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Metabotropic Glutamate Receptors Effects on Feeding

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Neuroscience

by

Jonathan Rene Charles Cano

March 2015

Dissertation Committee:

Dr. B. Glenn Stanley, Chairperson
Dr. Kelly Huffman
Dr. Todd Fiacco

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The Dissertation of Jonathan Rene Charles Cano is approved:

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Committee Chairperson

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Parts of this dissertation are re-prints as they appear elsewhere. Chapter 2 is re-printed from Neuropharmacology, volume 79, pages 59-65, “Activation of lateral hypothalamic mGlu1 and mGlu5 receptors elicits feeding in rats” by Jonathan R. Charles, Mark A. Duva, Gerardo J. Ramirez, Raul L. Lara, Charles R. Yang, and B. Glenn Stanley, Copyright 2014, with permission from Elsevier. Chapter 3 is reprinted from Physiology & Behavior, volume 139, pages 261-266, “Site selective activation of lateral hypothalamic mGluR1 and R5 receptors elicits feeding in rats” by Jonathan R. Charles, Everlinda Hernandez, Amy Winter, Charles R.Yang, and B. Glenn Stanley, copyright 2015, with permission from Elsevier. The co-author Glenn Stanley supervised and directed the research reported in this dissertation. Other co-authors listed gave technical assistance to various different aspects of the research presented here.

ABSTRACT OF THE DISSERTATION

Metabotropic Glutamate Receptors Effects on Feeding

by

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University of California, Riverside, March 2015
Dr. B. Glenn Stanley, Chairperson

Every living organism has two biological driving forces: the need to reproduce and the need to feed. The need to feed is not merely a balancing act between energy expended and caloric intake. Ingestion of calorically sufficient and palatable foods is mediated by the central nervous system and its subsidiaries. The hypothalamus is the main mediator of homeostasis and the lateral hypothalamus (LH) is of particular interest in regards to feeding. Glutamate (Glu) is present throughout the mammalian brain and within the LH Glu influences feeding. Previously ionotropic glutamate receptors were shown to facilitate this response. Here, we have attempted to identify and demonstrate that metabotropic glutamate receptors (mGluRs) also influence feeding. Intracranial injections of drugs either activating or blocking mGluRs increases or suppresses food

intake. These effects were limited to the LH, effects seen in different brain regions can be attributed to the diffusion of the agonist to the LH. Although blockage of mGluRs during a time period of high extracellular glutamate concentration is ineffective in suppressing feeding, we discuss possible alternative mechanisms for these observations. None the less our findings suggest that group I mGluRs are involved in feeding.

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Chapter 1 – Introduction

General Information

Food is essential for survival. It is the main source of energy for the animal and is needed at routine intervals in order to sustain adequate function and ultimately life. It is the fuel for the maintenance of a high metabolic rate in mammals, as well as the main nutrient source for proper development. Importantly, an imbalance of energy intake and expenditure causes serious problems. If energy expenditure exceeds energy intake (i.e. food) then the animal will have to utilize energy from internal stores of either fat or muscle. When available, organisms typically eat enough calories to replace the energy expended. If energy intake exceeds energy expenditure, then the animal has excess energy which is stored as fat. If excessive amounts of fat are not metabolized the end result is obesity.

Obesity has become a major health problem affecting a large portion of our population. In the US, an estimated 32.7 percent of U.S. adults 20 years and older are overweight, 34.3 percent are obese and 5.9 percent are extremely obese (Centers for Disease Control and Prevention 2009) and these percentages continue to grow. Obesity has been linked to a variety of secondary health problems, including coronary heart disease, type 2 diabetes, cancers (endometrial, breast, and colon), hypertension (high blood pressure), stroke, liver and gallbladder disease, sleep apnea and respiratory problems (Must et al 1999, Allison et al 1999). As of 2005, obesity has been estimated to

account for 21% of medical spending or $190 billion (Cawley and Meyerhoefer 2012), which if unchecked could increase $48 to $66 billion annually (Wang et al 2011). Ever increasing sedentary lifestyles and high caloric foods catalyze this upward trend (Rolls 2005, Drapeau 2007). Understanding the mechanisms by which the brain responds to body weight changes and the physiological processes involved in body weight regulation are a necessity to counteract this pandemic.

The traditional view of food intake is that animals only eat when they need calories to sustain metabolic homeostasis; however, this view does not take into account data suggesting that animals may eat even when there is no metabolic need. For example, people tend to eat in social situations even though they do not need the calories. If food intake was controlled independently by the metabolic need of cells, obesity would not be a problem. This leads to the idea that there must be multiple mechanisms controlling food intake.

Neurocircuitry

The brain is a complex system of interconnected neural circuits, some of which can sense circulating metabolic fuels and related signals such as glucose, insulin and leptin, which regulate feeding accordingly (Leibowitz and Stanley 1986, Oomura 1986). A convergence of classical and recent evidence reveals that the lateral hypothalamus (LH) is an important link in brain mechanisms of food intake control (Berthoud and

Munzberg, 2011; Jennings et al., 2013). Initially it was hypothesized that the hypothalamus was the controller of both the initiation and cessation of feeding behavior (Stellar 1954, Anand 1961). Anand and Brobeck (1951a) found that lesioning the LH led to severe aphagia and body weight loss. This prompted Stellar's (1954) 'dual center hypothesis' which proposed that the LH was a "feeding center," which contained a basic mechanism of food intake while the ventromedial nucleus (VMN), a "satiation center," contained an inhibitory mechanism of food intake. This theory has since then been rejected, as current research suggest that there is not a primary site of feeding, rather the control mechanisms are a distributed network (for review see Broberger 2005).

Nevertheless, early lesion and stimulation studies of the lateral hypothalamus have demonstrated its role in feeding regulation (Anand et al 1951a, 1951b, Delagado et al 1953). Destruction of the LH results in aphagia, adipsia and decreased body weight (Anand et al 1951b, Hoebel 1965, Grossman et al 1978, Boyle et al 1975, Dunnett et al 1985, Winn et al 1990), while electrical stimulation initiated eating for the duration of the stimulus (Delagado et al 1953, Wyrwicka and Dobrzecka 1960) and produces orofacial behaviors typically associated with aversive tastes (Berridge and Valenstein, 1991). Lesioning the LH resulted in such severe aphagia that animals without the aid of tube feeding would starve to death (Anand and Brobeck 1951b). It has been postulated that the lesioning of the LH establishes a lower body weight set point which may be maintained due to increased metabolism along with lowered food consumption (Bernardis and Bellinger 1993; Corbett and Keesey 1982).

Initially it was thought that destruction of the LH, via electrolytic lesions, led to aphagia due to the LH's influence as the "feeding center." However, contrasting hypotheses were that the observed effects were attributed to the loss of motor control or to actions on fiber tracts traversing the LH (Millhouse 1979). Findings from Grossman and others demonstrate that destruction of intrinsic cell bodies in the LH while sparing fibers of passage result in aphagia similar to that of electrolytic lesions (Aou et al 1984, Grossman et al 1978, Gossman and Grossman 1982, Stricker et al 1978, Winn et al 1990) arguing for a role for LH neurons in feeding control.

In addition, lesions of the LH led to generalized sensory neglect (Marshall et al 1971, Rodgers et al 1965), which was hemisphere specific. Meaning that when lesioned unilaterally, animals manifested neglect on the contralateral side of the body while exhibiting normal responses to sensory stimuli presented ipsilateral to the LH lesion (Marshall et al 1971). Findings such as these suggested that the LH is multifunctional; actually containing neurons that are components of overlapping neurocircuits, some of which control feeding and others which have other functions (Berthoud 2002). The LH has also been implicated in the circadian rhythm of feeding, wherein LH neuronal spike frequency firing rates increased at night and decreased during the day (Ono et al 1981, Ono et al 1986), similar to the rat's normal feeding pattern (Baker 1953, Siegel 1961, Siegel et al 1947). So even though the LH is no longer considered a feeding center, it still plays a prominent role in regulating feeding.

The LH has a vast array of efferent projections; these range from the hippocampal formation, extended amygdala, basal ganglia and thalamus, the midbrain and pons, to the brainstem and spinal cord, as well as other intrahypothalamic regions (Berthoud 2002, Duva et al 2005). Many of these have been linked to feeding behavior. Descending projections from the LH to the nucleus of the solitary tract (NTS) contain orexin and MCH, which will be discussed later on. Many such MCH fibers innervate NTS neurons which are activated by gastric loads (Peyron et al 1998, Zheng et al 2005). The LH receives reciprocal afferent input from certain areas such as the hippocampal formation and the ventral striatum/nucleus accumbens and the amygdala. The amygdala, which is typically associated with the limbic system, is essential for the formation of conditioned taste aversion. In addition the LH receives input from the prefrontal/orbito-frontal, insular, and olfactory cortex (Simerly 1995). The orbitofrontal cortex is a multimodal area which integrates taste (Rolls et al 1990) with olfaction (Rolls 2001), texture (Rolls et al 2003), even vision (Barbas 1993) before projecting to the LH (Rolls 2004). Similar to LH neurons, these orbitofrontal cortex neurons respond to the stimuli associated with food in a hunger dependent manner and lesioning of this area results in an inability to discriminate between food reward and non-reward cues (Rolls 2004). A well characterized connection between the LH and both the nucleus accumbens (NA) and ventral tegmental area (VTA) has been described by our lab (Stanley et al 2011). There are also a great variety of other brain areas that have

connectivity with the LH, but whose functional role is yet to be determined (Duva et al 2005). Some such areas will be discussed later.

Intrahypothalamical Areas in Feeding Control

Neurochemical and neurophysiological studies have begun to provide insights into the variety of hypothalamic areas which mediate energy balance. Several brain areas which are consistently activated following food consumption are the arcuate nucleus (ARC), LH, ventro-medial nucleus (VMN), paraventricular (PVH), dorso-medial hypothalamus (DMH) and the dorsal vagal complex of the caudal brainstem (DVC), which includes the nucleus of the solitary tract of the caudal brainstem (NTS), the dorsal motor vagal nucleus (DMX) and the area postrema (Johnstone et al 2006). The VMH receives input from the ARC and projects to many hypothalamic and extra-hypothalamic areas such as the ARC, PVH, LH, DMH and the NTS (Sternson et al 2005, Canteras et al 1994). It is thought to exert anorexic effects via brain-derived neurotrophic factor (BDNF) (Unger et al 2007). As the ARC is in close proximity to the 3rd ventricle and a semi permeable blood brain barrier (BBB), it is considered a primary nutrient sensing center of the hypothalamus. Supporting this idea is the evidence that subsets of neurons within the arcuate nucleus sense and respond to local levels of glucose, leptin and a variety of other feeding related circulating hormones (Elias et al 1999, Muroya et al 1999, Kamegai et al 1996, Hisano et al 1988). Activation of neurons in dorsomedial portion of the arcuate nucleus elicits eating, while activation of neurons in the ventrolateral portion of

the arcuate nucleus produces satiety (Elias et al. 1998a, 1998b, Elias et al 1999). Interestingly the satiety hormone leptin acts on both subsets of neurons, suppressing the activity of the feeding stimulatory neurons and activating the satiety neurons (Cowley et al 2001, Elias et al 1999). Interestingly, both types of arcuate neurons project directly to the LH, as well as to the PVN (Elias et al 1998b, Elias et al 1999).

LH Neurons: Responses to Food

Neural activity within the LH can be modified by visual and taste cues. Subsets of LH neurons within fasted but not satiated squirrel monkeys responded with changes in action potential production when the monkey looked at food or tasted food, as well as acquiring food like responses to neutral stimuli associated with food, like a feeding syringe (Rolls et al 1976, Burton et al 1976). Later it was determined that the neurons which responded to the sight and taste of food became unresponsive when the animal was fed to satiety on that particular food. This was termed sensory-specific satiety (Rolls 1986). The animal would feed on that food item until it became satiated, at which point the food would be actively rejected. This behavioral response correlated well with neuronal activity, where upon presentation of the novel food LH neuronal firing decreased as the animal became satiated. An increase in firing rates would then be observed after feeding resumed due to presentation of another food item. This sensory specific satiety may be an underlying cause of obesity in our society, as we are constantly exposed to novel food items in our everyday life. Further support for this

idea has been shown as rats exposed to a variety of palatable foods are more likely to become obese than their non-varied diet counterparts (Rolls et al 1983). In addition, humans show habituation to food wherein their response to liking the food which was constantly presented decreases over time (Ernst and Epstein 2002).

Rudimentary Control of Feeding Via Brainstem Mechanisms

Many nuclei can exert control over feeding. For example, nuclei in the hindbrain can maintain rudimentary feeding motor patterns even in the complete absence of forebrain inputs (Powley et al 1986, Grill 1986). When forebrain connections to the brainstem are severed, as in the mesencephalic rat, where the brainstem and spinal cord retain functional control over ingestive behaviors through autonomic and somatic innervation, the hindbrain nuclei appear to be sufficient to regulate feeding at a very rudimentary level (Grill and Norgren 1978, Grill et al 1992). Decerebrate rats show normal facial and mouth movements representative of ingestion when pleasant tastes are applied to the mouth and characteristic rejection responses such as gaping and head shaking when unpleasant foods, such as quinine, are tasted (Grill and Norgren 1978). Interestingly these animals can, in a basic form, regulate their food intake. Specifically, food deprived animals will ingest liquid food administered directly into the mouth and satiated rats would reject it. This suggests that fundamental ingestive processes can be carried out by nuclei within the brainstem independently from forebrain nuclei. This is a very basic form of feeding however and does not take into consideration the more

complex attributes of food consumption controlled by areas such as the lateral hypothalamus (Ernst and Epstein 2002), acting at least in part via these brainstem mechanisms. Examples of such complex attributes are feeding pattern correlation with circadian periodicity, where food intake is at its highest during the nocturnal phase (Riley et al 1981, Schmitt 1973) and food learning, such as taste aversion, where LH lesioned animals could not learn to avoid food paired with lithium chloride (LiCl), a poison, or an electric shock (Roth et al 1973). In addition to integrating these complex systems, the LH also incorporates the information provided by circulating metabolic fuels such as glucose and leptin.

