connects to multiple brain regions that are necessary for food reward.

A primary circuit responsible for reward and motivation is referred to as the mesocorticolimbic system (MCL). The MCL consists largely of dopaminergic projections from the ventral tegmental area (VTA) to the amygdala, nucleus accumbens, and prefrontal cortex (PFC) (Saper et al., 2002). The nucleus accumbens and particularly the nucleus accumbens shell have been shown to play a role in mediating feeding behavior. Inhibition of the nucleus accumbens elicits food intake and this feeding effect can be blocked via inhibition of the lateral hypothalamus (Urstadt et al., 2013). Further, clinical and pre-clinical research has demonstrated that dopamine deficiencies in the

reward system are linked to increased feeding and obesity (Wang et al., 2001; Volkow et al., 2011). Food exposure alone is linked to increases of dopamine in the ventral tegmental area, the striatum, and the nucleus accumbens, that decrease with repeated exposure, suggesting that overstimulation in these areas could lead to dopamine deficiencies (Volkow et al., 2011). It should be evident that there are multiple brain regions and systems that mediate and modulate feeding behavior. Research into the anatomical and functional connections between these systems can shed light on the mechanisms responsible for complex feeding behavior and disordered feeding.

Opioid receptor agonists such as morphine can increase food intake and opioid receptor antagonists can decrease feeding (Gosnell & Levine, 2009). Areas of the brain referred to as opioid hedonic hot spots can amplify or suppress the hedonic impact of natural rewards like feeding (Pecina et al., 2006). These hot spots include the rostral nucleus accumbens (NAc), the ventral pallidum, VTA, and the amygdala. When these brain regions are injected with μ-opioid agonists like morphine or [D-Ala$^2$, *N*-MePhe$^4$, Gly-ol]-enkephalin (DAMGO) feeding is typically elicited (Castro & Berridge, 2014). Rats will also self-administer morphine into the septal region (Le Merrer et al., 2007), suggesting a role for septal μ receptors in reward mechanisms. Opioid agonists, morphine, [D-Ala2]-methionine enkephalinamide (DALA), and MR2034 elicited increased food intake when injected into several hypothalamic and nonhypothalamic regions of the brain, including the paraventricular, dorsomedial and LH, amygdala and the LS (Stanley et al., 1988). The increased feeding was particularly robust in the animals that received drug directly into the LS.

The LS has a high density of opioid receptors (Risold & Swanson, 1997b). There are three typically expressed opioid receptors in the brain namely μ, δ, and κ opioid subtypes. Endogenous opioids that bind with δ and μ receptors include endorphins and enkephalins, while endogenous dynorphins bind with κ opioid receptors (Bodnar, 2004). Studies using in situ hybridization and immunohistochemistry found a high density of enkephalinergic neurons in the rostral lateral septum, with lower concentrations towards the caudal regions; dynorphins were also present but in lower concentrations (Risold and Swanson, 1987b). Interestingly, there is evidence that the hypothalamus contains enkephalinergic neurons that project to the LS (Sakanaka, 1982), suggesting that the hypothalamus may be a source of feeding stimulatory opioid afferents to the LS.

**The Lateral Septum Neuroanatomy**

As the name indicates, the LS is the lateral portion of the septum, a subcortical region in the forebrain that is a part of the basal ganglia (Swanson & Risold, 2000a). It is anatomically connected to multiple brain areas that are known to regulate motivation and emotion, as well as learning, memory, and social behavior. The lateral septum's main sources of input are glutamatergic projections from cortical regions including the hippocampus, entorhinal cortex, and PFC (Risold & Swanson, 1997a). Regions of the brain stem such as locus coeruleus and nucleus of the solitary tract also project to the LS. It has strong reciprocal connections to the amygdala and the bed nucleus of the stria terminalis, periaqueductal gray, and the raphe nucleus. Most if not all projections from the LS are inhibitory. Of interest here are the dense reciprocal connections that the LS

shares with the hypothalamus. Regions through the LS project primarily GABAergic neurons to the lateral hypothalamus. The medial hypothalamus including the arcuate nucleus project back primarily to the rostral and ventral LS.

The LS can be broken down into three main parts including the ventral LS (VLS), rostral (RLS), and caudal (CLS). Each of these areas projects to parts of the lateral hypothalamus (LH), which is known to be involved in the control of feeding. More specifically, the LS projects topographically to the LH, rostral parts of the LS connect to rostral parts of the LH, beginning with the lateral preoptic area and then progress to more medial and caudal parts of the hypothalamus. Each of these hypothalamic areas' projects back to the LS (Swanson & Risold, 1997b). Projections from the hypothalamus to the LS contain peptides like corticotropin-releasing factor, enkephalin, ghrelin, and orexin (Gong et al., 2013; 2014; Risold & Swanson, 1997a; Sakanaka et al, 1982). The LS has primarily GABAergic projections the hypothalamus and adjacent areas of the septum, the caudal LS projects heavily to the adjacent medial septum (MS). However, the ventral LS projects mostly to somatostatin releasing neurons to the periventricular nucleus (Swanson & Risold, 1997a). The periventricular zone of the hypothalamus projects in-turn to sympathetic and parasympathetic regions in the brain stem that is important in the regulation of feeding and drinking (Leibowitz, 1977).

The hippocampus projects heavily to the LS and those projections are largely glutamatergic neurons terminating in the dorsal part of the LS and the medial septum (Jakob & Leranth, 1995). The dorsal part of the LS represents an area that receives strong excitation from the hippocampus and is populated by both ionotropic and

metabotropic glutamate receptors and is also densely populated by both $GABA_A$ and $GABA_B$ receptors to inhibit this strong excitatory input. Additionally, the LS projects to and inhibits the medial septum which projects to the hippocampus, terminating on GABAergic and cholinergic neurons. Strong inhibition of the medial septum with morphine interrupts spatial acquisition on a Y-maze while morphine in the LS enhances spatial acquisition (Cazala & Norena, 1998). The LS receives vasopressin input from the amygdala and cholinergic and monoaminergic input from areas in the brain stem (Risold & Swanson, 1997b). In addition, while GABAergic receptors are widespread in the septum, the LS, CLS, and VLS, as well as the MS, are dense with mu receptors while the DLS and RLS have very few (Erbs et al., 2015; Mansour et al., 1994; Risold & Swanson, 1997). All the above evidence suggests that the lateral septum is a complex structure connecting many areas of the brain and using a range of neurotransmitters and peptides. It is no wonder that the lateral septum has been implicated in a wide variety of behaviors.

**The Lateral Septum – Role in Defensive Behaviors**

As its anatomical connections might suggest, the LS has been linked to a variety of affective and motivated behaviors, such as aggression, anxiety, depression, and reward. The LS has long been associated with anxiety but there is conflicting evidence about its role in fear, defensive behavior, and anxiety. Electrolytic lesions of the LS have led to a phenomenon dubbed septal rage (Brady & Nauta, 1953; Sheehan, 2004; Urstadt & Stanley, 2013). Septal rage consists of increased defensive but not aggressive behaviors in rats which suggests an exaggeration of fear and anxiety (Adams, 1979;

Thomas & Evans, 1983). Excitotoxic lesions of the LS increase behavioral and neuroendocrine stress response. More specifically, LS lesioned animals had an increased behavioral stress response, as measured by the forced swim test; they gave up trying to escape the forced swim in significantly less time and had significantly higher corticosterone and ACTH levels than unlesioned animals (Singewald et al., 2011). Conversely, septal lesions can decrease apparent anxiety, as measured by the elevated plus-maze and probe burying tasks in rats (Menard & Treit, 1996; Pesold & Treit, 1992). Also, dominant rats with septal lesions show a decrease in aggressive behaviors (Blanchard et al., 1977).

Given the conflicting data on the LS and anxiety, it is difficult to develop a parsimonious explanation of the lateral septum's role, but nevertheless Gray & McNaughton (1982) have proposed an explanatory model. They refer to this model as a "behavioral inhibition system" which is composed of projections between the septum and the hippocampus commonly called the septohippocampal system. This septohippocampal system is referred to as the behavioral inhibition system because it assesses incoming stimuli associated with threat or punishment and may inhibit any concurrent behaviors to allow the animal to pay attention and appropriately respond to stimuli.

**The Septum and Reward.**

The LS has also long been associated with reward. The septum is sometimes referred to as the first reward "center" discovered by Olds & Milner (1954). They serendipitously found that rats would "self-stimulate" i.e., perform an operant response that produced electrical stimulation of the septum. The LS has also been associated with the environmental or cue-induced reward. Rats show increased cFos immunoreactivity in the LS after acute injections with cocaine, and they show a similar increase in cFos after exposure to cocaine-related cues (Brown et al., 1992). Inhibition of the RLS attenuates cocaine preference overall, as do orexin antagonists in the LH (Sartor & Aston-Jones, 2012). Further, RLS neurons terminate on orexin neurons of the LH that are specifically involved in models of reward and addiction. Rats will self-administer morphine into the septal region (Le Merrer et al., 2007). Indeed, mice that received morphine injections into the LS learn to self-administer faster and have a higher rate of self-administration than animals that can directly self-administer into the NAc, commonly considered to be a major site mediating reward (Le Merrer et al., 2007).

Morphine injections into the LS increase cFos in the NAc, suggesting a role in disinhibition of reward and motivational circuitry in the brain (Varoqueaux & Leranth, 1997). Stimulation of LS neurons can increase neural firing rates in the VTA (Maeda & Mogenson, 1981). Stimulation of the LH promotes self-stimulation in both the LS and MS (Miller & Mogenson, 1971; Sheehan et al., 2004). Generally, mice with increased LS volume show better memory, less anxiety, and less susceptibility to substance abuse, (Talishinsky & Rosen, 2012).

The LS is also thought to play a role in social behavior which is supported by its reciprocal connections to the medial preoptic area of the hypothalamus, an area known to be associated with sexual and maternal behaviors and there is evidence of androgen and estrogen receptors within the LS (Risold & Swanson, 1997b; Sheehan & Newman, 2000). The lateral septum's connections to the amygdala, the MCL, and to the hypothalamus make it an interesting structure to consider regarding motivated behaviors like social and feeding behaviors.

**The Lateral Septum and Feeding**

The Lateral Septum is heavily connected to the hypothalamus and is known to modulate some hypothalamic behaviors. Miller and Mogenson (1971) found that electrical stimulation of the septum could facilitate or inhibit the electrical self-stimulation of the LH. Interestingly, when current levels in the LH were low septal stimulation increased self-stimulation in the LH but when current levels were high septal stimulation decreased LH self-stimulations, suggesting a modulatory role of the LS on the LH. Another study by Miller and Mogenson (1972) linked LH with the LS using electrophysiology/self-stimulation techniques. Low-intensity electrical stimulation of the septum caused increased self-stimulation and increased firing of neurons in the LH. High or intense electrical stimulation of the septum inhibited neurons in the LH and decreased self-stimulation. Electrical stimulation in the dorsal midline region of the septum in rats stimulates drinking behavior while stimulation of the more ventral lateral region inhibits drinking (Miller & Moganson, 1971). Furthermore, LH orexin neurons

are activated by RLS afferents during cocaine conditioned place preference, while inhibition of RLS afferents inhibits orexin neurons (Sartor and Aston-Jones, 2012). The hypothalamus is not only influenced by the LS but can influence the LS via reciprocal connections. According to Leranth and Vertes (2000), the hypothalamus influences the septohippocampal system in a few ways, including its direct projection to the hippocampus and medial septum and its reciprocal connections to the LS. In this way, the hypothalamus could influence hippocampal memory formation via the LS.

The anatomical, chemoarchitectural, and behavioral data taken together suggests that the LS, functionally a part of the basal ganglia and limbic system, acts as an interface between areas of the brain associated with motivation, memory and cognition and areas of the brain responsible for autonomic and endocrine processes. The lateral septum's role in eating could be crucial with regard to risk assessment and fear memory associated with a food source. Still, little is known about how the LS modulates feeding, and given the robust increase in eating in response to opioidergic drugs and GABAergic drugs, it is important to use these drugs to investigate the role of their corresponding receptors in eating.

## Chapter 2: Opioid elicited feeding in the Lateral Septum


### Abstract

After finding that morphine could elicit a robust feeding effect when injected into the lateral septum (LS), an area the brain associated with numerous behaviors involving reward, anxiety-like behavior, and learning and memory, I became interested in its role in feeding. Up to that point in 2013, there had been very little research done on its role in feeding. I first sought to replicate the robust feeding effect found by Stanley et al. (1988). I was able to replicate that effect with a lower dose of morphine (5 µg), which I discovered produced a stable feeding response with repeated injections. Specifically, across five days of repeated injections, there was no increase or sensitization effect, nor a decrease in feeding or tolerance. Additionally, I found that I was able to decrease the feeding effect of morphine with the mu-selective opioid receptor antagonist, naloxone. These experiments suggested that opioids did have a role in controlling food intake and are detailed below.


### Introduction

While researching the literature for a dissertation project back in 2013 I was struck by the serendipitous finding by Stanley et al. in 1988 that robust feeding was produced by morphine injected into the LS. The LS is an area of the brain that is most closely associated with anxiety and defensive behaviors, known to both inhibit and stimulate anxiety-like behaviors (Thomas & Evans, 1983). However, the LS was also considered to be an early reward center as animals would lever press to self-stimulate

(Olds & Milner, 1954). Aside from anxiety and its early status as a reward center, the LS is associated with social reward and learning and memory, but little research had been done on feeding up to that point.

As the LS has dense interconnections between homeostatic, hedonic, and affective regions in the brain, my hypothesis is that this brain area's role in feeding reflects an integration of these functions to control feeding behavior. Septal lesions have been found to both increase and decrease feeding behavior in rats. Flynn et al. (1986) found that rats with septal lesions showed interruptions during normal feeding due to increased locomotor activity, resulting in less meaningful and more frequent bouts of eating. On the other hand, Oliveira et al. (1990) found that LS lesions increased feeding post electrical stimulation in the LH, suggesting feeding suppressive role for LS in feeding behavior. Chemogenetic or optogenetic stimulation of septal GABAergic neurons or their projections to the LH decreased feeding, while chemogenetic inhibition of GABA neurons in the LH increased eating. This suggests that GABA neurons projecting from the LS stimulate GABAergic neurons in the LH to decrease feeding (Sweeny & Yang, 2016). Sweeny and Yang (2016) also discovered a hippocampal-LS circuit that controls feeding. They found that optogenetic activation of the ventral hippocampus projections to the LS reduces feeding, while optogenetic inactivation increases it, and that inactivation of the LS neurons disrupts these effects. These studies suggest that mostly GABA containing neurons in the LS play a role in feeding behavior. There is evidence that opioid receptors in the LS also play a role.

