Analysis of Ipsilateral Functional and Anatomical Associations Between the Nucleus Accumbens Shell and the Lateral Hypothalamus: Relations With Feeding Behaviors

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I dedicate this dissertation and the work within it to neuroscience and the ceaseless expansion of its research. I also thank the undergraduates, graduate students, and faculty at UCR and elsewhere who aided me in my research. Specifically, I thank my mentor/advisor, Glenn Stanley, for being there to provide answers but allowing me to take my own path. Thanks also go out to my other committee members, Kelly Huffman and Edward Korzus, whose courses have helped me diversify my knowledge. Further, I thank Khaleel Razak, Arshad Khan, and Richard Thompson, who had given essential the technical advice for tract tracing work and immunofluorescence staining. Lastly, I thank family, friends, and my partner in crime Andrew who have all supported my endeavors during the past 5+ years.

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ABSTRACT OF THE DISSERTATION

Analysis of Ipsilateral Functional and Anatomical Associations Between the Nucleus Accumbens Shell and the Lateral Hypothalamus: Relations With Feeding Behaviors

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The nucleus accumbens shell (AcbSh) and the lateral hypothalamus (LH) are associated in the control of feeding. Functional and anatomical evidence links these two areas in their control of food intake, and further studies implicate glutamate and GABA as major signaling factors in this circuit. However, it is unclear 1. whether inhibition of the LH decreases AcbSh-mediated intake in a behaviorally-specific manner, 2. if this behavioral specificity is regulated by an ipsilateral-only circuit, and 3. what neuron types send feedback signals from the LH to the AcbSh. In this dissertation, the background knowledge of these two areas and their roles in feeding and reward are reviewed. Then, in a series of studies, the three above issues are addressed. The AcbSh was inhibited by unilateral injection of muscimol or DNQX, and the LH was inhibited bilaterally, contralateral, or ipsilateral to the AcbSh injection site with D-AP5 or muscimol. Food intake and behaviors were monitored. Bilateral LH inhibition resulted in decreased AcbSh-mediated intake, but did so by inducing sleep. Contralateral LH inhibition had little effect on AcbSh mediated intake or behaviors, while ipsilateral LH inhibition decreased...
food intake by selective reducing feeding behavior only. In a separate group of rats, Fluorogold was iontophoretically injected into the AcbSh, and coronal sections with the LH were immunohistochemically stained for orexin, melanin-concentrating hormone (MCH), cocaine and amphetamine-regulated transcript (CART), nesfatin-1, vesicular GABA transporter (VGAT), vesicular glutamate transporters 2 and 3 (VGlut2 & VGlut3), glutamic acid decarboxylase (GAD67), and phospho-STAT3 (pSTAT3). LH neurons projecting to the AcbSh rarely were distributed in the ventrolateral LH, rarely co-localized with orexin, MCH, or pSTAT3 staining, occasionally co-localized with CART or VGlut3, and typically co-localized with nesfatin, VGAT, or GAD67. The body of evidence presented herein demonstrates a feeding-specific descending connection between the AcbSh and the LH, and with anatomical evidence suggests an inhibitory feedback circuit that may rely on decreasing food intake by acting on AcbSh sites that regulate aversive behaviors.
# Table of Contents

Chapter 1 – Introduction ................................................................. 1

Chapter 2 – Literature Review

Characterization of the Nucleus Accumbens and Its Involvement with Feeding ........................................ 4

Features of the Accumbens ........................................................................ 6

Accumbens Associations with Brain Areas ........................................ 12

Differences Between the Accumbens Shell and the Accumbens Core in the Regulation of Feeding ............ 14

Food Addiction via Dysregulation of Accumbens Shell Signaling – Clinical Implications ......................... 16

Characterization of the Lateral Hypothalamus and Its Involvement with Feeding ..................................... 18

Features of the Lateral Hypothalamus .................................................................................. 20

Lateral Hypothalamic Associations with Brain Areas ................................................................. 28

Dysregulation of Energy Balance via Hypothalamic Signaling Dysfunctions – Clinical Implications ......... 30

Implications of the Accumbens Shell - Lateral Hypothalamus Connection in the Specific Regulation of Feeding ........................................................................ 33

References ................................................................................................................. 37

Chapter 3 – Ipsilateral Feeding-Specific Circuits Between the Nucleus Accumbens Shell and the Lateral Hypothalamus: Regulation by Glutamate and GABA Receptor Subtypes

Abstract ................................................................................................................. 58

Introduction ............................................................................................................. 59

Methods .................................................................................................................... 61

Results ....................................................................................................................... 63

Discussion .................................................................................................................. 66
Chapter 4 – Behaviorally Specific Versus Non-Specific Suppression of Accumbens Shell-Mediated Feeding by Ipsilateral Versus Bilateral Inhibition of the Lateral Hypothalamus

Abstract........................................................................................................... 82

Introduction.................................................................................................. 83

Methods........................................................................................................ 85

Results........................................................................................................ 88

Discussion.................................................................................................... 93

References.................................................................................................... 100

Figures and Tables....................................................................................... 104

Chapter 5 – Distribution and Characterization of Ventral Lateral Hypothalamic Neurons Projecting to the Nucleus Accumbens Shell

Abstract........................................................................................................... 113

Introduction.................................................................................................. 114

Methods........................................................................................................ 116

Results........................................................................................................ 123

Discussion.................................................................................................... 126

References.................................................................................................... 130

Figures and Tables....................................................................................... 134

Chapter 6 – Conclusion ................................................................................ 143

References.................................................................................................... 145

Figure........................................................................................................... 146
List of Tables

Table 1.1 – Abbreviations Used in This Dissertation................................. 3
Table 3.1 – Injection Paradigms................................................................. 76
Table 4.1 – Injection Paradigms................................................................. 104
Table 4.2 – Food Intake Characteristics.................................................... 105
Table 5.1 – Brain Region Abbreviations.................................................. 134
List of Figures

Chapter 3

Figure 3.1 – Diagram of Central Injection Sites........................................... 77
Figure 3.2 – Histological Examples of Injection Sites................................. 78
Figure 3.3 – Food Intake for Bilateral LH Experiments (1A-D).................. 79
Figure 3.4 – Food Intake for Experiment 2A............................................. 80
Figure 3.5 – Food Intake for Experiment 2B............................................. 80
Figure 3.6 – Food Intake for Experiment 2C............................................. 81
Figure 3.7 – Food Intake for Experiment 2D............................................. 81

Chapter 4

Figure 4.1 – Food Intake for all Experiments.......................................... 106
Figure 4.2 – Behavioral Data for Experiment 1.......................................... 107
Figure 4.3 – Behavioral Data for Experiment 2.......................................... 108
Figure 4.4 – Behavioral Data for Experiment 3.......................................... 109
Figure 4.5 – Behavioral Data for Experiment 4.......................................... 110
Figure 4.6 – Diagram for Central Injection Sites...................................... 111
Figure 4.7 – Histological Examples of Injection Sites................................. 112

Chapter 5

Figure 5.1 – AcbSh Injection Sites.............................................................. 135
Figure 5.2 – Distribution of Retrograde Labeling in the LH....................... 136
Figure 5.3 – LH Injection Sites and AcbSh Anterograde Labeling.............. 137
Figure 5.4 – Orexin or MCH with Retrograde Labeling in the LH............. 138
Figure 5.5 – CART or Nesfatin with Retrograde Labeling in the LH......... 139
Figure 5.6 – GAD67 or VGAT with Retrograde Labeling in the LH.......... 140
Figure 5.7 – pSTAT3 or VGluT3 with Retrograde Labeling in the LH......141

Figure 5.8 – VGluT2- or VGluT3-Positive Neurons in other brain areas.................................................................142

Chapter 6

Figure 6.1 – Diagram of AcbSh-LH Neurocircuitry..............................................146
Chapter 1 - Introduction

Eating is considered a simple behavior by the general population. When hunger occurs, eating results; when satiety or nausea sets in, eating ceases and is avoided. However, the bodily signaling factors control eating are numerous and alarmingly complex. Many physiological factors, including but not limited to circulating blood glucose levels, bodily adiposity, gastric distention, and stress hormones, are integrated with motivational factors such as the rewarding properties of the food, ease of access, palatability, social incentives, and so on (Berthoud, 2002). All of these signals converge on the main processing center and output station of all behaviors: the brain.

The brain is one particular research and treatment target for modifying behavior. Of notable interest is the brain's control over food intake. The rise in obesity rates in the U.S. (Flegal et al., 2002) has driven research toward unraveling the brain's regulation of hunger and motivation to procure foods. Through this line of research, many brain regions have been associated with the control of what at first appears to be a simple behavior. One area that has received much focus over decades of research in the feeding field is the lateral hypothalamus (LH). This brain region, once dubbed the “hunger center” of the brain, was later found to be more of a signal-integrating hub for numerous and diverse neural inputs that regulate its output – the initiation of food procurement and consumption (Wise, 2013). Though other nuclei in the hypothalamus appear to also be associated with food intake, the LH is a major downstream target of many of these regions.

Functional studies of the LH have demonstrated how it controls feeding behaviors. One study utilized electrical stimulation of the LH in goats, which resulted in food consumption almost immediately (Wyrwicka and Dobrzecka, 1960). Lesions of the LH result in profound, sometimes lethal, aphagia, depending on the extent and cause of the lesion (Schallert and
Whishaw, 1978). Further insight was gathered from electrophysiological monitoring of LH neurons during certain food-related activities. Some LH neurons fire from the sight of food (Mora et al., 1979), others fire from decreases in blood glucose (Oomura et al., 1974), and others fire from food-associated cues (Mora et al., 1976). Consumption of food typically results in a decrease of LH activity (Mora et al., 1979). This collection of evidence demonstrates that the LH regulates feeding behaviors.

Another brain region that signals the LH, the nucleus accumbens shell (AcbSh), is classically associated with reward behaviors (Broekkamp et al., 1975). However, this region was later shown to influence feeding. Lesion of the AcbSh increases food intake, while stimulation decreases it (Ramaswamy et al., 1998, van der Plasse et al., 2012). Pharmacological AcbSh inhibition with GABA receptor agonists and certain glutamate receptor antagonists initiate feeding specifically (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1997; Stratford et al., 1998). Further, neurons in this region pause their firing patterns when drinking of a sucrose solution is initiated (Krause et al., 2010). Also, expression of genes associated with neurotransmitters that control feeding in the AcbSh are upregulated by food deprivation (Kelley et al., 2003). Thus, prior research has solidified the AcbSh's role in feeding behaviors.

Some research has linked the AcbSh and the LH in the role of feeding. Functional evidence describes how bilateral LH inhibition suppresses AcbSh-elicited intake (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1999). Also, unilateral AcbSh inhibition results in increased c-fos expression in the LH of the ipsilateral brain hemisphere (Stratford, 2005). Anatomical studies demonstrate, using anterograde or retrograde tracing, how the AcbSh sends projections to the LH (Duva et al., 2005; Usuda et al., 1998) and how the LH projects back to the AcbSh (Brog et al., 1993; Kampe et al., 2009). These studies suggest that the AcbSh relies on the LH to regulate its aspects of feeding, and that the LH can send feedback signals to the AcbSh.
However, there are some issues with the interpretation of data from food intake studies. Bilateral LH lesions cause deficits in movement and attention, not just feeding (Schallert and Whishaw, 1978). The same issues may temporarily occur from bilateral LH pharmacological inhibition. Thus, it cannot be confirmed that bilateral LH inhibition specifically halts AcbSh-mediated feeding as the aforementioned studies suggest. Further, it is unknown if bilateral LH inhibition results in the same behavioral deficits as seen from bilateral LH lesions. Lastly, the ascending LH to AcbSh projection has not been well characterized; its anatomical elucidation may clarify its role in feeding. In the series of studies described in this dissertation, the issues will be addressed using stereotaxic surgery, central injections of glutamate and GABA agonists and antagonists, intake and behavior monitoring, tract tracing, and immunofluorescence.

Table 1.1
Abbreviations used in this dissertation
Acb – nucleus accumbens
AcbC – nucleus accumbens core
AcbSh – nucleus accumbens shell
ACh – acetylcholine
AMPA – alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CART – cocaine and amphetamine regulated transcript
GABA – gamma-aminobutyric acid
GABA_A_R – GABA receptor subtype A
GAD – glutamic acid decarboxylase
ICSS – intracranial self-stimulation
LH – lateral hypothalamus
MCH – melanin-concentrating hormone
NMDAR – N-methyl-D-aspartate receptor
PFC – prefrontal cortex
VGAT – vesicular GABA transporter
VGluT – vesicular glutamate transporter
VMH – ventromedial hypothalamic area
VTA – ventral tegmental area

Note: Reference information for this chapter can be found starting on page 69.
Chapter 2 - Literature Review

Characterization of the Nucleus Accumbens and Its Involvement with Feeding

The boundaries of the nucleus accumbens and its subregions had only begun to be clarified in the latter half of the twentieth century. The term nucleus accumbens (Acb) was coined in the early 1900s, and despite much prior knowledge of the features of the globus pallidus and striatum, the accumbens and its subdivisions only began to resolve after the 1950s (Koikegami et al., 1967). What likely spurred the clearer parcellation of the Acb may have been functional evidence instead of anatomical data. It had been discovered that the Acb was a key site for intracranial self-stimulation (ICSS). One early ICSS study noted that rats highly preferred to press a lever that stimulated the septal region in the forebrain compared to stimulation of various other brain regions (Olds and Milner, 1954). This evidence suggested the involvement of the septal region (and neighboring areas such as the Acb) in the perception of reward.

Building off of this work, central injection studies showed that the Acb, and not the surrounding caudate-putamen, was found to be an effective brain site for self-administration of amphetamine (Hoebel et al., 1983). Further study using microdialysis showed that dopamine output increased dramatically in the Acb, far more than in other regions, when cocaine or amphetamine was injected via the dialysis probe (Hernandez and Hoebel, 1988; Hernandez et al., 1987). Around the same time, anatomical evidence revealed projection patterns that differed not only between the accumbens and the striatum, but also between subregions of the accumbens (Groenewegen and Russchen, 1984; Swanson and Cowan, 1975). These and many other studies at the time helped solidify the Acb as a functionally and anatomically distinct subdivision of the ventral striatum. Further, the idea of similarly distinct subdivisions within this nucleus was also presented.
The Acb had been then subdivided into the shell (AcbSh) and core (AcbC) subregions. A collection of evidence urged this division; differences existed in cytochemical characteristics, inputs and outputs, and functional properties (Brog et al., 1993; Deutch and Cameron, 1992; Groenewegen and Russchen, 1984; Jongen-Rêlo et al., 1994). Of particular note were the functional differences. Several studies determined that the AcbC was utilized for acquisition of reward-motivated behaviors, while the AcbSh was used for the execution of these behaviors (Corbit et al., 2001; Maldonado-Irizarry and Kelley, 1995; Parkinson et al., 1999). This distribution of functions between the two regions drove research to clarify effects that had only been mapped to the Acb as a whole.

Interestingly, the Acb was also associated with regulation of motivation to eat foods (Koob et al., 1978). The idea had been proposed due to the Acb having projections to limbic targets such as the hypothalamus, and also because of associations between drugs of abuse and disturbances in eating patterns and/or body weight regulation (Bendotti et al., 1986; Eichler and Antelman, 1979; Groenewegen and Russchen, 1984). Though prior studies posed that the Acb had some supplementary role in feeding via its reception of dopamine, its ability to directly initiate and drive feeding behavior was discovered years later by accident. In an attempt to disrupt reward-cue learning in the Acb via glutamate receptor blockade, Kelley and others found that the drug injections caused rats to eat significant amounts of food despite the rats being sated prior to the experiment (Kelley et al., 2005a; Maldonado-Irizarry et al., 1995). This finding opened the avenue for the role of the AcbSh in directly regulating feeding.

Those working with Kelley followed this line of research and tested various glutamate and GABA agonists and antagonists within the AcbSh in a variety of feeding conditions. The effects of both AMPA receptor (AMPAR) antagonism and GABA subtype A receptor (GABA$_A$R) activation were mapped to the AcbSh throughout its rostrocaudal extent (Maldonado-Irizarry et
al., 1995; Stratford and Kelley, 1997). Unexpectedly, activation or blockade of AcbSh NMDA or dopamine receptors had little effect on this phenomenon (Maldonado-Irizarry et al., 1995). It was thus suggested that glutamate input, likely from cortical areas, and GABA input, likely from subcortical areas, initiated or ceased feeding behaviors (Kelley et al., 2005a). There was correlational data from other labs to support this – glutamate levels fall within the Acb prior to meal onset (Rada et al., 1997). Further, pauses in Acb neural firing occur before intake of sucrose solutions (Krause et al., 2010). Thus, the Acb had a significant role in feeding, one which relied on changes in glutamate and GABA transmission far more than changes in dopamine input.

Features of the Accumbens

Behavioral Deficits from Accumbens Lesions

Initially, lesion research on the accumbens tended to follow two lines of methodology – specific destruction of dopaminergic fibers from the VTA, or traditional lesions (electrolytic or excitotoxic chemical-based). Dopaminergic fibers can be destroyed via site-specific injections of 6-hydroxydopamine (6-OHDA). The substance 6-OHDA is absorbed through dopamine transporters and destroys dopaminergic fibers/neurons through extensive oxidative stress and subsequent apoptosis (Glinka et al., 1997). As the mesolimbic system relies on secretion of dopamine to the accumbens, this tool was ideal for selective removal of that neurotransmitter from an otherwise intact system.

Accumbens lesions induced by 6-OHDA resulted in a subset of behavioral deficits. Rodents with this type of lesion exhibit hypoactivity in open field tests (Koob et al., 1981), generally reduced locomotion (Kubos et al., 1987), reduced exploratory and hoarding behavior (Choulli et al., 1987), and decreases in drug self-administration (Smith et al., 1985) and LH ICSS (Stellar and Corbett, 1989). Accumbal dopamine depletion also diminished the increased
locomotor and stereotyped behavioral responses seen in control rats from amphetamine administration. Further, these lesioned animals became highly sensitized to dopamine agonists compared to their sham-operated counterparts (Kelly et al., 1975). However, an issue with accumbens injection of 6-OHDA was that it destroyed norepinephrine-containing fibers as well (Carter and Pycock, 1980). Despite this projection degeneration, animals eventually recover by having re-innervation of any structures within which the dopamine (or norepinephrine) fibers were destroyed (Choulli et al., 1987). This specific type of chemical lesion revealed the role of the accumbens in various exploratory, locomotive, and reward functions.