Metabolic Fuels and Hormones as Mediators of Feeding

Some hypothalamic neurons can detect and integrate visceral signals, such as glucose, to induce food intake. Feeding patterns correlate with circulating levels of glucose. In studies where glucose was investigated in relation to feeding, each meal was preceded by a decrease in blood glucose and each decrease in blood glucose was followed by a meal. If this decrease in blood glucose was attenuated by intravenous injection of glucose the animals did not eat (Campfield et al 1985). Glucose is a major source of energy within the mammalian body and decreases in glucose naturally occur as an animal metabolizes it for energy. Changes in glucose are detected in the periphery as well as in the central nervous system. When an animal becomes hypoglycemic, neurons within the LH, termed glucose sensitive neurons, fire more frequently (Oomura

1981) thus contributing to the feeding response. Electrophysiological studies have shown that these LH neurons decrease their firing rate in the presence of glucose (Aou et al 1984, Oomura et al 1974) via an ATP-dependent Na^+ pump (Shibata et al 1982, Silver et al 1998). Interestingly other neurons whose firing rate increases in response to glucose, termed glucose-responsive neurons, have also been identified. These neurons however seem to be located primarily in the VMH (Ono et al 1982). If glucose was the main determinant of food intake, these types of neurons may have provided support for the dual hypothesis model mentioned earlier.

Circulating hormones that influence feeding include leptin, insulin, Ghrelin, Neuropeptide Y (NPY) and CCK. The lipostatic hypothesis by Gordon Kennedy in 1953 describes the idea that the brain monitors the amount of body fat and works to maintain this level. This implied communication by the body's fat stores and feeding behavior. The general mechanism by which this occurred was uncovered in 1960 by Doug Coleman using two strains of mice. These were *ob/ob* (for obese) and *db/db* (for diabetic). When parabiosed, joining two animals anatomically and physiologically, thus allowing them to share a functioning circulatory system, the *db/db* mice gain weight while their normal counterpart stops eating and essentially starves to death. When normal mice are paired with *ob/ob* mice they gain weight while the *ob/ob* mice lose weight. Then when *ob/ob* and *db/db* mice are paired, the *ob/ob* mice lose weight and the *db/db* mice gain weight. From these data, Doug Coleman suggested that *ob/ob* were deficient in a satiety hormone and *db/db* mice were deficient in the receptors for

this satiety hormone. It was later discovered that the hormone mediating these effects was leptin (Zhang et al., 1994). Leptin is secreted by fat cells and circulating levels are approximately proportional to levels of body fat (Considine et al 1996). Peripheral or central injections of leptin suppress feeding (Mistry et al 1997, Campfield et al 1995). For those reasons, leptin is considered to be a link between fat and eating, providing a plausible mechanism in support of the lipostatic hypothesis. As for the LH, leptin is linked to LH control of feeding due to leptin receptors being expressed by melanin concentrating hormone (MCH) and orexin (OX) neurons in the LH, as well as by findings showing that leptin administration within the LH decreases food intake (Satoh et al 1997, Håkansson et al 1998, 1999). The LH also receives neural inputs from leptin sensitive neurons in the arcuate nucleus (Broberger et al 1998). Rather than taking a top down approach in determining what ligand and receptor pair was mediating the behavior, as with leptin, we opted for a bottom up approach to uncover the behavioral effect of LH mGluR activation.

Cytoarchitecture and Borders of the LH

The LH is a relatively cell sparse zone, it contains scattered cells of different sizes and shapes with cell density variability at different sites of the LH (Palkovits and Van 1980). The somas of LH cells are not alike but can be fusiform, triangular, or spherical ranging from 50µm (only 5%) to 15-25µm or even smaller (Millhouse 1979). The neurons have long, tapering processes whose broad dendritic trees are arranged

perpendicularly with the medial forebrain bundle (MFB), a major fiber tract, in a similar fashion as Purkinje cells in the cerebellum (McMullen and Almli 1981, Millhouse 1979, Palkovits and Van 1980). A single axon emerges from a primary dendrite of these LH neurons and travels rostrally, caudally or medially. As mentioned earlier, the LH encompasses several axon tracts including the medial forebrain bundle, the fornix, and the stria terminalis (Millhouse et al 1979). In terms of scope and boundaries, the lateral hypothalamic area merges rostrally into the preoptic area and caudally into the ventral tegmental area. It is bordered medially by the dorsomedial, ventromedial, and arcuate nuclei, anteriorly by the anterior hypothalamic and medial preoptic areas, and laterally by the internal capsule, the optic tract, and more caudally by the subthalamic nucleus, consisting of numerous distinct nuclei (Swanson et al 2005, Saper et al 1979). Originally, the cells within the LH had been classified into three groups based on size, shape, spatial arrangement and density: those that are obvious clusters; those that are less obvious; and, those that are loosely organized (Geeraedts et al 1990). Now, generally the lateral hypothalamic area is divided into anterior, tuberal (roughly at the level of the ventromedial hypothalamus) and posterior portions based on its efferent connectivity as first described by Saper (1979). Recently the LH has been further subdivided by its different neuroanatomical connections (Hahn and Swanson 2012).

Neurotransmitters

The LH is the sole source of the two distinct groups of peptidergic neurons, one group containing MCH and the other group containing OX, both of which play significant roles in energy balance (Sakurai et al 1998) and arousal (Hagan et al 1999, Ida et al 1999, Li et al 2002). The LH contains several other peptides, including NPY, galanin, neurotensin, proopiomelanocortin (POMC), agouti-related peptide (AgRP) and amphetamine-related transcript (CART), as well as other hormonal factors such as CCK and Ghrelin (for Review see Meister 2007). All of these aforementioned neurotransmitters influence feeding in one form or another. For example, when injected into the paraventricular nucleus, galanin stimulates consumption of food, particularly high-fat diets, and alcohol (Barson et al 2010). In contrast, intracerebroventricular CART inhibits food intake (Kristensen et al 1998). Orexins exert direct excitatory actions on ARC NPY/AgRP neurons (Van den Top et al 2004) and indirect inhibitory actions on ARC POMC neurons (Ma et al 2007) and both actions apparently combine to increase eating behavior. MCH is important for feeding as overexpression of the MCH gene leads to obesity (Ludwig 2001). Additionally, MCH receptors are spread throughout the brain in areas such as the nucleus accumbens, striatum, hippocampus, locus coeruleus, including the cortex within the orbitofrontal, prelimbic, sensorimotor, motor and piriform cortex (Saito et al 2001), as well as being reciprocally connected with OX neurons (Guan et al 2002). While worth noting for context, the contributions of all these neurotransmitters

and hormones is vast and beyond the scope of my dissertation. More relevant are the ubiquitous amino acid neurotransmitters, glutamate and γ-aminobutyric acid (GABA).

LH GABA and Satiety

GABA is thought to be the most abundant neurotransmitter in the mammalian brain (Decavel and van den Pol 1990, Kimura and Kuriyama 1975). Within the confines of the hypothalamus, the LH has the highest levels of GABA (Kimura and Kuriyama 1975) along with the expression of both $GABA_a$ and $GABA_b$ receptors (Backberg et al 2004). Extracellular LH GABA fluctuates in a circadian pattern, where levels are low during the dark phase, when rats consume most of their food, and high during the light phase when they consume very little (Cattabeni et al 1978). In addition, in vivo microdialysis measurements indicate that GABA levels are low prior to a meal, steadily rise in the last third of the meal and are maintained during the post meal period, thus apparently acting as a meal termination signal (Rada et al 2003). LH injection of GABA receptor antagonists such as picrotoxin or bicuculline induce feeding, while LH administration of the $GABA_AR$ agonist muscimol will block feeding for up to 24 hrs. Prolonged activation of $GABA_A$ receptors via muscimol will actually lead to a reduction of body weight during the course of the injections, with a rapid rebound as soon as administration ceases. These effects appear to be $GABA_A$ receptor specific as $GABA_B$ receptor agonists and antagonists had no apparent effect (Turenius et al 2009a, 2009b). Collectively, these findings indicate that feeding at the onset of a meal may be facilitated by the low levels

of endogenous GABA disinhibiting feeding stimulatory LH neurons, while satiety near the end of a meal may be partly due inhibition of these feeding stimulatory LH neurons by high levels of endogenous GABA.

LH Glutamate and Hunger

Despite being the most abundant excitatory neurotransmitters in the mammalian brain, glutamate's involvement in food intake has only recently begun to be investigated. Glutamate is found to be localized in synaptic terminals of the LH (van den Pol et al 1993) and LH neurons are known to possess several types of glutamate receptors, specifically NMDA, AMPA and KA. Injections of glutamate within the LH induced a short latency feeding response (Stanley et al 1993a), suggesting a possible role in the control of feeding behavior. It was also shown that LH injection of the specific NMDA, AMPA and KA ligands each elicited food intakes in a dose dependent fashion (Stanley et al 1993b). A microdialysis probe mapping study of different subregions within the LH determined that the most sensitive site for NMDA administration to elicit eating was the tuberal LH (Duva et al 2002). It was later determined that feeding was induced via the specific NR2A, NR2B and AMPA/KA receptors within the LH. Additionally, LH injection of NMDA receptor antagonist blocked the feeding stimulatory effect of LH NMDA, and also suppressed feeding produced by fasting or that occurring at the onset of the circadian feeding cycle. Thus suggesting that glutamate and NMDA receptors in the LH are involved in natural feeding. (Hettes et al 2003, Khan et al 2000,

Khan et al 1999). In addition, increases in extracellular glutamate coincide with feeding, wherein glutamate levels start high in the LH prior to eating a meal and levels fall off rapidly as the animal eats and becomes satiated (Rada et al., 1997). Collectively, these finding strongly suggest that glutamate and its receptors participate in the regulation of eating behavior. This indicates that glutamate plays a crucial role in inducing food intake.

Metabotropic Glutamate Receptors (mGluRs)

A role for LH glutamate has been established for the initiation of feeding via ionotropic receptors, however not much research has been conducted on the other glutamatergic receptors, the metabotropic glutamate receptors, present within the LH. Within the LH there is a range of relative densities among ionotropic glutamate receptors and metabotropic glutamate receptors. NMDA receptors have the highest density, followed by mGluRs, while AMPA/KA receptors have the lowest density (Meeker et al 1994). Large amounts of mGluRs suggest a possible functional role within the LH in eliciting feeding.

MGluRs comprise a family of eight structurally related receptors, which can be divided into three pharmacological groups on the basis of sequence homology, agonist selectivity, intracellular signaling mechanisms and differential targeting in neurons (Pin and Duvoisin 1995). Group I mGluRs, mGluR1 and mGluR5, are positively coupled to

phospholipase C (PLC), activation of which leads to the formation of IP3 and diacylglycerol, release of Ca^{2+} from intracellular stores and stimulation of protein kinase C (PKC), while Group II receptors, mGluR2,3, and Group III, mGluR4,6,7, 8, are negatively coupled, i.e. an inhibitory enzyme, to adenylyl cyclase and cyclase respectively. The precise consequence of activating Group 1 mGluRs varies widely, depending on the neuron and the circuit in which it resides. Even though glutamate can have the same relative affinity among group 1 receptors (Pin and Duvoisin 1995), it will be important to differentiate mGluR1 and mGluR5's specific contributions as they can have different effects within different cells.

Previous preliminary evidence from our lab suggested that activation of group I/II mGluRs is sufficient to elicit food intake. Specifically, injection of the mGluR agonist 1S, 3R-1-aminocyclopentance-1,3-dicarboxylic acid (ACPD) elicits eating when injected directly into the LH. The agonist elicited a dose-dependent eating response that was significant at every dose above 10 nmol. When administering the inactive isomer (1R, 3S-ACPD) however only a small eating response was observed, indicating ACPDs effects were stereochemically specific (Duva et al 1998). As ACPD broadly affects multiple mGluR receptors, the individual contribution of each receptor must be explored. The focus of this dissertation will be on group I mGluRs. As group II and III mGluRs are mostly coupled with inhibitory G-proteins (G_i) and presynaptic their primary role would be to inhibit neurotransmitter release, in this case glutamate, and impede LH feeding rather than promote it.

Within the LH, mGluR1 and mGluR5 have been shown to be present postsynaptically (van den Pol 1994a, van den Pol et al 1995). Not much research has examined the specific roles of group I mGluRs in food intake. Mice with the mGluR5 gene knocked out show deficits in body weight (Bradbury et al 2005), which has since sparked interest in the mechanisms by which mGluRs may act to modulate body weight. Electrophysiological studies indicate that activation of group I mGluRs can potentiate NMDA synaptic currents. These effects were demonstrated on MCH neurons and possibly orexin neurons (Huang and van den Pol 2007). Support for the interaction of mGluRs and NMDA receptors comes from structural localization of these receptors. Group I mGluRs are concentrated in a ring around the periphery of the Post Synaptic Density (PSD) near NMDA receptors (Nusser et al 1994, Luján et al 1997). This suggests that synaptic transmission may be regulated in such a way wherein low concentration of glutamate act on ionotropic receptors and higher concentrations additionally act on the metabotropic receptors. As NMDA involvement has been previously been shown to influence food intake and group I mGluRs may be linked to NMDA receptors, group I mGluR involvement in food intake merits further investigation.