Decreased feeding and weight loss produced by fluoxetine (a selective serotonin reuptake inhibitor used to treat anxiety and depression) are associated with decreased levels of opioid receptors in the LS (Churruca et al., 2006). Finally, as noted previously, Stanley et al. (1988) found that μ opioid agonists, morphine, DALA, and MR2034 elicited food intake when injected into several hypothalamic and extra-hypothalamic regions of the brain including the paraventricular, dorsomedial and LH, amygdala and the LS. Importantly, the increased feeding effect was particularly robust in the animals that received drug directly into the LS. The evidence presented here suggests that there is a septal-hypothalamic circuit in control of feeding behavior that is regulated by opioid receptors in the lateral septum.

I sought to replicate the increased feeding in response morphine injections into the LS. I additionally sought to assess the effects of repeated administration of morphine injections into the LS on feeding to assess behavioral tolerance or sensitization. Next, I sought to test the receptor specificity and block the effect of morphine on food intake using naloxone (a competitive opioid receptor antagonist).

## Methods

### *Subjects and Surgery*

Adult male Sprague Dawley rats weighing 350-450 grams were used for all experiments. Rats were bred and tested in an on-campus vivarium on a 12-hour light-dark cycle and single housed starting a week prior to surgery. Animals were anesthetized

for surgery using intraperitoneal (IP) sodium pentobarbital at 50 mg/kg of body weight, preceded by an IP injection of 0.25 ml of atropine sulfate (0.54 mg/kg). A single 18 mm long, 26-gauge stainless steel guide cannula was stereotaxically implanted 1 mm dorsal to the target site in the LS, 8.7 mm anterior to the interaural line, 0.7 mm lateral to the midsagittal sinus and 4.7 mm ventral to the surface of the skull. Cannulas were held in place by dental acrylic and 6 stainless steel screws penetrating the skull. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California Riverside.

**Procedure**

Animals were maintained on Purina rat chow and water *ad libitum* until three days prior to testing when they were switched to mash diet consisting of Purina rat chow (500 g), evaporated milk (354 ml) and sugar (400 g). Animals were tested under satiated conditions; they were given fresh mash one hour prior to testing. Unless otherwise indicated, tests consist of a single injection through a 32-gauge injector that projects 1 mm past the cannula directly onto the targeted brain region. Animals were handled and given mock injections daily for three days prior to experiments.

Injections consisted of 0.3 µl of vehicle alone or the vehicle with a dissolved drug. Vehicle was artificial cerebrospinal fluid (aCSF) that consists of 147 mM $Na^+$, 154 mM $Cl^-$, 3.0 mM $K^+$, 1.2 mM $Ca^{2+}$, and 0.09 mM $Mg^{2+}$, dissolved into sterile water unless otherwise stated.

For all experiments, animals were satiated prior to injections and food was weighed 0, 60, 120, and 180 minutes post-injection, as well as 24 hours post-injection.

Experiment 1: Fourteen rats were used in the first experiment; they were randomly assigned to receive either aCSF (n=7) or morphine 5 µg/0.3 µl (n=7). Repeated injections across five days with one day in-between each injection of morphine and aCSF were performed to assess whether the eating response would exhibit tolerance or sensitization.

Experiment 2: Ten rats were assigned in a counterbalanced order across four conditions to receive either aCSF or naloxone ten minutes prior to receiving morphine (5 µg) or aCSF.

Histological Analysis: After behavioral testing, animals were deeply anesthetized and transcardially perfused with 10% formaldehyde. Unless otherwise stated, their brains sectioned into 100 µm coronal brain slices, and Nissle stained with cresyl violet to ensure that the cannula guides are on target. The cannula guide was on target if the tip of the cannula track was within 0.2 mm of the intended brain region and only rats meeting this criterion were included in the statistical analyses.

**Analyses**

A Two-way repeated-measures ANOVA was used to analyze the feeding effects of morphine compared to aCSF across days for each time point at 30, 60, 120, and 180 minutes. A two-way repeated-measures ANOVA was used to assess the effects of naloxone on the feeding effects of morphine. Significant ANOVAs were followed by pairwise comparisons of each drug dose to its vehicle control using the Bonferroni multiple comparison test.

## Results

Experiment 1: As shown in Fig. 1, animals that were given LS morphine injections ate more than animals given aCSF injections. Specifically, at 120 minutes post-injection, the morphine injected animals ate significantly more than the aCSF group on days 1 and 4 with p values of $p = 0.006$ & $p = 0.04$, respectively. Although they all approach significance, the effect on days 2, 3, and 5 did not reach statistical significance. At 180 minutes post-injections, animals that received morphine ate significantly more than the aCSF group on days 1, 3, and 5, but not other days.

**Figure 1.**

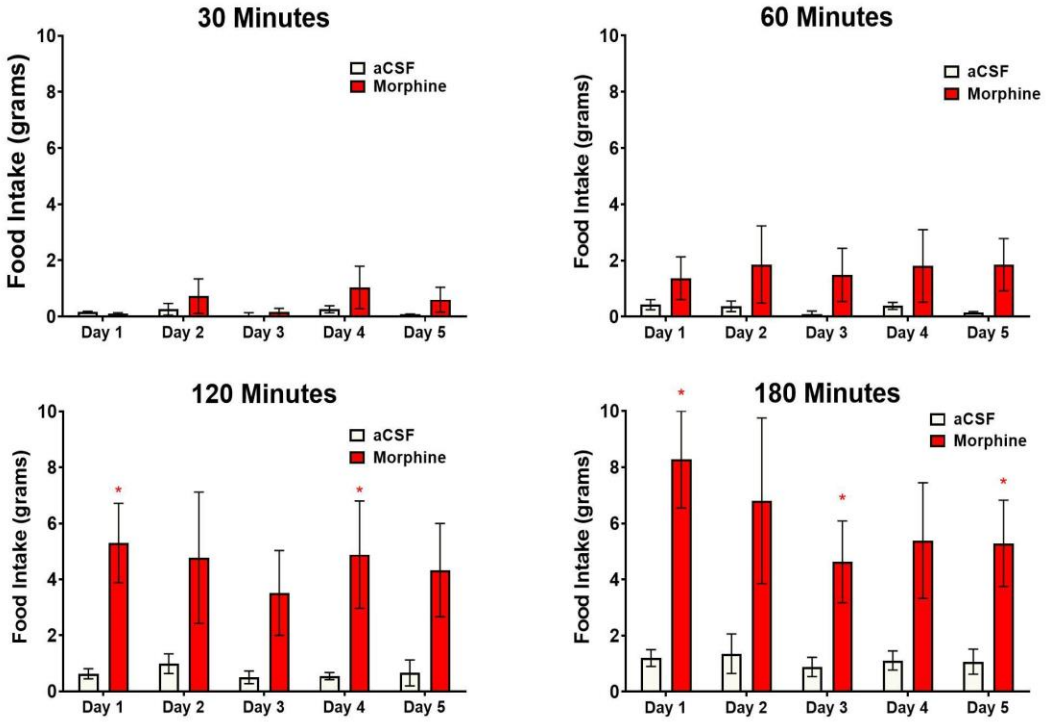


*Figure 1.* Mean cumulative food intake in grams ± SEM across days for animals that received either aCSF or 5 µg/0.3 µl of morphine (*<.05 indicates a significant difference from control).

Experiment 2: As shown in Fig. 2, morphine again increased feeding compared to aCSF at 120- and 180-minutes post-injection. Importantly, pretreatment with naloxone blocked morphine's eating stimulatory effects

**Figure 2**

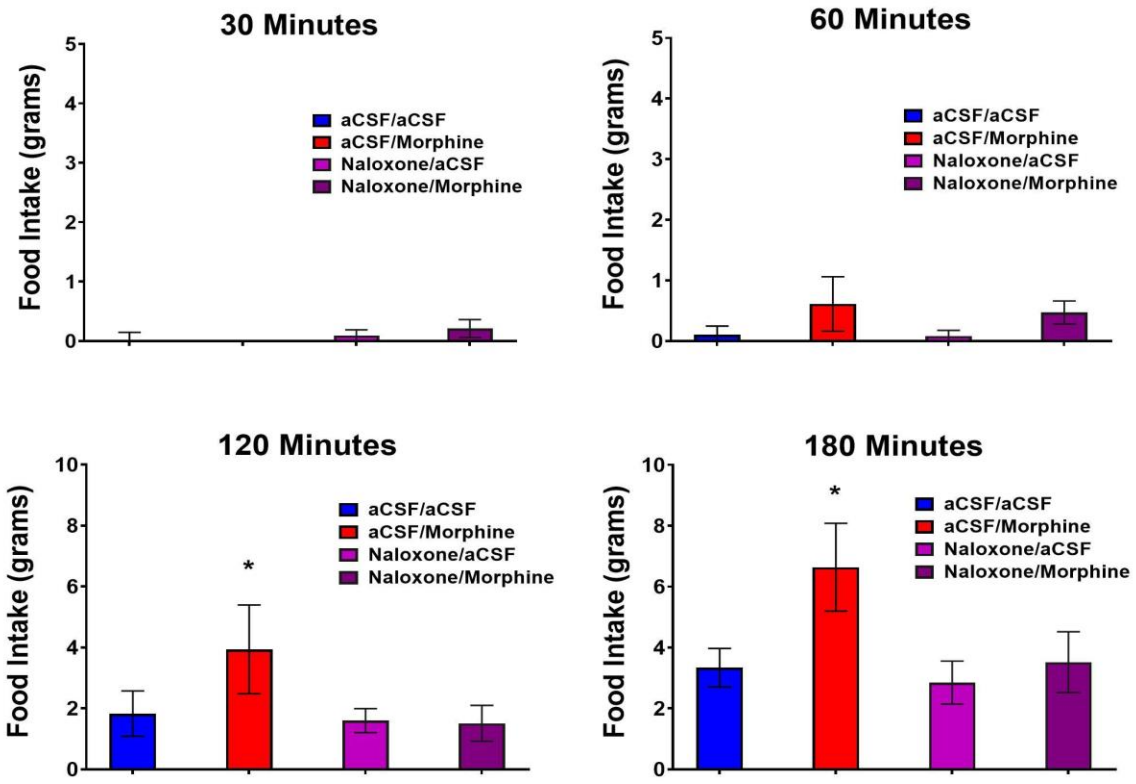


*Figure 2:* Cumulative food intake (mean grams ± SEM) across treatment days for animals that received either aCSF, 5 µg of Morphine alone, 10 µg of Naloxone alone, or Naloxone & Morphine directly into the LS 120 & 180 minutes post-injection (*<.05 different from all other conditions).

**Discussion**

These data replicate the findings of Stanley et al., 1988 showing the morphine injected into the LS elicits feeding in satiated rats and expand on those findings by showing that a lower (5 μg) dose is effective. Moreover, my findings show that that morphine injected into the LS increased feeding behavior across days and that that the magnitude of the effect was stable over time. Specifically, there was no decrease or increase in the trajectory of feeding stimulation over the five test days. This is important as opioid receptors are all G-protein coupled and are known to have long term effects and the behaviors related to opioid receptors including locomotor behavior and analgesia are subject to tolerance and sensitization.

As shown in Figure 2, pretreatment with LS injection naloxone blocked the eating elicited by the local injection of morphine. Naloxone is a broad-spectrum antagonist of opioid receptors. The opioid antagonist naloxone blocked the feeding effects caused by morphine suggesting that septal feeding is caused by the effects of morphine on opioid receptors. Morphine is commonly identified as a μ opioid agonist, but it does bind with less affinity to both κ and δ receptors (Bodnar, 2004). Naloxone is a general competitive antagonist; it binds with higher affinity than morphine and blocks all three major subtypes of opioid receptors. This experiment offers additional evidence that the increased feeding effect seen in rats after LS injections of morphine is caused by its actions on opioid receptors. Using more highly selective drugs can more definitively attribute these effects to an opioid receptor.

## Chapter 3: Lateral septum mu opioid receptors in stimulation of feeding


### Abstract

Stimulation of mu opioid receptors using drugs like morphine can increase eating when injected into multiple brain regions including the lateral septum (LS). The LS has been classically associated with reward, anxiety, and fearful behaviors but more recently has also received attention with regard to control of feeding. To investigate the role of LS opioid receptors in feeding, I injected mu, delta, and kappa-opioid receptor agonists and a mu specific receptor antagonist directly into the LS of rats. I expected that if feeding is mu receptor-specific then only mu receptor agonists would increase feeding. I also hypothesized that mu receptor antagonists would suppress the feeding elicited by mu receptor agonists like morphine. Further, because the LS is densely populated with GABA receptors, I used the $GABA_A$ receptor agonist muscimol to assess the effect of inhibition of LS neurons on feeding. Our results show that the mu receptor agonist morphine and the specific mu agonist DAMGO reliably and significantly increase feeding behavior across doses tested, while delta and kappa agonists were ineffective. CTAP, a specific mu receptor antagonist, at low doses unexpectedly increased morphine-elicited feeding but at high doses decreased morphine's effect, consistent with mediation by mu receptors. Finally, muscimol rapidly elicited feeding, suggesting a role for LS $GABA_A$ receptors in feeding stimulation. These findings suggest that mu opioid receptors in the LS play complex roles in feeding and that neural inhibition may be a mechanism by which they elicit feeding.

Keywords: Lateral Septum, Feeding, Opioids, GABA, Morphine

## Introduction

Mu opioid receptor agonists, such as morphine, can increase food intake when given systemically or when injected directly into one of several brain regions, including the nucleus accumbens (NaC), ventral tegmental area (VTA), amygdala, or hypothalamus (Bodnar, 2004 Gosnell & Levine, 2009; Peciña, et al., 2006). Stimulation of each of the major opioid receptor subtypes including mu, delta, and kappa receptors increased food intake, as well as hedonic reactions to palatable food, in the rostrodorsal part of the NaC shell (Castro & Berridge, 2014). Opioid agonists, morphine, [D-Ala2]-methionine enkephalinamide (DALA), and MR2034 elicited food intake when injected into several hypothalamic and extra hypothalamic regions of the brain, including the paraventricular hypothalamus, dorsomedial hypothalamus, lateral hypothalamus, amygdala and the LS (Stanley et al., 1988). Increased feeding was particularly robust in the animals that received drug directly into the LS, the focus of the current study. These findings suggest a potential role for opioids within the LS in the regulation of feeding.