Electrolytic and excitotoxic lesions to the accumbens have some different and some matching effects compared to effects seen from 6-OHDA lesions. Whole accumbens lesions appear to induce hyperlocomotion (Kubos et al., 1987) and prevent extinction in an operant task (Reading and Dunnett, 1991). Electrolytic or excitotoxic lesions to the AcbSh disrupt the integration of reward valuation and reward-seeking behavior (Albertin et al., 2000), impede latent inhibition (Tai et al., 1995), and induce overeating (Maldonado-Irizarry and Kelley, 1995; Ramaswamy et al., 1998). However, electrolytic lesions specifically to the AcbSh also appear to impede VTA stimulation-induced eating (Trojniar et al., 2007). Ablation of the AcbC reduces body weight (Maldonado-Irizarry and Kelley, 1995), impairs pre-pulse inhibition (Jongen-Rêlo et al., 2002), and decreases operant responses in a heroin self-administration paradigm (Alderson et al., 2001). Most interesting and relevant, lesions of the AcbSh or the AcbC had different effects on specific aspects of food anticipatory behaviors in rats under restricted feeding schedules. That specific study suggests that these two subregions work together to establish a “feeding rhythm” (Mendoza et al., 2005). In summary, the extensive accumbens lesion data demonstrates a partly dissociable set of functions for general accumbens activity, activity within specific accumbens subregions, and activity specifically regulated by dopamine input. Such evidence, in part, further
indicates the AcbSh's role in regulating food intake.

**Accumbens Activity and Manipulations**

Electrical stimulation of the accumbens results in a few behavioral effects that are mostly food or reward-based. Electrical stimulation halts feeding and/or curbs food reward (van der Plasse et al., 2012), decreases osmotic thirst (Szczepańska-Sadowska et al., 1978), increases sexual behaviors (Rodríguez-Manzo and Pellicer, 2010), and induces escape behaviors (Murer and Pazo, 1993). Other than accumbens stimulation translating to reward in certain contexts, the behavioral effects of its low intensity stimulation are minute in primates (Li et al., 2013). The lack of direct behavioral effects resulting from accumbens electrical stimulation may be due to the fact that it is an ensemble of neighboring circuits. Stimulation of all of them at once may result in non-specific signaling that the nucleus cannot translate to action (Pennartz et al., 1994).

However, the accumbens is one of many sites in the brain that promotes intracranial self-stimulation (ICSS). When an electrode is implanted into the accumbens and its stimulation is coincident with lever pressing, rats will lever press for it as they would for other rewards (Schaefer and Michael, 1992). This effect is intensified with amphetamine treatment and reduced by naltrexone treatment, indicating the involvement of dopamine and opioids in this reward circuitry (Phillips et al., 1975; West and Wise, 1988). Also, accumbens ICSS responding is differentially mediated by AMPARs, depending on the AMPAR's subunit composition (Todtenkopf et al., 2006); this evidence implicates glutamate neurotransmission to the accumbens in ICSS reward. Depriving rats of an accumbens ICSS testing session results in quicker responses when re-tested (similar to craving behaviors, described later) and progressive testing led to higher incidence and severity of seizures (a sensitization effect) (Jenkins et al., 1983). These features mimic those of drug abuse and to some degree “food addiction” (also described later) (Avena et
al., 2008a). However, accumbens ICSS may functionally dissociate from food intake - satiety does not decrease ICSS responding in monkeys (Mora et al., 1979). Accumbens ICSS may then be a reward-centric phenomenon not directly associated with food intake.

Accumbens neurons fire selectively, mostly to reward-related contexts. As expected, many neurons respond to the receipt of reward (Apicella et al., 1991a, 1991b). Some accumbens neurons fire prior to reward; this effect is not directly associated with planning or movement, and no longer occurs with repeated omission of a reward in the same testing context (Schultz et al., 1992). Accumbens neurons otherwise seem to have very varied responses to stimuli, motor control, and other behaviors not clearly directed toward a goal or reward (Pennartz et al., 1994). However, it was noted in more recent research that accumbens neuron pause their signaling before sucrose drinking commences (Krause et al., 2010). The above evidence insinuates that activity patterns of accumbens neurons are goal-directed, firing either in an anticipatory manner or in the procurement of rewards.

The immediate early gene protein product c-fos is typically used as a marker of neuronal activity (Dragunow and Faull, 1989). This marker suggests that certain neuron types had produced action potentials within the last 1-1.5 hours (MacDonald et al., 1993). The c-fos marker is expressed in the accumbens in a variety of situations, which include contextual fear (Beck and Fibiger, 1995), training for food rewards in operant tasks (Figlewicz et al., 2011), removal and subsequent return of rat pups to their mother shortly after birth (Fleming and Walsh, 1994), repeated amphetamine administration (Morshedi and Meredith, 2008), palatable meal-associated environments (Park and Carr, 1998), food hoarding (Yang et al., 2011), and re-exposure to drug reward after withdrawal and abstinence (Todtenkopf et al., 2002). Much of the c-fos upregulation seen in the accumbens is reward-based.

AcbSh c-fos increases from receipt of food reward, but this increase shifts over to the
AcbC as food reward cues are repeatedly presented (Schiltz et al., 2007). Meal patterns can entrain accumbens activity - rhythmic patterns in c-fos expression may be regulated by a clock gene peptide Per1 that is upregulated during regimens providing palatable foods at consistent times (Angeles-Castellanos et al., 2008). Further, in rats on a food-restricted schedule, c-fos expression increases in the AcbSh during feeding while expression in the AcbC increases in anticipation of food (Mendoza et al., 2005). Interestingly, food restriction-induced hyperactivity may alter accumbens reward circuitry, as animals under this regimen present increased c-fos that is diminished by access to sucrose solution (Duclos et al., 2013); in normal rats, appearance of sucrose reward normally increases c-fos (Figlewicz et al., 2011). These studies represent only a fraction of research assessing immediate early gene expression in the accumbens. However, from what can be deduced from these results, the AcbSh may respond to procurement of rewards while the AcbC responds to reward-related cues.

Central injection studies have shown a wealth of data indicating how various neurotransmitters can affect food intake and food palatability via the accumbens. As mentioned before, glutamate and GABA play key roles - AcbSh AMPAR antagonism and GABA_A_R stimulation induces feeding, as does GABABR activation (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1997). Meanwhile, AMPAR activation suppresses feeding (Stratford et al., 1998). It should be noted, however, that GABA_A_R activation in the caudal AcbSh does not produce robust feeding but instead produces defensive and aversive behaviors, suggesting a different neuron ensemble may be located there (Reynolds and Berridge, 2001).

Other neurotransmitters affect feeding in the accumbens as well. Antagonism of the receptor for the lateral hypothalamic peptide, MCH, decreases intake (Georgescu et al., 2005). Acetylcholine (ACh) also plays a prominent role, likely via muscarinic receptors - injection of scopolamine, a muscarinic antagonist, into the AcbSh potently reduces food intake for up to 24
hours, but does not diminish food seeking behaviors (Pratt and Blackstone, 2009; Pratt and Kelley, 2004). Further, synaptic release of acetylcholine in the accumbens increases immediately prior to cessation of a meal (Avena et al., 2008b; Rada et al., 2005). Another group of transmitters that mediate AccbSh feeding are the opioid peptides. Ligands for mu opioid receptors increase palatability responses when injected into the AccbSh and increase food intake when injected into the AccbC (Peciña and Berridge, 2005; Zhang and Kelley, 2000). Accb injection of ligands for delta receptors result in a modest increase in food intake, while kappa agonists have no effect (Bakshi and Kelley, 1993). Antagonism (or more accurately, inverse agonism) of these receptors using naltrexone will halt or reduce food intake initiated by deprivation (Kelley et al., 1996).

Evidently, an array of neurotransmitters can either modulate, drive, or halt feeding via the accumbens, and these systems likely interact (Echo et al., 2001; Kelley et al., 2000; Sears et al., 2010; Znamensky et al., 2001).

**Accumbens Cytoarchitecture**

The cell types found in the Accb are similar to those found elsewhere in the striatum – a vast majority are medium spiny GABAergic projection neurons and a small population are large cholinergic interneurons (for review, see Tepper et al., 2010). Until other markers had been determined, the Accb was difficult to distinguish cytochemically from the rest of the striatum. Cell morphology differed subtly – the medium spiny neurons in the dorsal striatum are larger, have more dendritic branching, and have more spines than their ventral striatal counterparts (Meredith et al., 2008). Also subtle was the differences in chemical markers between ventral striatum and dorsal striatum – dorsal striatum may stain more darkly for enkephalin and substance P than the ventral striatum does (Voorn et al., 1989). However, certain cell type markers were found that divided the AccbSh and AccbC more clearly that total Accb versus dorsal striatum. The AccbC is rich
in calbindin yet poor in Substance P and cholinesterase staining, while the AcbSh is the reverse for all three of these markers (Jongen-Rêlo et al., 1994).

**Accumbens Associations with Other Brain Areas**

*Afferents to the Accumbens Shell*

Many sources of glutamate input to the AcbSh, some of which are subcortical. The cortex is a prominent source of glutamatergic neurotransmission in the mammalian brain (Herzog et al., 2004), so it is not surprising that the most prominent Acb glutamate source is a cortical area, specifically the prefrontal cortex (PFC; Christie et al., 1985; Mitrano et al., 2010; Zangen and Hyodo, 2002). Other glutamate inputs may arise from the VTA (Yamaguchi et al., 2011), basolateral amygdala (Härtig et al., 2003; Wright et al., 1996), lateral hypothalamic orexin neurons (Baldo et al., 2003; Rosin et al., 2003), the parabrachial nucleus (Kaur et al., 2013; Saper and Loewy, 1980), the paraventricular thalamus (Kiss et al., 2011; Moga et al., 1995), and/or the hippocampus (Matthews et al., 2004).

The known sources of such GABA input to the AcbSh are, compared to its glutamatergic inputs, more limited and subcortical. Such afferents include lateral hypothalamic MCH neurons (Bittencourt et al., 1992; Elias et al., 2008), the ventral pallidum (Chen et al., 2003; Haber et al., 1985) or the VTA (Van Bockstaele and Pickel, 1995; Swanson, 1982). Stimulation or inhibition of some of these areas has been tied to AcbSh-mediated food intake (Mena et al., 2013; Trojniar et al., 2007).

It would appear that the accumbens receives signals conveying decision-making processes, reward incentives, fear, arousal level, signals relayed from various other brain regions, and cues from memory. Given that AMPAR blockade or GABA$_A$R activation within the AcbSh induces feeding, AcbSh-mediated feeding likely requires cessation of glutamatergic input or
initiation of GABAergic input from some of the aforementioned sources. Interestingly, each of these areas receives projections from the LH (Fadel and Deutch, 2002; Schmitt et al., 2012; Villalobos and Ferssiwi, 1987). It seems feasible that as the LH receives communication from AcbSh, the LH may project to these other regions and then feedback indirectly to the AcbSh.

Some additional attention should be directed at the involvement of VTA-AcbSh circuitry in the control of feeding. The VTA secretes dopamine to the AcbSh, and this dopamine signal is thought to code for rewarding stimuli (Tsai et al., 2009). Indeed, much literature indicates the importance of dopamine input to the AcbSh in order to regulate reward-motivated behaviors (for review, see Salamone et al., 2007). However, dopamine does not substantially change food intake, but perhaps instead changes the rewarding value of food (Covelo et al., 2012; Zhang et al., 2003). However, other studies show evidence that AcbSh dopamine influx has no effect on food procurement behavior or on learning food procurement based tasks (Hanlon et al., 2004; Stratford and Wirtshafter, 2012).

Aside from glutamate and GABA, other neurotransmitters modulate food intake or food-seeking behavior via the AcbSh, and these neurotransmitters originate from many of the same sources. Opioids, which generally act to increase intake when acting in the accumbens, may originate from the bed nucleus of the stria terminalis, amygdala, and certain hypothalamic regions including the LH, among other sources (Fallon and Leslie, 1986). MCH and orexin originate from the LH (Baldo et al., 2003; Saito et al., 2001). Acetylcholine is secreted by large neurons indigenous to the accumbens and striatum (Tepper et al., 2010). Such cholinergic neurons are likely activated by direct glutamatergic input from paraventricular thalamic neurons (Ligorio et al., 2009). These and aforementioned afferents to the AcbSh, by secreting a variety of neurotransmitters, can finely regulate food intake, reward, and seeking behaviors.

Efferents of the Accumbens Shell

13
The AcbSh is principally constituted of GABAergic neurons, thus projections of the AcbSh are GABAergic in nature. The GABAergic output of the AcbSh projects primarily into the prefrontal cortex, VTA, LH, ventral pallidum, bed nucleus of the stria terminalis, cingulate gyrus, and lateral preoptic area (Heimer et al., 1991; Zahm and Heimer, 1993). By innervating these targets, the accumbens can regulate reward, feeding, decision-making, motivation, and other similar limbicly-related processes.

It should be noted that there are two groups of medium spiny neurons in the accumbens – those that secrete enkephalin and those that secrete dynorphin as co-transmitters. The enkephalin population largely projects to limbic targets, while the dynorphin population projects to motor-related regions (Zhou et al., 2003). Indeed, some investigators propose that these two cell types may form separate “direct” and “indirect” accumbal efferent pathways (James et al., 2013; Kelley et al., 2005b). Peptidergic co-transmission may thereby discriminate accumbal projection patterns between what may otherwise appear to be a heterogeneous population of GABAergic medium spiny neurons.

**Differences Between the Accumbens Shell and Accumbens Core in the Regulation of Feeding**

Although the AcbSh has been implicated in control of feeding, its counterpart the AcbC does not have a notable role in driving primary amino acid neurotransmitter-based food intake (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1997). Instead, it appears to be a more sensitive target for only opioid-mediated feeding, moreso than the AcbSh (Zhang and Kelley, 2000). This finding is mirrored by the evidence that AcbC, but not AcbSh, ablation diminishes responding for heroin self-administration, which acts on opioid receptors (Alderson et al., 2001). Opioids do not drive substantial food intake when injected into the AcbSh, but in this region they
do modulate palatability responses (Peciña and Berridge, 2005). In this way, opioids may serve to modulate AcbSh-mediated feeding without directly evoking it, whereas such opioids could directly drive feeding through actions on the AcbC.

Considering the differences in outputs of AcbSh versus AcbC, it may seem unusual that the AcbC could regulate feeding. The AcbC is more integrated with motor-like systems, while the AcbSh is more limbically-situated (Heimer et al., 1991; Zahm and Heimer, 1990). How may the AcbC regulate feeding? Some have proposed that the effects rely upon the large cholinergic interneurons native to most of the striatum (Kelley et al., 2005b). In that proposal, it is suggested that these cholinergic neurons possess mu opioid receptors, and that mu opioid agonists inhibit ACh release from these neurons. Further, others suggest that there are extensive AcbC-AcbSh interconnections (van Dongen et al., 2005). Such interconnectivity suggests that, in order to regulate feeding, the AcbC routes signals through the AcbSh. This would allow the AcbC to indirectly communicate with limbic areas that regulate food intake.

In regard to differences in inputs, the AcbC receives inputs that are either motor-associated or reward cue-associated. The AcbSh receives some of the same reward-related inputs, but fewer of the motor-related inputs (Brog et al., 1993). Considering the interconnectivity between AcbC and AcbSh, the AcbC may receive signals that inform the nucleus of movement states and incentive cues. The AcbC may then convert these signals into a fixed action pattern that is output via the AcbSh. Phasic dopamine release from the VTA to the accumbens initially occurs when a reward is given, but this release occurs to the onset of a cue instead as a cue is repeatedly associated with the reward (Stuber et al., 2008). Then, the Acb entrains reward-motivated behaviors in response to this phasic dopamine release from the VTA (Tsai et al., 2009). Glutamate action on NMDA receptors is required to facilitate dopamine-based appetitive learning (Smith-Roe and Kelley, 2000), likely from the PFC. Thus, the AcbC may be a node by which
signals from the VTA and the PFC that entrain motivated behaviors can be translated into action. However, the VTA and PFC may use communications with the AcbSh to bypass the AcbC and initiate feeding behaviors. Lesions to the AcbC do not affect VTA stimulation-induced feeding, but lesions to the AcbSh do (Trojniar et al., 2007). Considering that such stimulation may activate VTA GABA neurons, the VTA may use this projection to directly drive food intake. Another input likely drive food intake via the AcbSh - the PFC, which normally emits glutamate onto the AcbSh, may have such glutamate release halted by mu opioid receptor activation in the PFC (Mena et al., 2013). As shown by AMPAR antagonism of the AcbSh (Maldonado-Irizarry et al., 1995), blocking excitatory input in this region serves to drive food intake. Other inputs to the AcbSh have yet to be explored in their role in driving AcbSh-mediated food intake.

**Food Addiction via Dysregulation of Accumbens Shell Signaling - Clinical Implications**

As the accumbens is one locus upon which drug addiction may be mediated, it seems reasonable to suspect other addictions may also be regulated by this brain area. Indeed, mounting evidence points toward a “food addiction” phenomenon (Avena et al., 2008a). The support for this claim indicates similarities in behavioral responses between drug addiction and addiction to palatable food. Such similarities include bingeing, withdrawal, and craving behaviors in addition to cross-sensitization effects. A collection of evidence, described below, demonstrates how food addiction may contribute to cases of obesity arising from consumption of foods far beyond caloric needs.

Food binge behaviors in rats are characterized in studies primarily centered around dopamine neurotransmission and limited food access. Binge eating paradigms used on rats feature having limited access to both chow and sucrose solution (12 hours per 24 hour period) while being food deprived for the remainder of the day, including part of the dark phase (Avena et al.,
It was determined via microdialysis that for the first hour sucrose is available, a spike in dopamine efflux is seen. This dopamine increase was substantially larger than that seen in non-deprived rats given access to chow and sucrose or rats given sucrose only twice to retain its novelty. Further, the typical increase in accumbens ACh levels that precedes the termination of the meal was delayed in these “sucrose-dependent” rats (Rada et al., 2005).