Short term effects on food intake and body weight are produced by chronic injections of muscimol, a $GABA_A$ agonist, and DAP5, an NMDA antagonist, as both decreased food intake and body weight during the course of the injections, but these levels began to return to baseline after the cessation of injections (Turenius et al 2009b, Stanley et al 1996). Glutamate agonist injection within the LH causes an extended

increase in food intake (Stanley et al 1993b). As ionotropic responses are typically electrophysiologically fast and short, the long-lasting effect of mGluR activation on NMDA responses (Huang and van den Pol 2007) could contribute to the long lasting effects on feeding of these glutamate agonists. The underlying mechanism of this NMDA enhancement is not well known, but I suspect that mGluRs may also be involved in even longer lasting effects within the LH. MGluRs are known to act via G proteins and second messenger cascades which can lead to long-term cellular effects. MGluR5 may be an important modulator of feeding and energy balance in rodents, as mice lacking this gene, mGluR5-/- , weighed less than their control littermates (mGluR5+/+) even when food was present ad libitum (Bradbury et al 2005). In addition chronic i.p. mGluR5 antagonist injections with MTEP, were shown to decrease body weight in mice (Bradbury et al 2005).

Just as glutamate is widely distributed throughout the brain, so are mGluRs. (Shigemoto et al 1992). Anatomical studies indicate that mGluR1 and mGluR5 are present within the LH (van den Pol 1994a, van den Pol et al 1995). Additionally mGluR4 and mGluR7 are also expressed within the LH but to a lesser extent and each having different intracellular effects. MGluR1 RNA was detected in the embryonic hypothalamus and by the first day of birth, with both mGluR1 RNA and protein being strongly expressed throughout the hypothalamus (van den Pol et al 1994b)

In addition mGluRs have possible interactions with other ionotropic glutamate receptors. Group I mGluRs have been found to be post-synaptically present within the LH (van den Pol 1994a, van den Pol et al 1995). As mGluR act via G-coupled proteins, they may have multiple intracellular sites of action. One such site may be NMDA receptors. Group I mGluRs may enhance cell excitability through NMDA receptors within the rat spinal cord (Jones and Headley 1995). It has also been suggested that mGluR1s may potentiate responses specifically by NMDA receptors (Huang and van den Pol 2007).

This receptor interaction is consistent with other studies which found that antagonism of ionotropic glutamate receptors was more effective than antagonism of metabotropic glutamate receptors in reducing food reward (Popik et al 2011, Bisaga et al 2008). Specifically when peripherally administering an NMDA antagonist, rats ate less of a palatable food (lard) than rats which received the mGluR5 antagonist, MPEP. This trend seems to be conserved in higher order primates, as baboons sought out a rewarding treat such as a lollipop less than MPEP treated animals (Bisaga et al., 2008). Therefore the effects seen in the nocturnal and deprivation studies may be attributed to activation of ionotropic receptors and not the mGluRs themselves. As mentioned earlier, mGluRs are distributed throughout the brain and other brain areas, such as the NA, therefore antagonism of such brain areas may aid in suppressing normal feeding more effectively. However this should not devalue the importance of mGluRs in eliciting feeding.

Zalewska and Wisniewski (1997) found that ICV administration of the group I/II agonist, ACPD, significantly improved consolidation of affectively-motivated memory, but failed to effect recognition memory. Later on they observed similar results with the group I agonist, DHPG (2000). Given these data and the association between mGluR activation of OR and MCH neurons as previously stated (Huang and van den Pol 2007) lend support to the notion that mGluRs may be involved in memory formation. Additionally, Petrovich and colleagues showed that orexin neurons, but not melanin concentrating hormone neurons are involved in Pavlovian learning (Petrovich et al 2012, Reppucci and Petrovich 2012, Petrovich et al 2007). Group I mGluRs have been implicated in modulating learning via long term potentiation (LTP) and long term depression (LTD) within the hippocampus (Manahan-Vaughan 1997, Riedel et al 1996, Ménard and Quirion 2012). Of particular interest for future studies, work from the Rolls lab has implicated the LH in neuronal learning, where LH firing was associated with the observation of a feeding syringe alone (Burton et al 1976). Petrovich and Gallagher (2007) have also shown that the LH is involved in Pavlovian food association learning. Therefore with mGluRs link to long term potentiation (Mukherjee and Manahan-Vaughan, 2013), mGluRs may be a possible mechanism for such learning, providing additional knowledge to the framework of feeding behavior.

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Chapter 2 – Activation of lateral hypothalamic mGlu1 and mGlu5 receptors elicits feeding in rats.

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Abstract

Metabotropic glutamate receptors (mGluRs) have been popular drug targets for a variety of central nervous system (CNS) disease models, ranging from seizures to schizophrenia. The current study aimed to determine whether mGluRs participate in lateral hypothalamic (LH) stimulation of feeding. To this end, we used satiated adult male Sprague-Dawley rats stereotaxically implanted with indwelling bilateral LH guide cannulas to determine if injection of (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), a broad mGluR group I and II agonist, would elicit feeding. Administration of 100 nmol ACPD induced feeding with a short latency. Similarly, unilateral LH injection of the selective mGluR group I agonist (S)-3,5-dihydroxyphenylglycine (DHPG) elicited significant feeding beginning 60 minutes postinjection and continuing until 4 hr postinjection. Administration of the mGluR5 agonist, (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) produced a smaller delayed feeding response. These

delayed but prolonged eating responses suggest that activation of LH mGluR1 and/or mGluR5 might be sufficient to elicit feeding. To determine which subtypes were involved, LH DHPG injections were preceded by LH injection of either the group I antagonist n-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide (PHCCC), the mGluR1 antagonist 6-amino-n-cyclohexyl-n,3-dimethylthiazolo[3,2-a]benzimi dazole-2-carboxamide hydrochloride (YM-298198) or the mGluR5 antagonist 3-((2-methyl-4-thiazolyl)ethynyl)pyridine (MTEP), and food intake was measured. PHCCC blocked DHPG elicited feeding, and each of the other antagonists produced significant feeding suppression. These findings suggest roles for mGluR1 and/or mGluR5 in lateral hypothalamic circuits capable of stimulating feeding behavior.

Introduction

Glutamate is a major excitatory neurotransmitter in the mammalian nervous system and previous findings strongly suggest a role for ionotropic glutamate receptors in feeding, especially within the lateral hypothalamus (LH; Stanley et al., 2011). Specifically, injections of glutamate or glutamate receptor agonists n-methyl-d-aspartate (NMDA), 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) or kainic acid (KA) into the LH induce short latency feeding responses, while NMDA receptor antagonists suppress both the NMDA-elicited and natural feeding (Stanley et al., 1993a, 1993b, and 1996). Cannula and microdialysis probe mapping studies showed the most

sensitive site for these agonists to elicit feeding is in the tuberal LH (Duva et al., 2002, Stanley et al., 1993a). In addition, increases in extracellular glutamate levels within the LH coincide with feeding initiation, whereas LH glutamate levels fall rapidly as the animal eats and becomes satiated (Rada et al., 1997). This increase in glutamate release is feeding specific and does not occur with drinking (Thongkhao-on et al., 2008). This indicates glutamate plays a crucial role in inducing food intake in the LH. However, the role of metabotropic glutamate receptors (mGluRs) within the LH has yet to be demonstrated.

The family of mGluRs is composed of eight structurally related receptors, divided into three groups on the basis of sequence homology, agonist selectivity, intracellular signaling mechanisms and differential targeting in neurons (Pin and Duvoisin, 1995). Group I mGluRs, mGluR1 and mGluR5, are positively coupled to phospholipase C (PLC), activation of which leads to the formation of IP3 and diacylglycerol, intracellular release of Ca^{2+} and stimulation of protein kinase C (PKC), while group II receptors, consisting of mGluR2 and 3, and group III receptors, consisting of mGluR4,6,7, and 8, are negatively coupled to adenylyl cyclase and cyclase respectively. Different subtypes in each mGluR group have different functional roles. For example, within group 1 mGluRs, mGluR1 modulate intracellular Ca^{2+} concentration, while mGluR5 are additionally linked to potentiation of NMDA receptors (Mannaioni et al 2001). Support for the interaction of mGluRs and NMDA receptors comes from structural localization of these receptors. Group I mGluRs are concentrated in a ring around the periphery of the post synaptic

density near NMDA receptors (Nusser et al., 1994, Lujan et al., 1997). Therefore as NMDA receptors have previously been implicated in feeding stimulation and group I mGluRs are linked to NMDA receptors, group I mGluR involvement in food intake merits investigation. This study aims to determine if LH mGluRs provide a functional role in feeding and to identify which receptors may mediate this behavior.

Materials and Methods

Subjects and stereotaxic surgery

Adult male Sprague-Dawley rats weighing 350-500 g at the time of surgery were used. These rats were bred in the University of California, Riverside, Psychology Department vivarium and were descended from rats obtained from Charles River, Inc. They were individually housed in a temperature controlled vivarium (21°C) on a 12:12 hr light:dark cycle, and allowed free access to standard Purina rat chow pellets and water.

Surgery to implant an 18 mm long, 26 gauge (o.d.=0.46 mm) stainless steel indwelling guide cannula within the LH was performed under barbituate anesthesia (Nembutal, 50 mg/kg body weight, i.p.). The stereotaxic coordinates were: 6.1 mm anterior to the interaural line, +1.8 mm lateral to the midsagittal sinus (±1.8mm when implanting bilaterally), and 8.2 mm ventral to the surface of the skull. Coordinates were initially based on the atlas of Paxinos and Watson (2007) then modified empirically; the

incisor bar was placed at –3.3 mm during all surgeries. Cannulas were held in place by dental cement affixed to four stainless steel screws imbedded in the skull and protected with a plastic guard. To prevent clogging a 33-guage stainless steel obturator was placed within the cannula. The animals were given a minimum of seven days to recover from surgery before proceeding with testing. During this timeframe all animals were handled to adapt them to testing procedures. Procedures were approved by the University of California Riverside Institutional Animal Care and Use Committee. Three days prior to testing, the standard pellet food was replaced with a milk-mash diet consisting of Purina rat chow (500 g), sucrose (400 g), and Carnation evaporated milk (354 ml).

Central injections

Tests consisted of unilateral or bilateral injection within the LH. Solutions were injected via a 33-gauge needle that projected 1.0 mm beyond the ventral tip of the cannula. Each injection consisted of a 0.3 µl volume of either the mGluR group I and II agonist, (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD); mGluR1/5 agonist, (*S*)-3,5-dihydroxyphenylglycine (DHPG); the mGluR5 agonist, (RS)-2-chloro-5-hydroxyphenylglycine (CHPG); the mGluR5 antagonist, (3-((2-methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride (MTEP); the mGluR1 antagonist, 6-amino-n-cyclohexyl-n,3-dimethylthiazolo[3,2-a]benzimi dazole-2-carboxamide hydrochloride (YM-298198), the group I mGluR antagonist, n-Phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide (PHCCC) at varying doses or the vehicle (VEH), artificial cerebrospinal fluid

(ACSF) where indicated. The ACSF consists of (in mM): Na^+ (147), Cl^- (154), K^+ (3.0), Ca^{2+} (1.2) and Mg^{2+} (0.9) with a pH of 7.4, except for ACPD administration, when 0.1M NaOH was used to improve the solubility of ACPD. All drugs were purchased from Tocris (St. Louis, MO). Drug doses were extrapolated from previous findings. Drug dilutions were prepared one day prior to the first test day and aliquots stored at -80 ^oC. When tested under satiated conditions, fresh mash was given at least one hour prior to testing, at the onset of the light cycle. For food deprived or nocturnal feeding conditions, fresh mash was given immediately after injection at the commencement of the dark cycle. For all experiments, doses were given in counterbalanced order across test days. No single rat was administered more than 16 individual injections. Food intake was typically measured at 15 min and 30 min as well as 1, 2 and 4 hr post-injection. All Subjects were fed within their homecage and food bowls weighed on a daily calibrated scale. Between tests, rats had at least one undisturbed day with ad libitum access to food and water unless otherwise noted.

Experiment 1: Bilateral activation of LH mGluR group I/II receptors elicits feeding

In order to determine whether broad activation of mGluRs elicits feeding, a group of 8 rats was given bilateral injections of ACPD, the mGluR group I/II agonist, at 100 nmol or VEH. Additionally, to determine if specific mGluR group I activation was sufficient to induce food intake, DHPG at 1 nmol or ACSF was administered bilaterally

within the LH of 6 different rats. These injections were bilateral to maximize their effectiveness and food intakes were measured from 15 min to 2 hr post-injection.

Experiment 2: Unilateral activation of LH mGluR1/5 elicits feeding

To determine whether group I mGluR activation in the LH elicits eating, rats were given unilateral injections of DHPG at 0.1 nmol, 0.5 nmol, 1nmol, 5 nmol, 10 nmol, 25 nmol, 50 nmol or ACSF. Due to the wide range of drug concentrations, half the doses were tested in one group of 11 rats and the other half were tested in another group of 11 rats. Unilateral injections were employed to test the effectiveness of single site administration and to limit tissue damage. The data sets were then combined post hoc.

Experiment 3: Unilateral activation of mGluR elicits eating

To determine if specific activation of mGluR5 increased food intake, a group of 19 rats was given unilateral LH injections of CHPG at 0.1 nmol, 0.5 nmol, 1 nmol, 5 nmol, 10 nmol or ACSF. As these doses were ineffective, a second batch of 19 rats was given unilateral LH injections of CHPG at 25 nmol or 50 nmol.

Experiment 4: Does blocking mGluR1, mGluR5 or both receptors prevent DHPG-induced feeding within the LH?

To confirm receptor selectivity, a group of 11 rats was given unilateral injections of MTEP, a mGluR5 antagonist, (0.1 nmol, 1 nmol, 10 nmol) or YM-298198, a mGluR1 antagonist, (0.1 nmol, 1 nmol, 10 nmol) 5 min before injection of 1 nmol DHPG. An

additional group of 7 rats was given unilateral injections of PHCCC (10 nmol) 5 min before LH injection of 1.0 nmol DHPG.