As the name indicates, the LS is the lateral portion of the septum, a subcortical region in the forebrain considered a part of the basal ganglia and limbic system (Risold & Swanson, 1997a). While the LS receives inputs from and projects outputs to many brain areas, it is most densely and reciprocally connected to the hypothalamus (Risold & Swanson, 1997a). These dense reciprocal connections, particularly those from the lateral hypothalamus, support a potential role for the LS in the regulation of feeding behaviors.

In addition to the hypothalamic connections, the LS is also strongly connected to areas of the brain that regulate reward and has been long associated with reward. Indeed, the septum is sometimes referred to as the first reward "center" based on a serendipitous discovery by Olds & Milner in 1954. They found that rats would "self-stimulate" i.e., repeatedly perform an operant response that produced electrical stimulation of the septum. The LS is interconnected with the VTA and also projects to the NaC; both regions are integral parts of a reward pathway (Risold and Swanson, 1997a). Furthermore, mice will self-administer morphine into the septal region and learn this response faster than they learn to self-administer it into the NaC, commonly considered to be a major site mediating reward (Le Merrer et al., 2007). Taken together, this evidence suggests that the LS may play a role in reward-linked behaviors like eating.

The LS's connections to the VTA and NaC as well as the hypothalamus make it an interesting structure to consider regarding motivated behaviors like feeding. As noted previously, Stanley et al. (1988) found that mu opioid agonists, morphine, DALA, and MR2034 elicited food intake when injected into several hypothalamic and extra-hypothalamic regions of the brain and the feeding effect was particularly robust in the animals that received drug directly into the LS. The purpose of the current experiments was to determine the receptor specificity of the opioid-induced feeding effect using our standard palatable mash diet (Stanley et al., 1988). The main types of opioid receptors in the LS are the mu, delta, and kappa subtypes. Morphine is primarily a mu receptor agonist, but it also binds to both kappa and delta receptors with lower affinity (Feng et al., 2012). Accordingly, selective opioid agonists for mu, delta and kappa receptors were

compared to aCSF control to determine receptor specificity of the septal feeding. Furthermore, if septal feeding is due to binding of mu opioid receptors in the LS then I expected to suppress the effect with a mu specific antagonist. Morphine's primary direct action on neurons is inhibitory and the increase in feeding could generalize to other inhibitory agonists. To examine this possibility, a gamma-aminobutyric acid (GABA) receptor agonist was injected directly into the LS and food intake was measured.

## Results

### The mu receptor agonist reliably increased feeding while the delta and kappa agonists were ineffective.

As shown in Fig. 3, DAMGO elicited significant feeding across multiple doses at every time tested, from 30 to 180 minutes post-injection. In the early, 30- and 60-minute post-injection periods only the two highest doses were statistically significant, whereas almost all doses were statistically significant in the latter, 120- and 180-minute post-injection periods. In contrast, neither the delta nor the kappa agonists elicited significant feeding. A two-way repeated measures ANOVA revealed that while there was no significant effect of DAMGO on food intake ($F(3,36)=1.89$, $p=0.15$), there was a significant effect of time ($F(3,36)=30.16$, $p<0.0001$) and a significant dose by time interaction ($F(9,108)=2.51$, $p=0.01$). Pairwise comparisons of dose to vehicle justified by the dose x time interaction revealed numerous statistically significant effects of DAMGO, as specified in Fig. 3.

In contrast, DPDPE had no statistically significant effect on feeding. Specifically, two-way ANOVA revealed that there was no significant effect of DPDPE ($F(3,36)=.775$, $p=0.51$), a significant effect of time $F(3,36)=24.70$, $p<0.0001$ and no dose x time interaction ($F(9,108)=1.58$, $p=0.12$).

Similarly, the kappa receptor agonist U50488 had no statistically significant effect on feeding. Two-way ANOVA revealed no significant effect of U50488 ($F(3,36)=.70$, $p=0.55$), a significant effect of time ($F(3,36)=20.41$, $p<.0001$) and no interaction of time and dose ($F(9,108)=0.43$, $p=0.91$).

As illustrated in Fig. 4a, the injection sites for the subjects tested with these opioid agonists were clustered within the LS, with none centered more than 0.2 mm outside the border of this brain region.

As shown in Fig. 5, DAMGO again increased food intake. The initial effects were small, with increases in food intake of approximately 2 grams over vehicle 30 minutes post-injection, and maximal increases of approximately 5 grams 180 minutes post-injection. Further, the feeding stimulation was dose-dependent at 120- and 180-minutes post-injection, but at the initial 30-minute post-injection period showed that only the intermediate doses (0.15 and 0.6 ugs) were effective. ANOVA revealed no significant effect of dose ($F(4,32)=2.07$, $p=0.10$) but there was a significant effect of time ($F(3,24)= 12.51$, $P<.0001$) and a dose by time interaction $F(12,96)=2.07$, $p=0.02$.

Morphine similarly increased food intake, with strong dose-dependent effects 120- and 180-minutes post-injection, but only a single dose (1.5 ugs) produced an effect at an earlier time (see Fig. 5). Two-way repeated-measures ANOVA revealed a significant effect of dose ($F(4,32)=8.11$, $p<0.0001$), time ($F(3,24)=23.93$, $p<0.0001$) and a significant dose by time interaction ($F(12,96)=4.87$, $p<.00001$). The injection sites for these subjects are illustrated in Fig. 4b.

**Figure 3**

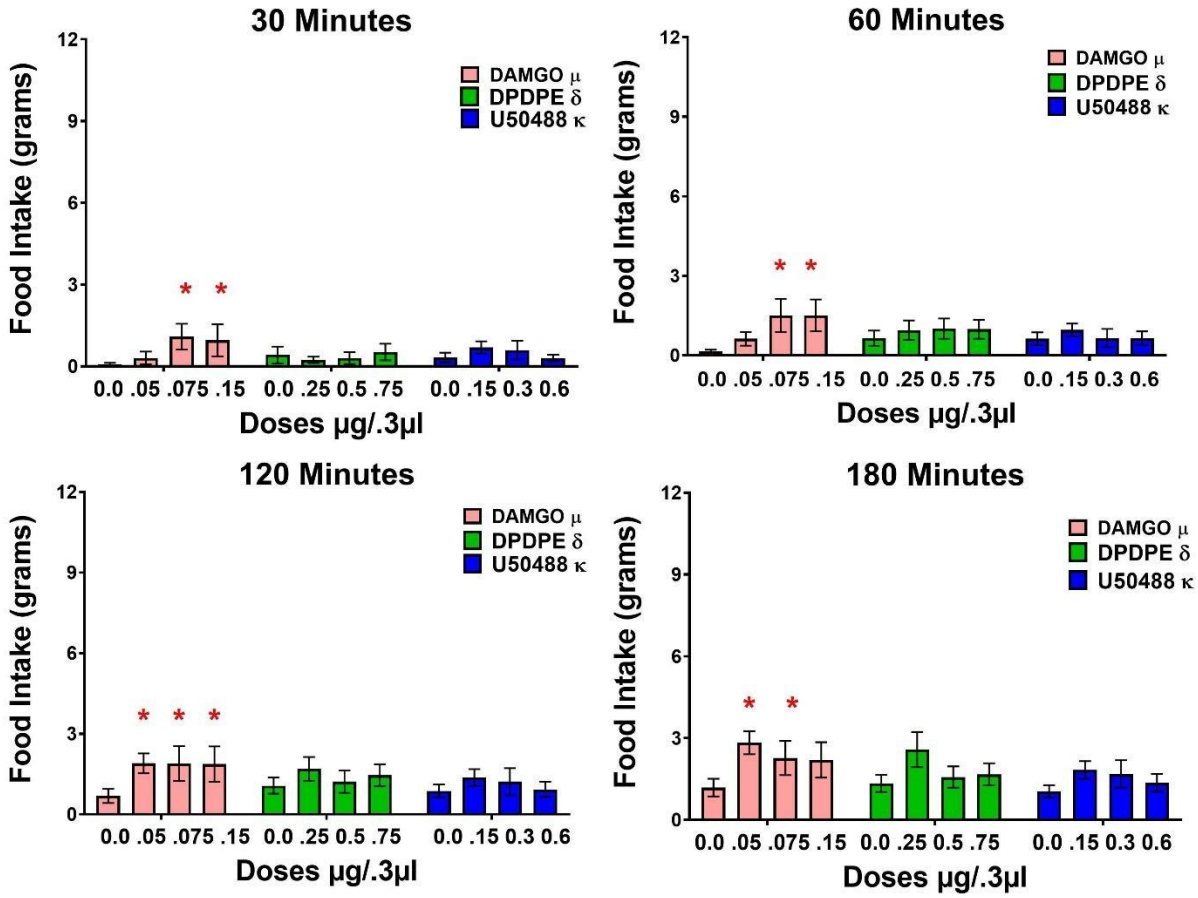


*Figure 3: Cumulative food intake (mean ± SEM) in response to LS injections of mu, delta, and kappa opioid receptor agonists 30, 60, 120- and 180-minutes post-injection.* * *represents $p<0.05$ by Bonferroni multiple comparison tests compared to aCSF at the same post-injection time.*

**Figure 4**

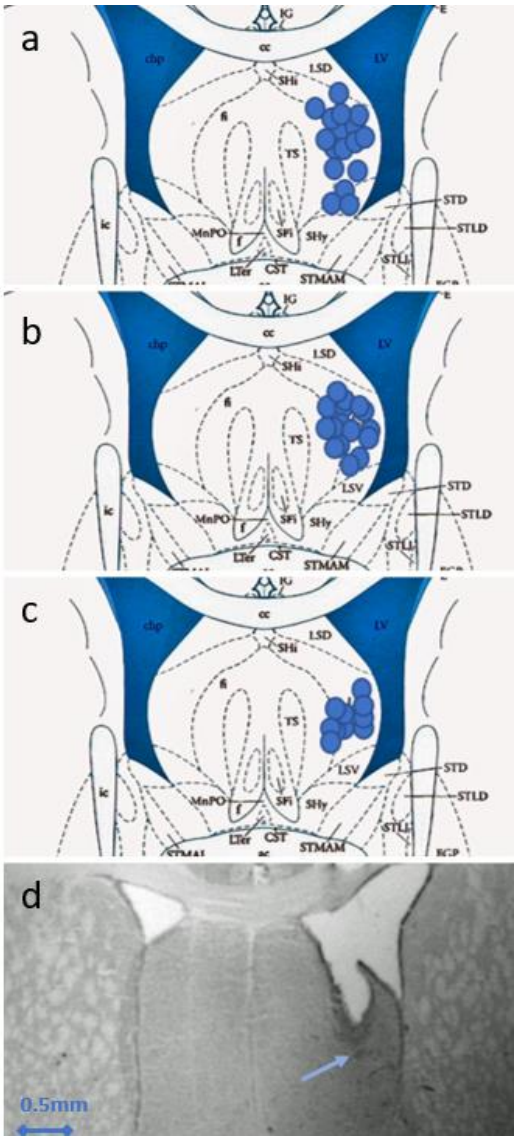


*Figure 4: Injection sites mapped onto figures from the stereotaxic atlas of Paxinos and Watson (2007). a) Injection sites of 39 animals represented by dots from Phase 1 of Experiment 1. b) Injection sites of 18 animals represented by dots from Phase 2 of Experiment 1. c) Injection sites of 12 animals from Experiment 2. d) Histological image showing a representative injection site (marked by the arrow) within the lateral septum of a cresyl violet stained section. The scale bar = 0.5 mm.*

**Figure 5**

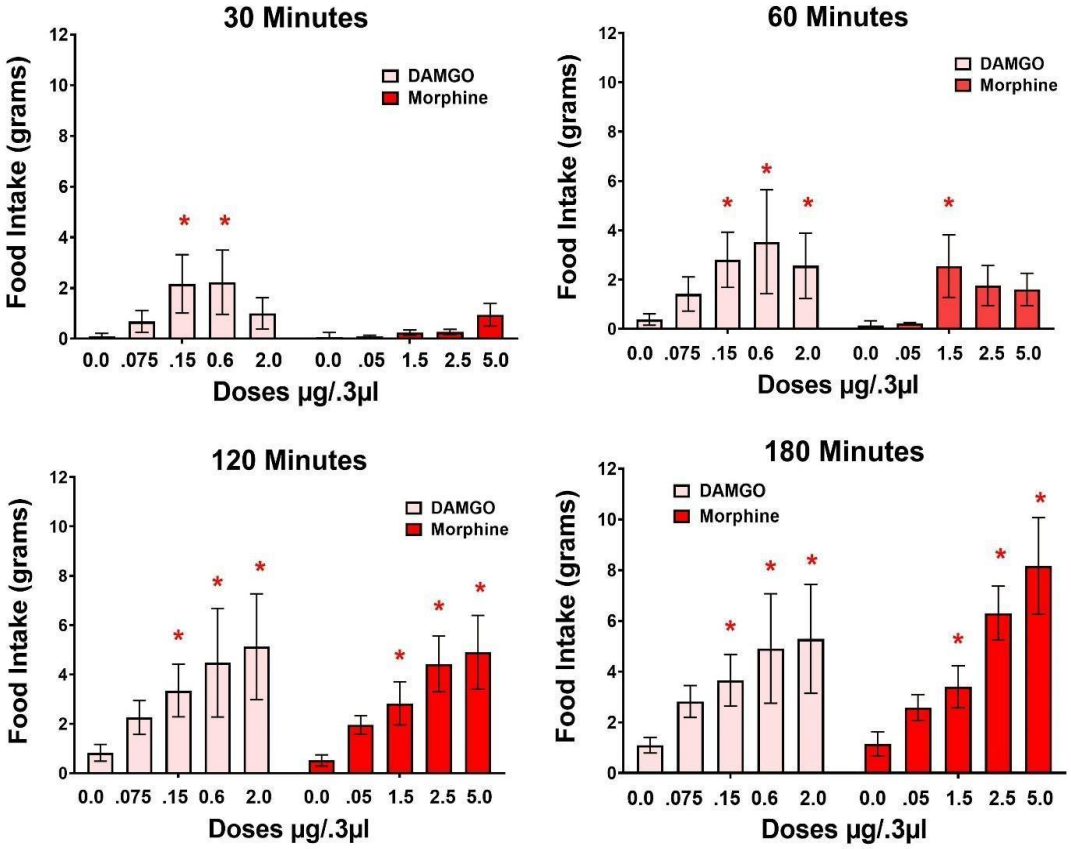


*Figure 5: Cumulative food intake (mean ± SEM) in response to mu receptor agonists DAMGO and morphine at 30, 60, 120, and 180 minutes post-injection.* * *represents $p<0.05$ by Bonferroni multiple comparison tests compared to aCSF at the same post-injection time.*

**The bidirectional effects of mu receptor antagonist on morphine-elicited feeding**

Unexpectedly, the lowest dose of CTAP (0.05 ug) increased morphine-induced feeding 180 minutes post-injection (see Fig. 6). Intermediate doses of CTAP had no statistically significant effect on morphine-elicited feeding and the highest dose of CTAP suppressed morphine-elicited feeding. Similar trends were evident 120 minutes post-injection, except that morphine's effect, was not statistically significant at this time. Two-way repeated-measures ANOVA revealed a significant effect of dose ($F(5,55)=3.86$, $p=0.004$), time ($F(3,33)=41.14$, $p<0.0001$) and a significant dose by time interaction ($F(15,165)=5.58$, $p<0.0001$). The injection sites for these subjects are illustrated in Fig. 3c.