Chronic food restriction, body weight loss, and more restricted access to sucrose exaggerates these neurotransmitter patterns; dopamine efflux increases even more while the ACh response fails to increase in proportion (Avena et al., 2008b). Additionally, sucrose intake escalated in these studies much like cocaine self administration does when given in limited access (Ahmed and Koob, 1998). The neurotransmitter dynamics in the accumbens resulting from sugar bingeing are mimicked by systemic morphine treatment; dopamine increases and ACh decreases (Rada et al., 1991). These studies demonstrate how food binges may mimic drug binges due to similar accumbens signaling.

Other drug-like effects are seen with palatable food addiction. Drug withdrawal, typically characterized in rats by amotivation, depression, and anxiety (Schulteis et al., 1998; De Vries and Shippenberg, 2002), can also exhibited by sucrose deprivation in “sucrose-dependent” rats (Avena et al., 2008c). Such deprivation decreases extracellular dopamine and increases extracellular ACh within the accumbens (Avena et al., 2008c). These neurotransmitter changes are also exhibited in drug withdrawal (Rada et al., 2004). Craving behaviors are also induced by both drug abstinence and abstinence from food to which a rat has become dependent. Craving is exhibited by increased effort to obtain the abused substance (Koob and Le Moal, 2005), increased responses in tasks with drug-associated cues despite absence of reward (Bienkowski et al., 2004), and consumption of the substance upon relapse that surpasses the amount consumed prior to abstinence (Heyser et al., 1997). These features are seen after sucrose abstinence in sucrose-
dependent rats (Avena et al., 2005).

Most importantly, systems activated during drug abuse appear to overlap with some food intake regulatory circuitry. This effect can be noted in cross-sensitization paradigms. Sucrose-dependent rats will respond with increased locomotion to low amphetamine doses that have little effect on non-sucrose-dependent animals (Avena and Hoebel, 2003). Sucrose intake also sensitizes animals to cocaine (Gosnell, 2005). In sucrose-dependent rats, withdrawal symptoms can be precipitated by administration of naloxone, an opioid antagonist. This withdrawal results in decreases in accumbens extracellular dopamine and increased ACh, suggesting the development of a dependence on chronically-increased endogenous opioid neurotransmission (Colantuoni et al., 2002). Cross-sensitization provides further validity to the concept of food addiction.

These effects normally associated with drugs of abuse – binge behavior, withdrawal, cravings, and cross-sensitization – are mirrored in cases where subjects become dependent on foods. By these mechanisms, an individual's caloric needs are overridden by the high salience and rewarding value of foods. This drives an individual to consume in excess, and thus this model may serve as one mechanism by which obesity may develop. In light of the mechanics involved, it behooves neuroscientists to understand accumbens circuitry further in order to develop treatments to combat this pathological overconsumption of foods.

**Characterization of the Lateral Hypothalamus and Its Involvement with Feeding**

The lateral hypothalamic area may have been delineated as a distinct subregion of the hypothalamus as early as the beginning of the 1900s, though the changes in nomenclature make this difficult to determine (Krieg, 1932). As with the accumbens, the behavioral effects that occurred from LH manipulations likely guided its parcellation from the rest of the hypothalamus
more than differences in cytoarchitecture. Early lesion research demonstrated a loss of various behaviors, including feeding, locomotion, and arousal (Von Der Porten and Davis, 1979). Damage to other hypothalamic areas had other profound but not as lethal consequences. It was from this lesion data that the LH became a center of attention for researchers interested in investigating homeostatic and motivational processes.

Research on the LH was primarily popularized by and grew from the development of the dual center hypothesis (Stellar, 1954). The proposal was based on lesion research showing that ventromedial hypothalamic (VMH) lesions resulted in severe overeating and body weight gain, whereas LH lesions resulted in anorexia and potentially lethal weight loss. From this evidence, the author posed the idea that the LH is a “hunger” center while the VMH is a “satiety” center, and that the destruction of either one results in the removal of its function. Though this is an interesting theory, there is more to the LH than controlling hunger. Later research demonstrated that the LH is a hub for various homeostatic and motivational signals.

The LH regulates or at least modulates many bodily functions. The homeostatic signals that may converge on this region include (but are not limited to) signals from the arcuate nucleus that indicate circulating leptin, ghrelin, and cholecystokinin levels, circulating blood sugar, insulin, and fatty acids, bodily hydration, circadian rhythm, gastric distention via the nucleus of the solitary tract, sense of nausea or detection of toxins via the area postrema, and taste information via the parabrachial nucleus (Anand and Pillai, 1967; Håkansson et al., 1998; Kelly and Watts, 1996; Ono et al., 1986; Oomura and Kita, 1981; Orsini et al., 1990; Pedigo and Brizzee, 1985; Saper and Loewy, 1980; Valassi et al., 2008). Other input to the LH that drives behavior may include visual or taste information, fear or emotional distress, hedonic incentives, level of arousal, and executive functions from cortical areas (Briski and Gillen, 2001; Burton et al., 1976; Fukuda et al., 1986; Morshed and Meredith, 2008; Szymbusiak and McGinty, 2008;
Torterolo et al., 2011). Indeed, a variety of signals may be integrated through the LH in order to make the final “decision” to initiate feeding by activating hindbrain nuclei and motor systems required for feeding.

The primary amino acid neurotransmitters glutamate and GABA are likely secreted into the LH in order to communicate many of the above signals. These neurotransmitters can subsequently control feeding via the LH. Our lab has shown feeding-specific responses to injections of glutamate and GABA. Injections of glutamate and its agonists induce feeding, and will do so in a specific manner (Duva et al., 2002; Stanley et al., 1993). The same effects are seen from injection of GABA receptor antagonists such as picrotoxin or bicuculline (Turenius et al., 2009a, 2009b). Injection of the N-methyl-D-aspartate receptor (NMDAR) antagonist D-AP5 or the GABA_A receptor agonist muscimol will cease feeding, and may do so for a full 24 hours at higher doses (Stanley et al., 1996; Turenius et al., 2009b). As glutamate and GABA receptors are relatively ubiquitous in the rat brain, these feeding effects could be argued to result from injections spilling over into neighboring regions. However, further studies from our lab indicate that the feeding effects of NMDA and of picrotoxin are specific to the LH and not surrounding regions (Duva et al., 2002; Turenius et al., 2009a). In light of these results, our lab proposed that there is a careful balance of the synaptic release of glutamate and GABA to the LH in order to regulate feeding (Stanley et al., 2011). Maintaining this balance within the LH is of utmost importance for the proper maintenance of food intake and body weight.

**Features of the Lateral Hypothalamus**

*Behavioral Deficits from Lateral Hypothalamic Lesions*

Lesion studies spawned the concept of a hypothalamic dual center hypothesis in body weight regulation. In this hypothesis, the LH was considered the hunger center of the brain while
the VMH was considered the satiety center (Anand and Brobeck, 1951; Hetherington and Ranson, 1940; Stellar, 1954). The data supporting this argument showed that ablation of the LH disrupted ability to feed and ablation of the ventromedial hypothalamus caused massive overeating. This was later supported by results from electrical stimulation of these areas in goats, such that LH stimulation initiated feeding and VMH stimulation ceased it (Wyrwicka and Dobrzecka, 1960). However, further study suggested that this hypothesis was too simplified. Other evidence began to emerge that suggested the LH was not the “hunger center” of the brain. First, other regions of the brain were found to regulate feeding, so the LH and VMH could not be the “central” controllers of feeding (Elmquist et al., 1999). Second, though bilateral lesions of the LH can be lethal if an animal is left alone, the animal can regain independent feeding ability through consistent force-feeding over a few weeks (Corbett and Keeseey, 1982). If the LH were a hunger center, its destruction should abolish all eating. Third, a variety of other deficits are seen from LH lesions other than inability to eat. Depending on the type of lesion, there may be deficits in arousal, inability to properly react to tactile cues, deficits in social interactions, body temperature dysregulation, adipsia, amotivation, and hypolocomotion (McMullen and Almli, 1980; Schallert, 1982; Schallert and Whishaw, 1978). As such, the LH was proposed to be a locus that integrated various elements of homeostatic information with signals that drive motivated behaviors (Berthoud, 2002). These issues will be addressed below.

Other brain regions can regulate food intake. For example, decerebrate animals can still consume foods and even respond to taste stimuli. Complete cuts that sever the rostral half of the brain from the caudal half reveal that rostral brain areas are not required for the most basic functions of consumatory behavior. Animals with this surgery will eat foods placed in the mouth, and will reject bitter quinine solutions (Grill and Norgren, 1978). This is not to say that the LH is unnecessary. Without these forebrain connections, animals become purely reactive, do not seek
out foods even in deprived states, and are unable to learn taste aversion. However, those authors also noted that the animals were at least able to ambulate in response to stimuli, maintain posture, and produce orofacial responses to certain tastants (Grill and Norgren, 1978). Such evidence, in combination with LH lesion results, suggested that the hypothalamus works together with other forebrain regions in order to motivate animals to seek out and procure foods in order to meet homeostatic needs or hedonic incentives. Also, the hindbrain was shown to contain nuclei that are important for taste responses and orofacial movements required for ingestion. Thus, the LH, though important, does not act alone in regulating intake.

Rats with LH lesions recover feeding ability as long as they are force-fed for a few weeks (Bernardis and Bellinger, 1996). Because of this occurrence, the LH cannot be considered as a hunger center, as hunger recovers despite the region's destruction. Instead, some propose that LH ablation establishes a lower body weight set point, and that it may be maintained due to increased metabolism in addition to lowered food consumption (Bernardis and Bellinger, 1993; Corbett and Keesey, 1982). The recovery phase is also interesting – lesioned rats will only eat palatable food items initially before being able consume regular chow (Mrosovsky and Hallonquist, 1986). Apparently, even in the LH lesioned rat, both hedonic-based and regular caloric-need based feeding can still occur, insinuating that eating is not abolished by LH lesions and the region cannot be considered a hunger center of the brain (or the only one, at least).

In regards to other homeostatic and behavioral deficits seen from LH lesions, there are a myriad of them depending on the nature of the lesion. For example, electrolytic lesions ablate not only all cell bodies, neuron and glia alike, but they also destroy fibers of passage. Though the glial tissue can regenerate to some degree, the destruction of circuitry both native to the lesioned area and the pathways simply on route through it are permanently removed. Thus, the effects seen from LH lesions can not be attributed to only the LH but may also be due to destruction of
dopaminergic projections to basal forebrain areas (Ungerstedt, 1970). The range of deficits seen, including hypophagia, thermal dysregulation, amotivation, and hypolocomotion, would not be representative of the normal functions of the LH alone. However, chemical lesion methods or methods of pharmacological inhibition (what some may consider a “temporary lesion”) can delineate which of the above specific functions are LH-centric, and which are due to destruction of fibers of passage.

Popular chemical brain lesions utilize excitotoxins, such as ibotenic acid. This substance is a potent glutamate agonist that will cause either necrosis or apoptosis of neurons while sparing fibers of passage and most other non-neuronal cell types (Ankarcrona et al., 1995; Jarrard, 1989). Using this method, behavioral functions that required the LH alone were determined. Ibotenic acid lesions of the LH resulted in similar but less intense adipsia and aphagia as seen from electrolytic lesions. However, these acid-lesioned animals did not have the same movement deficits and responded to dopamine agonists with hyperlocomotion, an effect usually impaired by electrolytic LH lesions (Winn et al., 1984). Furthermore, using this method, the LH was determined to be the locus of LH ICSS. Animals that had electrodes implanted into the LH did not self-stimulate after ibotenic acid lesions (Velley et al., 1983). This evidence show that the LH must be essential in regulating certain aspects of reward-motivated behaviors. The aforementioned research implicates the LH in some of the homeostatic and motivation-associated functions that electrolytic lesions had first determined. However, movement control may be regulated by other circuits not directly integrated with LH functions.

**LH Activity and Manipulations**

As mentioned before, electrical stimulation of the LH can result in food intake (Wyrwicka and Dobrzechka, 1960). However, depending on where specifically within the LH the
stimulation occurs, many other behaviors can manifest. Stimulation of certain LH sites within the
cat brain can result in predatory attack behaviors or defensive aggressive behaviors (Brown et al.,
1969). This result is partly mimicked in rats; stimulation of certain LH areas can result in jumping
avoidance behavior, attacking stimuli including other rats, or defensive treading (Kruk et al.,
1983). Some other behaviors manifested by LH stimulations include hyperlocomotion, grooming,
drinking (Mogenson and Stevenson, 1967; Singh et al., 1996; Velley, 1985), though it should be
noted that hyperlocomotive responses may be due to stimulation of the medial forebrain bundle
and not the LH (Velley, 1985). Many of these behaviors are exhibited by LH infusions of
 glutamate agonists (Duva et al., 2001, 2002; Hettes et al., 2007), corroborating these effects. Such
corroboration is necessary in confirming that these effects are LH-specific and not due to the
electrical stimulation of fibers of passage. Central injections of neurotransmitter
agonists/antagonists serve to confirm this as they do not act on fibers of passage. Summarily,
electrical stimulation of the LH demonstrates how this brain region regulates many behavior
responses, mostly those on the axis with food procurement and defensive behaviors.

The LH is also a site that effectively promotes ICSS when rats have an electrode
implanted into it. As mentioned before, this ICSS response effect is not due to medial forebrain
bundle fibers passing through the LH, as chemical ablation of LH neurons hinders the rewarding
properties of LH ICSS (Velley et al., 1983). Further work using lesions and ICSS in the LH
suggested that this effect may be localized to the tuberal LH (Velley, 1986). Drug withdrawal
increases LH ICSS responses (Schaefer and Michael, 1983), demonstrating the association of the
LH with reward circuitry activated by drugs of abuse. LH ICSS also is affected by food
consumption or deprivation; overconsumption of food (Hoebel and Teitelbaum, 1962) or
systemic administration of an appetite suppressant (McClelland et al., 1989) hinders LH ICSS,
while food deprivation increases it (Carey and Goodal, 1975). These data suggest that the
rewarding properties resulting from electrical stimulation of the LH may intersect with or even utilize the same neural circuits that mediate drug reward and feeding.

Electrophysiological recordings reflect some of these behavioral control attributes of the LH and reveal additional attributes. Certain LH neurons are inhibited by direct application of glucose, suggesting the role of the LH in glycemic responses (Orsini et al., 1990). LH neurons fire to the sight and taste of known foods (Burton et al., 1976; Fukuda et al., 1986). Taste-responding LH neurons only respond during hunger but not satiety (Rolls et al., 1980). Also, food-related cue learning can occur within the LH. LH neurons will fire to non-food cues when continually paired with foods (Mora et al., 1976). LH neurons will also be inhibited by gastric distention (Anand and Pillai, 1967). Additionally, LH neurons fire to certain food-related odors (Shiraishi, 1988). These electrophysiological studies reveal the consumption-oriented activity profile of LH neurons.

Many studies have used c-fos immunohistochemistry to study LH neuron activity. Staining for a marker of neuron activity within the LH is ideal as it allows for additional staining to further identify the activated cells. In many cases, orexin neurons or neurons that utilize other neuropeptides may be activated in certain situations. The c-fos protein is expressed in LH neurons from consumption of a palatable meal (Park and Carr, 1998), interruption of sleep (Castillo-Ruiz and Nunez, 2011), systemic insulin treatment (Bahjaoui-Bouhaddi et al., 1994), morphine withdrawal (Georgescu et al., 2003), and food and drug reward cues (Harris et al., 2005). In some of these cases, some of the neurons activated were orexin neurons. These markers of activity provide evidence for the role of specific LH neuron populations in modulating arousal, reward-seeking, and food procurement.

LH central injection studies revealed much in the way of the brain area's pharmacological workings. As stated previously, the LH is a primary site within the hypothalamus for glutamate
receptor agonist- and GABA_A antagonist-elicited eating (Duva et al., 2002; Stanley et al., 1993; Turenius et al., 2009a). Also, recent evidence shows that metabotropic glutamate receptors play a part in regulating feeding via the LH (Charles et al., 2013). Other neurotransmitters and their receptors play a role as well. Injection of neuropeptide Y, agouti-related peptide, or orexin into the LH induces feeding (Kim et al., 2000; Kotz et al., 2002; Stanley et al., 1985), while injection of leptin, nesfatin, and alpha-melanocyte stimulating hormone suppress feeding (Adan et al., 2006; Chen et al., 2012; Faouzi et al., 2007; Satoh et al., 1997). Some classical neurotransmitters impact LH-mediated feeding as well. Dopamine agonist injections into the LH may elicit or curb feeding, depending on the receptor the agonist acts on (Chen et al., 2013; Parada et al., 1992), serotonin may inhibit food intake (Parada et al., 1992), and norepinephrine may act via beta adrenergic receptors to reduce feeding (Leibowitz, 1970). This myriad of neurotransmitters act in concert to regulate food intake through the LH.

**LH Cytoarchitecture**

Early investigation utilized Nissl and Golgi staining to define cell shapes. Research demonstrated that the LH is one of the largest hypothalamic nuclei and yet the one with the lowest neuron density. The majority of the neurons in this nucleus are large, stellate, and have long dendritic processes that weave carefully through the medial forebrain bundle that traverses that region (Krieg, 1932). Because of this association with the medial forebrain bundle, the neurons in the LH are arranged to receive an array of signals. The number of cells in this region – about 250,000 – is modest considering the large size of the area. This moderate number, in tandem with the integration with the medial forebrain bundle, suggests that the LH is adept for integrating signals incoming from various brain regions (Palkovits and Van Cuc, 1980).

The LH, though originally treated as a homogenous region due to feeding being elicited
by electrical or chemical stimulation within much of its extent, is being increasingly parceled in recent anatomical studies. Initially, it was divided into three parts: anterior, tuberal, and posterior (Saper et al., 1979). However, closer study resulted in more parcellation: magnocellular, periformal, tuberal, anterior, and peduncular subdivisions arose from one brain atlas (Paxinos and Watson, 2005). Others utilize a brain atlas in which the LH has been divided into far more numerous subregions based on differences in cell structure, afferents, and efferents (Swanson, 2004). Such subdivisions include the anterior dorsal, anterior ventral, anterior intermediate, juxtadorsomedial, dorsal, juxtaventromedial dorsal, juxtaventromedial ventral, magnocellular, supraforminal, juxtaparaventricular, posterior, motor-related, parvicellular, ventromedial, ventrolateral, subforminal anterior, subforminal posterior, and subforminal premamillary subregions. Such a large array of subdivisions of the LH speaks to its potential diversity of functions and its precisely compartmentalized nature.