Experiment 5: Does antagonism of mGluR1/5 in the LH suppress spontaneous food intake?

Animals implanted with bilateral LH cannulas were tested during the dark phase (n=11). Bilateral injection of antagonists was employed to block the effects of bilateral release of endogenous glutamate. Specifically, satiated animals were tested 0-30 min before the onset of the dark phase with bilateral LH injection of either MTEP (0.1 nmol, 1 nmol or 10 nmol), YM-298198 (0.1 nmol, 1 nmol or 10 nmol) or ACSF and given fresh mash post-injection. A second group of rats (n=9) were similarly tested with PHCCC (1 nmol or 10 nmol). Food intakes were measured 15 min to 4 hr post-injection and a rest day was given between injections.

The animals used for the nocturnal PHCCC tests were food deprived for 24 hr and once again injected in counterbalanced order with PHCCC (1 nmol, 10 nmol, 50 nmol) or ACSF. Fresh mash was given post-injection. Food intakes were measured 15 min to 4 hr post-injection with a day between injections.

Histology and statistics

After the final behavioral test, animals were deeply anesthetized with Nembutol and perfused transcardially with 4% formaldehyde. The brains were removed from the

skull and placed in the same fixative for at least 24 hr. Using dry ice, brains were frozen, 100 µm thick coronal sections were cut and mounted on polarized glass slides. Slides were Nissil stained and coverslipped for later examination. Accuracy of placements was determined by comparing the image obtained using a projection microscope and the rat brain atlas of Paxinos and Watson (2007). Data from any placements 1.0 mm or more outside the borders of the intended LH injection site were discarded.

Food intake data were analyzed using ANOVA followed by Fisher's LSD (Least Significant Difference) test. A standard significance level of $p<0.05$ was used for all tests.

Results

Experiment 1: Bilateral activation of LH mGluR group I/II receptors elicits feeding.

Representative cannula placements are presented in Figs 2.1A and B. Bilateral administration of ACPD, the broad mGluR group I and II agonist, within the LH of satiated animals elicited feeding at a dose of 100 nmol. As shown in Fig 2.2A animals ate an average of 6.7 g within 15 min of the injection with a maximum intake of 9.5 g. Repeated measures two-way ANOVA revealed significant effects of bilateral ACPD treatment [$F_{1,63}$ =6.6, $p<0.05$], time [$F_{3,63}$ =3.7, $p<0.05$] and interaction of both [$F_{3,63}$ =4.1, $p<0.05$]. In comparison, bilateral administration of 1 nmol DHPG produced significant food intake 1hr post-injection with a total of about 2.3 g at 2 hr (Fig 2.2B). Repeated measures two-

way ANOVA revealed significant effects of DHPG treatment [$F_{1,47}$ =4.3, p<0.05], time [$F_{3,47}$ =10.8, p<0.01] and interaction of both [$F_{3,47}$ =6.1, p<0.01].

Experiment 2: Unilateral activation of LH mGluR elicits feeding

As shown in Fig 2.3, unilateral LH injection of DHPG elicited feeding in a dose-dependent manner. The minimally effective dose was 0.5 nmol, 1 nmol produced a strong consistent effect and 25 nmol appeared to be nominally effective. With a single exception the elicited intakes were not statistically significant until 60 min post-injection, when the magnitudes were modest (approximately 2-4 g). However, intakes continued to increase thereafter, until 4 hr post-injection, reaching magnitudes over 8 g. Repeated measures two-way ANOVA revealed significant effects of DHPG treatment [$F_{7,703}$ =15.3, p<0.001], time [$F_{3,703}$ =146.7, p<0.001] and interaction of both [$F_{21,703}$ =18.6, p<0.001]. Behavioral hyperactivity was also evident after the 25 nmol and 50 nmol dose injection throughout the course of the experiment.

Experiment 3: Unilateral LH activation of mGluR5 induces eating

To address whether activation of LH mGluR5 receptors is sufficient to elicit feeding, unilateral injections of CHPG were administered in satiated rats. While the doses from 0.1 nmol to 10 nmol were ineffective (data not shown), doses of 25 nmol and 50 nmol elicited feeding (Fig 2.4). Mean cumulative food intake was 3.1 g at the 50 nmol dose. Repeated measures two way ANOVA revealed significant effects of higher dose

CHPG treatment [$F_{2,284}$ =5.1, p=0.01], time [$F_{4,284}$ =27.7, p<0.001] and interaction of both [$F_{8,284}$ =2.1, p<0.05].

Experiment 4: Does blocking mGluR1, mGluR5 or both receptors prevent DHPG-induced feeding within the LH?

As shown in Fig 2.5, pre-treatment with the group I mGluR antagonist, PHCCC, blocked the feeding produced by DHPG. Repeated measures two-way ANOVA revealed significant effects of PHCCC pretreatment [F2,104 =11.3, p<0.002], time [F4,104 =11.2, p<0.001] and interaction of both [F8,104 =4.5, p<0.001]. This indicates involvement of mGluR1 and/or mGluR5 in feeding.

To determine whether mGluR1 or mGluR5 mediates DHPG-elicited feeding, unilateral LH administration of YM-298198 or MTEP was given prior to LH DHPG injection. As shown in Fig 2.6, the mGluR5 antagonist, MTEP at 10 nmol, strongly suppressed DHPG-induced feeding from 2-4 hr post-injection. Repeated measures two-way ANOVA revealed significant effects of MTEP pretreatment [$F_{4,274}$ =3.1, p<0.05], time [$F_{4,274}$ =26.1, p<0.001] and interaction of both [$F_{16,274}$ =2.3, p=0.005].

Similarly, as shown in Fig 2.7, the mGluR1 antagonist, YM-298198 at 0.1 to 10 nmol, suppressed DHPG-elicited food intake from 2 h to 4 h postinjection. Repeated measures two-way ANOVA revealed significant effects of YM-298198 pretreatment [$F_{4,274}$ =2.976, p=0.03], time [$F_{4,274}$ =25.6, p<0.001] and interaction of both [$F_{16,274}$ =2.8,

p<0.001]. The antagonist data are consistent with roles for both mGluR1 and mGluR5 in feeding.

Experiment 5: Does antagonism of mGluR1/5 in the LH suppress natural feeding?

As shown in Table 2.1, PHCCC at 1 and 10 nmol doses injected bilaterally into the LH did not suppress nocturnal eating (treatment [$F_{2,134}$ =1.0, p=0.375]). Similarly injection of MTEP did not reduce nocturnal feeding nor did YM-298198. We also tested to determine whether bilateral LH PHCCC would suppress eating elicited by 24 hr food deprivation, and while there appeared to be a trend of slight food intake suppression (Table 2.2), this was not statistically significant.

Discussion

The present study demonstrates that agonists for mGluRs injected directly into the LH consistently and reliably elicited feeding in satiated rats. In addition, prior LH injection of antagonists for mGluRs blocked or suppressed the agonist-elicited feeding responses. To the best of our knowledge this is the first evidence for hypothalamic, and more specifically lateral hypothalamic, mGluRs involvement in feeding stimulation. These findings support and expand our earlier evidence that LH injection of glutamate and agonists of ionotropic glutamate receptors elicit feeding (Stanley 1993a, b) and LH injection of NMDA receptor antagonist suppress natural feeding (Stanley 1996). Our

results also extend earlier research suggesting a role for mGluR5 in feeding stimulation. Specifically, Bradbury et al. 2005 showed reduced body weight in mice in which the mGluR5 gene had been knocked out. Additionally, the anorectic effect of peripheral injection of the mGluR5 antagonist MTEP was abolished in these knock-out mice. Moreover, intracereberoventricular (i.c.v.) injection of the mGluR5 agonist, CHPG, has been shown to elicit feeding in rats, suggesting stimulation of CNS mGluRs may elicit feeding (Ploj et al. 2010). While previous data suggest a role for brain mGluR5 in eating control, the present study provides two lines of pharmacological evidence that Group I mGluRs in the LH are involved in mediating feeding. Specifically: 1) LH injections of ACPD, DHPG and CHPG, three chemically different agonists which act in distinct way on mGluRs all elicit feeding, and 2) LH injection of PHCCC, YM-298198 and MTEP, three chemically distinct mGluR antagonists suppress or block the agonist-elicited feeding. This evidence supports a role for mGluR involvement in feeding within the CNS, specifically through the LH. Furthermore, the larger feeding responses produced by agonists which act on multiple mGluRs suggest that feeding may be elicited by concurrent stimulation of multiple subtypes of mGluRs.

Which mGluR subtypes mediate feeding in the LH?

Our evidence suggests eating can be elicited by activation of either LH mGluR1 or mGluR5, with activation of both receptors combining to produce the largest effect. The initial support for the possibility that one or both Group I receptors is involved in feeding

was that unilateral LH injection of DHPG, a mGluR1 and R5 agonist, produced a strong eating stimulatory effect within one hour, which was blocked by pretreatment with PHCCC, a mGluR Group I antagonist (Fig. 2.3). The effectiveness of PHCCC in blocking the elicited feeding by DHPG (Fig. 2.5) strongly argues that eating by DHPG is mediated in a receptor specific manner, rather than by a non-specific chemical action, such as a change in local osmotic pressure or pH.

As for the specific involvement of the mGluR5 subtype in feeding, first we demonstrated that CHPG, a mGluR5 agonist, stimulated eating when administered within the LH (Fig 2.4). Additional support for brain mGluR5 in eating stimulation was provided by (Ploj et al., 2010) demonstrating intraventricular injection of the mGluR5 agonist, CHPG, induces feeding. Although CHPG has been classified as a mGluR5 specific agonist (Doherty et al., 1997 and Ploj et al., 2010), a recent report indicates that CHPG may activate both mGluR1 and mGluR5 (Kammermeier 2012). Second, we showed that pretreatment with LH injection of the mGluR5 antagonist MTEP suppressed eating elicited by subsequent LH injection of DHPG (Fig 2.6). More generally, blockade of mGluR5 receptors, via intraperitoneal MTEP injection, has been shown to prevent food-seeking (Eiler et al., 2011), nicotine-seeking (Tronci et al., 2010) and ethanol-seeking (Kelley and Berridge, 2002; Backstrom et al., 2004; Cowen et al., 2005) behaviors, suggesting a motivation-controlling role for mGluRs as well. Further, Bradbury et al (2005) have shown mGluR5 gene knockout mice are lighter than their wild type

counterparts and peripheral injection of MTEP suppressed eating in wild type but not mGluR5 knock-out mice.

As mGluR5 are widely distributed throughout the brain (Shigemoto et al 1992), brain areas other than the LH may contribute to the overall influence of mGluRs on feeding. The lower food intake produced by LH injection of CHPG may be attributed to activation of mGluRs specific to LH neurons, whereas the larger food intake seen by Ploj et al (2010) may have been due to activation of other brain regions, such as the nucleus accumbens, along with or aside from the LH.

Our data also indicate that eating can be produced by activation of the mGluR1 subtype. Specifically, the effectiveness of LH pretreatment with the mGluR1 antagonist, YM-298198, in suppressing eating elicited by DHPG, suggests that mGluR1 within the LH may influence eating (Fig 2.7). Dose response relationships were not always robust and the underlying reasons are unclear at this time. Supporting physiological roles for LH mGluR1 and mGluR5 is evidence the LH contains significant quantities of mGluR1 and mGluR5, with mGluR1 being present in the highest concentrations (Shigemoto et al 1992, 1993). The convergent evidence by stimulation of feeding with DHPG, and the reduction of elicited feeding by mGluR1 and mGluR5 antagonists, suggests that the observed feeding was mediated by mGluRs, specifically the R1 and R5 subtypes.

In addition to mGluR1 and mGluR5 subtypes involvement in eating stimulation, we have evidence suggesting that Group 2 mGluRs might contribute. Specifically, the combined group 1 and group 2 agonist ACPD produced substantially larger feeding

responses at shorter latencies than the group 1 specific agonist DHPG. In particular, most of the ACPD-elicited eating response occurred within 15 min of injection, while DHPG rarely elicited any feeding response until 1 hour of injection (Fig 2.2). These differences suggest that activation of multiple mGluRs may produce more profound effects, or even effects of a different nature, than activation of single mGluR subtypes and this may be physiologically relevant, as glutamate binds to all subtypes of these receptors (Conn and Pin, 1997). In addition, rather than exciting LH neurons directly, as with Group I mGluRs, other mGluR groups may influence LH neurons in a different or even opposite manner. For example, orexin neurons which are specifically localized within the LH and are highly involved in motivated feeding (Cason et al., 2010) have been suggested to be tonically inhibited by group III mGluRs (Acuna-Goycolea et al., 2004).

What is mGluRs involvement in Natural Feeding?

mGluRs role in natural feeding remains unclear. In this study, bilateral LH injection of mGluR antagonists did not suppress deprivation-induced or nocturnal feeding (Table 2.1 and 2.2), which might imply that mGluRs in the LH do not mediate these forms of natural feeding. While possible, this seems unlikely given: Glutamate is released within the LH during the course of a meal (Rada et al., 1997), LH injection of glutamate or NMDA elicited eating (Duva et al., 2002, Stanley et al., 1993a, 1993b), LH injection of NMDA receptor antagonists markedly suppress natural feeding (Stanley et al., 1996), mGluRs are abundant in the LH (Meeker et al., 1994, van den Pol 1994, and

van den Pol et al., 1995), and that LH injections of mGluR agonists elicit feeding. Given the likely involvement of LH mGluRs in feeding and the evidence for NMDA receptors mediation of natural feeding, it may be interesting to note that the mGluRs may be providing an indirect contribution toward activation of ionotropic receptors, such as NMDA receptors. For example, mGluR5 is associated with potentiation of NMDA receptor-mediated currents and Huang and van den Pol (2007) demonstrated that activation of mGluR5 can modify NMDA synaptic currents within LH melanin-concentrating hormone (MCH) neurons. Whereas stimulation of mGluR1 leads to other intracellular effects (Mannaioni et al., 2001, Jones and Headley, 1995, Doherty et al 1997). Such modulatory effects of mGluR activation may contribute to the delayed eating responses elicited by LH injection of DHPG and CHPG and to the failure of LH injections of mGluR antagonists to suppress the forms of natural feeding we tested.