**Figure 6**

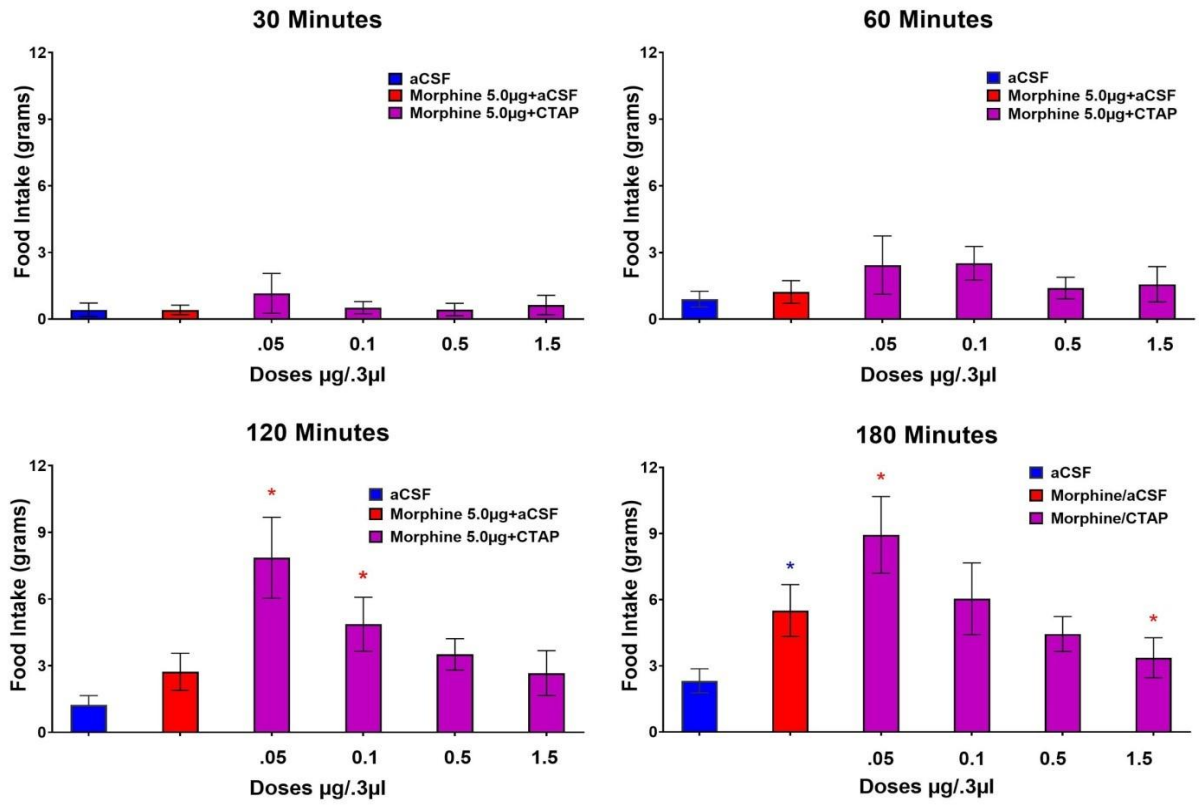


*Figure 6: Cumulative food intake (mean ± SEM) at 30, 60, 120- and 180-minutes post-injection in response to LS injections of morphine+aCSF, morphine+CTAP at 0.05, 0.1, 0.5 and 1.5 ug. * represents p<0.05 difference from morphine+aCSF and * represents a p<.05 difference from aCSF alone, both by Bonferroni multiple comparison tests compared at the same post-injection time.*

**LS Injection of the $GABA_A$ Agonist Muscimol Elicits Eating**

Muscimol, like DAMGO, significantly increased feeding at each post-injection time. The effects were not clearly dose-dependent, with the lowest and highest doses producing greater effects than the intermediate dose (see Fig. 7). Two-way repeated measure ANOVA showed a significant effect of dose ($F(3,33) = 3.17$, $p = 0.03$) and time ($F(3,33) = 9.78$, $p < 0.001$) but no interaction effect ($F(9,99) = 1.17$, $p = 0.32$).

**Figure 7**

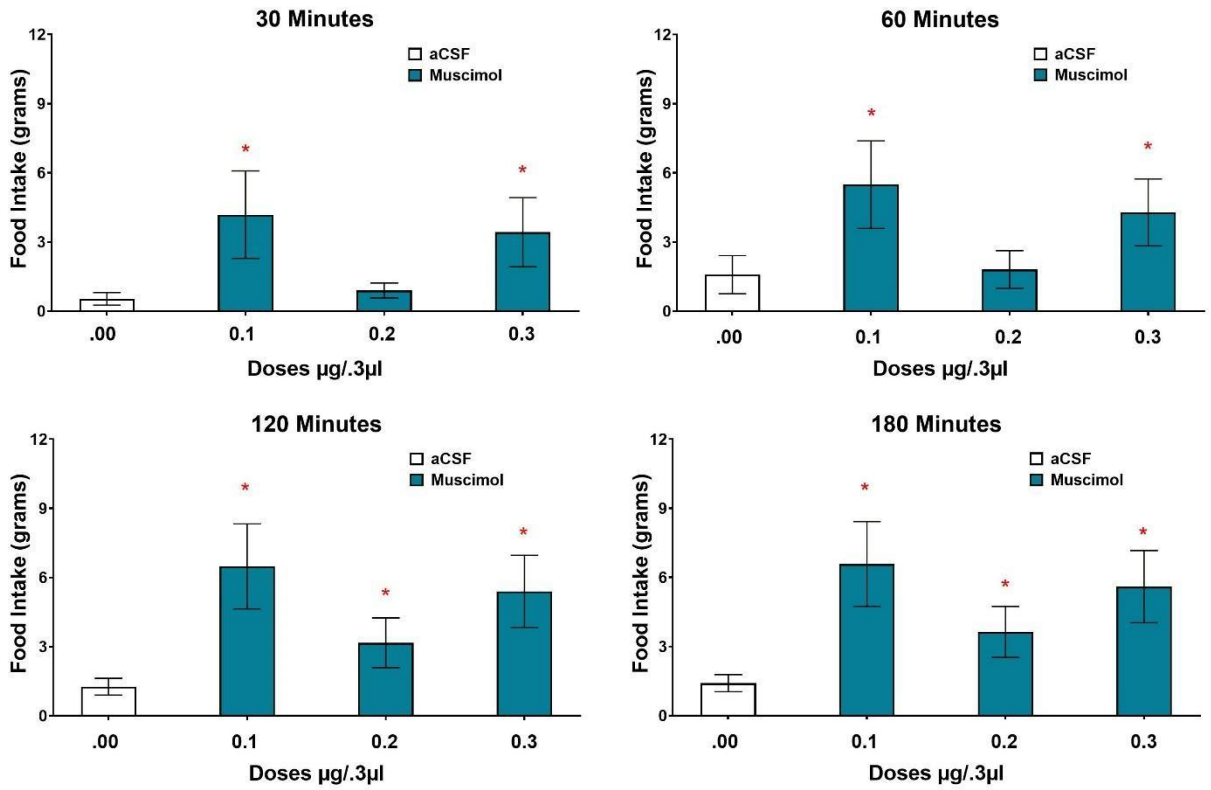


*Figure 7: Cumulative food intake (mean ± SEM) in response to muscimol (0.1, 0.2, & 0.3 µg) at 30, 60, 120- and 180-minutes post-injection. *represents $p<0.05$ by Bonferroni multiple comparison tests compared to aCSF at the same post-injection time.*

## Discussion

Previous studies have shown that morphine injected directly into the LS of satiated rats produces a robust but delayed eating response (Stanley et al., 1988) and our core goal in the present study was to provide insights into the receptor subtype or subtypes mediating this response. To that end, I compared the ability of opioid receptor

agonists selective for mu, delta, and kappa subtypes to elicit feeding when injected into the LS. I found that the mu receptor agonists DAMGO and morphine both elicited similarly robust and dose-dependent patterns of eating behavior, while kappa and delta receptor agonists were ineffective (see Figs. 3 & 5). Noteworthy is that the doses of these drugs were tested in ascending order, an approach I have previously used to establish initial dose ranges (e.g. Stanley et al., 1993). While this confounding of dose with sequence might impact the shape of the dose-response curve, it is unlikely to impact the core issue, which agonists are effective versus which are ineffective. The robust, reliable, and receptor-specific effect of LS mu agonists suggests that stimulation, specifically of mu opioid receptors in or near the LS, can produce an eating response in satiated rats of a palatable mash diet.

To test this possibility further, I attempted to suppress the morphine-elicited feeding by LS injection of CTAP, a potent and specific mu opioid receptor antagonist. The lower doses of CTAP I employed unexpectedly increased morphine-elicited feeding 120- and 180-minutes post-injection (see Fig. 5). If due to a mu opioid receptor antagonist effect, the apparent stimulatory effect of low doses of CTAP on morphine-elicited feeding may suggest that subsets of mu opioid receptors exist in the septum whose activation by endogenous ligands or by morphine is actually acting to suppress feeding, an effect unmasked by the low doses of CTAP. As these tests did not include a CTAP alone condition, it is alternatively possible that CTAP alone elicited feeding, rather than enhancing morphine-elicited feeding. If so, CTAP, like morphine, elicited feeding only after a two-hour delay. In contrast to this apparent feeding stimulatory effect, the

highest dose of CTAP suppressed morphine-elicited eating at 180 minutes post-injection. That the mu opioid receptor antagonist suppressed morphine-elicited feeding suggests that mu opioid receptors in the LS mediate morphine-elicited feeding. More broadly, the antagonist and agonist data collectively provide a convergence of evidence suggesting that stimulation of mu opioid receptors in the LS can produce a substantial feeding effect. Supporting this possibility is prior data suggesting mu opioid receptors are abundant in the LS (Mansour et al., 1994).

As previously stated, the LS has dense and reciprocal connections to the hypothalamus. The rostral LS projects primarily to the lateral preoptic area of the hypothalamus via GABAergic neurons, while the caudal LS projects to the adjacent medial septum (MS) (Risold & Swanson, 1997a). As for inputs to the LS, the anterior parts of the hypothalamus, including the preoptic nucleus, project mainly to the rostral/ventral part of the LS. Additionally, the lateral hypothalamus projects to areas throughout the LS but with very few projections directly to the ventral portion. These dense reciprocal connections, particularly those from the lateral hypothalamus, support a potential role for the LS in the regulation of feeding behaviors. A new finding is that the $GABA_A$ receptor agonist muscimol rapidly elicits feeding with injection into the LS. This finding suggests that LS $GABA_A$ receptors and perhaps GABAergic inputs may have roles in stimulating feeding behavior. Supporting this possibility are previous studies demonstrating the existence of $GABA_A$ receptors in the LS and suggesting the existence of GABA terminals in the LS (Mansour et al., 1994; Risold & Swanson, 1997b). More specifically, as $GABA_A$ receptor activation hyperpolarizes membrane

potential, these finding suggest that the muscimol-elicited feeding may be consequent to inhibition of LS neural activity (Bazemore et al., 1957). Given that LS injection of either $GABA_A$ or mu opioid receptor agonists elicits feeding, it seems likely that mu opioid receptor activation also elicits eating by neural inhibition. All three opioid receptors are similar: they are G-protein coupled receptors that interact with potassium channels to hyperpolarize the cell (Feng et al., 2012; Stein, 2016).

It may be noted that while muscimol-elicited feeding was complete within 30-60 minutes of LS injection, the full cumulative response to morphine and DAMGO appeared to be relatively delayed. Given that mu opioid agonist and $GABA_A$ receptor agonist are generally similar in rapidly hyperpolarizing membrane potential and reducing action potential production, the delayed feeding produced by mu opioid receptor agonists may be due to effects other than neural inhibition. Perhaps relevant is that opioid receptors may also inhibit pre and postsynaptic calcium channels (Feng et al., 2012), which could inhibit the release of LS neurotransmitters, including GABA.

These data suggest a role for the lateral septal opioid receptors in the regulation of feeding. Previous research supports this possibility, demonstrating that LS has a high density of opioid receptors (Risold & Swanson, 1997b). Similarly, studies using in situ hybridization and immunohistochemistry found a high density of enkephalinergic neurons in the rostral LS, with lower concentrations towards the caudal regions; dynorphins were also present but in lower concentrations (Risold and Swanson, 1987b). Previous research further suggests that opioid receptors in the LS have a role in

rewarding behavior. Indeed, mice will self-administer morphine into the LS, and morphine in the LS increases Fos expression in the NaC, suggesting a role in disinhibition of reward and motivational circuitry in the brain (Le Merrer, 2007). The LS is interconnected with the VTA and also projects to the NaC, integral parts of the reward pathway, and rats show increased cFos immunoreactivity in LS neurons after acute systemic injections with cocaine and after exposure to cocaine-related cues (Brown et al., 1992; Risold & Swanson, 1997a). Inhibition of the rostral LS attenuates cocaine preference overall and stimulation of LS neurons can increase neural firing rates in the VTA (Maeda & Mogenson, 1981; Sartor & Aston-Jones, 2012).