The LH is also known for its diversity in neurons that utilize peptide neurotransmitters. The most prominent populations in the literature are orexin and melanin-concentrating hormone (MCH) neurons. These co-distribute primarily in the tuberal LH around the fornix and zona incerta (Hahn, 2010). Other moderately-sized neuron populations exist in the LH; there are those that secrete cocaine and amphetamine regulated transcript (CART) (Broberger, 1999), nesfatin-1 (Foo et al., 2008), and a specific population receptive to leptin that do not contain orexin or MCH but do contain galanin (Laque et al., 2013). Within the LH, some nesfatin neurons contain CART and/or MCH, and most CART neurons contain MCH (Broberger, 1999; Foo et al., 2008). However, both nesfatin and CART are more widely distributed throughout the LH than MCH and exist in various other brain regions.

Unfortunately, the LH will only be subdivided into the periformal and lateral subregions
in Chapters 3 and 4 of this dissertation. This is due to the use of central drug injections in much of the research described in this dissertation. Such injections can diffuse across distances of 1 mm or more in the rat brain, thereby easily encompassing most of the smaller subdivisions described by the aforementioned brain atlases. Because of this drug spread, it would be misleading to claim that the effects described in Chapters 3 and 4 act on a specific single or specific subset of these LH subdivisions unless prior research specifically determined otherwise. Nonetheless, it is clear that at least some of the LH subregions listed above, and multiple specialized neuron populations, are the loci for primary amino acid-mediated feeding.

Lateral Hypothalamic Associations with Other Brain Areas

Afferents to the Lateral Hypothalamus

The LH receives projections from numerous areas throughout the brain (Duva et al., 2005). A number of glutamate inputs exist among them, including (but not limited to) projections from the PFC (Fremeau et al., 2004; Mena et al., 2013), hippocampus (Walaas and Fonnum, 1980), VTA (Swanson, 1982), parabrachial nucleus (Niu et al., 2010; Saper and Loewy, 1980), ventral pallidum (Gritti et al., 2006), paraventricular thalamus (Kiss et al., 2011; Moga et al., 1995), septal regions (Lin et al., 2003), and amygdala (Niu et al., 2012; Petrovich et al., 2001). Many GABAergic sources innervate the LH as well. These consist primarily of the AcbSh (Oertel and Muggaini, 1984; Usuda et al., 1998), ventral pallidum (Gritti et al., 2006), VTA (Barrot et al., 2012), amygdala (Nakamura et al., 2009), bed nucleus of the stria terminalis (Jennings et al., 2013), and lateral septum (Zhao et al., 2013). This collection of inputs allows the LH to receive information about decisions for food procurement, cues indicating food, reward-related cues, taste properties, motivational control information, indirectly relayed signals, and stimuli associated with fear. These inputs likely modulate or direct primary amino acid-regulated feeding
through the LH.

The inputs to the LH use a variety of other neurotransmitters to either activate or cease food intake. The orexigenic peptides NPY and AgRP originated from the arcuate nucleus and orexin from within the LH (Baldo et al., 2003; Funahashi et al., 2003; Kim et al., 2000). The feeding-suppressive peptides projecting to the LH – alpha-melanocyte-stimulating hormone, nesfatin, and glucagon-like peptide 1 (Adan et al., 2006; Chen et al., 2012; Shughrue et al., 1996) are found via the arcuate, via various brain regions, and via the nucleus of the solitary tract, respectively. The classic neurotransmitters serotonin from the raphe nucleus, dopamine from the VTA, and norepinephrine from the locus coeruleus and hindbrain sources also influence feeding behavior regulated by the LH (Meister, 2007). The fact that such a diverse array of neurotransmitters modulate LH activity implies that the region carefully balances numerous specific signals in order to maintain body weight and control food intake.

**Efferents of the Lateral Hypothalamus and Their Neurotransmitters**

The lateral hypothalamus innervates numerous sources, in some cases via specific well-described neuron populations. Not all glutamatergic LH efferents have been described, but those in which glutamate is used as a co-transmitter have been identified. Specifically, orexin co-releases glutamate (Rosin et al., 2003), and orexin neurons project to various regions including the nucleus accumbens shell (but not core), ventral tegmental area, ventral pallidum, various hypothalamic regions, paraventricular thalamus, central amygdala, lateral septum, substantia nigra, locus coeruleus, raphe nucleus, and nucleus of the solitary tract (Baldo et al., 2003). Thus orexin, and likely glutamate, are poised to interact with various systems regulating reward, motivation, arousal, feeding, and energy homeostasis.

GABAergic efferents of the LH are equally understudied, but the efferents of specific
populations of neurons that co-transmit GABA are more understood. MCH neurons co-transmit GABA (Elias et al., 2008) and they project predominantly to the basolateral amygdala, hippocampus, various hypothalamic areas, nucleus accumbens, and various hindbrain feeding regulators (Saito et al., 2001). Also, a leptin-receptive population within the LH that does not contain orexin or MCH has been identified. These neurons are GABAergic (Leinninger, 2011). Although the specific projections of this leptin-receptive LH neuron population have not been identified, certain brain regions receive input from leptin-receptive neurons originating from various brain areas. The terminals primarily innervated the accumbens, ventral tegmental area, multiple hypothalamic areas, periaqueductal gray area, claustrum, bed nucleus of the stria terminalis, central amygdala, substantia nigra, dorsal raphe nucleus, and the nucleus of the solitary tract (Patterson et al., 2011). These GABAergic LH neuron populations summarily innervate regions associated with arousal, ingestion, reward, fear, stress, metabolism, and movement.

**Dysregulation of Energy Balance via Hypothalamic Signaling Dysfunctions – Clinical Implications**

As obesity is a major concern in the United States, much research has been aimed at investigating its sources. Some propose that dysfunctions in hypothalamic signaling are responsible (Davis et al., 2010). The arcuate nucleus has received much focus as it receives signals from several circulating hormones, especially the adipose tissue hormone leptin (Berthoud, 2002). Leptin has been proposed as one mode by which central signaling may not accurately convey the amount of energy stores in the periphery. Leptin is secreted by adipose tissue into the bloodstream, and it is secreted in amounts proportional to the amount of adipose tissue found in the body (Sinha et al., 1996). Leptin acts on the central nervous system, likely via
the arcuate nucleus, in order to inhibit food intake if there is an overabundance of body fat (Satoh et al., 1997). Leptin inhibits food intake when acutely administered either to the ventricles, to the LH, or to the arcuate in normal weight rats (Satoh et al., 1997). However, overweight animals demonstrate blunted or lacking response to leptin, likely attributable to its chronic overabundance and receptor desensitization, a phenomenon termed leptin resistance (Myers et al., 2008) or otherwise due to the saturation of a leptin transporter system (Banks et al., 1996). Another mechanism by which leptin has diminished effects could be due to high fat diets inducing apoptosis of intake-regulating LH neurons (Moraes et al., 2009). Thus, leptin has much importance in balancing energy state, and determining how to reverse leptin resistance is of much interest.

Though the arcuate nucleus is one predominant recipient of circulating leptin, a population of leptin-receptive neurons have been recently characterized in the LH (Leinninger, 2011). These neurons are distributed throughout the lateral and perifornical hypothalamic regions and are intermingled with orexin and MCH neurons. They appear to use GABA and the anorectic peptide galanin as transmitters (Laque et al., 2013). Leptin may act directly on this population of neurons or on orexin and MCH neurons (Håkansson et al., 1998; Leinninger, 2011). Additionally, leptin-receptive LH neurons communicate with the VTA and modulate reward and feeding (Leinninger et al., 2009). Dysfunctions in leptin signaling may prevent these leptin-receptive neurons from being active, and thus other neuron populations are chronically released from that source of inhibition. These downstream populations, when active, may serve to increase food intake, arousal and secondarily food intake, metabolism and secondarily food intake, or rewarding properties of food and secondarily hedonic food intake.

Another set of neuron populations that regulate feeding beyond caloric need are orexin and MCH neurons. As mentioned in regards to their effects in the AcbSh, orexin and MCH not
only induce food intake but also enhance reward-motivated behavior when administered to the AcbSh (Georgescu et al., 2005). This function can also occur indirectly via the VTA. Orexin input to the VTA increases dopamine output to the AcbSh, and MCH knockout mice possess dysfunctional reward signaling (Borgland et al., 2009; Pissios et al., 2008). Orexin and MCH also have effects on both arousal and metabolism. Increases in orexin output or absence of MCH output increase arousal (Hagan et al., 1999; Marsh et al., 2002). Orexin and MCH also regulate thermogenesis, thereby modulating metabolism (Glick et al., 2009; Madden et al., 2012). As orexin and MCH neuron activity modulates a diverse set of homeostatic and reward functions, it is clear that disturbances in the balance of their activity can have substantial consequences on food intake and energy balance.

Lastly, the LH serves as an integration point through which signals indicating the need to feed are routed. It is true that other regions in the hypothalamus and hindbrain communicate certain intake-regulating signals to each other (Grill and Norgren, 1978). However, these signals, either directly or indirectly, must be transferred or relayed through the LH. The LH is poised to finely regulate food intake, especially on a carefully temporally-controlled basis via a balance of synaptic emission of glutamate and GABA (Stanley et al., 2011). Thus, although the proper functioning of other intake-regulating brain regions is essential for maintaining normal amounts of food consumption and body weight, the LH is also indispensable. Understanding and controlling LH neurocircuitry will aid in counteracting the dysfunctions of other feeding-associated nuclei.
Implications of the Accumbens Shell - Lateral Hypothalamus Connection in the Specific Regulation of Feeding

The collection of aforementioned evidence in this chapter implies an integration of hedonic and homeostatic systems which involves, among a myriad of other circuits, a connection between the AcbSh and the LH. There are a few lines of evidence that support the concept of a connection between these regions. Anatomical data, using either retrograde tracer infusion into the LH or anterograde tracer injection into the AcbSh, reveals a direct projection from the AcbSh to the LH (Duva et al., 2005; Usuda et al., 1998). Efferents from the AcbSh are primarily GABAergic (Oertel and Mugnaini, 1984; Walaas and Fonnum, 1979). Behavioral studies assessing feeding behaviors have shown that AcbSh inhibition will drive feeding, while bilateral LH inhibition (pharmacological silencing of neuron firing within the LH in each hemisphere) will halt AcbSh-mediated feeding (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1999). Also, AcbSh inhibition induces c-fos only in the LH of the hemisphere ipsilateral to the AcbSh injection (Stratford, 2005), and some of these activated LH neurons likely contain orexin (Baldo et al., 2004). Because of these effects, some suggest that inhibition of AcbSh neurons projecting to the LH may serve to release the LH from inhibition and subsequently activate or perhaps modulate feeding behaviors (Kelley et al., 2005b). The AcbSh regulates feeding, and seems to require normal function of the LH in order to execute food intake.

Additionally, the LH may direct feedback signals to the AcbSh; LH neuropeptides associated with food intake appear to influence reward-motivated behaviors. MCH injected into the AcbSh increases food intake and its antagonist decreases food intake (Georgescu et al., 2005), MCH knockout disrupts mesolimbic dopamine signaling (Pissios et al., 2008), and MCH precursor protein knockout decreases the motivation to procure palatable foods (Mul et al., 2011). Such MCH signaling appears to rely on modulating AMPAR function (Sears et al., 2010),
implicating glutamate in AcbSh food reward circuitry. Orexin may have a less direct but equally impactful role. Orexin neurons appear to regulate the motivation to procure food via the VTA (Borgland et al., 2009). As AcbSh inhibition activates orexin neurons (Baldo et al., 2004) and orexin neurons project to the VTA (Fadel and Deutch, 2002), the VTA like influences the drive to procure and consume food via projections to the AcbSh (MacDonald et al., 2004; Martínez-Hernández et al., 2006). By further understanding how the AcbSh and the LH communicate using either primary amino acid neurotransmitters or neuropeptides, much can be learned about how food intake and food reward are controlled by the brain.

One issue that obfuscates the role of AcbSh to LH signaling in feeding stems from the use of manipulations that impede food intake or food procurement. It should be noted that food intake can be halted in a variety of ways. For instance, if an animal is sedated, it would be unable to seek out food and eat. Similarly, a paralyzed animal would be equally unsuccessful in being able to consume food, regardless of arousal level. Other possible factors that would impede food intake include (but are not limited to) dysfunctional locomotion, lack of motivation to procure food, seizures, and nausea. In experiments solely monitoring food intake and not the behavior of the animal, such behavioral effects would go unnoticed. However, the result will appear the same – the experimental manipulation suppressed food intake. In the case of prior feeding research implying the AcbSh - LH descending connection, past experiments did not assess whether bilateral LH inhibition halted AcbSh-mediated feeding in a behaviorally-specific manner, nor did they assess what behaviors resulted from bilateral LH inhibition. Indeed, it is possible that the conclusion stating “the LH regulates AcbSh-mediated feeding” may be falsely claimed.

There is much evidence suggesting how behaviorally non-specific effects can occur from manipulating certain brain regions bilaterally or even unilaterally. Arousal in particular can be influenced; unilateral injections of tumor-necrosis factor alpha into the preoptic area (anterior of
the LH) promotes non-rapid eye movement sleep (Kubota et al., 2002). Further, an animal can still be active yet have locomotion impede the ability to feed. For instance, unilateral activation of GABA receptors in different components of the basal ganglia can result in circling behaviors (Ikeda et al., 2010). This phenomenon is not restricted to typical motor-associated areas; unilateral disruption of NPY signaling in ventral thalamic areas using an NPY antibody also can cause circling as well as barrel rolling (Walter et al., 1994). Less noticeable effects of LH inhibition could potentially occur - nausea may be induced as the LH is associated with the nausea-regulating area postrema (Baird et al., 2009; Pedigo and Brizzee, 1985), or lethargy may be induced through inhibition of LH orexin neurons that regulate arousal (Tsunematsu et al., 2011).

One other question remains mostly unresolved – what signals other than orexin or MCH does the LH use to communicate directly with the AcbSh? The descending AcbSh to LH anatomical connection has been well-explored, but the reciprocal connection has received less attention. Studies using anterograde tracing from the LH or retrograde tracing from the suggest that this connection does exist (Brog et al., 1993; Goto et al., 2005). The neurochemical content of some of these LH neurons projecting to the AcbSh has been defined – some contain orexin, and others contain MCH (Kampe et al., 2009). However, these neurotransmitter peptides only comprise some of the types utilized throughout the LH. Also, no one has explored the potential for either a glutamatergic or GABAergic LH to AcbSh connection. Determining whether these primary amino acid neurotransmitters are used in this ascending connection is important as such transmitters have been shown to regulate food intake and food procurement behaviors in the AcbSh (Stratford and Kelley, 1997; Stratford et al., 1998). Lastly, the destination of specific LH projections into the AcbSh needs to be clarified. One study suggests that the LH only projects to the caudal AcbSh (Brog et al., 1993), and this area produces different (primarily aversive)
behaviors compared to the rostral AcbSh (Reynolds and Berridge, 2001). Closer scrutiny of LH projections to the AcbSh may allow for a clearer concept of that ascending connection's behavioral purpose.

In the works described in this dissertation, I aim to resolve these issues. I will address the following research questions: 1. Does unilateral LH inhibition specifically halt feeding mediated by ipsilateral AcbSh inhibition?, 2. What behaviors are expressed or suppressed during bilateral and unilateral LH inhibition, and how may either manipulation halt AcbSh-mediated feeding?, and 3. What types of LH neurons project to the AcbSh, and what is the specific topography in these projection patterns? These questions will be answered using central injections of glutamate and GABA agonists and antagonists, food intake measurement, behavioral monitoring, tract tracing, and immunofluorescence. The subsequent chapters will promote the case for an important role of glutamate and GABA neurotransmission within the AcbSh to LH circuit, how this communication may regulate feeding specifically, and how the reciprocal LH to AcbSh connection may serve to regulate feeding or aversive behaviors.
References


Chapter 3 - Ipsilateral Feeding-Specific Circuits Between the Nucleus Accumbens Shell and the Lateral Hypothalamus: Regulation by Glutamate and GABA Receptor Subtypes

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Abstract

The nucleus accumbens shell (AcbSh) and the lateral hypothalamus (LH) are both involved in the control of food intake. Activation of GABA\textsubscript{A} receptors or blockade of AMPA and kainate receptors within the AcbSh induces feeding, as does blockade of GABA\textsubscript{A} receptors or activation of NMDA receptors in the LH. Further, evidence suggests that feeding induced via the AcbSh can be suppressed by LH inhibition. However, it is unclear if this suppression is specific to feeding. Adult male Sprague-Dawley rats with 3 intracranial guide cannulas, one unilaterally into the AcbSh and two bilaterally into the LH, were used to explore this issue. DNQX (1.25 \(\mu\)g) or muscimol (100 ng) infused into the AcbSh unilaterally elicited feeding, and this elicited intake was suppressed by bilateral LH injection of D-AP5 (2 \(\mu\)g) or muscimol (25 ng). The effectiveness of D-AP5 or muscimol infusion into either the LH site ipsilateral or contralateral to the AcbSh injection was compared. Ipsilateral LH injection of D-AP5 or muscimol was significantly more effective than contralateral injection in suppressing food intake initiated by AcbSh injection of DNQX or muscimol. These results are consistent with prior evidence that inhibition of the LH through pharmacological modulation of NMDA or GABA\textsubscript{A} receptors specifically suppresses feeding initiated by AcbSh inhibition, and that these two regions communicate via an ipsilateral circuit to specifically regulate feeding.
Introduction

The lateral hypothalamus (LH) is important in the control of feeding and has thus been the focus of much research over the past few decades. The LH is classically associated with the initiation of feeding behavior (Wyrwicka and Dobrzecka, 1960). Subsets of neurons in this area may regulate food intake by integrating circulating metabolic factors like blood glucose (Orsini et al., 1990) and leptin (Håkansson et al., 1998) with extrasensory information such as sight or taste of food (Rolls et al., 1976). Manipulations of this region may dramatically effect consumption. Bilateral lesions of the LH markedly suppress food intake and reduce body weight (Von Der Porten, 1978), while electrical stimulation may elicit eating (Wyrwicka and Dobrzecka, 1960). Furthermore, food intake controlled through the LH can be regulated by the primary amino acid neurotransmitters glutamate and gamma-aminobutyric acid (GABA). Glutamate or N-methyl-d-aspartate (NMDA) injected specifically into the LH elicits robust feeding, whereas NMDA receptor (NMDAR) antagonists injected into this region block this elicited feeding and suppress natural feeding (Stanley et al., 1993, 1996). Conversely, agonists of GABA subtype A receptors (GABA\textsubscript{A}Rs) suppress natural feeding while GABA\textsubscript{A}R antagonists elicit feeding when administed to the LH (Turenius et al., 2009). These findings suggest that natural feeding may, in part, reflect the balance in the synaptic release of glutamate and GABA in the LH. Specifically, meals may be initiated when synaptic release of glutamate increases while meals may be terminated when synaptic release of GABA increases in this region (Stanley et al., 2011). This balance of glutamate and GABA indicates that a certain level of neural firing within this region can regulate a complex behavior as food seeking and ingestion. The nucleus accumbens shell region (AcbSh) is a logical candidate that provides GABA input to the LH to regulate food intake.