Perspectives

These findings implicate mGluRs in feeding. A number of other amino acid and peptide neurotransmitters, such as GABA, orexin, and NPY have also been implicated in LH feeding mechanisms (for review see Meister 2007). However, as the dominant excitatory neurotransmitter in the LH (van den Pol and Trombley 1993) glutamate may play a prominent role. Understanding mGluRs involvement in LH control of food intake may generate new insights about the underlying mechanisms and also lead to new

therapeutic applications for obesity, as approximately 30% of all current commercially available drugs target G-protein coupled receptors (Groom and Hopkins, 2002).

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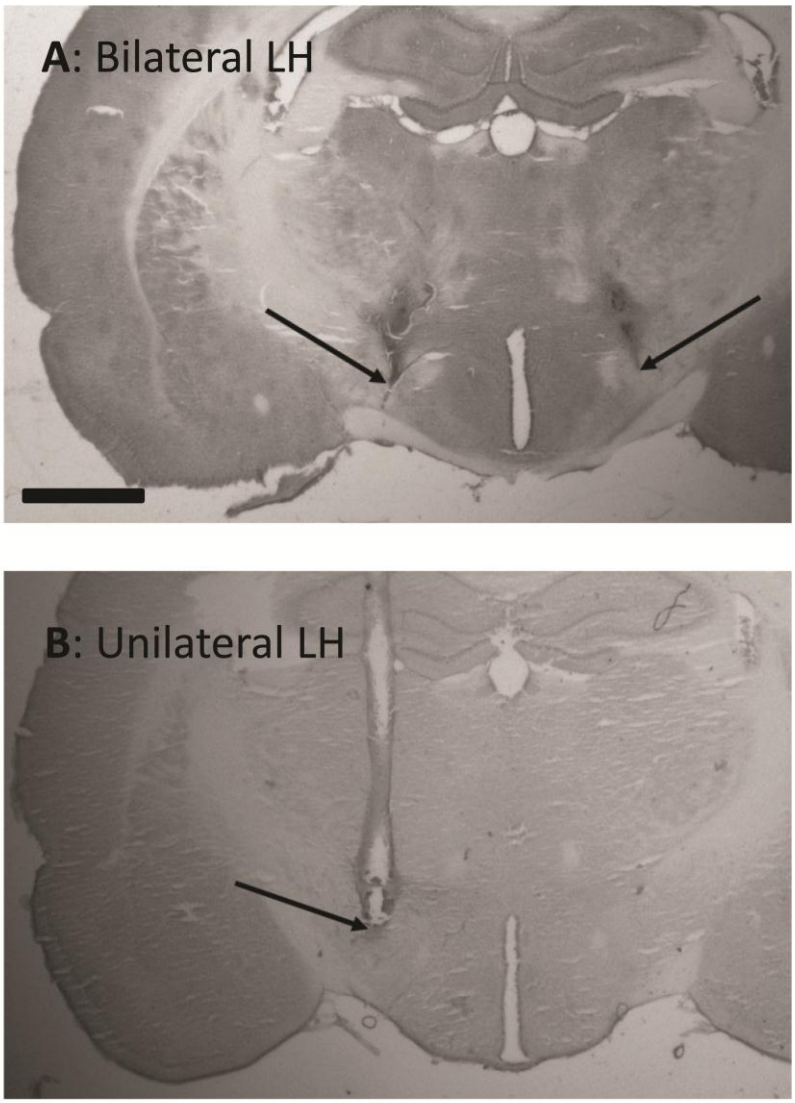

Figure 2.1. Photomicrographs of cannula placements. (A) Bilateral cannula tracks within the LH. (B) Unilateral cannula track within the LH. Scale bar is 2mm.

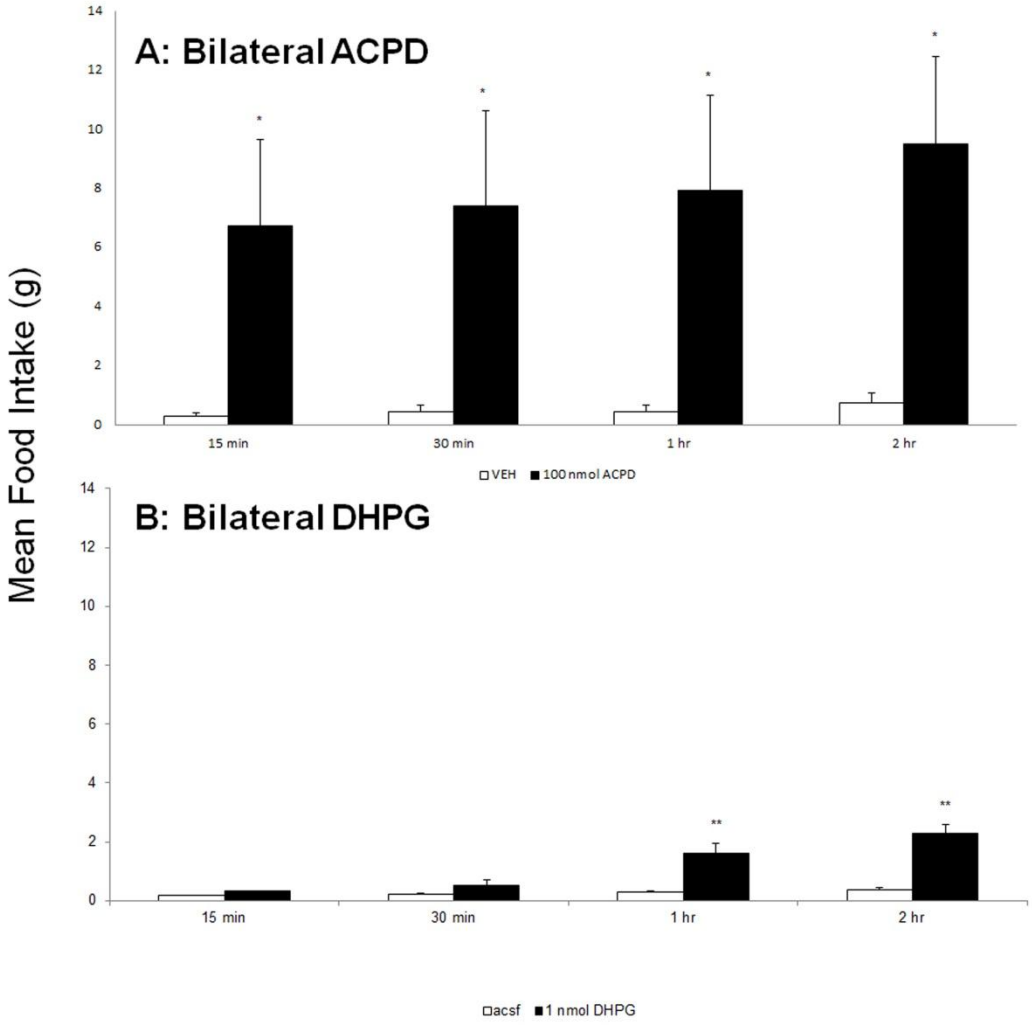

Figure 2.2. Bilateral administration of group I/II mGluR agonists induced food intake. (A) Satiated animals began feeding within 15min of ACPD (100 nmol). n=8.(B) Injection of DHPG (1 nmol) elicited feeding after 1hr. n=6. Cumulative food intakes ± SEM(*p<0.05,**p<0.01).

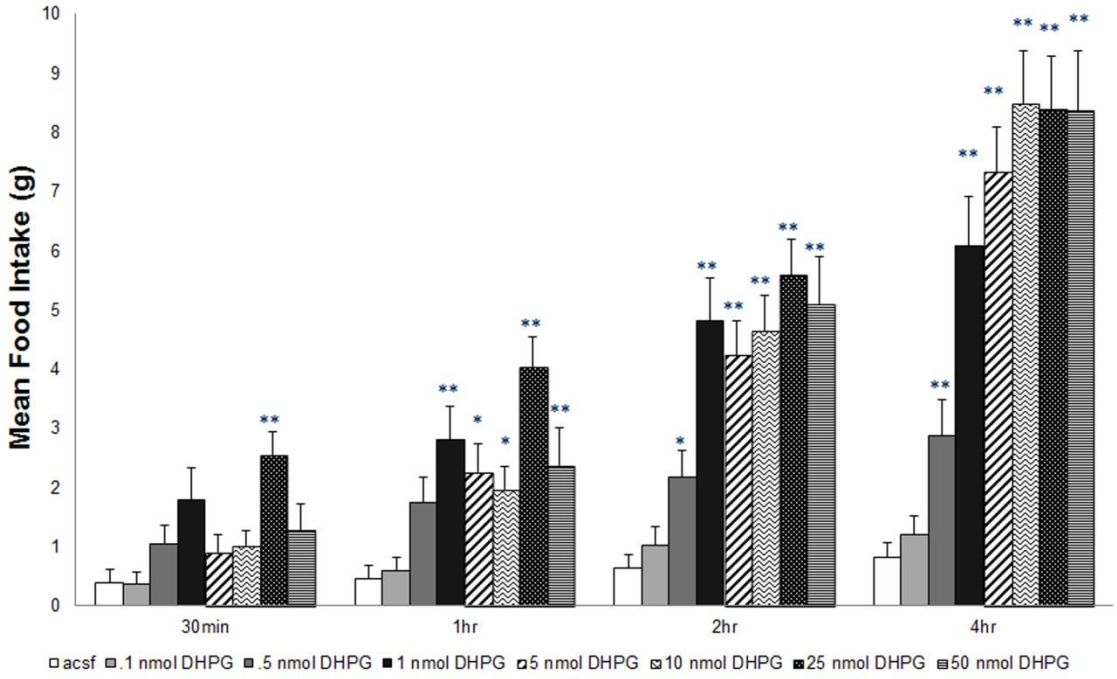

Figure 2.3. DHPG dose-dependently elicited significant food intake within 1 hr of injection, with further increases at subsequent times. n=11. Cumulative food intakes ± SEM (*p<0.05,**p<0.01).

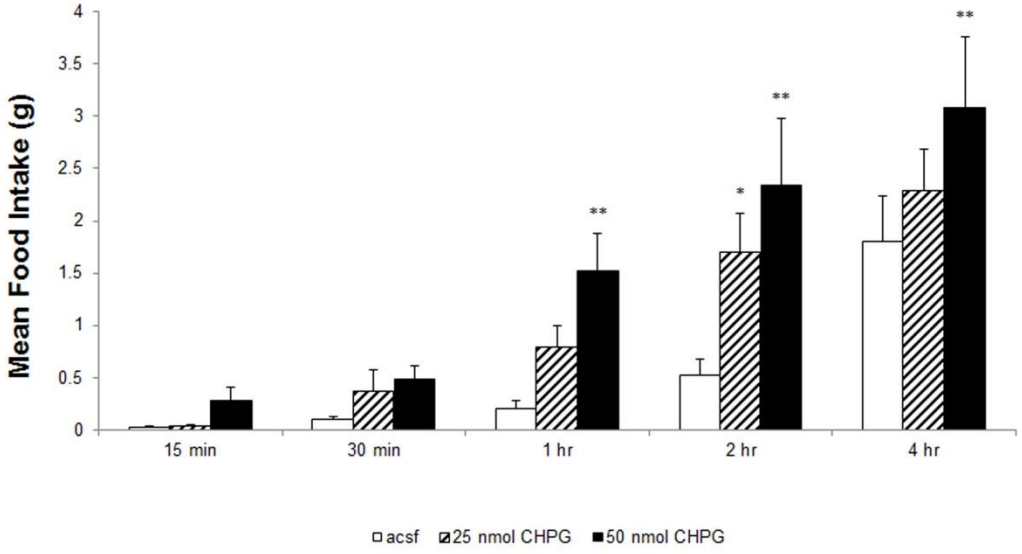

Figure 2.4. CHPG, an mGluR5 agonist, significantly increased food intake. n=19. Cumulative food intakes ± SEM (*p<0.05,**p<0.01)

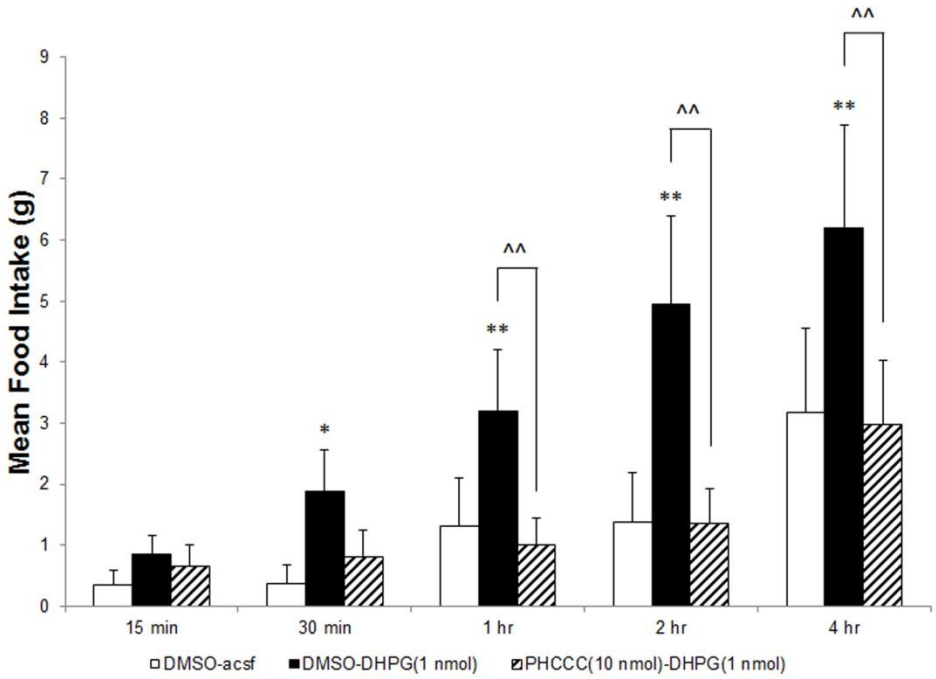

Figure 2.5. The broad group I mGluR receptor antagonist, PHCCC, blocked DHPG induced feeding. n=7. Cumulative food intakes ± SEM (*p<0.05,**p<0.01; by Fisher LSD post hoc test compared to ACSF controls; ^^p<0.01).