Previous research has shown that opioid hot spots, where injections of mu opioid drugs produce increases in feeding and hedonic responses, exist in the NaC and the ventral pallidum (Castro & Berridge, 2014; Peciña & Berridge, 2000). As the NaC is comparatively close to the LS it is conceivable that the opiate-elicited feeding observed might have been consequent to diffusion to NaC hot spots. However, this seems unlikely as the NaC also contains opioid feeding suppressive cold spots and these are much closer to the LS than the hot spots, which are in the rostral portions of the NaC (Castro & Berridge, 2014). Similarly, a major contribution of ventral pallidum hot spots to the feeding produced by LS opiate injection seems unlikely given that those hot spots are well over 2 mm from the LS injections sites. Moreover, the doses of opioid drugs and volumes used in the present study are comparable to those used in previous research, which found that the drugs diffused less than 1 mm from injection sites (Castro & Berridge, 2014; Peciña & Berridge, 2000). Further, that the eating stimulatory effects are

due to local actions is supported by unpublished cannula-mapping data from our lab showing that opioid drugs are less effective in the rostral part of the lateral septum than the medial caudal regions.

A role for LS opioids in feeding behavior is further supported by evidence that the LS is densely populated by mu opioid receptors and there are enkephalin containing projections from the hypothalamus to the LS (Risold & Swanson, 1997a; Sakanaka et al., 1982). Stimulation of the lateral hypothalamus promotes self-stimulation in both the LS and MS (Miller & Mogenson, 1971; Sheehan et al., 2004). Taken together, the evidence suggests that the hypothalamus may be a source of feeding stimulatory opioid afferents to the LS and that opioids have a meaningful role in the regulation of feeding.

Interestingly, septal lesions have been found to both increase and decrease feeding behavior in rats. Flynn et al. (1986) found that rats with septal lesions showed interruptions during normal feeding due to increased locomotor activity, resulting in less meaningful and more frequent bouts of eating. On the other hand, Oliveira et al. (1990) found that LS lesions increased feeding post electrical stimulation in the LH, suggesting a feeding suppressive role for the LS in feeding behavior. More recently, chemogenetic or optogenetic stimulation of septal GABAergic neurons or their projections to the LH were shown to decrease feeding, while chemogenetic inhibition of GABA neurons in the LH increased eating, suggesting that GABA neurons projecting from the LS act on GABAergic neurons in the LH to decrease feeding (Sweeny & Yang, 2016). Sweeny and Yang (2017) also discovered a hippocampal-LS circuit that controls feeding. They found

that optogenetic activation of the ventral hippocampus projections to the LS reduces feeding, while optogenetic inactivation increases it, and that inactivation of the LS neurons disrupts these effects, suggesting that most GABA-containing neurons in the LS play a role in feeding behavior. Further, studies have shown that the LS contains gastric distention responsive and ghrelin releasing neurons that project to the arcuate nucleus of the hypothalamus and increase gastric motility (Gong et al., 2013; 2014). Other studies show that activation of glucagon like peptide 1 (GLP-1) receptors in the LS can decrease overnight food intake and sucrose intake while blockade of these receptors can increase fat intake and motivation to obtain food (Terrill et al., 2016). This suggests complex roles for the LS in feeding behaviors involving multiple mechanisms.

This study found that mu opioid agonists reliably elicit feeding while kappa and delta agonists do not. The feeding effect in response to a mu opioid antagonist is initially increased at low doses and suppressed at higher doses. Furthermore, I found a feeding response to $GABA_A$ agonist that is comparable to the response elicited by morphine and DAMGO. The evidence presented here suggests that there is a septal-hypothalamic circuit in control of feeding behavior that is regulated by opioid receptors in the LS and that they may do this via inhibition of the LS and disinhibition of regions within the hypothalamus. Furthermore, it will be important to determine if there are other opioidergic pathways that terminate in the LS to shed light on how opioids modulate feeding behaviors.

## Methods

### Subjects and Surgery.

Adult male Sprague Dawley rats weighing 350-450 grams were used for all experiments. Rats were bred and tested in an on-campus vivarium on a 12-hour light-dark cycle and single housed starting a week prior to surgery. Animals were anesthetized for surgery using intraperitoneal (IP) sodium pentobarbital at 50 mg/kg of body weight, preceded by an IP injection of 0.25 ml of atropine sulfate (0.54 mg/kg). A single 18 mm long, 26-gauge stainless steel guide cannula was stereotaxically implanted 1 mm dorsal to the target site in the LS, 8.7 mm anterior to the interaural line, 0.7 mm lateral to the midsagittal sinus and 4.7 mm ventral to the surface of the skull. Cannulas were held in place by dental acrylic and 6 stainless steel screws penetrating the skull. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California Riverside.

### Procedure.

Animals were maintained on Purina rat chow and water *ad libitum* until three days prior to testing when they were switched to our standard palatable mash diet *ad libitum* consisting of Purina rat chow (500g), sugar (400g), and Carnation evaporated milk (354 ml). Animals were handled and given mock injections daily for three days prior to experiments.

Animals were tested under satiated conditions; they were given fresh mash one hour prior to testing no more than 1-3 hours after the beginning of the 12-hour light cycle. Tests consisted of a single or two sequential injections through a 32-gauge injector projecting 1 mm past the cannula directly onto the LS. Injections consisted of 0.3 µl of vehicle or the vehicle with a dissolved drug. Vehicle was artificial cerebral spinal fluid (aCSF) consisting of 147 mM $Na^{+}$, 154 mM $Cl^{-}$, 3.0 mM $K^{+}$, 1.2 mM $Ca^{2+}$, and 0.09mM $Mg^{2+}$, dissolved into sterile distilled water. The aCSF solution was mixed in the lab from reagent grade chemicals from Sigma-Aldrich. Food was weighed 0, 60, 120 and 180 minutes, and 24 hours post-injection. After behavioral testing, animals were deeply anesthetized with IP injection of sodium pentobarbital and transcardially perfused with 10% formaldehyde. Brains were sectioned into 100 µm thick coronal brain slices and Nissl stained with cresyl violet to ensure that the cannulas were on target. The cannula guide was on target if the tip of the cannula track is within 0.2 mm of the intended brain region. A representative histological photomicrograph and the injection sites are illustrated in Fig. 2.

**Analysis.**

Two-way repeated-measures ANOVAs were used to analyze each drug's feeding effects across time. Significant ANOVAs were followed by pairwise comparisons of each drug dose to its vehicle control using Bonferroni multiple comparison test.

**LS Mu, Delta, and Kappa opioid agonist effects on feeding**

**Animals**. A total of 57 rats were used in this experiment. For the first phase, 39 rats were randomly assigned to one of three groups, 13 animals/group. For the second phase, there were 9 animals in the DAMGO conditions and 9 in the morphine conditions.

**Drugs**. For the first phase, the three treatment groups were: DAMGO (0, 0.05, 0.075, 0.15 µg/0.3 µl), U50488H (0, 0.15, 0.3, 0.6 µg/0.3 µl) or DPDPE (0, 0.25, 0.50, 0.75 µg/0.3 µl) with doses tested in ascending order. The second phase used DAMGO (0, 0.075, 0.15, 0.60 & 2.0 µg/0.3 µl) and morphine (0, 0.50, 1.5, 2.5, 5.0 µg/0.3 µl). Drugs were purchased from Sigma-Aldrich. (The doses were derived from those used by Calcagnetti et al., 1988; Castro & Berridge, 2014; Kapusta et al., 1993; Randall-Thompson et al., 2010.)

**LS Mu opioid antagonist on the feeding effect of morphine**

**Animals and Drugs**. 12 rats received morphine 5 µg/0.3 µl or vehicle followed ten minutes later by an injection of CTAP (0.05, 0.1, 0.5, 1.5 µg/0.3 µl) or vehicle (doses derived from Castro & Berridge, 2014; Wilson & Junor, 2008). As morphine's feeding stimulatory effect is typically delayed beyond 60 minutes post-injection, CTAP was injected after morphine to enhance the duration of its action. Post agonist injection is justified by the therapeutic effectiveness of opiate antagonists in rescuing victims of opiate overdose (e.g., Skolnick, 2018).

## LS GABA Agonist Stimulation of feeding

**Animals and Drugs.** 12 rats received injections of aCSF or muscimol (0, 0.1, 0.2, 0.3 µg/0.3 µl) (doses from Urstadt et al., 2013a, b) in ascending order across four treatment days.


## Acknowledgments

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## Chapter 4: Behavioral effects of mu and GABA receptor activation in the lateral septum


### Abstract

Given that opioids and the lateral septum (LS) are associated with anxious and defensive behaviors, social behaviors, locomotor activity, and reward as well as learning and memory it is important to evaluate behaviors that may be co-occurring with feeding in response to opioid or $GABA_A$ agonist injection into the LS. So, I assessed behavior for three hours after injection of aCSF, DAMGO, morphine, and muscimol. The assessments included latency to eat, food intake, and the amount of time spent feeding, drinking, grooming, active, resting, and sleeping. I found that both morphine and muscimol decreased the latency to eat, and all drugs tested increased food intake. The feeding occurred within 30 minutes of muscimol injection but was delayed after opioid injections. The minutes spent feeding, drinking, grooming, and general active behavior were all unaffected by drug conditions. There was an increase in time spent sleeping subsequent to the eating elicited by muscimol, and an increase in the time spent resting prior to the eating elicited by DAMGO. The absence of increases in goal-oriented behavior like drinking or grooming or behavioral hyperactivity is supportive of a primary effect of muscimol and the opioids on brain mechanisms of feeding control and argues against the feeding being secondary to behaviorally non-specific effects.

## Introduction

The data in Chapter 3 showed in satiated rats that LS injections of muscimol elicit feeding rapidly, while morphine or DAMGO elicited a delayed feeding response. The purpose of the experiments in the current chapter was to assess a broad range of the behaviors exhibited by rats after septal injections of these substances in order to determine the extent to which their effects are, or are not, specific to feeding behavior. The underlying issue is whether the feeding was elicited by direct action on feeding control neurocircuits, or instead, whether the feeding might have been secondary to impacts on other behaviors, with feeding being a secondary consequence. This is a pertinent issue as it has been demonstrated that eating may be induced by manipulation as nonspecific as gentle and prolonged tail pinch (Levine & Morley, 1982). This concern is exacerbated by the demonstrations that many drugs of abuse, including opioids like morphine and DAMGO, induce locomotor activation when given systemically (Babbini & Davis, 1972). More specifically, morphine intraperitoneally injected in mice can increase their locomotor activity for up to three hours post-injection, which matches the time course of my feeding data. It is therefore important for me to assess multiple behaviors post-injection for three hours. If the increase in eating is solely due to an increase in a locomotor activity then I expect to see increases in active behaviors such as exploring, sniffing, and grooming, as well as eating and drinking. I also expect to see a decrease in non-active behaviors like sleeping and resting. However, if the increase in feeding is not accompanied changes in other behaviors then that would suggest that mu

and $GABA_A$ receptor activation in the lateral septum is specific to feeding behavior and thus is likely due to direct actions on feeding control neurocircuits.

**Methods**

**Subjects & Surgery**

16 Adult male Sprague Dawley rats weighing 350-450 grams were used for all experiments. Rats were bred and tested in an on-campus vivarium on a 12-hour light-dark cycle and single housed starting a week prior to surgery. Animals were anesthetized for surgery using intraperitoneal (IP) sodium pentobarbital at 50 mg/kg of body weight, preceded by an IP injection of 0.25 ml of atropine sulfate (0.54 mg/ml). A single 18 mm long, 26-gauge stainless steel guide cannula was stereotaxically implanted 1 mm dorsal to the target site in the LS, 8.7 mm anterior to the interaural line, 0.7 mm lateral to the midsagittal sinus and 4.7 mm ventral to the surface of the skull. Cannulas were held in place by dental acrylic and 6 stainless steel screws penetrating the skull. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California Riverside.

**Drugs.** All animals received either aCSF, DAMGO, morphine, or muscimol on test days. Vehicle was artificial cerebrospinal fluid (aCSF) consisting of 147 mM $Na^+$, 154 mM $Cl^-$, 3.0 mM $K^+$, 1.2 mM $Ca2^+$, and 0.09 mM $Mg2^+$, dissolved into sterile distilled water. Drugs were purchased from Sigma-Aldrich.

**Procedure.**

Animals were maintained on Purina rat chow and water *ad libitum* until three days prior to testing when they were switched to our standard palatable mash diet *ad libitum* consisting of Purina rat chow (500 g), sugar (400 g), and Carnation evaporated milk (354 ml). Animals were handled and given mock injections daily for three days prior to experiments. Animals were tested under satiated conditions; they were given fresh mash one hour prior to testing no more than 1-3 hours after the beginning of the 12-hour light cycle. Tests consisted of a single injection through a 32-gauge injector projecting 1 mm past the cannula directly onto the LS. Injections consisted of 0.3 µl of the vehicle or the vehicle with a dissolved drug. Food was weighed 0, 60, 120, and 180 minutes, and 24 hours post-injection. Additionally, the animals were observed every minute for 180 minutes post-injection and their behavior was coded each minute as eating, drinking, grooming, resting, sleeping, or active.

Histology: After behavioral testing, animals were deeply anesthetized with IP injection of sodium pentobarbital and transcardially perfused with 10% formaldehyde. Brains were sectioned into 100 µm thick coronal brain slices and Nissl stained with cresyl violet to ensure that the cannulas were on target. The cannula guide was on target if the tip of the cannula track is within 0.2 mm of the intended brain region.

**Statistical Analysis.** Latency to eat was analyzed by a one-way repeated measures ANOVA. Food intake was analyzed using a two-way repeated-measures ANOVA. Time spent eating, drinking, grooming, being active, resting, and sleeping was

each analyzed by one-way repeated measures ANOVA comparing each time point using Bonferroni comparisons

## Results

### Latency to Eat

As shown in Fig. 1A, morphine ($p=0.009$), and muscimol ($p=0.005$) significantly decreased the latency to eat compared to aCSF. DAMGO also decreased the latency to eat but the effect did not reach statistical significance ($p=0.06$).