Kelley and colleagues demonstrated that blockade of 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid and kainic acid receptors (AMPARs and KARs) or GABA\textsubscript{A}R
activation in this site can specifically induce food intake (Maldonado-Irizarry et al., 1995; Stratford et al., 1998; Stratford and Kelley, 1997). Such manipulations in the accumbens core or other neighboring nuclei were ineffective. The AcbSh and the LH have been linked by evidence that bilateral blockade of LH NMDARs or bilateral activation of LH GABA\textsubscript{A}Rs halts AcbSh-initiated feeding (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1999). Further, AcbSh GABA\textsubscript{A}R activation induces c-fos expression exclusively in the LH of the ipsilateral hemisphere (Stratford, 2005). Thus, it is likely that these two regions communicate to regulate feeding.

Some concern exists regarding the behavior specificity of the aforementioned food intake suppression. Early studies showed that LH lesions can substantially decrease general activity, reduce arousal, and produce sensory neglect or sensory aversion (Levitt and Teitelbaum, 1975; Schalert and Whishaw, 1978). Some of these deficits may be tied to ablation of orexin-producing neurons within the LH, which may regulate arousal and motivation (Hagan et al., 1999, 2005; Thannickal et al., 2000 and 2008). Also, these orexin neurons contain NMDARs and GABA\textsubscript{A}Rs (Yamanaka et al., 2003). Thus, bilateral LH inhibition via NMDAR blockade or GABA\textsubscript{A}R activation might affect overall arousal instead of solely affecting consumatory behaviors.

To examine this issue, Stratford and Wirtshafter (2012) had used unilateral LH cytotoxic lesions to suppress unilateral AcbSh-mediate food intake and found that LH lesions ipsilateral to the AcbSh injection site were more effective than contralateral LH lesions. We extend this finding by examining how ipsilateral LH NMDAR or GABA\textsubscript{A}R modulation may suppress AcbSh mediated feeding. We hypothesize that feeding elicited by AcbSh injection of a GABA\textsubscript{A}R agonist or AMPAR/KAR antagonist will be suppressed by ipsilateral but not contralateral LH injection of an NMDAR antagonist or a GABA\textsubscript{A}R agonist. First, we utilize a design that inhibits the AcbSh unilaterally to elicit feeding and inhibits the LH bilaterally to halt this feeding. This manipulation provides a baseline for subsequent experiments. Second, we inhibit the AcbSh unilaterally to
elicit feeding and inhibit the LH ipsilateral or contralateral to the AcbSh injection site to observe which manipulation halts feeding.

**Methods**

**Subjects and Housing**

Adult male Sprague-Dawley rats, weighing 350-450 grams at the time of surgery, were individually housed and tested in wire mesh cages in a temperature-controlled vivarium at 21 °C with a 12 hour light-dark schedule, with lights on at 9 AM. These subjects were maintained and tested on a sweetened milk-mash diet, which was presented to the animals in plastic food bowls and consisted of 39.3% Purina rat chow powder, 31.4% sugar, and 29.3% evaporated milk (all percentages by mass). All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California – Riverside.

**Surgical Implantation of Guide Cannulas**

Subjects under pentobarbital anesthesia (50 mg/kg body weight, i.p.) were stereotaxically implanted with three 18 mm length, 26 gauge stainless steel guide cannulas, one directed at the AcbSh unilaterally (0.9 mm lateral, 2.3 mm anterior, and 5.8 mm ventral to bregma; Fig. 1A) and two into the LH bilaterally (±1.8 mm lateral, 2.9 mm posterior, and 8.2 mm ventral to bregma; Fig. 1C). Cold cure dental acrylic, anchored to the skull by stainless steel screws, secured the cannulas in place. A plastic guard was embedded in the dental cement to protect the cannulas, and removable 33 gauge stainless steel obturators sealed the cannulas to prevent occlusion. To ensure full recovery and avert transient post-operative confounds, a minimum of seven days passed before performing central injection tests. Prior to central injection tests, animals were handled multiple times and mock injected to adapt them to testing procedures.
Central Injection Technique

During central injections, 0.3 μL of vehicle only or vehicle containing a dissolved drug was injected into the brain tissue via 33 gauge injectors protruding 1 mm beyond the end of the guide cannula. Vehicle consisted of artificial cerebrospinal fluid (aCSF), containing 147 mM Na⁺, 154 mM Cl⁻, 3 mM K⁺, 1.2 mM Ca²⁺, and 0.9 mM Mg²⁺. For DNQX injections and their control vehicle counterparts, a 1:1 mixture of aCSF and dimethylsulfoxide (DMSO) was used. Hypothalamic DMSO and aCSF vehicles produced comparable feeding effects in a previous study (Blevins et al., 2002). Drug and injection site combinations for each experiment are given in Table 3.1. Drug doses for muscimol and D-AP5 in the LH were selected based on their maximal suppressive effect of AcbSh-mediated food intake (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1999) without disrupting food intake past the 4 hour experimental period (Stanley et al., 1995; Turenius et al., 2009). The AcbSh muscimol dose was selected based on maximal efficacy in a prior study (Stratford and Kelley, 1997). The AcbSh DNQX dose selected was higher than that used in a prior study (Maldonado-Irizarry et al., 1995) due to weak effects on food intake from unilateral injections (data not shown). Except where indicated (Table 3.1), animal groups were naïve to the treatment they received.

Experimental Design for Food Intake Measurement

At the start of the light cycle, subjects were fed fresh mash diet for one hour to maximize satiety. Injections commenced immediately afterward. Subjects were tested in every condition in counterbalanced order over multiple days. Food and water were available ad libitum and tests were separated by one or more “rest” days. Food consumption was measured 0.5, 1, 2, and 4 hours post-injection.
Nissl Stain Histology

Cannula placement was determined after completion of the behavioral tests. Subjects were anesthetized by overdose of sodium pentobarbitol (400 mg/kg body weight, i.p.) and transcardially perfused with a 4% formaldehyde solution. Brains were post-fixed in this solution for a minimum of 2 days, frozen with dry ice, sliced on a microtome into 100 μm thick coronal sections, and mounted onto glass microscope slides. Once dried, brain slices were stained with cresyl violet and coverslipped with Permount (Fisher Scientific). The Nissl stained sections were then studied to assess placement of guide cannulas. The sections were compared against templates made from the Paxinos and Watson brain atlas (2007); examples of these sections can be seen in Figure 3.1B and 3.1D. Subjects possessing cannulas located more than 1 mm outside of the intended injection site (either LH or AcbSh) were excluded.

Statistical Tests

The SigmaStat statistical program was used to determine significant differences in these experiments. A two-way repeated measures analysis of variance (ANOVA) was used to determine the significance (p < 0.05) of differences in food intakes between treatment groups in each experiment. When ANOVA results were statistically significant, significant (p < 0.05) differences in food intake between treatment groups at matched times were assessed with post-hoc Student-Newman-Keuls pairwise comparison tests.

Results

Experiments 1A-1D: Bilateral LH inhibition suppresses unilateral AcbSh inhibition-induced food intake

Experiments 1A-1D (Fig. 3.3) used unilateral AcbSh inhibition via DNQX or muscimol
with concurrent bilateral LH inhibition using D-AP5 or muscimol to assess effects on food intake. For all experiments, significant differences were observed between treatment conditions. Experiments 1A (Fig. 3.3A) and 1B (Fig. 3.3B) used unilateral AcbSh injection of the AMPAR/KAR antagonist DNQX to elicit feeding, whereas Experiments 1C (Fig. 3.3C) and 1D (Fig. 3.3D) used unilateral AcbSh injection of the GABA₄R agonist muscimol to elicit feeding. All AcbSh drug injections produced significant food intake as early as 30 minutes post-injection. Bilateral LH drug injections were given simultaneously to halt this elicited eating. For this purpose, Experiments 1A (Fig. 3.3A) and 1C (Fig. 3.3C) used bilateral LH injection of the NMDAR antagonist D-AP5 whereas Experiments 1B (Fig. 3.3B) and 1D (Fig. 3.3D) used bilateral LH injection of muscimol. Both D-AP5 and muscimol injected bilaterally into the LH halted AcbSh DNQX- or muscimol-elicited eating. LH drug injections reduced AcbSh-mediated feeding to baseline intake amounts. Significant ANOVA testing results showed differences between treatments (Exp 1A: F(3,30) = 4.125, p = 0.015; Exp. 1B: F(3,39) = 7.358, p < 0.001; Exp. 1C: F(3,30) = 3.578, p = 0.025; Exp. 1D: F(3,51) = 8.195, p < 0.001), across testing times (Exp. 1A: F(3,30) = 9.18, p < 0.001; Exp. 1B: F(3,39) = 25.635, p < 0.001; Exp. 1C: F(3,30) = 17.233, p < 0.001; Exp. 1D: F(3,51) = 40.745, p < 0.001), and in the treatment by time interactions (Exp. 1A: F(9,30) = 3.019, p = 0.003; Exp. 1B: F(9,39) = 2.728, p = 0.006; Exp. 1C: F(9,30) = 3.195, p = 0.002; Exp. 1D: F(9,51) = 2.919, p = 0.003). No effects on food intake were produced by co-administration of AcbSh vehicle with bilateral LH D-AP5 or muscimol in any experiment.

Experiments 2A-2D: Ipsilateral LH inhibition is more effective in reducing AcbSh inhibition-induced feeding than contralateral LH inhibition

To investigate the behavioral specificity of feeding-suppressive LH NMDAR and
GABA\(_A\)R modulation, these receptors were modulated unilaterally instead of bilaterally within the LH. We elicited feeding via unilateral AcbSh DNQX infusion, then injected D-AP5 (Experiment 2A, Fig. 3.4) or muscimol (Experiment 2B, Fig. 3.5) into the LH ipsilateral or contralateral to the AcbSh injection site. In both experiments, significant differences were seen between treatments (\(F(5,65) = 3.215, p = 0.012\) for Exp. 2A and \(F(5,50) = 6.502, p < 0.001\) for Exp. 2B), times (\(F(3,39) = 27.796, p < 0.001\) and \(F(3,30) = 17.779, p < 0.001\)), but only treatment by time interaction for Experiment 2A (Exp. 2A: \(F(15,65) = 2.211, p = 0.007\); Exp. 2B: \(F(15,50) = 1.282, p = 0.22\)). Unilateral AcbSh DNQX administration significantly increased food intake within the first half hour. Notably, D-AP5 infused into the ipsilateral LH blocked AcbSh DNQX feeding, whereas contralateral LH D-AP5 had no effects on this elicited feeding (Fig. 3.4). Similarly, ipsilateral LH muscimol injection reduced AcbSh DNQX-induced food intake while contralateral LH muscimol again had no effect on the elicited intake (Fig. 3.5). Thus, D-AP5 or muscimol, when placed into the ipsilateral LH, were more effective in reducing feeding induced by AcbSh DNQX than contralateral LH drug injections.

We next aimed to determine whether ipsilateral LH drug injections were more effective than contralateral injections in suppressing AcbSh muscimol-induced feeding. Experiments 2C (Fig. 3.6) and 2D (Fig. 3.7) show significant differences between treatments (\(F(5,75) = 9.627, p < 0.001\) for Exp. 2C and \(F(5,65) = 4.616, p = 0.001\) for Exp. 2D), times (\(F(3,45) = 15.249, p < 0.001\) and \(F(3,39) = 25.822, p < 0.001\)), and treatment by time interactions (\(F(15,75) = 2.763, p < 0.001\) and \(F(15,65) = 2.277, p = 0.006\)). Unilateral AcbSh muscimol injection significantly increased intake within a half hour post-injection. Ipsilateral LH administration of D-AP5 halted this feeding (Fig. 3.6). Contralateral LH D-AP5 also somewhat reduced the elicited feeding, as the intake was still significantly greater than control treatment intakes. Importantly, ipsilateral LH D-AP5 was significantly more effective than contralateral LH D-AP5 in suppressing AcbSh
muscimol-elicited feeding. Lastly, AcbSh muscimol was administered in tandem with ipsilateral or contralateral LH muscimol. Ipsilateral LH muscimol transiently reduced the elicited feeding while contralateral LH muscimol did not (Fig. 3.7). In Experiments 2A – 2D, food intake was unaffected by ipsilateral or contralateral LH injections of D-AP5 or muscimol in tandem with vehicle administration into the AcbSh.

**Discussion**

These experiments provide evidence for an ipsilateral feeding-specific brain circuit connecting the AcbSh and the LH. First, we replicated results showing that injection of the AMPAR/KAR antagonist DNQX or the GABA\(_A\)R agonist muscimol into the AcbSh increases food intake (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1997). We also showed that both of these elicited feeding effects are blocked by bilateral LH administration of an NMDAR antagonist or a GABA\(_A\)R agonist. Prior evidence had shown that AcbSh DNQX-elicited feeding can be blocked with bilateral LH muscimol and AcbSh muscimol-elicited feeding can be blocked with bilateral LH D-AP5 (Maldonado-Irizarry et al., 1995; Stratford and Kelly, 1999). We replicated these findings and provided new data showing that that AcbSh DNQX-elicited feeding can be blocked with bilateral LH D-AP5 (Fig. 3.3A, Experiment 1A) and that AcbSh muscimol-elicited feeding can be blocked with bilateral LH muscimol (Fig. 3.3D, Experiment 1D). This combination of results suggests that bilateral NMDAR inactivation or GABA\(_A\)R activation of the LH suppresses feeding mediated by either AcbSh AMPAR/KAR inactivation or GABA\(_A\)R activation. Furthermore and most importantly, we report the novel finding in support of our hypothesis that ipsilateral LH NMDAR antagonism halts and LH GABA\(_A\)R activation reduces AcbSh-mediated food intake, while contralateral LH drug injections showed little or no effect on feeding.
An unexpected finding was that contralateral LH D-AP5 reduced AcbSh muscimol feeding (Experiment 2C, Fig. 3.6). It might be possible that the contralateral effect was due to a partially hemisphere-crossed circuit from the AcbSh to the LH. However, the intake reducing effect of contralateral LH D-AP5 was not observed with AcbSh DNQX-elicited feeding (Fig. 4) and thus the cross-hemisphere proposal seems unlikely. Another possibility is AcbSh muscimol increased food intake enough to allow a weak intake-suppressive effect of contralateral LH D-AP5 to become apparent.

As AMPAR/KAR antagonists and GABA\(_A\)R agonists are likely to decrease activity in AcbSh neurons expressing GABA\(_A\)Rs, AMPARs, or KARs, inhibition of the AcbSh appears to stimulate food intake. That AcbSh-mediated feeding may be inhibited by NMDAR antagonists or GABA\(_A\)R agonists in the LH suggests hyperpolarization of LH neurons may suppress food intake. Assuming inhibition of the LH halts feeding induced by AcbSh inhibition, our findings suggest that AcbSh-mediated feeding is dependent on LH activation. Most importantly, our results suggest that unilateral AcbSh-mediated feeding requires normal activity of the ipsilateral LH, whereas activity of the contralateral LH is less critical for this feeding.

Research from our lab has suggested that the balance of glutamatergic and GABAergic transmission within the LH is essential for food intake regulation, especially through NMDARs and GABA\(_A\)Rs (Stanley et al., 1993, 1996; Turenius et al., 2009). We have suggested that synaptic release of LH glutamate participates in the initiation of natural feeding via NMDARs and that synaptic release of LH GABA participates in the termination of natural feeding via GABA\(_A\)Rs (Stanley et al., 2011). The origins of glutamatergic and GABAergic synaptic inputs into the LH are numerous. Of these inputs, the AcbSh seemed a likely source of GABAergic input based on the evidence for a functional association between the two regions (Maldonado-Irizarry et al., 1995; Stratford and Kelly, 1999).
Anatomical evidence suggests that there is a strong, if not exclusive, ipsilateral bias to the AcbSh-LH circuit. Firstly, a collection of evidence shows projections originating from the AcbSh and terminating in the LH (Duva et al., 2005; Usuda et al., 1998). Further, these projections to the LH arise specifically from the medial AcbSh and not the accumbens core (Heimer et al., 1991; Usuda et al., 1998; Zahm and Heimer, 1993). The AcbSh contacts more limbically-situated targets such as the LH while projections of the accumbens core are more basal ganglia-oriented. Importantly, unilateral retrograde Fluorogold labeling revealed that the ipsilateral nucleus accumbens has a moderate number of neurons projecting to the injection site whereas the accumbens contralateral to the injection showed few or no retrogradely-labeled neurons (Duva et al., 2005). This collection of anatomical evidence is supported by a functional study in which unilateral AcbSh inhibition causes elevated ipsilateral LH c-fos (Stratford, 2005). Collectively, these findings support the proposal made by Kelley et al. (2005), among others, that the AcbSh may utilize a direct pathway to communicate with the LH. In light of these ideas, we sought to determine whether there is an ipsilateral bias in the ability of LH inhibition to halt AcbSh-mediated feeding through specific receptor subtypes, most importantly those acted on by glutamate and GABA.

A recent study demonstrated this functional ipsilateral bias by showing that unilateral AcbSh inhibition-induced feeding is suppressed more effectively by ipsilateral cytotoxic LH lesions than by contralateral LH lesions (Stratford and Wirtshafter, 2012). This study provided evidence for the functional lateralization of the AcbSh-LH circuit that had been suggested by prior anatomical studies. However, the role of different receptor subtypes in this ipsilateral circuit had not been addressed. Also, lesions are a chronic manipulation that may induce unforeseen long term changes in communications between brain regions, whereas injections are acute and may not precipitate such unknown changes. We employed an ipsilateral versus contralateral injection
design to temporarily and specifically modulate ionotropic primary amino acid neurotransmitter receptors. Using this design, we sought to determine whether an ipsilateral bias exists in their communications between the AcbSh and the LH. To this end, our results suggest that glutamate and GABA neurotransmission in the AcbSh and LH are components of an ipsilateral neurocircuit that plays a role in food seeking and consumatory behaviors.