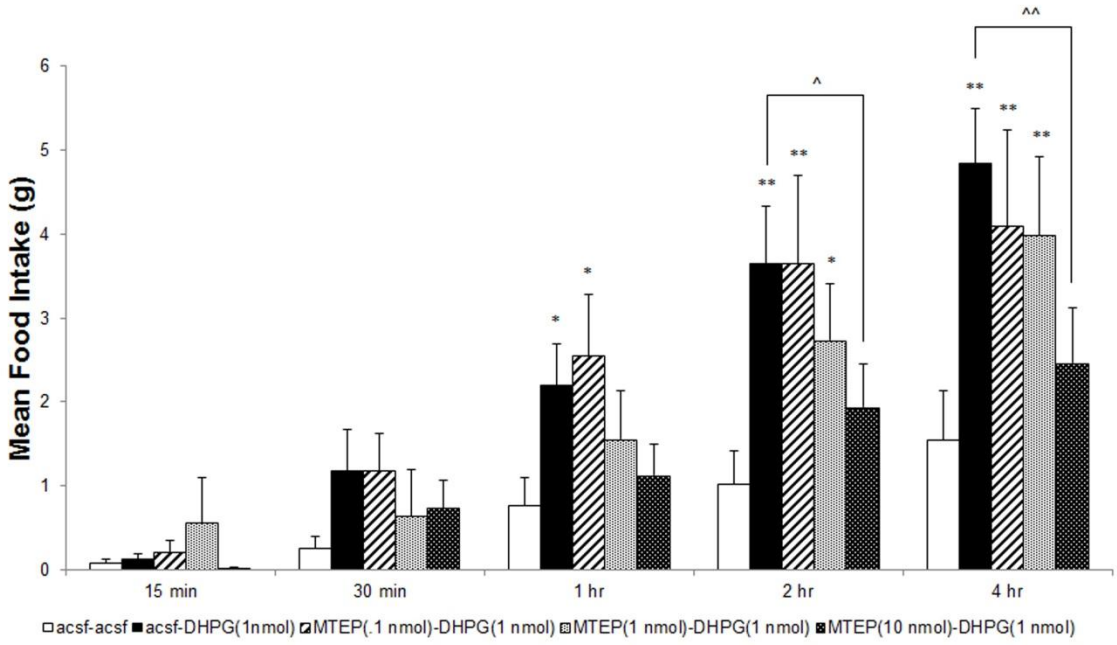

Figure 2.6. Pre-treatment with MTEP, an mGluR5 antagonist, suppressed DHPG induced food intake. n=11. Cumulative food intakes ± SEM (*p<0.05,**p<0.01; by Fisher LSD post hoc test compared to ACSF controls; ^p<0.05, ^^p<0.01).

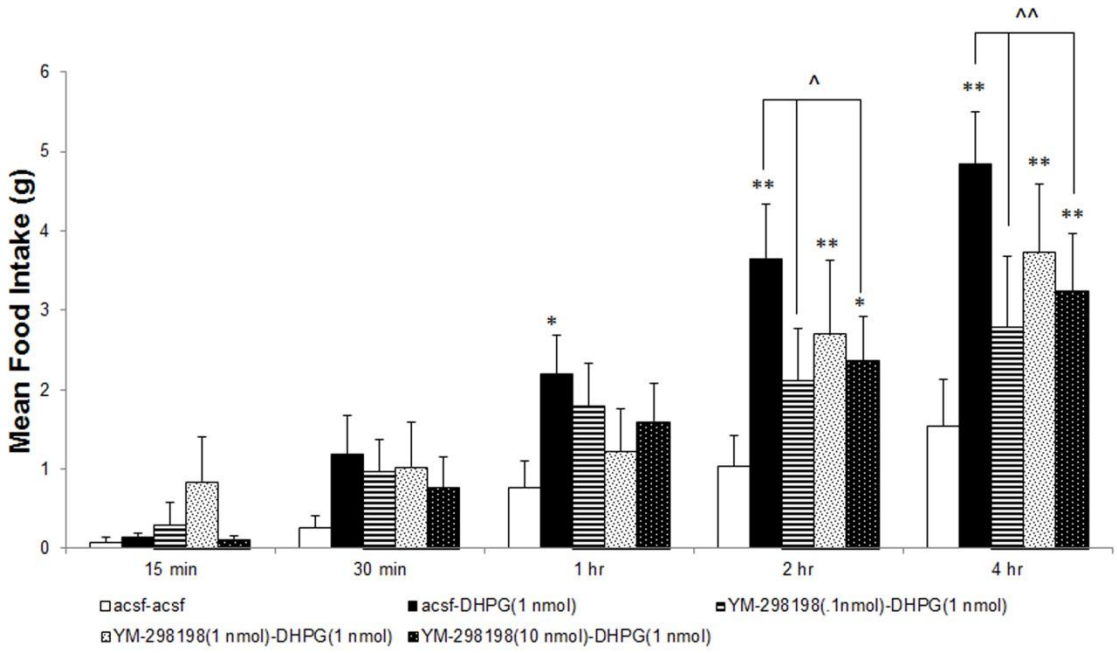

Figure 2.7. Pre-treatment with YM 298198, an mGluR1 antagonist, suppressed DHPG elicited food intake. n=11. Cumulative food intakes ± SEM (*p<0.05,**p<0.01; by Fisher LSD post hoc test compared to ACSF controls; ^p<0.05, ^^p<0.01).

Table 1.

Antagonism of LH group I mGlu receptors does not affect nocturnally driven food intake.

Time post injection (min)	Food intake										
	PHCCC			MTEP				YM-298198			
	Vehicle	1nmol	10nmol	acsf	0.1nmol	1nmol	10nmol	acsf	0.1nmol	1nmol	10nmol
15	2.4 ± 1.1	2.1 ± 0.9	1.9 ± 0.6	4.2 ± 0.9	2.3 ± 0.6	3.3 ± 0.7	4.5 ± 1.7	4.2 ± 0.9	4.4 ± 1.0	3.7 ± 1.4	2.3 ± 0.7
30	3.0 ± 1.2	2.2 ± 0.9	2.5 ± 0.8	4.6 ± 1.2	3.1 ± 0.6	4.0 ± 0.8	4.9 ± 1.7	4.6 ± 1.2	4.5 ± 1.0	3.7 ± 1.0	4.0 ± 0.8
60	3.9 ± 1.3	4.0 ± 0.9	3.7 ± 1.1	5.0 ± 1.2	4.2 ± 0.6	5.1 ± 1.0	5.3 ± 1.6	5.0 ± 1.2	5.0 ± 1.1	5.4 ± 1.1	4.5 ± 0.9
120	8.1 ± 1.8	5.6 ± 1.1	6.7 ± 1.5	7.9 ± 1.2	7.4 ± 1.3	7.3 ± 0.9	8.7 ± 1.7	7.9 ± 1.2	8.9 ± 1.5	8.0 ± 1.0	8.1 ± 1.0
240	11.3 ± 1.5	10.2 ± 1.0	11.2 ± 1.5	12.7 ± 1.4	11.2 ± 1.6	11.4 ± 0.8	13.2 ± 1.6	12.7 ± 1.4	12.5 ± 1.9	14.4 ± 1.7	11.7 ± 1.4

Table 2.1. Cumulative food intakes (mean ± SEM in grams) 15, 30, 60, 120, and 240 min post-injection in subjects at the nocturnal phase of the light:dark cycle.

Table 2.
Antagonism of LH group I mGlu receptors does not affect food deprivation induced food intake.

Time post injection (min)	Food intake		
	PHCCC		
	Vehicle	1nmol	10nmol
15	4.2 ± 0.6	3.1 ± 0.8	3.2 ± 0.4
30	10.8 ± 1.0	8.8 ± 1.4	9.4 ± 0.9
60	13.2 ± 0.8	11.5 ± 1.4	11.4 ± 1.4
120	15.0 ± 1.2	14.0 ± 1.5	13.8 ± 1.4
240	16.1 ± 1.0	14.3 ± 1.6	14.7 ± 1.5

Table 2.2. Cumulative food intakes (mean ± SEM in grams) 15, 30, 60, 120, and 240 min post-injection in subjects fasted for 24 hr. MTEP and YM-298198 were not tested as PHCCC was ineffective at preventing food intake during the food deprived condition in addition to the previous nocturnal condition.

All efforts in the preceding experiments were made to minimize animal suffering. Experiments were approved by the institutions Animal Care and Use Committee.

Chapter 3 - Site selective activation of lateral hypothalamic mGluR1 and R5 receptors elicits feeding in rats.

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Abstract

Recent findings from our lab indicate that metabotropic glutamate receptor (mGluR) activation elicits eating, and the goal of the current study was to specify whether the lateral hypothalamus (LH) is the actual brain site mediating this effect. To examine this issue we injected the selective mGluR group I agonist (*S*)-3,5-dihydroxyphenylglycine (DHPG) unilaterally into the LH and surrounding regions (n=5-8 subjects/brain site) of satiated adult male Sprague-Dawley rats and measured elicited feeding. We determined that 1.0 nmol elicited food intake only within the LH. Increasing the dose to 10 or 25 nmol produced a more sustained effect in the LH, and also elicited eating in several other brain sites. These results, demonstrating that the LH mediates the eating elicited by low doses of DHPG, suggest that the LH may contain mGluR whose activation can produce eating behavior.

Introduction

A convergence of classical and recent evidence reveals that the LH is an important link in brain mechanisms of food intake control (Berthoud and Münzberg 2011, Jennings et al 2013). Glutamate within the LH appears to play an important role as a feeding stimulatory neurotransmitter. Within the LH increases in extracellular glutamate coincide with feeding, with glutamate levels in fasted rats rising in the LH prior to eating a meal and then declining rapidly as the animal eats and becomes satiated (Rada et al 1997). Glutamate has been localized in synaptic terminals of the LH (Van den Pol and Trombley 1993) and many LH neurons are known to possess several types of glutamate receptors, including n-methyl-d-aspartate (NMDA), 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid (AMPA) and kainic acid (KA). We have shown that LH injection of glutamate, as well as NMDA, AMPA or KA receptor ligands elicit food intake in a dose-dependent fashion (Stanley eta l 1993a, Stanley et al 1993b) and that LH injection of NMDA receptor antagonists suppress natural feeding (Stanley et al 1996). This indicates that glutamate plays a crucial role in inducing food intake.

While a role for LH ionotropic glutamate receptors in the induction of feeding has been clearly established, a role for metabotropic glutamate receptors has received little attention. MGluRs are widely distributed in the mammalian brain, each having distinct effects on behavior. Focusing on the particular contribution of group 1 mGluRs, mGlu1R and 5 receptors, within the hippocampus group 1 mGluRs have been linked

with long term potentiation (LTP) and memory formation (Mukherjee and Manahan-Vaughan 2013), while in the frontal cortex they have been associated with schizophrenia-like symptoms (Herman et al 2012). Within the LH the relative density of glutamate receptors is: NMDA receptors have the highest density, followed by mGluRs, while AMPA/KA receptors have the lowest density (Meeker et al 1994). The relatively high densities of mGluRs suggest functional roles within the LH.

Anatomical studies indicate that mGluR1 and mGluR5 are present within the LH (van den Pol 1994, van den Pol et al 1995). However, little research has examined the functionality of these receptors, much less the possible contribution of group I mGluRs in food intake. Mice with the mGluR5 gene knocked out show deficits in body weight (Bradbury et al 2005), which has since sparked interest in the mechanisms in which mGluRs may act to modulate body weight.

Initial findings from our lab suggest that Group I mGluR activation within the LH can initiate food intake. Significant increases in food intake were observed with both 1 nmol and 25 nmol doses of DHPG. This agonistic induction of food intake could also be suppressed using mGlu1 and 5 receptor antagonists, suggesting a role for LH mGluRs in regulating feeding (Charles et al 2014). Although this evidence may suggest that feeding is elicited by activation of group I mGluRs within the LH, the anatomical specificity of such effects has not been addressed and the injected agents may have acted in sites other than or in addition to the LH to stimulate eating behavior. Agonists may diffuse and thereby produce behavioral effects not specific to the LH or may even enter the

ventricles and diffuse throughout the brain. Therefore it is important to identify the anatomical location which may be susceptible to mGluR activation of food intake. The purpose of this study is to determine whether mGluR-induced feeding is limited to the LH.

Materials and Methods

Subjects and stereotaxic surgery

Adult male Sprague-Dawley rats weighing 350-500 g at the time of surgery were used. These rats were bred in the University of California, Riverside, Psychology Department vivarium and were descended from rats obtained from Charles River, Inc. They were individually housed in a temperature controlled vivarium (21°C) on a 12:12 hr light:dark cycle, and allowed free access to standard Purina rat chow pellets and water.