### Food Intake

As shown in Fig. 1B, muscimol elicited feeding rapidly, with statistically significant increases 30 minutes post-injection and at each subsequent interval. In contrast, morphine and DAMGO did not elicit statistically significant feeding until 120- and 180-minutes post-injection.

### Observational Measures of Behavior

As shown in Fig. 9A, muscimol significantly increased the amount of time that eating was observed 30 minutes post-injection, but no further increases were observed thereafter. As shown in Fig. 10A, muscimol also produced a statistically significant suppression of observed time resting 30 minutes post-injection, and at all but one other post-injection interval. Notably, as shown in Figs. 10B and 11D, muscimol also produced a dramatic increase in the observed amount of time spent sleeping at every

post-injection time from 90 to 180 minutes post-injection. These data suggest that the increase in the time spent sleeping was at the expense of the amount of time spent resting (Fig. 10A and 11D). As shown in Fig. 9B, 9C & 9D, there were no statistically significant effects by any drug at any time on observed time drinking, grooming, or the time observed in active behavior.

As for other effects, as shown in Fig. 10A, DAMGO significantly increased resting at 60- and 90-minutes post-injection and, as shown in Fig. 10B, morphine significantly decreased sleeping at 60, 90, 120, and 150 minutes post-injection. Noteworthy, is that morphine and DAMGO, which produced increases in cumulative food intake as measured by food weights (Fig. 8B), did not produce statistically significant increases in feeding as measured by minute-by-minute observation of behavior (Fig 9A), suggesting that this is a less sensitive measure of feeding behavior.

**Figure 8**

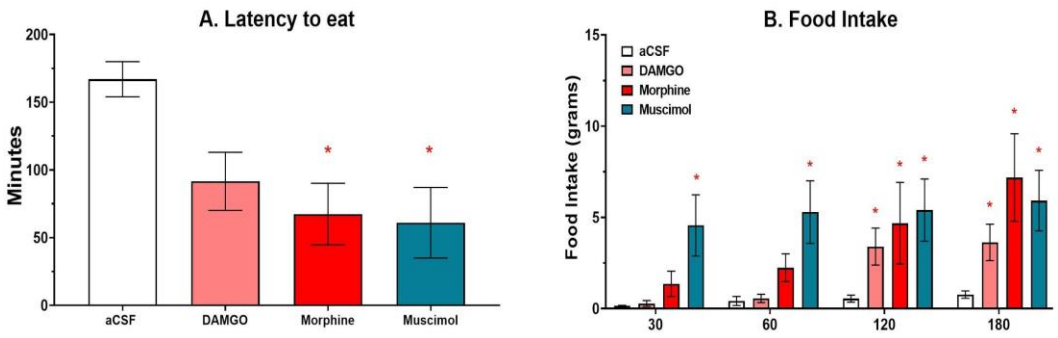


*Figure 8: A. Latency to eat (mean minutes ± SEM) following injection of aCSF, DAMGO, morphine or muscimol. B. Cumulative food intake (grams ± SEM) for all conditions at 30, 60, 120- and 180-minutes post-injection. * signifies a significant effect compared to aCSF at p<.05. Bonferroni multiple comparison test were used.*

**Figure 9**

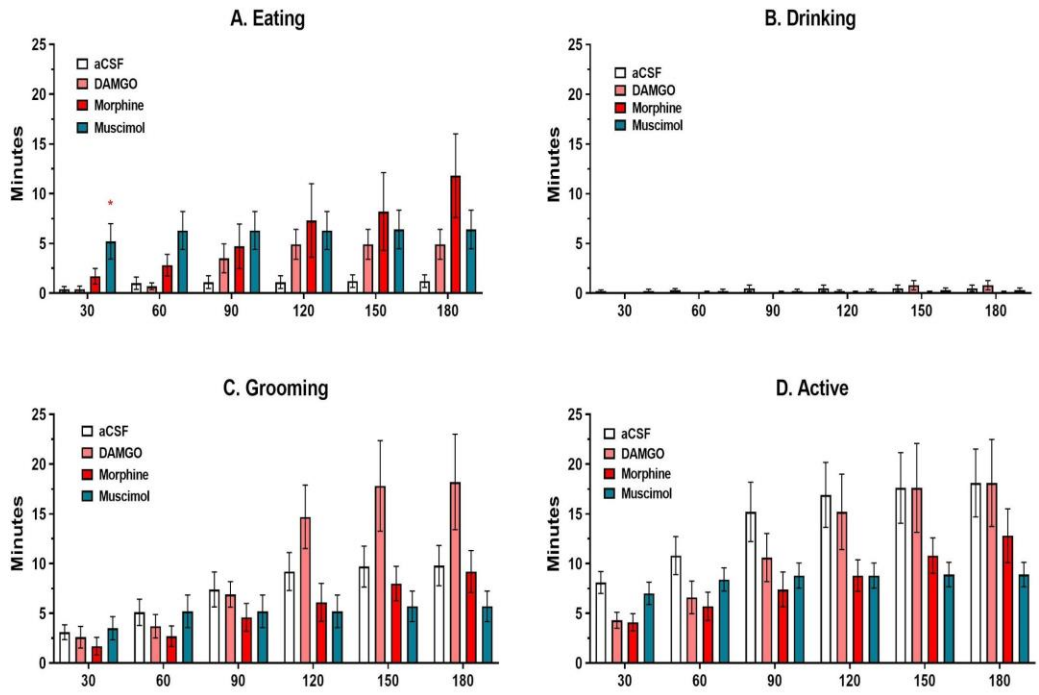


*Figure 9: Cumulative time (mean minutes ± SEM) spent A. Eating, B. Drinking, C. Grooming, and D. Active in response to aCSF, morphine, DAMGO & muscimol. * represents a significant difference from aCSF at p<.05. Bonferroni multiple comparison test were used.*

**Figure 10**

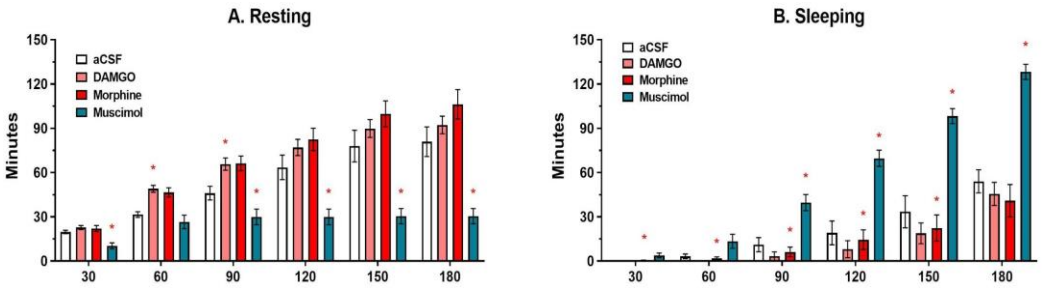


*Figure 10: Cumulative time (mean minutes ± SEM) spent A. Resting and B. Sleeping, following aCSF, morphine, DAMGO & muscimol. * represents a significant difference from aCSF at p<.05. Bonferroni multiple comparison test were used.*

**Figure 11**

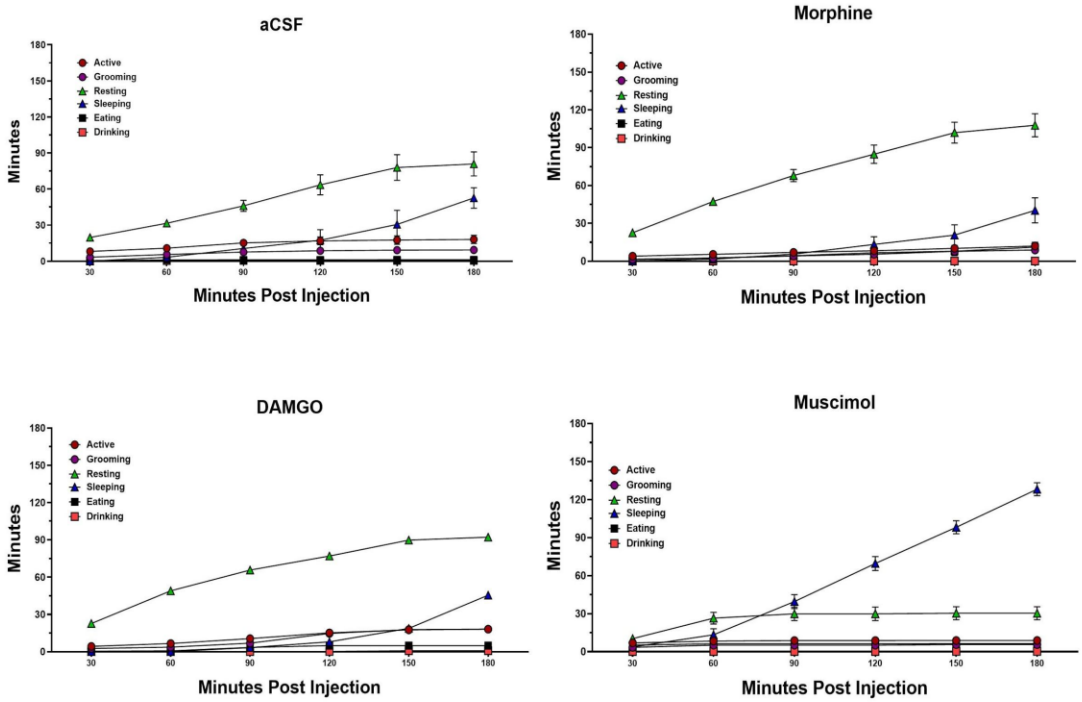


*Figure 11: Cumulative time (mean minutes ± SEM) in each observed behavior following LS injection of aCSF, morphine, DAMGO & muscimol.*

**Discussion**

The results of this experiment show that direct LS injection of DAMGO, morphine or muscimol decreased the latency to eat and increased food intake with no significant effects on any other active behavior, including drinking, active exploring, or grooming. The eating elicited by muscimol predominantly occurred in the initial 30-minute post-injection period, while the eating produced by morphine and DAMGO was delayed, with the first significant increases occurring two hours post-injection. While the eating behavior measured by direct minute-by-minute observation also increased, these increases were not statistically significant except for muscimol, which significantly increased the amount of time exhibiting eating behavior 30 minutes post-injection. Muscimol additionally produced a delayed and prolonged increase in the amount of time spent sleeping, with a commiserate decrease in the amount of time spent resting. As for the opioids, DAMGO additionally produced some increases in the time spent resting, and morphine produced some decreases in the time spent sleeping.

The fundamental question being addressed by these studies was whether and to what extent the eating behavior produced by LS injection of these GABA and opioid agonists was due to the direct actions of these agonists on eating control circuits or instead to the indirect activation of these circuits via a non-specific route of action, one occurring as a secondary consequence of actions on other behaviors. The answer seems clearest for the $GABA_A$ receptor agonist muscimol. This agonist elicited a strong eating response with a short latency and no other behavioral effect during the 30-minute period during which almost all the muscimol-elicited eating occurred. Further, muscimol

produced no effect at any time on active behaviors, including drinking, grooming, or on levels of behavioral activity. The only other major effect of muscimol was a sustained increase in the amount of time spent sleeping. Notably, studies of the sequence of behaviors related to spontaneously occurring eating have shown that increases in sleeping normally follow eating in rats. Indeed, this is part of what has been termed the post-prandial satiety sequence (Gibbs & Smith, 1982). This suggests that the increase in sleeping following LS injection of muscimol may have been a natural consequence of the prior eating elicited by this drug. Collectively, these behavioral data are supportive of the possibility that some $GABA_A$ receptors in the septum are components of neurocircuits controlling eating behavior.

As stated above, systemic administration of many drugs of abuse including opioids can increase locomotor activity. More specifically, morphine can elicit increased locomotor activity for up to three hours post injection (Babbini & Davis, 1972). Additionally, the LS is connected to areas of the hypothalamus that are thought to control locomotor behavior related to eating. Given these facts, it was important to investigate all behavioral effects of direct injections of morphine and DAMGO in the LS to address the possibility that increased feeding was due to increased locomotor activity. The data argue against this possibility. Specifically, as shown in Fig. 9D there was no apparent or statistically significant increase in behavioral activity produced by either morphine or DAMGO, and indeed morphine tended to produce a decrease in the time spent expressing behavioral activity. Consistent with this, neither of the opioids produced a decrease in the amount of time spent resting. Indeed, DAMGO produced an increase in the time spend

resting (Fig. 10A). Taken together, these data indicate that the increased feeding effect of each drug condition is not due to increased locomotor activity, consistent with a specific role in feeding. Furthermore, lateral septal mu or GABA receptor stimulation could disinhibit locomotor behaviors specifically linked to feeding. Parts of the LS project to areas of the motor areas hypothalamus and to motor areas of the brain stem (Hunt et al., 2010; Nieh, et al., 2016; Risold & Swanson, 1997a).

All known opioid receptors are inhibitory G-protein-coupled receptors, while GABA receptors activated by muscimol are inhibitory ion channels. In general, the actions of ion channels are quick but brief, in which G-protein-coupled receptors acts have a slower onset and prolonged action. If inhibition of neural activity the LS causes the increase in feeding, then these receptor differences may explain why muscimol acts more quickly than mu receptor agonists DAMGO or morphine. This may also be indicative of the long-term role that opioid receptors have in the lateral septum versus GABA receptors. GABA receptors are largely ionotropic and inhibit immediately while opioid receptors may induce long term changes based on experience. While these receptor differences may contribute to the differences in the time-course of muscimol and opioid-elicited feeding in the LS, electrophysiological studies show that opioid receptor activation can produce effects in seconds to minutes, which is considerably quicker than the delayed feeding produced by opioid injection into the LS. Therefore, additional mechanisms are needed to fully explain the delay in feeding elicited by LS opioid injections.