Suppression of unilateral AcbSh-elicited feeding with ipsilateral and not contralateral LH inhibition suggests the effect is feeding-suppressive and not does not impair other behaviors. If LH inhibition had resulted in non-specific motor or arousal confounds, AcbSh-mediated food intake would have decreased equally from unilateral LH injections to either hemisphere. Decreasing arousal is one example of a non-specific means that can suppress food intake. The LH does regulate arousal using orexin neurons. As orexin is tied to both feeding behaviors (Sweet et al., 1999) and wakefulness (Tsunematsu et al., 2011), it is difficult to discern which of these two roles is predominantly affected during bilateral LH inhibition. Inhibiting orexin neurons or blocking orexin function does promote sleep. Bilateral optogenetic silencing of orexin neurons is sufficient to promote slow wave sleep in mice and administration of an orexin antagonist can induce sleep even in the dark phase (Brisbare-Roch et al., 2007; Tsunematsu et al., 2011). However, the results of our research oppose the argument of feeding suppression through non-specific means: ipsilateral LH inhibition successfully suppresses AcbSh mediated feeding whereas contralateral does not. Thus, AcbSh-LH communication may regulate feeding and not these other behaviors.

As the AcbSh directly innervates the LH (Duva et al., 2005) and efferents from the AcbSh are primarily GABAergic (Oertel and Mugnaini, 1984), inhibition of AcbSh GABAergic neurons projecting to the LH may release the LH from inhibition and activate or modulate feeding behaviors (Kelley et al., 2005). Further, glutamatergic efferents to the LH from sources
such as the frontal cortex or thalamus (Duva et al., 2005; Fremeau et al., 2001; Fremeau et al., 2004) may also be released from inhibition presynaptically. This dis-inhibition would allow LH synaptic glutamate output to rise and may activate LH NMDARs. Alternatively, initiation of feeding via the LH may require a simultaneous halting of GABAergic input from the AcbSh and an increase in glutamate input from other sources. Nonetheless, current knowledge suggests that the AcbSh is poised to modulate LH-mediated feeding via its GABAergic or potentially peptidergic outputs.

We support the idea that inhibition of the AcbSh or excitation of the LH will result in feeding. However, the neuropharmacological effects on feeding through these regions may be more complicated than simply turning brain sites on or off. Some studies have shown food intake effects from drugs acting on GABA\(_B\)Rs or NMDARs in the AcbSh and other substances acting on AMPARs/KARs in the LH (Stanley et al., 1993; Stratford and Kelley, 1997; Echo et al., 2001; Hettes et al., 2003; Miner et al., 2010;). In a specific case, AcbSh injection of NMDA or AMPA can induce intake (Echo et al., 2001). Further, AcbSh AMPAR and mu opioid receptor (MOR) co-stimulation synergize to induce greater food intake than MOR stimulation alone. Considering these effects, we elected to use DNQX or muscimol in the AcbSh and D-AP5 or muscimol in the LH due to the consistency of their effects on food intake in prior studies (Maldonado-Irizarry et al., 1995; Stanley et al., 1996; Stratford et al., 1998; Stratford and Kelley, 1999; Stratford, 2005; Stratford and Wirtshafter, 2012; Turenius et al., 2009). We nonetheless acknowledge the idea that other receptor types also play an important and complex role in food intake control through these regions.

Although there may be direct functional connections between the AcbSh and the LH, there are likely also indirect functional pathways between these regions. Evidence implicates the ventral pallidum (VP) as a likely intermediate area, as VP disinhibition induces eating (Stratford
et al., 1999), AcbSh inhibition activates the VP ipsilaterally (Stratford, 2005), and the AcbSh projects to the medial VP, which in turn projects to the LH (Heimer et al., 1991; Haber et al., 1985; Groenewegen et al., 1993). While some data supports an intermediate functional role for the VP (Stratford and Wirtshafter, 2012), other research suggests the nucleus accumbens and the VP work in parallel, not serially, to induce food consumption (Taha et al., 2009). This discrepancy may be due to the different neurotransmitter systems activated in these studies, specifically MORs versus GABA₃Rs. These different neurotransmitter receptors exhibit different loci of action within the accumbens (accumbens core versus AcbSh; Zhang and Kelley, 2000; Stratford and Kelley, 1997). Thus, accumbens MOR-mediated feeding may not require the VP, but AcbSh GABA₃R mediated feeding likely does. What remains unclear is whether the circuit simply utilizes the VP as an intermediate, or if the direct AcbSh to LH pathway and the indirect AcbSh to VP to LH pathway must both be intact to execute feeding behaviors. The latter idea is supported by anatomical data showing the existence of both pathways.

Our results, in conjunction with prior research, argue for a functional, ipsilateral AcbSh to LH circuit utilizing glutamatergic and GABAergic signaling. By utilizing this ipsilateral versus contralateral injection design, concerns of non-specific behavioral effects are largely alleviated. The essential role of glutamate and GABA in controlling feeding through specific receptor subtypes in both of these regions is reinforced by our findings. As the communication between the AcbSh and the LH appears to be heavily geared toward food intake and perhaps to some degree appetitive responses through its associations with reward, this pathway may be an ideal target for novel treatments in the prevention of obesity.
References


### Table 3.1: Injection Paradigms

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Figure</th>
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<th>Injection sites and drugs used</th>
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<td>11</td>
<td></td>
<td>DNQX (1.25 µg)</td>
<td>D-AP5 (2 µg bilaterally)</td>
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<tr>
<td>1B</td>
<td>3B</td>
<td>14</td>
<td></td>
<td>DNQX (1.25 µg)</td>
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<tr>
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<td>D-AP5 (2 µg bilaterally)</td>
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<tr>
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<tr>
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<td>14*</td>
<td>Muscimol (100 ng)</td>
<td>Muscimol (25 ng unilaterally)</td>
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List of individual experiments, their respective figures, subject pool sizes, drugs injected unilaterally into the AcbSh, and drugs injected into the LH bilaterally or unilaterally. All drug amounts are per hemisphere. Control treatments featured vehicle (DMSO for DNQX, aCSF for all others) without the listed drug. An asterisk in the subjects column indicates where some animals were used for two experiments.
Figure 3.1: Representation of cannula tip locations in the AcbSh (A-C) and the LH (D-F) for all animals used in this study. AcbSh diagrams represent coronal slices at 2.52 (A), 2.16 (B), and 1.80 (C) um anterior of bregma. LH diagrams represent slices at 2.40 (D), 3.00 (E), and 3.36 (F) um posterior of bregma. Individual cannula placements are marked by either circles for subjects in Experiments 1A-1D or triangles for subjects in Experiments 2A-2D. These shapes are much smaller than the area of tissue damage on the original brain tissue and should be considered as the centerpoints of where injections terminated. Images were modified from pages in the Paxinos and Watson (2005) brain atlas.
Figure 3.2: Photographs of Nissl-stained coronal sections are shown for the AcbSh (A) and the LH (B), with cannula scarring and injector tip bruising denoted by arrows. Note that the section shown in (A) does not feature a connected corpus callosum – the AcbSh cannula was aimed at 2.3 mm anterior of bregma, right before the corpus callosum appears.
Figure 3.3: Food intake over time for Experiments 1A-1D. DNQX (A, B) or 100 ng muscimol (C, D) injection elicits feeding when delivered to the AcbSh unilaterally (*, p < 0.05; **, p < 0.01), and D-AP5 (A, C) or 25 ng muscimol (B, D) infused bilaterally into the LH halts this increase (^, p < 0.05; ^^, p < 0.01). Treatment groups are listed on the x axes. All AcbSh injections are unilateral, and all LH injections are bilateral. Mean total amount consumed is represented over progressive times post-injection, by bar fill color (see legend). DMSO is used for brevity to denote the AcbSh vehicle injection composed of the aCSF/DMSO mixture. Error bars are +/- 1 SEM.
Figure 3.4, Experiment 2A: Ipsilateral LH D-AP5 blocks feeding spurred by unilateral AcbSh DNQX injection. A ° symbol denotes intake suppression compared with AcbSh DNQX + Contralateral LH D-AP5 (p < 0.05) while a # symbol denotes intake suppression in comparison to AcbSh DNQX + Bilateral LH aCSF (p < 0.08). Treatment with contralateral LH D-AP5 does not suppress unilateral AcbSh DNQX feeding. Graph conventions similar to prior figures.

Figure 3.5, Experiment 2B: AcbSh DNQX elicits significant feeding, and ipsilateral LH muscimol treatment (25 ng) reduces this intake increase. Contralateral LH muscimol fails to suppress feeding as intake is still significant. Graph conventions similar to prior figures.
Figure 3.6, Experiment 2C: Unilateral AcbSh inhibition with muscimol (100 ng) produces significant food intake, and ipsilateral LH D-AP5 blocks this increase. Ipsilateral D-AP5 has more efficacy in suppressing this increase compared to contralateral D-AP5 (°, p < 0.05). Although contralateral LH D-AP5 partially reduces the elicited feeding (as denoted by + and ++ when compared against AcbSh muscimol + Bilateral LH aCSF, p < 0.05 and p < 0.01 respectively), this feeding is still significantly greater than baseline. Graph conventions similar to prior figures.

Figure 3.7, Experiment 2D: Ipsilateral LH muscimol (25 ng) is effective in reducing the significant increase in feeding resulting from unilateral AcbSh muscimol (100 ng). Contralateral LH muscimol is ineffective in reducing the increase in intake. Graph conventions similar to prior figures.
Chapter 4 - Behaviorally Specific Versus Non-Specific Suppression of Accumbens Shell-Mediated Feeding by Ipsilateral Versus Bilateral Inhibition of the Lateral Hypothalamus

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Abstract

The nucleus accumbens shell (AcbSh) and lateral hypothalamus (LH) are linked in the control of food intake. Pharmacological inhibition of the LH may block AcbSh-elicited feeding, but the behavioral phenotype associated with this feeding suppression is unknown. To examine this phenotype, adult male Sprague-Dawley rats were implanted with three cannulas - one unilaterally in the AcbSh and two bilaterally in the LH - to allow for central drug injections. The AcbSh received injections of the AMPA receptor antagonist DNQX or the GABA\textsubscript{A} receptor agonist muscimol while the LH received injections of the NMDA receptor antagonist D-AP5 or muscimol. Eating, drinking, grooming, locomotion, quiescence, and sleeping behaviors were measured every minute for 60 minutes post-injection. From this observational data, feeding bout durations, feeding frequency, and latency to feed were determined. AcbSh muscimol or DNQX increased food intake by increasing feeding bout durations and frequency and decreasing latency to feed. D-AP5 or muscimol, injected into the LH bilaterally or ipsilateral to the AcbSh injection, reversed these AcbSh-mediated effects. Though bilateral LH D-AP5 or muscimol injections blocked feeding responses, they also hastened onset of sleep. In contrast, ipsilateral LH D-AP5 or muscimol injections suppressed AcbSh-mediated feeding behaviors without substantially altering sleeping or other behaviors. These results suggest bilateral LH inhibition via NMDA receptor blockade or GABA\textsubscript{A} receptor activation produces behavioral effects that might indirectly suppress feeding, but ipsilateral LH inhibition through these receptors suppresses AcbSh AMPA
and GABA<sub>A</sub> receptor-mediated feeding specifically. This evidence strengthens the concept of a feeding-specific association between these regions.

**Introduction**

The prevalence of obesity in developed nations has led to much research that investigates the neural circuitry governing food intake. A notable intake-controlling brain region is the lateral hypothalamus (LH). Early studies showed that LH lesions produce hypophagia, while electrical stimulation of the LH elicits eating (Von Der Porten and Davis, 1979; Wyrwicka and Dobrzecka, 1960). Studies from our lab implicate the LH as a key brain region through which glutamate and gamma-aminobutyric acid (GABA) regulate feeding. Specifically, activation of N-methyl-D-aspartate receptors (NMDARs) or blockade of GABA subtype A receptors (GABA<sub>A</sub>Rs) will induce feeding, and will do so with behavioral specificity (Duva et al., 2001; Stanley et al., 1993; Turenius et al., 2009a). Mapping studies show that the LH is a primary site for these effects (Duva et al., 2002; Turenius et al., 2009b). Also, LH administration of the NMDAR antagonist D-AP5 or the GABA<sub>A</sub>R agonist muscimol will suppress drug-elicited, nocturnal, and deprivation-induced feeding in rats (Stanley et al., 1996; Turenius et al., 2009a). Food intake mediated through the LH is thought to be regulated in part by the balance between synaptic glutamate and GABA levels in this region (Stanley et al., 2011). Thus, glutamate and GABA act within the LH to coordinate the initiation and cessation of appetitive responses.

Although initially regarded as a regulator of reward, substantial research also implicates the nucleus accumbens shell subregion (AcbSh) in controlling food intake (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1997; Stratford et al., 1998), with glutamate and GABA also mediate feeding through this region. Within the AcbSh, blockade of 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid receptors (AMPARs) with DNQX or activation of
GABA_ARs with muscimol induces food intake, an effect not mediated by surrounding brain regions (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1997). Further data demonstrates that the induced food intake is specific to eating and not due to behavioral hyperactivity (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1997). Additionally, action potential activity ceases in certain AcbSh neurons prior to initiation of sucrose intake (Krause et al., 2010), further solidifying the AcbSh's role in natural feeding processes.

Much research links the AcbSh and the LH in the control of eating, especially studies in which bilateral inhibition of the LH with D-AP5 or muscimol blocks AcbSh DNQX- or muscimol-elicted feeding (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1999; Urstadt et al., 2013). Also, unilateral AcbSh muscimol induces c-fos expression in the ipsilateral but not contralateral LH (Stratford, 2005). Retrograde tract tracer infusion into the LH labels ipsilateral AcbSh neurons, suggesting that a connection between the nuclei may be direct and ipsilateral (Duva et al., 2005). Most importantly, unilateral AcbSh muscimol-elicited eating can be suppressed by ipsilateral LH lesions (Stratford and Wirtshafter, 2012) or by ipsilateral LH GABA_A, R activation or NMDAR blockade (Urstadt et al., 2013), an effect not shared by such receptor modulation in the contralateral LH. These studies suggest that the AcbSh requires normal LH functioning to produce feeding behaviors, and that these nuclei may have a feeding-specific relationship.

However, the LH controls functions in addition to feeding. Studies using bilateral lesions have shown behavioral deficits aside from hypophagia. For example, bilateral LH lesions may produce adipsia, hypolocomotion, increased sedation, and neglect of or aversion to stimuli (Levitt and Teitelbaum, 1975; Schallert and Whishaw, 1978). It has not been determined whether or not acute bilateral LH inhibition (via drug application) impacts behaviors other than feeding, thought we did incidentally observe a decrease in overall activity in our prior study (Urstadt et al.,
This issue is important when interpreting past studies that used bilateral LH inhibition to suppress natural, deprivation, or drug-induced food intake, as this suppression might have been an artifact of an indirect behavioral effect. These non-specific behavioral effects are not seen from unilateral LH lesions (Feeney and Wier, 1979; Stratford and Wirtshafter, 2012), but it is unclear if unilateral LH inhibition produces such non-specific effects, especially on AcbSh-mediated feeding. We hypothesize that bilateral LH inhibition with D-AP5 or muscimol halts AcbSh-mediated feeding non-specifically, whereas ipsilateral LH inhibition with D-AP5 or muscimol will halt this feeding specifically. Using behavioral assays, we assessed the behavioral phenotypes resulting from bilateral or unilateral LH D-AP5 or muscimol on normal and AcbSh DNQX- or muscimol-induced behaviors.

**Methods**

**Subjects and Housing**

Forty adult male Sprague-Dawley rats received surgery for the experiments described in this paper. However, only the data from twenty-nine rats were used, as the data from the remaining eleven were not included due to mistargeted guide cannulas. These subjects, weighing 350-450 grams at the time of surgery, were individually housed and tested in wire mesh cages in a temperature-controlled vivarium at 21 °C with a 12 hour light-dark schedule, with lights on at 9 AM. These subjects were maintained and tested on a sweetened milk-mash diet, which was presented to the animals in plastic food bowls and consisted of 39.3% Purina rat chow powder, 31.4% sugar, and 29.3% evaporated milk. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California – Riverside.
Surgical Implantation of Guide Cannulas

Subjects under pentobarbital anesthesia (50 mg/kg body weight, i.p.) were stereotaxically implanted with three 18 mm length, 26 gauge stainless steel guide cannulas, one directed at the AcbSh unilaterally (0.9 mm lateral, 2.3 mm anterior, and 5.8 mm ventral to bregma) and two into the LH bilaterally (±1.8 mm lateral, 2.9 mm posterior, and 8.2 mm ventral to bregma). Cold cure dental acrylic, anchored to the skull by stainless steel screws, secured the cannulas in place. A plastic guard was embedded in the dental cement to protect the cannulas, and 33 gauge stainless steel obturators were inserted into the cannulas to maintain patency. Central injection tests commenced seven days after surgery to ensure full recovery. Prior to central injection tests, animals were handled multiple times and mock-injected to adapt them to testing procedures.

Central Injection Technique

A drug solution or vehicle was injected into the brain tissue in 0.3 μL volumes via 33 gauge injectors protruding 1 mm beyond the end of the guide cannula. Vehicle consisted of artificial cerebrospinal fluid (aCSF), containing 147 mM Na\(^+\), 154 mM Cl\(^-\), 3 mM K\(^+\), 1.2 mM Ca\(^{2+}\), and 0.9 mM Mg\(^{2+}\). The GABA\(_A\)R agonist muscimol (100 ng), the AMPAR antagonist DNQX (1.25 μg), and the NMDAR antagonist D-AP5 (2 μg) were used in this study; doses are per brain hemisphere. DNQX was dissolved in a 1:1 mixture of dimethylsulfoxide (DMSO) and aCSF. Drugs and dosages are based on our prior study (Urstadt et al., 2013), except that the dose of muscimol in the LH was increased to more effectively suppress AcbSh-elicited eating. Drug doses, site combinations, and number of subjects in each experiment are given in Table 4.1.