Surgery for the implantation of an 18 mm long, 26 gauge (o.d.=0.46 mm) stainless steel indwelling guide cannula was performed under barbiturate anesthesia (Nembutal, 50 mg/kg body weight, i.p.). Cannulae were implanted into the LH or sites bracketing the LH by 1-2 mm. The stereotaxic coordinates for each site are in Table 3.1 with the incisor bar placed at –3.3 mm for all implantations. Cannulae were held in place by dental cement affixed to four stainless steel screws imbedded in the skull and protected with a plastic guard. To prevent clogging, a 33-guage stainless steel obturator was inserted into the cannula. Procedures were approved by the University of California

Riverside Institutional Animal Care and Use Committee. Three days prior to testing, the standard pellet food was replaced with a spill resistant and palatable milk-mash diet consisting of Purina rat chow (500g), sucrose (400g), and Carnation evaporated milk (354ml).

Central injections

Tests consisted of a single injection through the cannula into the target area, with cumulative food intake measured 0.5, 1, 2 and 4 hrs postinjection. Solutions were injected via a 33-gauge needle that projected 1.0 mm beyond the ventral tip of the cannula. Each injection consisted of a 0.3 µl volume bolus of either (*S*)-3,5-dihydroxyphenylglycine (DHPG) at varying doses or the control, artificial cerebrospinal fluid (aCSF). The aCSF consists of (in mM): Na+ (147), Cl- (154), K+ (3.0), Ca2+ (1.2) and Mg2+ (0.9). Injector tips were retained within the cannulae for 10 sec to allow for drug diffusion from the injector. On test days, rats were given fresh mash for at least one hour prior to testing. The drug doses ranged from 1 nmol to 25 nmol, based on the previously identified effective range (Charles et al 2014). Drug dilutions were prepared one day prior to the first test day and aliquots stored at -80 ^{o}C. For all experiments, control and drug injections were given in counterbalanced order across test days. Between tests, rats had at least one undisturbed day with ad libitum access to food and water unless otherwise noted.

Histology and statistics

After behavioral testing, all experimental animals were deeply anesthetized with Nembutal and perfused transcardially with 10% formalin. The brains were removed from the skull and placed in the same fixative for at least 24 h. Brains were frozen, 100 µm thick coronal sections were cut and mounted on polarized glass slides. Sections were Nissil stained and cover slipped for later examination. Injections sites were accurately determined by projecting the image onto illustrations from the rat brain atlas of Paxinos and Watson (2007). Placements further than 1 mm from the targeted brain site were disregarded. At the conclusion of the experiment a total of 36 animals were used.

Food intake data from subjects with injections in each brain site were analyzed using one-way and two-way ANOVA. When main effects were statistically significant by ANOVA, these were followed by Fisher's LSD (Least Significant Difference) tests with $p<0.05$ used as the minimal value of statistical significance in all tests.

Results

Experiment 1: Activation of LH mGluR Group I receptors elicits feeding.

Fig. 3.1 shows representative photomicrographs of the injection sites in each of the 6 targeted brain regions. These photomicrographs show that the injectors typically produced minimal apparent damage to the targeted site.

As shown in Fig. 3.2, within each region, all injection sites were tightly clustered in close proximity to each other. Specifically, LH injections sites were typically within the magnocellular nucleus of the lateral hypothalamus and the tuberal lateral hypothalamus (tuLH). The anterior injection sites were centered on the nucleus of the horizontal limb of the diagonal band (HDB). The medial injection sites were clustered near the ventral part of the dorsomedial hypothalamic nucleus (DMH). The lateral injection sites were clustered near the basolateral amygdaloid nucleus (AMY). The dorsal injection sites were located near the ventrolateral nucleus of the thalamus (THAL). The posterior injections, although more scattered than in other sites, were generally near the substantia nigra pars reticulata (SN) and within 0.5 mm of the posterior lateral hypothalamus. In the posterior SN region, there was some minor overlap between the posterior LH and the SN, as the SN in that region is relatively small (Fig 3.2A). Also illustrated in Fig. 3.2 is that the injection of 1 nmol DHPG into the LH elicited over 1 gm of food intake in 5 of the 8 individual subjects. In contrast, not a single subject injected with this dose of DHPG in any other brain area ate 1 g or more.

Fig. 3.3 shows that the low 1.0 nmol dose of DHPG elicit feeding only in the LH, while the higher doses were much less anatomically specific. At each time point two-way ANOVAs demonstrated significant effects of DHPG dose, site and a dose x site interaction (30 min, dose [$F_{3,143}$ =5.7,p<0.001], site [$F_{5,143}$ =5.4,p=0.001], dose x site [$F_{15,143}$ =2.2,p=0.008]; 1 hr, dose [$F_{3,143}$ =12.0,p<0.001], site [$F_{5,143}$ =5.0,p<0.001], dose x site [$F_{15,143}$ =3.5,p<0.001]; 2 hr, dose [$F_{3,143}$ =17.0,p<0.001], site [$F_{5,143}$ =7.9,p<0.001],

dose x site [$F_{15,143}$ =3.3,p<0.001]; 4 hr, dose [$F_{3,143}$ =29.5,p<0.001], site [$F_{5,143}$ =13.7,p<0.001], dose x site [$F_{15,143}$ =4.8,p<0.001]). As shown in the top panel (A) of Fig. 3.3, baseline eating after aCSF injection was low across all brain sites at all postinjection intervals, with mean cumulative intakes totaling less than 2 gm even 4 hrs postinjection. As shown in Figure 3.3B, DHPG at 1 nmol elicited significant feeding only when injected within the LH. Rats ate an average of 1.5 g within 30 min with a steady increase to 4.8 g at 4 hrs. For comparison, the mean cumulative food intake 4 hrs postinjection was a maximum of 1.3 g in the other injection sites. As shown in Fig. 3.3C, at 10 nmol of DHPG, food intake increased further with LH injections, and injections into the DMH and THAL also elicited significant feeding. At the 25 nmol dose of DHPG, there was less site specificity, with increases in feeding within the LH, SN, DMH and THAL but not with injections into the HDB or AMY (Figure 3.3D). As for behaviors other than feeding, the 25 nmol dose of DHPG within the SN induced hyperactive behaviors, wherein animals gnawed on the food bowl rather than feed on the mash and had increased locomotor activity, however these were not quantified.

Discussion

Previous studies focus primarily on ionotropic receptors, such as NMDA, AMPA and KA, in feeding stimulation (Stanley et al 1993a, Stanley et al 1993b, Stanley et al 1996, Stanley et al 2011). Here we provide evidence that activation of group I mGluRs,

specifically within the LH, stimulate feeding as well. These findings confirm our prior evidence (Charles et al 2014) suggesting that mGlu1 and 5 receptor activation within the LH elicits dose-dependent eating responses, and provide new data showing that injection into sites bracketing the LH is ineffective or less effective. Specifically, activation by the Group I mGluR agonist, DHPG, consistently induced feeding within the LH, while injection into other areas (i.e. HDB, DMH, AMY, THAL or SN) had no effect or elicited eating only at higher doses. As these bracketing sites are only 1-2 mm from the LH, these data strongly argue that mGlu1 and 5 receptors within the LH mediate the elicited feeding. The results from this study suggest that: 1) At low doses the LH is the major focus of mGluR feeding stimulatory effects and 2) The LH is but one of several sites capable of producing eating in response to mGluR activation. Each of these points will be addressed in order.

LH site sensitivity to Group I mGluRs

Mapping the hypothalamus with increasing doses of a mGluR group I agonist revealed site specific effects, with the LH being the most sensitive site for mGluR induced feeding. As injected substances can diffuse and produce behavioral effects at distant sites (Myers et al 1971), it is prudent to examine which brain area may actually be mediating the effect. As previous studies with mGluR5 agonist and antagonists used intracerebroventricular (i.c.v.) or intraperitoneal (i.p.) routes of injection, there is little

data to specify the brain sites mediating the elicited feeding (Bradbury et al 2005, Ploj et al 2010, Pan et al 2004). Mapping studies have shown that the LH is the most effective site for ionotropic glutamate receptor agonist or $GABA_A$ receptor antagonists to elicit feeding (Stanley eta l 1993a, Stanley et al 1993c, Duva et al 2002, Hettes et al 2003, Turenius et al 2009). As shown in Fig 3.2 of the present study, the LH was the most sensitive site tested for the group I mGluR agonist to induced feeding. Injections of 1 nmol DHPG specifically and consistently elicit feeding within the LH. In particular, injections tightly clustered in the LH consistently produced food intakes greater than 1g within 1 hr, while injection into the other tested sites failed to elicit eating. This is further demonstrated in Fig 3.3 which clearly shows that 1 nmol DHPG elicited feeding within the LH as compared with other brain sites and higher doses. This in agreement with the notion that drugs administered within the site of action should be effective at lower doses than those injected in adjacent tissue (Wise and Hoffman 1992).

Further support for LH site specificity was provided by our earlier finding (Charles et al 2014) that feeding was elicited by LH injection of CHPG, an mGluR5 and possibly 1R agonist (Kammermeier 2012). Earlier work had shown that i.c.v. CHPG elicits feeding (Ploj et al 2010). We observed a similar effect with LH injection of CHPG, however, LH CHPG induced feeding with shorter latencies and lower doses than i.c.v. injected CHPG (Charles et al 2014, Ploj et al 2010). In addition, LH administration of MTEP and YM 198298, mGluR5 and R1 antagonist respectively, suppressed DHPG induced food intake (Charles et al 2014) lending support to the idea that mGluRs mediate eating, as

previously published findings have indicated. Collectively, these findings suggest that the LH is a primary site of action for group I mGluR elicited feeding.

What are the mechanisms by which LH mGluR activation elicits eating? While it is expected that the mechanisms are multi-faceted, one possibility is that Group I mGluRs, may be acting on melanin concentrating hormone (MCH) containing neurons. MCH neurons have been implicated in feeding (Cone 2005) and the LH is virtually the sole site from which MCH neurons originate (Skofitsch et al 1985). In particular cultured MCH cells were depolarized in the presence of DHPG (Huang and van den Pol 2007) indicating that MCH cell express functional mGluRs. Huang et al. (2007), note that LH mGluRs may be influencing Orexin (OX) neurons as well. OX neurons originating from the LH have been shown to influence motivated feeding (Harris et al 2005, Williams 2014) and MCH and OX neurons are reciprocally connected (Guan et al 2002).

Thus far, Group I mGluRs have only been specifically associated with MCH and OX neurons within the LH, although it is likely that they are present within multiple types of neurons, and also likely that multiple types of LH neurons mediate the feeding stimulatory effects of mGluRs.

Broad distribution of feeding mGluRs

What mechanisms might account for the eating elicited by DHPG injected into sites other than the LH? More simply, was the eating produced by injection of DHPG

into areas such as the DMH, THAL, AMY, HDB and SN, the result of DHPG acting on mGluR within each of those specific brain regions or instead the result of DHPG diffusion to other regions, specifically the LH?

Intracranial injections are a pharmacologically direct way of examining the relative contributions to a behavior of specific receptors within a neural circuit in an awake, freely moving animal. Figs 3.1 and 3.2 indicate the degree of placement accuracy that was achieved with this technique. However, a limitation is the possible diffusion of a compound into nearby regions, which may be the actual site from which the behavior was elicited. Diffusional spread is affected by multiple factors, such as injection volume (Myers 1966) osmolarity and concentration (Bondareff et al 1970). Small injection volumes may minimize nonspecific drug site effects (Stanley et al 1993c). With time however post-injection drug diffusion increases and studies show that with increasing distance the drug concentration drops dramatically (Myers et al 1971, Stanley et al 1993c). Most relevant to the current study is that diffusion away from the injection site occurs with injections into the LH (Myers et al 1971). While, the specific diffusional properties of DHPG within the LH are unknown, these previously noted studies provide useful parameters. The minimum dose effective in the LH is 1 nmol, so if the LH is the actual site of action it stands to reason that injections of this dose outside of the LH will not activate LH neurons, as the drug concentration needed to elicit a response will not be reached. Administering concentrations larger than 1 nmol into surrounding regions increases the probability that agonist concentrations sufficient to elicit eating will be

reached in the LH, as was observed with the higher DHPG doses (Fig 3.3 C, D). Therefore, diffusion of DHPG to the LH may contribute to the feeding produced by injection of the higher doses into sites surrounding the LH. In addition, all tested areas show similar expression of mGluR5, with the HDB, AMY and DMH having lower concentrations of mGluR1 than the LH, SN, and THAL (Shigemoto and Mizuno 2000) indicating that all of the investigated regions have the same capacity for Group I mGluR binding, but the LH alone elicited feeding at a low dose.

Interestingly, each of the areas in which high doses of DHPG elicited eating also project with varying degree to the LH, in particular the tuLH (Duva et al 2005). The possibility that DHPG elicited eating by stimulating these anatomical projections to the LH cannot be excluded, especially as evidence exists that, for example, DMH neurons may participate in feeding control mechanisms (Yang et al 2009). As Duva et al (2005) has indicated the LH receives substantial projections from the THAL, it is likely that THAL relays activation to the LH. The feeding produced by SN injections might be partly due to general motor activation, as of group I mGluRs stimulation in this region may increase motor activity (Marino et al 2001). Rats administered 25 nmol of DHPG exhibited signs of hyperactivity and increased eating, whereas 1 nmol DHPG showed neither effect. Both diffusion of DHPG to the LH and to a lesser extent the activation of mGluRs within the SN, as well as the THAL and DMH, may have acted synergistically to produce the higher food intake observed.

Other mGluR effects

As mGluRs are expressed throughout the brain they likely have a myriad of effects (Shigemoto et al 1992, Romano et al 1995, Lavreysen et al 2004). MGluRs are present on astrocytes and other that inducing an influx of Ca^{2+}, their exact function is unknown. However, it has been suggested that they mediate reuptake transport of glutamate, and possibly glutamate release from glia (Bonansco et al 2011). As both post synaptic neurons and glial cells express mGluR1/5, there is no way at this point to differentiate their independent roles in feeding.