What are the mechanisms by which the LS elicits eating behavior? The LS projects to the PVN of the hypothalamus and this nucleus is involved in sympathetic and parasympathetic autonomic processes associated with defensive, reproductive, and feeding behaviors (Daviu, Füzesi,& Rosenegger et al., 2020; Risold & Swanson, 1997a) and this projection may contribute to the feeding produced by our LS manipulations. Perhaps also relevant is that Sweeny & Yang (2016) identified an inhibitory circuit from the LS to the LH, in which chemogenetic or optogenetic activation of the LS decreased eating, while inhibition of the LS increased eating. Another way the LS might control eating is through gastric motility and feelings of satiation or hunger. The LS has been associated with the enteric nervous system and is involved in the regulation of gastric motility. Gong et al. (2013) found that electrical stimulation of the lateral septum excited gastric distention sensitive neurons in the LS and increased gastric contraction. The same group also found that the LS has gastric distention sensitive neurons and ghrelin receptors (Gong et al., 2014). Ghrelin administration to the LS increased the firing rates of gastric distention inhibitory neurons and excitatory neurons. It would be interesting to see the effects of LS ghrelin on food intake. Conversely, previous research has established that the LS innervates the lateral hypothalamus, and inhibition of the rostral LS can inhibit fos activation of orexin neurons in the LH during conditioned placed preference for cocaine (Sartor, 2012). The LS also has a complex modulatory role on anxiety and defensive related behaviors. Lesions or excitation of the LS have been linked to both increases and decreases in anxiety like and defensive behaviors. Morphine specifically has been shown to cause anxiogenesis in the elevated plus maze and the hole board task (Le Merrer et al.,

2006; Menard & Treit, 2000). It brings up the question as to whether this feeding effect is due to anxiety. The conflicting findings in much of the LS research might suggest that its effects are context dependent or site-specific within the LS. Overall, these data suggest that the lateral septum can stimulate and inhibit behaviors related to feeding, pointing to a complex relationship between the LS and the LH in which the LS can inhibit or excite feeding related behaviors. Whatever the mechanisms might be, our data provide support for a role for LS GABA and opioids in feeding control mechanisms.

## Chapter 5: Site specificity of mu and $GABA_A$ receptor agonist-induced feeding

### Abstract

After establishing receptor and behavioral specificity of the lateral septal induced eating, these experiments were designed to compare the effects on feeding behavior of morphine and muscimol injections into specific areas outside of and within the septum, as a step towards identifying the locus of opiate and $GABA_A$ receptors involved in the stimulation of feeding behavior. For Experiment 1, I compared injections of morphine in the lateral septum (LS) to multiple regions of the brain surrounding the LS. Target areas were all $\geq$ 1 mm away from the LS site and included the ventricular injections, the PFC, parastrial nucleus (PSN), hippocampus and caudate nucleus. For Experiment 2, I compared the effects on feeding of morphine and muscimol injected into six subregions within the LS. I hypothesized that mu opioid and $GABA_A$ agonists in a single area within the lateral septum would produce a significantly greater feeding response than in any other site. I found that although there were increases in feeding in the LS and PSN there

was even more site-specificity within the LS, with the ventral and rostral LS showing the greatest increases in eating in response to injections of muscimol, while the lateral septum, medial septum, and caudal septum showed increases in feeding in response to morphine.

**Introduction**

I assessed the site-specificity of the increased feeding effect of morphine using two experiments. In Experiment 1, I sought to assess the anatomical specificity of the feeding effect of morphine using a cannula-mapping study. Eight brain areas were tested, bracketing the LS 1 to 2 mm ventral, anterior, or lateral to the target brain site. There are multiple hedonic hotspots in the brain which respond to opioids to induce increases in feeding, including but not limited to the rostral nucleus accumbens shell, the ventral pallidum, and the prefrontal cortex. (Bodnar, 2013; Castro & Berridge 2014). Additionally, given that systemic opioids typically increase feeding in rats, it is important to assess the effect of intraventricular injections of morphine (Pecina & Berridge, 1995; Sanger & McCarthy, 1980). Stanley et al., 1988 found that opioid agonists caused an increase in feeding in multiple hypothalamic and extrahypothalamic areas, including the amygdala and the septum. This increase in feeding was particularly robust in the septum. For this first experiment I expected to replicate the robust effects found by Stanley et. al., 1988 and show that the LS would show a robust feeding effect in response to morphine. The lateral septum was of particular interest because although it has been classically associated with anxiety and defensive behaviors, and it had been considered

an early reward center, it was, at the time largely unexplored with respect to feeding and motivation (Olds & Milner, 1954; Sheehan et al., 2004). I expected to find that the lateral septum would show a significant increase in feeding behavior after direct morphine injections. I further expected that the surrounding brain areas would show little to no significant increases in feeding after direct injections of morphine.

For Experiment 2, I compared sites within the septum including the medial septum, LS, dorsal, caudal, ventral, and rostral LS. This study was designed to compare the effects on feeding behavior of morphine injections into specific areas in the septum, as a step towards identifying the locus of opiate receptors involved in the stimulation of feeding behavior. I also tested muscimol given my recent evidence that this $GABA_A$ can elicit feeding with LS injection (Chapter 3; Calderwood et al., 2020). As previously stated, the septum can be divided up into two main regions, the medial and lateral regions, which can be further divided into four sub-regions, the dorsal, ventral, rostral, and caudal. Here I compared all four subregions of the LS, as well as the original site tested in the preliminary studies, and the medial septum. These regions differ in anatomical connections and neurochemical makeup (Mansour et al., 1994; Risold and Swanson 1998a,b). Due to these differences in anatomical and neurochemical connections, I expect that these areas may also have separate functions. This study is important and novel because, although the different regions of the septum have been recognized, the differences in function between the regions have been largely unexplored. I hypothesized that morphine in a single area in the LS will produce a significantly

greater feeding response than any other site and I predicted that the medial septum locations will produce no significant effect on feeding.

## Methods

### Subjects and Surgery.

*Experiment 1* – Comparing Feeding Elicited by LS versus Surrounding Brain Sites: This first cannula-mapping study used 54 adult-male Sprague Dawley rats weighing between 350-450 grams at the time of surgery. Rats were bred and tested in an on-campus vivarium on a 12-hour light-dark cycle and singly housed the week prior to surgery. Animals were anesthetized for surgery using intraperitoneal (IP) sodium pentobarbital at 50 mg/kg of body weight. 18 mm long, 26-gauge stainless steel guide cannulas were stereotaxically implanted 1 mm dorsal to the target site at coordinates listed in Table 1. Animals were given one week to recover from surgery before testing.

*Experiment 2* – Comparing eating elicited by injections within the Septum: Thirty-four adult male Sprague Dawley rats weighing between 350-450 grams were used. Rats were bred and tested in an on-campus vivarium on a 12-hour light-dark cycle and singly housed the week prior to surgery. Animals were anesthetized for surgery using intraperitoneal (IP) sodium pentobarbital at 50 mg/kg of body weight. 18 mm long, 26-gauge stainless steel guide cannulas were stereotaxically implanted 1 mm dorsal to the target site. See Table 2 for coordinates within the septum. Animals were given one week to recover from surgery before testing.

**Drugs.** All animals were injected through the cannula with either aCSF, DAMGO 2.0 µg/0.3 µl, morphine 5.0 µg/0.3 µl, or muscimol on test days. Vehicle was artificial cerebrospinal fluid (aCSF) consisting of 147 mM $Na^+$, 154 mM $Cl^-$, 3.0 mM $K^+$, 1.2 mM $Ca2^+$, and 0.09 mM $Mg2^+$, dissolved into sterile distilled water. Drugs were purchased from Sigma-Aldrich.

**Procedure**

Animals were maintained on Purina rat chow and water *ad libitum* until three days prior to testing when they were switched to a palatable mash diet consisting of Purina rat chow (500g), evaporated milk (354ml) and sugar (400g). Animals were tested under satiated conditions; they are given fresh mash one hour prior to testing. Unless otherwise indicated, tests consist of a single injection through a 32-gauge injector that projects 1 mm past the cannula directly onto the target brain region. Animals were handled and given mock injections daily for three days prior to experiments.

For all experiments, food was weighed 0, 60, 120- and 180-minutes post-injection, as well as 24 hours post-injection. After behavioral testing, animals were deeply anesthetized and transcardially perfused with 10% formaldehyde. Their brains were sectioned into 100 µm coronal brain slices and Nissle stained with cresyl violet to ensure that the cannula guides are on target. The cannula guide is on target if the tip of the cannula track is within 0.2 mm of the intended brain region.

**Table 1. Brain targets surrounding the lateral septum**

| Brain Region | Anterior | Lateral | Ventral | N |
|---|---|---|---|---|
| **LS** | 8.7 | 0.7 | 4.7 | 13 |
| **Ventricles** | 8.7 | 1.5 | 4.7 | 6 |
| **PFC** | 10.7 | 0.7 | 4.7 | 8 |
| **PSN** | 8.7 | 0.7 | 7.2 | 9 |
| **Hippocampus** | 6.7 | 0.7 | 4.7 | 7 |
| **Caudate** | 8.7 | 2-2.7 | 4.7 | 10 |

Table 1. Stereotaxic coordinates bracketing the lateral septum. These measurements are mm Anterior to the interaural line, mm Lateral to the midline, and mm Ventral to the surface of the skull.

**Table 2. Brain targets within the lateral septum**

| Brain Region | Anterior | Lateral | Ventral | N |
|---|---|---|---|---|
| **LS** | 8.7 | 0.7 | 4.7 | 7 |
| **Ventral LS** | 8.7 | 0.7 | 5.7 | 6 |
| **Dorsal LS** | 8.7 | 0.7 | 3.7 | 3 |
| **Caudal LS** | 7.5 | 0.7 | 4.7 | 5 |
| **Rostral LS** | 9.7 | 0.7 | 4.7 | 6 |
| **MS** | 8.7 | 0.2 | 4.7 | 7 |

Table 2: Stereotaxic coordinates of septal brain regions (mm Anterior to the interaural line, mm Lateral to the midline and, mm Ventral to the surface of the skull).

**Statistical Analysis.**

To test feeding response to morphine in areas bracketing the LS, I ran repeated measures ANOVAs comparing the effects of morphine and aCSF on food intake in each brain area at each. post-injection time.

To test feeding response to mu and GABA receptor stimulation in areas within the septum, I ran repeated measures ANOVAs at each post-injection time on the effects of morphine, DAMGO, or muscimol on food intake compared to aCSF control for each brain area tested within the septum.

## Results

### Experiment 1 - Feeding response to mu stimulation surrounding the LS

As shown in Fig. 12, there were significant increases in food intake in response to morphine injected into the parastrial nucleus 120- and 180-minutes post-injection, as well as in the lateral septum 180 minutes post-injection. No other increases were statistically significant.

**Figure 12: Food intake in response to morphine**

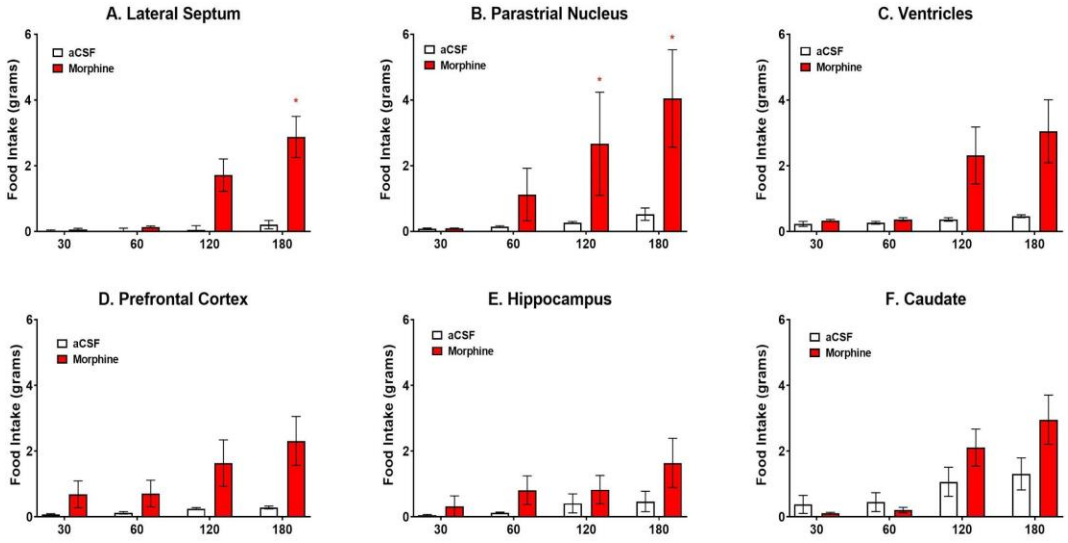


*Figure 12. Cumulative food intake* (grams ± SEM) *in response to morphine (5µg/0.3µl) injected directly into the lateral septum, parastrial nucleus, prefrontal cortex, hippocampus, lateral ventricles and caudate.* represents p<.05.*

**Experiment 2: Feeding in response to mu and GABA stimulation within the septum**

As shown in Figs. 13B and 13E, muscimol injections significantly increased eating when injected directly into the VLS, and RLS at every time from 30 minutes to 180 minutes post-injection.

In contrast, as shown in Figs. 13B and 13E, morphine has no statistically significant effects in these sites, nor in the DLS. Instead, as shown in Figs. 13A, 13D, and 13F, there were significant effects of morphine on eating when injected into the LS, and CLS at 180 minutes post-injections, and the MS and 120 and 180 minutes post-injection. DAMGO had no statistically significant stimulatory effects on eating, which was unexpected given our previous data showing such effects (Chapters 3 & 4). In

summary, while morphine and muscimol both elicited eating with injection into septal sites, the patterns were distinct in location and latency. Muscimol's effects were rapid and produced in the VLS and RLS, while morphine's effects were delayed and produced in the LS, CLS, and MS.