Experimental Design for Behavioral Assessments

Subjects received fresh mash diet at the start of the light cycle to maximize satiety and
injections commenced one hour later. For Experiments 1 and 2, rats received vehicle or drug unilaterally into the AcbSh, then vehicle or drug bilaterally into the LH. In Experiments 3 and 4, AcbSh injections were the same, but LH injections utilized either vehicle on both sides, drug in the ipsilateral hemisphere and vehicle contralaterally, or vehicle ipsilaterally and drug contralaterally. To simplify graphical representation of the behavioral results in Experiments 3 and 4, groups receiving vehicle in the AcbSh and drug in either the ipsilateral or contralateral LH were merged into an AcbSh vehicle – unilateral LH drug group after determining that there were no significant behavioral differences between these treatments. Subjects were tested in every condition in counterbalanced order over multiple days, each separated by a rest day without injections. Food intake was measured immediately prior to injections and one hour post-injection. Subjects were only used in one experiment before sacrifice.

Behavioral observations lasted 70 minutes, starting 10 minutes prior to injection to assess baselines and ending 60 minutes post-injection to assess drug effects. Subjects were observed in their home cages pre- and post-injection by a trained rater that was unaware of which rats received which treatments. Each subject was monitored for 7 second per minute in Experiments 1 and 2, and 5 seconds per minute in Experiments 3 and 4. These time lengths were determined by dividing 60 seconds by the number of subjects in each experiment; Experiments 1 and 2 had 8 subjects each, while Experiments 3 and 4 had 12 subjects each (Table 4.1). During the 5-7 second monitoring period, only the single dominant behavior observed (the behavior the rat spent the most time engaging in) was recorded for each rat. The same rater was used for a given experiment across all experimental days to avoid inter-rater discrepancies in the observations. Minute-by-minute counts for individual behaviors were combined into 5 minute bins.

Behaviors were classified as follows: eating - consuming food directly from the bowl or scooping food into paws and eating from them; drinking - licking water spout; grooming - licking
body fur, scratching at the head using paws, or cleaning the tail; locomotion - any form of ambulation around the cage, including rearing; sleeping - inactive with eyes closed and curled into species-typical sleeping posture; quiescence - inactive but visible wakefulness, eyes open, alert, not in sleeping posture.

Feeding characteristics such as average bout duration, maximum bout duration, number of bouts, and latency to eat were also assessed. A feeding bout was defined as the interval over which a subject was continually observed to be eating. Bout characteristics were derived from the observed times at which eating commenced and stopped for each rat. For each subject, an average of the durations of multiple feeding bouts defined average bout duration, while the duration of the largest observed feeding bout defined maximum bout duration. These values were averaged across subjects within each treatment.

Nissl Stain Histology

Cannula placement was determined after completion of the behavioral tests. Subjects were anesthetized by overdose of sodium pentobarbital (400 mg/kg body weight, i.p.) and transcardially perfused with a 10% formalin solution. Brains were post-fixed in this solution for a minimum of 2 days, frozen with dry ice, sliced on a microtome into 100 μm thick coronal sections, and mounted onto glass microscope slides. Once dried, brain slices were stained with cresyl violet and coverslipped with Permount (Fisher Scientific). The Nissl stained sections were then studied to assess correct placement of guide cannulas. The sections were compared against templates made from a brain atlas (Paxinos and Watson, 2005). Subjects possessing any cannula located more than 0.5 mm outside the LH or AccSh were excluded. This distance was determined using a reverse-projection microscope and printed brain atlas sheets scaled to the size of the
Nissl-stained slice projections. A rat required all cannulas to correctly target the AcbSh and the LH to be included in this study.

Statistical Tests

Differences within each behavior between treatment groups over time were assessed for each experiment using a two-way repeated measures ANOVA with a p < 0.05 criterion for significance. When a significant main effect of treatment or a significant treatment by time interaction was found, Tukey's pairwise comparisons were conducted to localize the specific differences. Food intake characteristics (intake amount, feeding bout durations, latency to eat, and number of feeding bouts) were assessed using a one-way repeated measures ANOVA. Significant ANOVA results were followed by Fisher's pairwise comparisons to localize the specific differences.

Results

Treatment Effects on Feeding Characteristics

AcbSh injections of DNQX or muscimol produced significant food intake in all experiments (Fig. 4.1A-1D). Significant differences were detected between groups in all experiments (Exp. 1: F(3,15)=8.7, p<0.001; Exp. 2: F(3,15) = 8.7, p<0.001; Exp. 3: F(4,28)=3.3, p<0.05; Exp. 4: F(4,32)=6.6, p<0.001). Bilateral (Fig. 4.1A) or ipsilateral (Fig. 4.1C) LH D-AP5 injection abolished AcbSh-mediated feeding, as did bilateral (Fig. 4.1B) or ipsilateral (Fig. 4.1D) LH muscimol.

In Experiments 1 and 2, ANOVA tests revealed significant effects of treatment on average feeding bout duration, maximum feeding bout duration, number of feeding bouts, and latency to eat (Table 4.2). In Experiment 1, AcbSh DNQX significantly increased all bout
characteristics and decreased latency relative to control. Concurrent bilateral LH D-AP5 injection blocked these changes. In Experiment 2, AcbSh muscimol treatment significantly increased bout characteristics but did not significantly alter latency. Concurrent bilateral LH muscimol treatment abolished the bout characteristic increases, and increased latency to eat relative to AcbSh muscimol alone.

ANOVA showed significant effects of treatment on average bout duration, number of bouts and latency to eat in Experiment 3, and on average bout duration, maximum bout duration, number of bouts, and latency to eat in Experiment 4 (Table 4.2). In Experiment 3, AcbSh DNQX alone and AcbSh DNQX with concurrent contralateral LH D-AP5 significantly increased average bout duration and number of bouts while decreasing latency to eat. Ipsilateral LH D-AP5, given concurrently with AcbSh DNQX, significantly abolished these effects. In Experiment 4, AcbSh muscimol alone significantly increased maximum bout duration and number of bouts while decreasing latency. AcbSh muscimol with contralateral LH muscimol increased both average and maximum bout duration but did not significantly affect number of bouts or latency. Ipsilateral LH muscimol, injected with AcbSh muscimol, significantly decreased average bout duration relative to AcbSh muscimol with contralateral LH muscimol and also decreased maximum bout duration relative to both other AcbSh muscimol groups. However, ipsilateral LH muscimol treatment did not significantly alter other AcbSh muscimol-mediated feeding changes.

Bilateral LH Inhibition Interferes with Feeding and Other Behaviors

In Experiment 1 (Figure 4.2), main effects of treatment were significant for eating (F(3,195)=8.9, p<0.001), grooming (F(3,195)=3.4, p<0.05), sleeping (F(3,195)=13.8, p<0.001), and quiescence (F(3,195)= 8.427, p<0.01), but not for locomotion or drinking (no drinking was observed). Time was significant for all behaviors (except drinking; not shown), and interaction
of treatment by time was significant for eating, grooming, and sleeping, but not for locomotion or quiescence. As shown in Fig 4.2A, AcbSh DNQX paired with bilateral LH vehicle produced feeding that was sustained from 5 to 20 minutes post-injection. Subsequently, a marked increase in sleeping was observed in this group (Fig. 4.2E). Importantly, co-treatment with bilateral LH D-AP5 suppressed this elicited feeding, but also accelerated the onset of sleep. Specifically, this treatment significantly increased sleep versus control from 20 to 40 minutes post-injection while significantly decreasing quiescence from 25 to 30 minutes post-injection. Both groups receiving D-AP5 bilaterally into the LH exhibited suppressed grooming relative to control from 20 to 25 minutes post injection (Fig. 4.2C). AcbSh vehicle with bilateral LH D-AP5 resulted in a substantial suppression of sleep versus control from 55 to 60 minutes (Fig. 4.2E), at which point quiescence was substantially increased versus both AcbSh DNQX groups (Fig. 4.2F). In summary, AcbSh DNQX induced feeding, while bilateral LH D-AP5 treatment suppressed AcbSh DNQX-induced feeding but also altered other behaviors.

In Experiment 2 (Figure 4.3), main effects of treatment were significant for eating ($F(3,195)=5.6, p<0.01$), grooming ($F(3,195)=5.5, p<0.01$), locomotion ($F(3,195)=3.7, p<0.05$), and sleeping ($F(3,195)=11.1, p<0.001$), but not in drinking or quiescence. ANOVA tests on all behaviors except drinking revealed a significant effect of time (not shown). Significant interaction of treatment by time were seen in locomotion ($F(39,195)=1.5, p<0.05$) and quiescence ($F(39,195)=1.4, p<0.05$) only. Experiment 2 showed similarities to Experiment 1 in feeding and sleeping. AcbSh muscimol alone increased feeding from 15 to 20 minutes post-injection (Fig. 4.3A). This treatment also increased locomotion at 60 minutes post-injection (Fig. 4.3D) and did not significantly change sleep versus control. Co-treatment with bilateral LH muscimol blocked the elicited feeding, substantially decreased locomotion from 10 to 20 minutes, decreased grooming from 30 to 35 minutes (Fig. 4.3C), and increased sleeping from 15 to 25
minutes post-injection (Fig. 4.3E). From 30 to 60 minutes post-injection, both groups receiving muscimol bilaterally to the LH exhibited increased sleeping relative to the AcbSh muscimol – LH vehicle group. Overall, AcbSh muscimol induced feeding and locomotive behaviors, while co-treatment suppressed this feeding and locomotion but also altered other behaviors.

**Ipsilateral LH Inhibition Suppresses Feeding Specifically**

Fewer behavioral effects were seen in Experiment 3 (Figure 4.4) compared to prior experiments. Main effects of treatment were significant only for grooming (F(4,364)=4.0, p<0.05) and quiescence (F(4,364)=2.7, p<0.05). Time had a significant main effect on all behaviors except drinking (not shown). The only significant interaction effect was on eating (F(13,364)=1.4, p<0.05). Both AcbSh DNQX alone and with contralateral D-AP5 increased eating relative to control at 10-15 minutes post-injection (Fig. 4.4A). Notably, ipsilateral LH D-AP5 blocked this AcbSh DNQX-elicited feeding. AcbSh DNQX alone also increased grooming behaviors at 25 and 40 minutes post-injection (Fig. 4.4C). Unilateral D-AP5, with AcbSh vehicle, elevated quiescence transiently at 35-40 minutes post-injection relative to AcbSh DNQX alone (Fig. 4.4F). However, no differences in sleeping were noted across treatments (Fig. 4.4E).

Comparable effects were observed in Experiment 4 (Figure 4.5). Main effects of treatment were significant for eating (F(4,416)=4.1, p<0.01), grooming (F(4,416)=7.9, p<0.001), and locomotion (F(4,416)=4.0, p<0.01) only. A significant main effect of time occurred in all behaviors except drinking (not shown). Significant interaction effects occurred for eating (F(52,416)=2.3, p<0.001) and grooming (F(52,416)=2.3, p<0.001) only. As shown in Fig. 4.5A, feeding was equivalently increased by AcbSh muscimol alone or when paired with contralateral LH muscimol from 5 to 30 minutes post-injection. Although ipsilateral LH muscimol did not suppress AcbSh muscimol-elicited eating at 10 minutes, this treatment did prevent eating at all
other times. Ipsilateral LH muscimol significantly suppressed AcbSh muscimol-elicited feeding compared to contralateral LH muscimol at 20 minutes post-injection. All treatment groups exhibited significantly less grooming than control at 10 and 50 minutes post injection (Fig. 4.5C). At 5 minutes post-injection, subjects given AcbSh muscimol alone exhibited less locomotion than AcbSh vehicle – unilateral LH muscimol and AcbSh muscimol – ipsilateral LH muscimol (Fig. 4.5D). The AcbSh muscimol – contralateral LH muscimol group had suppressed locomotion versus AcbSh vehicle unilateral LH muscimol at 20-25 minutes and versus control at 20 minutes, which coincides with increases in eating and sleeping. Again, no effect of treatment was noted on sleeping patterns (Fig. 4.5E).

**Histological Verification**

Of the 40 rats used for these experiments, 29 rats possessed cannulas correctly targeting both the AcbSh unilaterally and the LH bilaterally The distribution of identified injection sites for these 29 subjects is shown in Figure 4.6, with an example of a Nissl-stained pair of sections in Figure 4.7. As shown, the injection sites were within or proximal to the AcbSh and LH. The 11 additional rats used in these experiments possessed mistargeted cannulas, and their data were omitted from this study.

**Discussion**

Our findings replicated previous studies showing that AcbSh injection of DNQX or muscimol elicits feeding that can be blocked by concurrent LH injection of D-AP5 or muscimol (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1997; Urstadt et al., 2013). Further, while ipsilateral LH D-AP5 or muscimol was highly effective in suppressing feeding elicited by AcbSh DNQX or muscimol, contralateral LH administration of D-AP5 or muscimol was ineffective, as
we have shown previously (Urstadt et al., 2013). We expand upon these studies with new findings suggesting that AcbSh-elicited feeding can be suppressed in a behaviorally specific manner by ipsilateral LH administration of D-AP5 or muscimol. While ipsilateral LH injection of D-AP5 or muscimol strongly suppresses AcbSh-mediated feeding, they produced little or no effects on other behaviors. In contrast, bilateral LH injection of these drugs markedly increased sleeping. Specifically, subjects receiving bilateral LH D-AP5 or muscimol began sleeping only minutes post-injection during the time that AcbSh DNQX- or muscimol-elicited eating would have occurred. This behavioral evidence suggests that the suppression of the AcbSh-elicited eating by presumed bilateral suppression of LH neural activity may have been an artifact of the elicited sleeping response. In contrast, our data also suggest that ipsilateral LH inhibition suppressed feeding in a behaviorally specific manner. These experiments provide further evidence for an association between these brain regions as neural substrates in feeding control. However, we also demonstrate that bilateral LH inhibition can alter other behaviors, suggesting caution in interpreting findings that use bilateral manipulations to suppress food intake or other appetitive behaviors.

An intriguing finding is that bilateral LH injections elicited sleep, while unilateral LH injections did not. This contrast suggests that sustained LH activity in a single hemisphere may be sufficient to maintain wakefulness. Neurons that contain the orexins, arousal-associated neurotransmitter peptides, are likely involved as they are located exclusively in or near the LH (Hagan et al., 1999; de Lecea et al., 1998). Manipulations that activate these neurons promote wakefulness, while those that inhibit these neurons promote sleep (Adamantidis et al., 2007; Brisbane-Roch et al., 2007; Tsunematsu et al., 2011). In some cases, bilateral ablation or bilateral inhibition of orexin neurons is required to significantly affect sleep (Hara et al., 2001; Sasaki et al., 2011). Our bilateral LH drug injections may have inhibited these orexin neurons sufficiently
to cause sleep, whereas the unilateral injections were apparently insufficient. If feeding were interrupted by sleep onset in our ipsilateral-contralateral paradigm, both the ipsilateral and contralateral LH drug injection would have been equally effective in suppressing or reducing feeding. However, only ipsilateral LH injection suppressed feeding, while contralateral injection did not. Thus, we propose that the feeding-suppressive effects of ipsilateral LH drug injections acted specifically on feeding and not arousal.

It should be noted that bilateral LH D-AP5 with AcbSh vehicle did not induce sleep but instead promoted locomotion (Fig. 4.2D) or quiescence (Fig. 4.2F). This was an unexpected effect, considering that bilateral LH muscimol resulted in a clear increase in sleep (Fig 4.3E) regardless of AcbSh treatment. Moreover, this effect of LH D-AP5 was not seen when injected ipsilateral or contralateral to AcbSh vehicle. These results suggest that bilateral LH NMDAR antagonism may be associated with an alternate, yet-to-be-defined behavioral phenotype that may act on a different circuit than those influenced by GABA₄R activation.

Analysis of feeding characteristics reinforced prior findings and revealed more specific data on how feeding bouts were affected. Earlier findings demonstrated that AcbSh DNQX or muscimol decreased latency to feed while increasing feeding duration, leading to increased intake (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1997). We replicated these findings and add new data showing increased meal frequency from these AcbSh drug injections. The latency data revealed that feeding onset was later than that noted in prior studies (Stratford and Kelley, 1997; Stratford et al., 1998); this may be due to our use of unilateral instead of bilateral AcbSh drug injections. The increases in bout size and frequency and decrease in latency caused by AcbSh DNQX or muscimol mimic those seen from LH neuropeptide Y (NPY) injection (Marin Bivens et al., 1998). Marin Bivens et al. proposed that this NPY effect is mediated by brain
mechanisms mediating hunger, as these patterns also mimic those exhibited by diabetic or fasted rats. It is possible that AcbSh-mediated eating utilizes similar mechanisms.

We previously demonstrated that bilateral or ipsilateral LH D-AP5 injection suppresses AcbSh DNQX-elicited intake, and bilateral or ipsilateral LH muscimol suppresses AcbSh muscimol-mediated intake (Urstadt et al., 2013). Our current results show that the LH manipulations suppressed AcbSh-mediated eating by consistently lowering bout duration and number of bouts while raising latency to eat. It should be noted that in treatments where no animals ate, the latency was capped at the maximum of 60 minutes. Thus, latency differences between groups that fed and groups that did not feed may be larger than represented here. Also worth noting is that bilateral LH injections were stronger in their suppression of feeding characteristics than ipsilateral-only LH injections. This difference in effectiveness, based on our behavioral assays, may be due to both suppression of arousal and ingestive circuitry for bilateral injections, while ipsilateral LH injections primarily suppress the AcbSh-activated ingestive circuits.