Our findings suggest that group I mGluRs, specifically within the LH, can elicit feeding. This may be of particular interest given the evidence that the LH is involved in Pavlovian food association learning (Petrovich and Gallagher 2007) and that mGluRs have been linked to long term potentiation (Mukherjee and Manahan-Vaughan 2013).

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Table 1				
Brain Area	n	Anterior (mm)	Lateral (mm)	Ventral/Injector target (mm)
LH	8	6.1	1.8	8.2 (9.2)
HDB	5	8.1	1.8	8.2 (9.2)
SN	6	4.1	1.8	8.2 (9.2)
DMH	5	6.1	0.8	8.2 (9.2)
AMY	6	6.1	3.8	8.2 (9.2)
THAL	6	6.1	1.8	6.2 (7.2)

Table 3.1. Anterior, to interaural line; Lateral, to midline; Ventral, to skull surface. Numbers in parentheses indicate the intended location of the injector which extended 1 mm beyond the cannula.

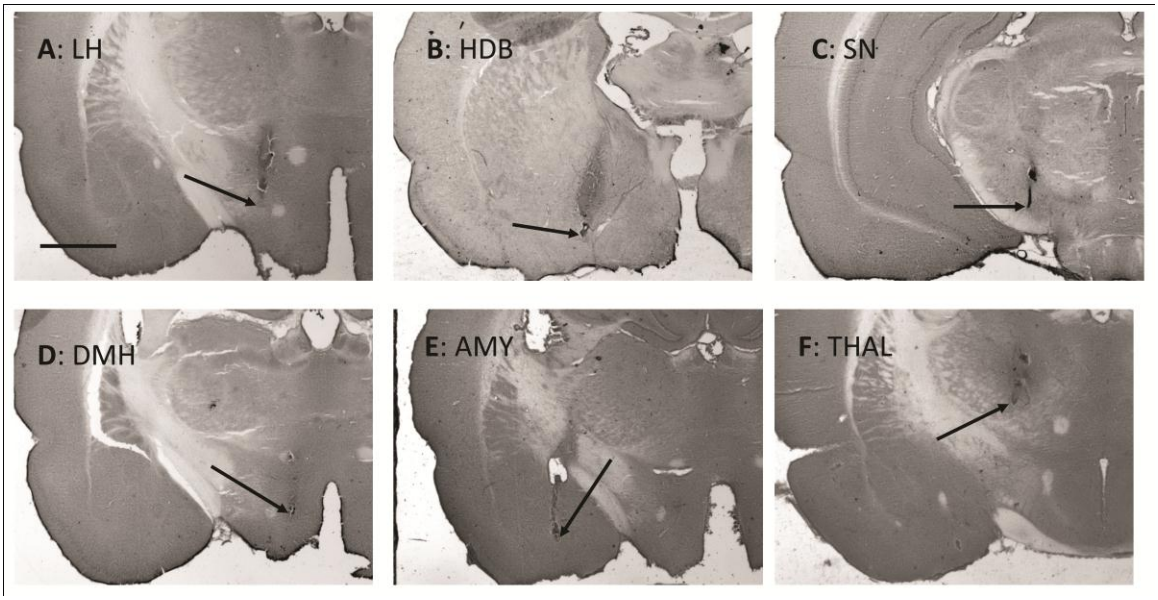

Figure 3.1. Representative photomicrographs of cannula placements. (A-F) Cannula tracks into the LH and five brain sites surrounding the LH, with injection sites indicated by arrows. (A) Lateral hypothalamus (LH). (B) Anterior target, Horizontal limb of the diagonal band (HDB). (C) Posterior target, Substantia nigra (SN). (D) Medial target, Dorsal medial hypothalamus (DMH). (E) Lateral target, Amygdala (AMY). Dorsal target, Thalamus (THAL). Scale bar is 2 mm.

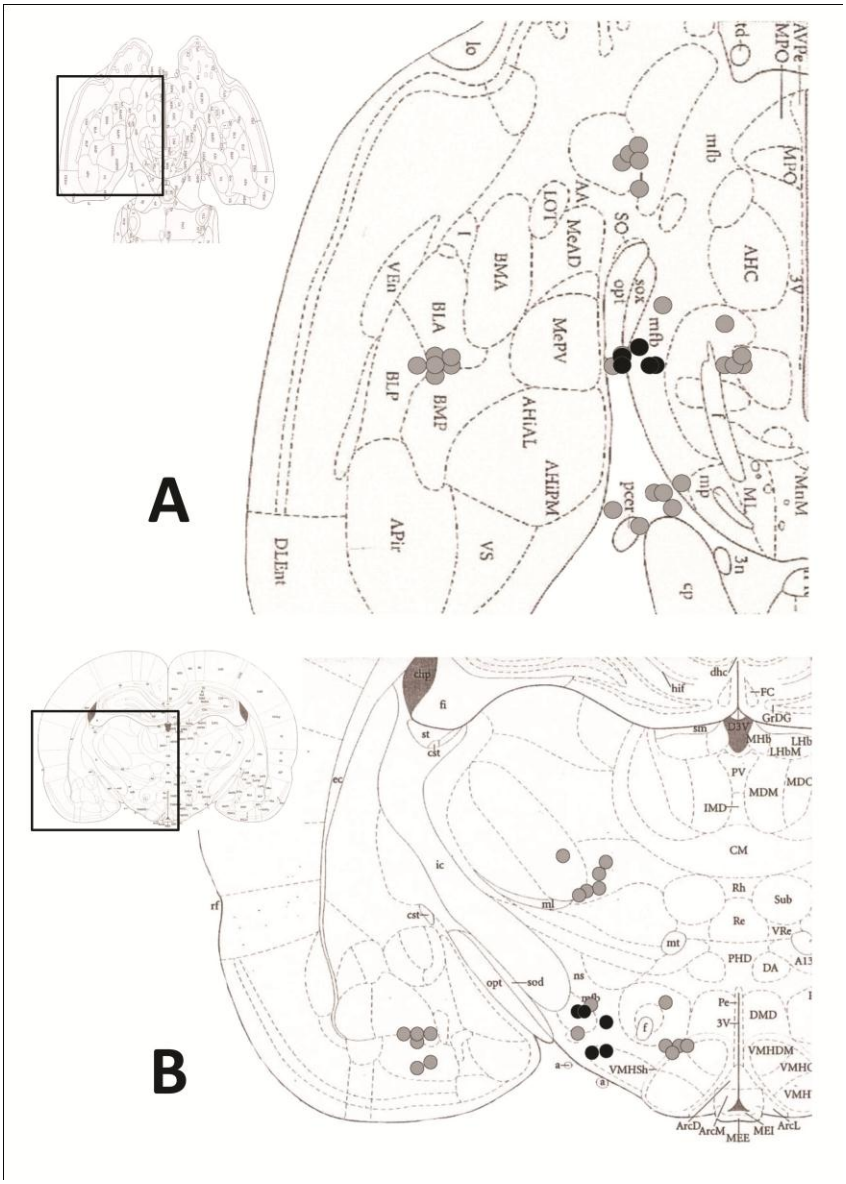

Figure 3.2. Schematic diagram relating food intake 1 hr after injection of 1 nmol of DHPG to injection site. Grey circles indicate food intakes less than 1 gram; black circles indicate food intakes greater than 1 gram (range 1.6 to 4.4 grams). For clarity, some symbols were moved 0.1 mm from the actual injection site. (A) Horizontal section at -8.82 mm below skull. Best possible cross sections. All injections hit targeted areas within 1 mm criteria. Substantia nigra injection sites out of plane. (B) Coronal section 6.08 mm anterior to interaural line. One of the LH injections was slightly more anterior than the other injections and not visible on this coronal section. Atlas sections adapted from Paxinos and Watson (2007).

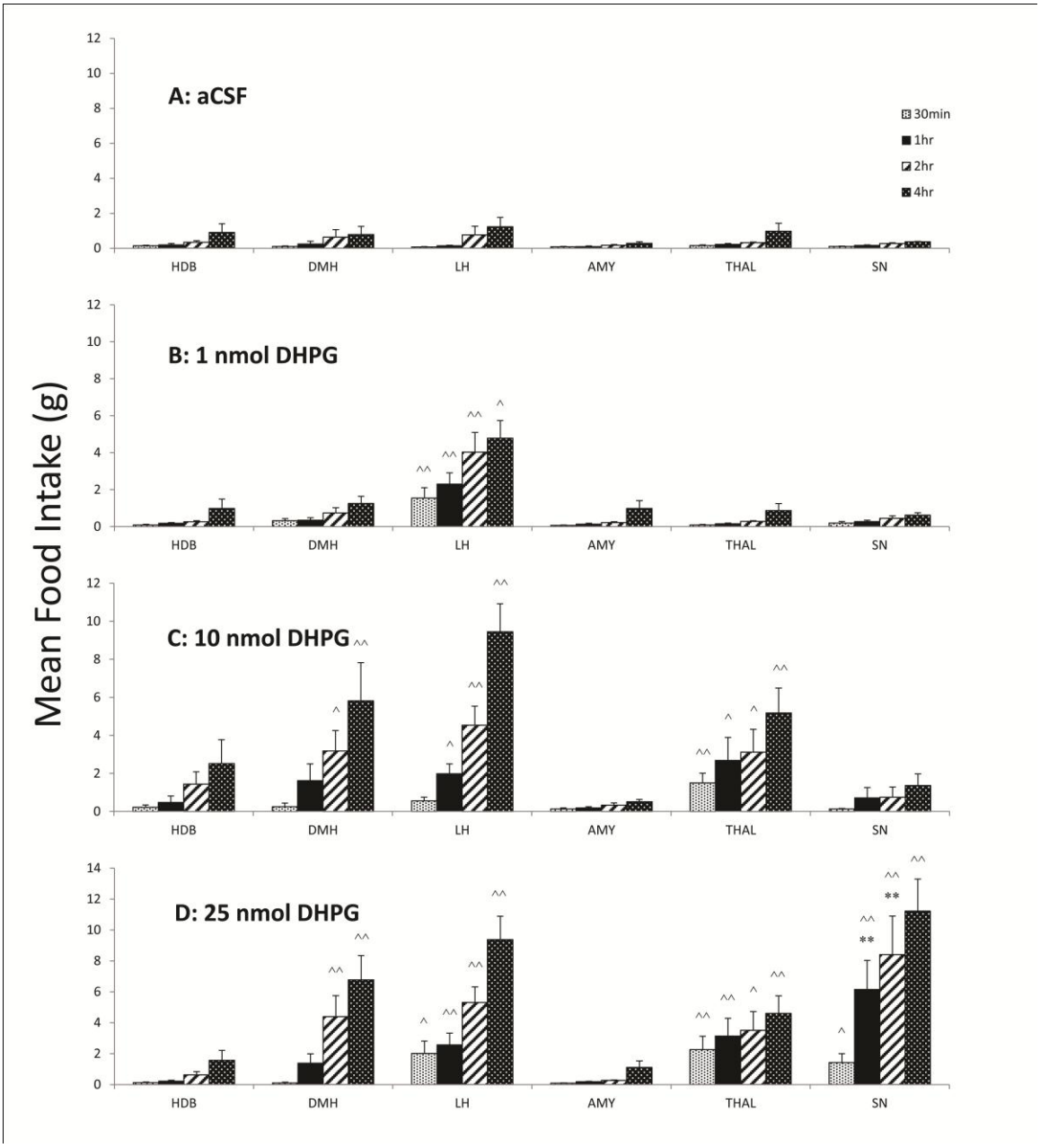

Figure 3.3. Cumulative food Intake as a function of injection site. At 1 nmol DHPG elicited feeding specifically within the LH. Bars represent mean ± SEM, cumulative food intake from 30 min to 4 hr after injection. Anterior [horizontal limb of the diagonal band (HDB)], Posterior [substantia nigra (SN)], medial [dorsal medial hypothalamus (DMH)], lateral [amygdala (AMY)] or dorsal [thalamus (THAL)] to the LH. **p<0.01; by LSD post hoc test LH compared to all other sites for matched treatment and time. ^p<0.05; ^^p<0.01 compared to aCSF control injection for matched site and time.

General Conclusions

Glutamate is one of the primary excitatory neurotransmitter within the mammalian nervous system and extensive research has focused on the involvement of ionotropic glutamate receptors in lateral hypothalamic control of food intake. Here we provide some of the first evidence that metabotropic glutamate receptors help mediate these effects as well. Using a variety of pharmacological tools we have shown a role for Group I mGluRs specifically in mediating food intake, and pointed to the LH as a major site of action. Activation of Group I mGluRs consistently induced an increase in food intake. As two general types of glutamate receptors are present within the brain, previous findings have focused primarily on the direct immediate effects by ionotropic receptors. Whereas the behavioral effects elicited by indirect modulation of mGluRs has only begun to unfold. The specificity of activating ionotropic receptors is also what could limit their potential usefulness. In trying to modify a neural circuit via NMDA, AMPA or KA receptor antagonism rapid, short term, even possibly excitotoxic effect may be seen. In a clinical perspective this may lead to severe side effects. Antagonism via mGluRs however, may lead to gradual, more long term effects leading to the increasing trend of g-protein investigation. A comparable analogy would be that instead of completely shutting off water flow with a dam (ionotropic antagonism), it would be more beneficial to have more of a gradual, sustained flow (mGluR antagonism).

It is also interesting to note that there is possibility that mGluRs may play role in food learning which may merit further investigation. The pharmacological manipulation

of such a system may lead to clinical applications. The precise physiological action of mGluRs is further convoluted by their abundant intracellular effects and presence on different neuronal populations as well as glia which were not tested here. Further studies with more precise techniques may aid in separating and clarifying these intricate functions.