**Figure 13: Food intake across time in response to mu agonist and GABA agonist**

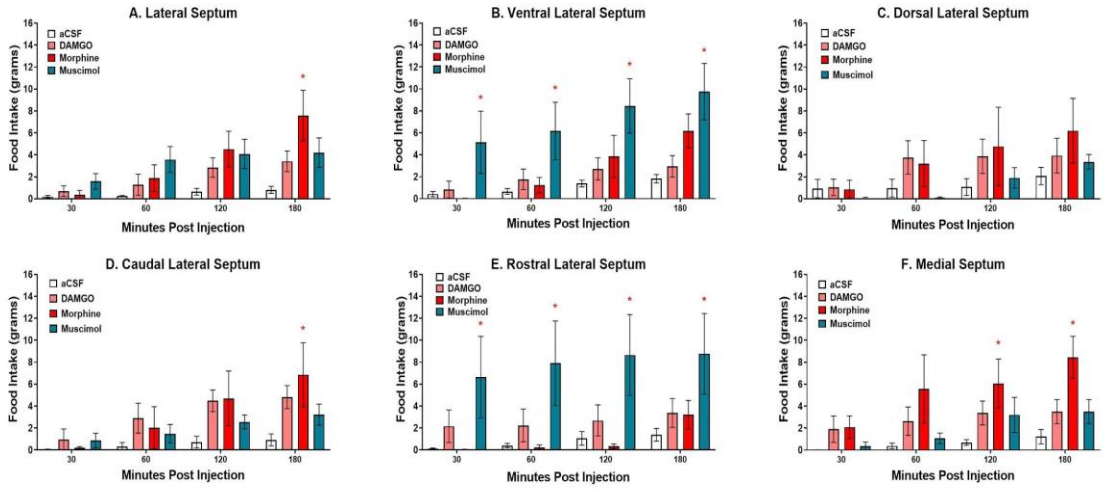


*Figure 13: Cumulative food intake (mean grams ± SEM) as a function of time post-injection in response to lateral septal injections of aCSF, DAMGO, morphine or muscimol. * represents p<.05.*

**Discussion**

As shown in Figure 12, morphine tended to elicit feeding with injection into multiple locations including, and most importantly with respect to these studies, the LS. While morphine tended to elicit feeding in all tested areas, the only statistically significant effect was in the LS and the parastrial nucleus, located approximately 1 mm ventral to the LS injection site. The LS and the nearby parastrial nucleus potentially

represent other areas in the brain that can increase feeding when exposed to opioidergic drugs. However, the robust effects of morphine are unlikely to be due to diffusion to areas outside of the septum, as the doses of opioid drugs and volumes used in the present study are comparable to those used in previous research, which found that the drugs diffused less than 1 mm from injection sites (Castro & Berridge, 2014; Peciña & Berridge, 2000).

The increase in eating seen in response to opioidergic drugs directly injected into septum might be thought to be an artifact of diffusion to the nearby NaC; however, that seems unlikely as the NaC also contains opioid feeding suppressive cold spots and these are much closer to the LS than the hot spots, which are in the rostral portions of the NaC posterior to the prefrontal cortex (Castro & Berridge, 2014). Similarly, a major contribution of ventral pallidum feeding hot spots to the feeding produced by LS opiate injection seems unlikely given that those hot spots are well over 2 mm from my LS injections sites.

Feeding in the LS can be blocked by the general opioid receptor antagonist naloxone (Chapter 2) and suppressed by high doses of the mu receptor specific antagonist CTAP, suggesting mediation by mu type opioid receptors (Calderwood et al., 2020 and Chapter 3). Furthermore, chemogenic and optogenetic stimulation of GABAergic projections from the LS to the LH decrease feeding (Sweeney & Yang, 2016). Morphine is self-administered by animals into the LS and has been shown to enhance the binding of muscimol and enhance its anxiogenic effect in a mouse model of anxiety (Le Merrer et al., 2006; 2007; Sasaki, 2001). Taken together, the data suggests that LS has some role in

feeding which could be due to opioid stimulation or general inhibition, or GABA stimulation. Although low doses of these drugs seem to cause different pattern of feeding and potentially sleeping, it is still unclear if they are interacting or how these systems interact within the lateral septum. Previous research indicates that while the septum is ubiquitously dense with GABA receptors, the distribution of mu opioid receptors varies with in the septum (Mansour et al., 1994). In order to test the effects of opioid stimulation within the septum, as well as the effects of GABA stimulation in the LS, it was necessary to map the feeding effect within the septum to better assess whether morphine has site specific effects.

Furthermore, as stated above, the septum is a subcortical region part of the basal ganglia. Projections from the septum are nearly exclusively inhibitory or disinhibitory and is associated with regulation emotions such as fear and motivated behaviors like eating reproductive behaviors (Risold & Swanson 1997a; Sheehan et al., 2004). The lateral septum receives mostly excitatory projections from cortical regions including the hippocampus and projects to the ventral pallidum. Regions of the lateral septum vary regarding connections from various cortical and subcortical regions. The lateral region can be divided up into caudal, rostral and ventral subsections. Its descending projections to the hypothalamus are topographically organized with the ventral part primarily projecting to the medial preoptic area and the periventricular zone of the hypothalamus (Risold & Swanson, 1997b). Based on the topographical organization, I expected there to be differences in the amount of eating based on injection site within the LS. Also, because opioids and $GABA_A$ agonist muscimol's primary action on neurons is to inhibit

them, I expect that the hypothalamic areas that correspond to the LS will be disinhibited. Specifically, I might expect that the caudal region would disinhibit the lateral hypothalamus and lead to feeding behavior, although our evidence suggests that the ventral lateral septum is more effective than the nearby central or original lateral septal injection site. Furthermore, the inhibitory electrophysiological effects of mu and GABA agonists are immediate but the increase in eating begins at least 30 minutes post injection and lasts up to three hours, so the feeding effect could be due to downstream signaling cascades, retroactive signaling or interactions between the opioids and another neurotransmitter systems. Determining the most effective site for inducing eating in the lateral septum can help us elucidate the specific mechanisms involved and shed light on the role of mu opioids of the LS in feeding.

An interesting and unexpected finding of Experiment 2 was that while morphine and muscimol both elicited feeding, those effects were due to actions within sites that did not overlap within the septum. While morphine elicited feeding with injections into the LS, the caudal LS and the MS, muscimol was ineffective in all three of those sites. In contrast to morphine, muscimol elicited feeding with injections into the ventral and rostral septum, sites where morphine was ineffective. These contrasts have several implications. One is that these agonists are not merely diffusing from their site of injection to a distant common site to elicit feeding; instead, their effects appear to be due to local actions within or very near their respective sites of injection. This in turn implies that morphine and muscimol are likely acting on anatomically distinct neurons within

different sub regions of the septum. If these agonists are acting on mu opioid and $GABA_A$ receptors respectively, then the subset of those different receptors would not appear to be localized on a common set of neurons, but rather on distinct neurons in different septal regions, or perhaps on distinct parts of large neurons with cell bodies and dendrites that span multiple regions within the septum. In short, the feeding behavior elicited by these two agonists is likely due to actions via distinct mechanisms. In support of this interpretation, the behavior effects of these two agonists is distinct as shown in Chapter 4. Specifically, while muscimol injection within the LS rapidly elicited feeding followed by increased sleeping, morphine injected into the LS decreased sleeping and produced a delayed stimulation of feeding behavior. An interesting question is whether these mechanisms interact within the septum? That seems likely given that both elicit the same behavior, feeding, and do so by actions within different sub regions of the same general brain structure, the septum. Exploring the nature of this putative interaction would seem to be an important avenue for future research.

My findings are especially interesting because I did find specific effects within the septum which could reflect the differences in receptor distribution throughout. These results support previous research that found mu receptors were heavily distributed throughout the medial septum but found primarily in the caudal portion of the lateral septum (Mansour et al., 1994).

As shown in Fig. 2, the increased feeding effect of morphine can be seen in the medial septum, medial lateral, and caudal lateral septum. Additionally, morphine injected into the LS increasing feeding, replicating our previous findings.

It is possible that stimulating opioid receptors in the medial and caudal lateral as well as the medial septum disinhibit areas of the hypothalamus related to eating. Alternatively, given that the mu receptor antagonists are G-protein coupled, the increase in eating might be changes in neurotransmitter release on hypothalamic areas responsible for feeding. In general, the lateral septum inhibits the ventral pallidum, the medial septal diagonal band of Broca, and the hypothalamus and it receives projections from the medial hypothalamus and paraventricular nucleus (Risold & Swanson, 1997a). Specifically, the caudal lateral septum that has previously been shown to be dense with mu and kappa receptors, projects to the lateral hypothalamus where activity or disinhibition from the lateral septum would increase feeding (Risold & Swanson, 1997b).

Muscimol was specific to the VLS and the RLS where it robustly increased feeding while both morphine and DAMGO were ineffective in these areas. What might be the neuroanatomical pathways mediating muscimol's feeding stimulatory effects? Interestingly, the ventral and rostral part of the lateral septum projects to the medial septum, the medial preoptic area and the paraventricular nucleus (Risold & Swanson, 1997a). The paraventricular nucleus has reciprocal projections to the ventral lateral septum and is directly involved with endocrine and autonomic processes. The projections from the PVN to the ventral lateral septum likely terminate on receptors for opioids, estrogen and somatostatin receptors. The lateral septum also has receptors for ghrelin, orexin and neurons that respond to gastric distention (Gong et. al., 2014; Risold & Swanson, 1997a). Animals in these experiments are all satiated and inhibition of gastric distention could contribute to an increase in food intake. Another possibility is

that muscimol or morphine in one or multiple sites directly inhibit an excitatory hippocampal to lateral septum neural circuit that suppresses feeding, previously identified by Sweeny & Yang (2016). It is clear that both muscimol and morphine can increase feeding when injected into specific areas of the lateral septum. It is still unclear which neural circuits are activated and how they function in the context of feeding.

## General Discussion

Lateral septal mu opioid receptor activation and GABA activation can stimulate feeding in satiated animals. This effect for morphine injected directly into the lateral septum is reliable across days and is not affected by tolerance or sensitization. The effects of morphine are blocked by opioid receptor antagonists and more specifically by highly selective mu opioid receptor antagonists and there seems to be a potentially interesting effect of dose such that CTAP may increase the effects of morphine at a very low dose but block it at higher doses. The behavioral effect of CTAP alone at the various doses used in this study needs to be assessed to better understand its effect on the morphine elicited feeding.

The increase in feeding seen in response to morphine and muscimol is not explained by the effects of morphine on locomotor activity, grooming, drinking, sleeping, or resting. While studies show that morphine can stimulate locomotor activity for up to three hours post injection when given systemically, animals in our study did not spend more time active in response to septal injections of morphine, DAMGO or muscimol. In fact, the animals increased resting and sleeping across three hours post injection of mu

opioid or GABA agonist. While time spent eating was increased by muscimol in the first 30 minutes post injection it was unaffected by the opioid drug conditions, food intake increased and latency to eat was decreased. The effect of muscimol appears to be more robust than morphine which may indicate that mu opioid receptors exert their effects by inhibition of neurons in the LS.

In addition to the possibility that muscimol is more effective and opioids are exerting the increase in feeding via general inhibition, it is possible that these interact to affect anxiety related and feeding behaviors. First, morphine increases the anxiolytic effects of muscimol when injected systemically (Sasaki, 2002), an effect not seen in mu opioid receptor knockout mice. Second, morphine when injected into the lateral septum has been shown to increase anxious-like behaviors in the elevated plus maze and the hole board task (Le Merrer et al., 2006; Menard & Treit, 2000). The lateral septum is classically linked to anxiety, defensive and motivational behaviors in the rat (Menard & Treit, 1996; Pesold & Treit, 1992; Singewald et al., 2011. It has been proposed that the lateral septum modulates eating related behavior in the context of fear (Gray & McNaughton, 1982). It would be interesting to investigate how these behaviors interact in response to mu receptor or GABA receptor stimulation in the LS. Novelty suppressed feeding is a model of anxiety in the rat, as these animals are reluctant to eat in novel spaces that elicit anxiety and it is a paradigm that is appropriate to test how manipulation of the lateral septum might affect eating in the context of fear (Blasco-Serra et al., 2017). The last interesting outcome of these studies is that the effectiveness of morphine, DAMGO, and muscimol varied depending on the injection site with morphine being most

effective in the medial septum and the medial and caudal lateral septum. Muscimol on the other hand was most effective in the ventral and rostral lateral septum.

The lateral septum exerts control over feeding and does so through potentially multiple mechanisms and pathways. The mechanism controlling feeding could be due to opioid control through the medial and caudal lateral septum or GABAergic via the rostral and ventral lateral septum. The lateral septum receives excitatory, mostly glutamate, control from the hippocampus, important for learning and memory especially in the context of fear or reward. It additionally receives monoamine and vasopressin control from the amygdala and the VTA, important for the assessment of fear and reward. The lateral septum receives cholinergic and serotonergic control from the brain stem area and hormonal control from the hypothalamus. The lateral septum has receptors for peptides typically associated with the hypothalamus including opioid control- POMC, estrogen, ghrelin, and orexin. This indicates the LS is influenced by emotion, memory, and cognition as well as the autonomic and endocrine systems.

The lateral septum then modulates other brain areas related to motivational behaviors including feeding and social behaviors as well as defensive behaviors and fear. The LS projects to the NaC and prefrontal cortex, both thought to be involved in motivation and reward. It projects to various areas of the hypothalamus and brainstem that are associated with control of feeding, social and locomotor behaviors.

Taken together this evidence shows that the lateral septum exerts some modulatory control overfeeding. The LS represents an area of the brain that can act as an interface between higher cognitive functions, motivation and memory with autonomic

and endocrine processes evident by its anatomical connections. The lateral septum has been linked to a variety of affective and motivated behaviors including aggression, anxiety, depression, reward, social behavior, feeding, and even learning and memory. The lateral septum's control of all these behaviors is supported by the anatomy of the lateral septum and neural correlates of behavior. A parsimonious hypothesis of how the LS mediates or controls many behaviors has yet to be found but the general aim of these studies is to come to a greater understanding of how lateral septum opioid receptors influence feeding behavior. The evidence suggests that the lateral septum is important for modulating rewarding behavior in different contexts like fear or hunger. Dysfunction can lead to a variety of behavioral problems in mood and motivation making it of interest in research for disordered eating like binge eating, anorexia, and bulimia that often co-occur with affective disorders such as anxiety and depression.

Our research leads to several interesting questions related to septal control of feeding that is mechanistic, anatomical, and behavioral. How might morphine affect the feeding effects of muscimol, can we expect synergistic effects of these? How does opioid stimulation in the MS and LS and GABA stimulation in the RLS and VLS affect response to palatable versus non-palatable food? Furthermore, does general inhibition or mu stimulation of the LS have the same effect? Is the increased feeding effect of muscimol due to disinhibition of the LH or increased inhibition of the MS? Does this increase in feeding reflect increased or decreased anxiety? How does increased anxiety affect the increase in feeding in response to septal inhibition or opioid stimulation? Whatever the answers to these questions my data placed septal opioids and GABA as

significant mechanisms involved in the lateral septal process of aggregating information from multiple brain regions to modulate motivational behaviors such as eating and socializing.

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