The ipsilateral LH manipulations used in this study to suppress AcbSh-mediated feeding may operate by reducing the drive to eat. Considering that the AcbSh projects directly to the LH (Duva et al., 2005), AcbSh projections are primarily GABAergic (Oertel and Mugnaini, 1984), and that inhibition of the LH via GABA$_\text{A}$Rs suppresses nocturnal, deprivation-induced, and AcbSh elicited feeding (Maldonado-Irizarry et al., 1995; Turenius et al., 2009a), the evidence supports the proposal that feeding occurs due to increases in appetite when the LH is released from AcbSh inhibition (Kelley et al., 2005). This possibility is supported by evidence showing that pauses in AcbSh neuron firing precede the onset of sucrose fluid intake (Krause et al., 2010). However, AcbSh manipulations that increase bout duration and frequency while decreasing latency, and the ipsilateral LH manipulations that reverse these effects, may also act on
motivation-controlling circuits (Berridge, 1996). Our ipsilateral LH drug injections may reduce the rewarding qualities of food that AcbSh drug injections have been shown to promote (Stratford et al., 1998; Wirtshafter and Stratford, 2010). One LH substrate through which these motivational effects may be mediated is orexin neurons, which are not only associated with arousal but also feeding and reward. Interesting evidence shows that AcbSh inhibition with muscimol activates orexin-containing neurons in the lateral periformal LH (Baldo et al., 2004). Food reward and drug reward cues preferentially activate this lateral orexin neuron group (Harris et al., 2005). Also, the AcbSh has been shown to project preferentially to orexin neurons in lateral subregion (Yoshida et al., 2006). Considering this evidence, it seems that AcbSh connections to the LH may regulate both motivation and appetite through lateral periformal orexin neurons, and inhibition of the LH may counteract such effects through these orexin neurons.

It is unclear whether the intake-controlling communication between the AcbSh and the LH is direct, indirect, or both. Growing evidence implicates the ventral pallidum (VP) as a possible functional and anatomical intermediate. Unilateral AcbSh muscimol induces c-fos expression in the ipsilateral VP and the ipsilateral LH (Stratford, 2005). Also, unilateral AcbSh muscimol-elicited feeding is suppressed by ipsilateral VP or LH lesions, and feeding elicited by VP administration of the GABA$_A$R antagonist bicuculline is blocked by ipsilateral LH lesions (Stratford and Wirtshafter, 2012, 2013). Anatomical evidence shows AcbSh projections to the VP that may secrete GABA (Heimer et al., 1991; Oertel and Mugnaini, 1984). The same VP subregion that receives AcbSh projections also appears to send projections to the LH (Duva et al., 2005; Groenewegen et al., 1993; Haber et al., 1985). Despite this evidence, it is still unclear whether or to what extent AcbSh-mediated feeding relies on the VP to relay signals to the LH. Retrograde labeling identified the tuberal LH, the LH locus regulating NMDAR and GABA$_A$R-mediated feeding (Duva et al., 2002; Turenius et al., 2009b), as the site that produced the most
retrograde labeling in the AcbSh, whereas tracer injections into the anterior or posterior LH labeled far fewer AcbSh cells (Duva et al., 2005). However, the VP appears to send equal amounts of projections throughout the anterior-posterior extent of the LH (Duva et al., 2005; Groenewegen et al., 1993; Haber et al., 1985). Anatomical evidence suggests that the VP may utilize different feeding or non-feeding circuitry through the LH in comparison to that used by the AcbSh, and AcbSh anatomical and LH functional evidence suggests that the direct connection between the AcbSh and the LH regulates feeding.

We have previously proposed that the balance in the synaptic release of glutamate and GABA within the LH is important in regulating feeding behavior (Stanley et al., 2011), and a similar balance has been suggested for the AcbSh's role in food intake (Kelley et al., 2005). Thus, in these experiments, we used drugs that act on ionotropic glutamate and GABA receptors. Although other neurotransmitter receptors play a role in AcbSh-LH food intake control, we selected AcbSh AMPARs and GABA\textsubscript{A}Rs in addition to LH NMDARs and GABA\textsubscript{A}Rs because of the consistent feeding-specific effects produced by modulating these receptors (Maldonado-Irizarry et al., 1995; Stanley et al., 1996; Stratford and Kelley, 1997; Stratford et al., 1998; Turenius et al., 2009a; Urstadt et al., 2013) and our current results reflect this effect. Within the AcbSh and the LH, these receptors are likely the targets through which the balance of glutamate and GABA regulates food intake specifically.

Increases or decreases in specific behaviors between treatment groups are likely directly caused by specific drug treatments. However, indirect effects can also occur – a treatment can cause one behavior to increase, thereby interrupting and decreasing competing behaviors. For example, AcbSh inhibition has been shown to increase locomotion in the absence of food (Stratford et al., 1998), but eating behavior likely prevented this increased locomotion (Fig. 4.5A and 4.5D, AcbSh muscimol – contralateral LH muscimol treatment). Also, one behavior caused
by a treatment directly can lead to an increase in a subsequent behavior. An example of this phenomenon is when food intake leads to post-prandial somnolence (Zammit et al., 1992), as seen in Experiment 1 (Fig. 4.2A and 4.2E). Despite the drug treatments having indirect effects, behaviors observed immediately after injection such significant elicited eating or significant elicited sleep were likely caused directly by the drug injections and were not caused by other behaviors.

Past studies using bilateral LH inhibition had suggested that the LH bilaterally governed elicited, nocturnal, or deprivation-induced food intake specifically (Maldonado-Irizarry et al., 1995; Stanley et al., 1996; Stratford and Kelley, 1997; Turenius et al., 2009a). However, our data suggest that bilateral LH inhibition may have suppressed feeding through non-specific behavioral mechanisms in these and other studies. Further, we suggest that certain types of elicited feeding, such as AcbSh-induced intake, can be controlled unilaterally/ipsilaterally through the LH in a specific manner. This evidence indicates that the AcbSh and the LH communicate using glutamate and GABA to specifically regulate feeding through an ipsilateral connection. Further study may yet demonstrate this connection as a clinical target to treat obesity or other eating disorders.
References


Table 4.1
Injection Paradigms

<table>
<thead>
<tr>
<th>Experiment</th>
<th>N</th>
<th>Injection sites and drugs used</th>
<th>AcbSh</th>
<th>LH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6/8</td>
<td>DNQX (1.25 µg)</td>
<td></td>
<td>D-AP5 (2 µg bilaterally)</td>
</tr>
<tr>
<td>2</td>
<td>6/8</td>
<td>Muscimol (100 ng)</td>
<td></td>
<td>Muscimol (100 ng bilaterally)</td>
</tr>
<tr>
<td>3</td>
<td>8/12</td>
<td>DNQX (1.25 µg)</td>
<td></td>
<td>D-AP5 (2 µg unilaterally)</td>
</tr>
<tr>
<td>4</td>
<td>9/12</td>
<td>Muscimol (100 ng)</td>
<td></td>
<td>Muscimol (100 ng unilaterally)</td>
</tr>
</tbody>
</table>

Drugs were injected unilaterally into the AcbSh and either bilaterally or unilaterally injected into the LH. All drug doses are per hemisphere. Vehicle was a DMSO/aCSF mixture for DNQX and aCSF for all other drugs. N represents the number of subjects whose data was used out of the total number monitored in each experiment (as some were excluded due to mistargeted cannulas). All subjects in each experiment were tested with all drug combinations.
Table 4.2
Food intake characteristics

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Treatment</th>
<th>Average bout duration (min)</th>
<th>Maximum bout duration (min)</th>
<th># of bouts</th>
<th>Latency to eat (min)</th>
<th># of subjects observed feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Across all treatments (ANOVA results)</td>
<td>F(3,15)=4.9, p&lt;0.05</td>
<td>F(3,15)=8.6, p&lt;0.001</td>
<td>F(3,15)=5.5, p&lt;0.01</td>
<td>F(3,15)=6.3, p&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>AcbSh Veh, Bilateral LH Veh</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>60 ± 0.0</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>AcbSh Veh, Bilateral LH D-AP5</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>60 ± 0.0</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>AcbSh DNQX, Bilateral LH Veh</td>
<td>3.5 ± 1.5*</td>
<td>5.6 ± 1.8*</td>
<td>1.8 ± 0.7*</td>
<td>22.8 ± 11.8*</td>
<td>4/6</td>
<td></td>
</tr>
<tr>
<td>AcbSh DNQX, Bilateral LH D-AP5</td>
<td>0.4 ± 0.4^</td>
<td>0.5 ± 0.5^</td>
<td>0.3 ± 0.3^</td>
<td>51 ± 9.0^</td>
<td>1/6</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th>Treatment</th>
<th>Average bout duration (min)</th>
<th>Maximum bout duration (min)</th>
<th># of bouts</th>
<th>Latency to eat (min)</th>
<th># of subjects observed feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Across all treatments (ANOVA results)</td>
<td>F(3,15)=4.7, p&lt;0.05</td>
<td>F(3,15)=4.7, p&lt;0.05</td>
<td>F(3,15)=6.8, p&lt;0.01</td>
<td>F(3,15)=3.9, p&lt;0.05</td>
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</tr>
<tr>
<td>AcbSh Veh, Bilateral LH Veh</td>
<td>0.5 ± 0.3</td>
<td>0.6 ± 0.4</td>
<td>0.6 ± 0.4</td>
<td>43.5 ± 10.7</td>
<td>2/6</td>
<td></td>
</tr>
<tr>
<td>AcbSh Veh, Bilateral LH Musc</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>60.0 ± 0.0</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>AcbSh Musc, Bilateral LH Veh</td>
<td>2.9 ± 1.1*</td>
<td>3.6 ± 1.4*</td>
<td>2.8 ± 0.8*</td>
<td>25.3 ± 10.5</td>
<td>5/6</td>
<td></td>
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<tr>
<td>AcbSh Musc, Bilateral LH Musc</td>
<td>0.0 ± 0.0^</td>
<td>0.0 ± 0.0^</td>
<td>0.0 ± 0.0^</td>
<td>60.0 ± 0.0^</td>
<td>0/6</td>
<td></td>
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<table>
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<tr>
<th>Experiment 3</th>
<th>Treatment</th>
<th>Average bout duration (min)</th>
<th>Maximum bout duration (min)</th>
<th># of bouts</th>
<th>Latency to eat (min)</th>
<th># of subjects observed feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Across all treatments (ANOVA results)</td>
<td>F(4,28)=3.0, p&lt;0.05</td>
<td>F(4,28)=2.1, p=0.10</td>
<td>F(4,28)=3.8, p&lt;0.05</td>
<td>F(4,28)=3.1, p&lt;0.05</td>
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</tr>
<tr>
<td>AcbSh Veh, Bilateral LH Veh</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>54.1 ± 5.8</td>
<td>1/8</td>
<td></td>
</tr>
<tr>
<td>AcbSh Veh, Unilateral LH D-AP5</td>
<td>0.3 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>51.1 ± 6.2</td>
<td>2/8</td>
<td></td>
</tr>
<tr>
<td>AcbSh DNQX, Bilateral LH Veh</td>
<td>2.0 ± 0.7*</td>
<td>3.0 ± 1.3</td>
<td>1.2 ± 0.3*</td>
<td>30.6 ± 8.7*</td>
<td>6/8</td>
<td></td>
</tr>
<tr>
<td>AcbSh DNQX, Contralateral LH D-AP5</td>
<td>2.3 ± 1.1*</td>
<td>3.6 ± 2.1</td>
<td>1.0 ± 0.3*</td>
<td>28.8 ± 9.5*</td>
<td>5/8</td>
<td></td>
</tr>
<tr>
<td>AcbSh DNQX, Ipsilateral LH D-AP5</td>
<td>0.1 ± 0.1^</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.2^</td>
<td>55.8 ± 4.2^</td>
<td>1/8</td>
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<table>
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<tr>
<th>Experiment 4</th>
<th>Treatment</th>
<th>Average bout duration (min)</th>
<th>Maximum bout duration (min)</th>
<th># of bouts</th>
<th>Latency to eat (min)</th>
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<tr>
<td></td>
<td>Across all treatments (ANOVA results)</td>
<td>F(4,32)=2.8, p&lt;0.05</td>
<td>F(4,32)=3.3, p&lt;0.05</td>
<td>F(4,32)=3.3, p&lt;0.05</td>
<td>F(4,32)=4.8, p&lt;0.01</td>
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<tr>
<td>AcbSh Veh, Bilateral LH Veh</td>
<td>0.4 ± 0.3</td>
<td>0.4 ± 0.3</td>
<td>0.2 ± 0.1</td>
<td>50.7 ± 6.2</td>
<td>2/9</td>
<td></td>
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<tr>
<td>AcbSh Veh, Unilateral LH Musc</td>
<td>0.7 ± 0.5</td>
<td>1.0 ± 0.6</td>
<td>0.4 ± 0.3</td>
<td>54.7± 3.5</td>
<td>2/9</td>
<td></td>
</tr>
<tr>
<td>AcbSh Musc, Bilateral LH Veh</td>
<td>6.4 ± 3.3</td>
<td>8.8 ± 4.3*</td>
<td>2.1 ± 0.7*</td>
<td>19.8 ± 7.9*</td>
<td>7/9</td>
<td></td>
</tr>
<tr>
<td>AcbSh Musc, Contralateral LH Musc</td>
<td>8.9 ± 4.3*</td>
<td>12.0 ± 5.4*</td>
<td>1.1 ± 0.4</td>
<td>34.3 ± 9.9</td>
<td>5/9</td>
<td></td>
</tr>
<tr>
<td>AcbSh Musc, Ipsilateral LH Musc</td>
<td>2.2 ± 1.2^</td>
<td>2.7 ± 1.4^</td>
<td>1.1 ± 0.4</td>
<td>32.0 ± 9.0</td>
<td>7/9</td>
<td></td>
</tr>
</tbody>
</table>

Excluding ANOVA results in the initial line for each experiment, the feeding bout durations, number of bouts, and latency to eat are mean values (plus or minus SEM) across all subjects, including those that did not eat. Subjects observed eating is given as a ratio of total subjects in the experiment. *: a significant difference versus control. ^: significant difference between feeding-eliciting and feeding-suppressive treatments.
Figure 4.1: Mean food intake through the duration of the monitoring period for Experiment 1 (A), 2 (B), 3 (C), and 4 (D). Error bars are ± 1 S.E.M. * denotes a significant increase in intake versus control group while # indicates a significant suppression of elicited intake.
Figure 4.2: Animal behaviors throughout the monitoring period in Experiment 1. Behavioral activity in each panel is represented as a percentage the total time spent in a particular behavior within each five minute interval, averaged across all subjects. AcbSh DNQX elicited eating soon after injection, while bilateral LH D-AP5 both halted this feeding and increased sleep. Gray lines represent AcbSh vehicle treatments while black lines represent AcbSh DNQX treatments. Squares and solid lines represent treatments that received bilateral LH vehicle, whereas triangles and dashed lines represent treatments with bilateral LH D-AP5. Error bars are ± 1 S.E.M. Error bars are colored and solid/dashed in the same manner as the line they are associated with. * denotes significant increase and # indicates significant decrease in a behavior versus control. ^ indicates a significant difference between treatment groups other than AcbSh Vehicle + LH Vehicle.
Figure 4.3: Behaviors exhibited in Experiment 2. Conventions are similar to Fig. 4.2, except that muscimol was used instead of DNQX or D-AP5. AcbSh muscimol increased feeding within 15 minutes after injection, while bilateral LH muscimol abolished this feeding and hastened onset of sleep.
Figure 4.4: Behavioral activity in Experiment 3. Conventions are similar to Fig. 4.2, though treatments differ (see legend). A fifth line, consisting of black inverted triangles and a dense dotted line, was added to represent the AcbSh DNQX – contralateral LH D-AP5 treatment. AcbSh DNQX increased feeding within 10 minutes post-injection, and contralateral LH D-AP5 co-treatment failed to suppress this feeding. Ipsilateral LH D-AP5 did suppress the feeding without altering sleep onset.
Figure 4.5: Behavioral changes in Experiment 4. Conventions are similar to Fig. 4.3, except that muscimol replaces both DNQX and D-AP5. AcbSh muscimol increased feeding soon after injection. Concurrent contralateral LH muscimol failed to suppress this feeding. Ipsilateral LH muscimol did partially suppress AcbSh muscimol-elicited eating without compromising wakefulness.
Figure 4.6: Schematics representing brain regions containing the AcbSh (A-C) and LH (D-F). Symbols represent injection sites for each cannula in each subject. Circles designate sites in animals used in Experiments 1 and 2, while triangles designate sites for animals used in Experiments 3 and 4. Images were modified from a brain atlas (Paxinos and Watson, 2005).
Figure 4.7: Photomicrographs of brain slices containing cannula tracts terminating in the AebSh (A) and the LH (B). Arrows indicate sites of injection.
Chapter 5 - Distribution and Characterization of Ventral Lateral Hypothalamic Neurons Projecting to the Nucleus Accumbens Shell

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Abstract

The nucleus accumbens shell (AcbSh) and the lateral hypothalamus (LH) interact to control feeding and reward along with dread and aggression, and are reciprocally connected. However, few studies identify the specific LH subregion locations and neurochemical phenotypes of AcbSh-projecting neurons. To explore this issue, 33 adult male Sprague Dawley rats received iontophoretic injections of Fluorogold (FG; 2\%) into either the anterior or posterior half of the medial AcbSh. After a 7-day survival period, their brains were extracted, fixed, and sliced into 40 um coronal sections. Coronal sections containing the LH were immunofluorescently-stained with antibodies against Orexin A, melanin concentrating hormone (MCH), cocaine and amphetamine regulated transcript (CART), nesfatin, phosphorylated signal transducer and activator of transcription 3 (pSTAT3), glutamic acid decarboxylase (GAD67), vesicular GABA transporter (VGAT) or vesicular glutamate transporters 2 or 3 (VGluT2 or 3). Analyses showed that FG-labeled cells were mainly distributed throughout the ventrolateral and subfornical LH subregions. These cells in these ventral LH subregions sometimes had overlapping distributions with orexin-, MCH-, pSTAT3-, and CART-positive cells, but these peptides rarely co-localized with FG labeling in the same neurons. However, almost all FG-labeled neurons were GAD67- or nesfatin-positive, and many were VGAT- or VGluT3 -positive. This data suggests although orexin and MCH may play a role in dorsal LH/PeFLH to AcbSh signaling as previously thought, the larger number of glutamatergic, GABAergic, and/or nesfatin-utilizing ventral LH neurons projecting to
the AcbSh suggests those neurotransmitters may have a prevailing role in this circuit. These LH neurons may drive aversive responses via the posterior AcbSh.

**Introduction**

Due to prevalence of obesity in certain developed nations, research has aimed to resolve the brain circuitry regulating not only food intake and metabolism, but also the hedonic drives to consume foods. Through this line of research, the lateral hypothalamus (LH) and the nucleus accumbens shell (AcbSh) have been implicated in controlling feeding behaviors and reward-based actions (Krause et al., 2010; Maldonado-Irizarry et al., 1995; Stanley et al., 1993; Wyrwicka and Dobrzecka, 1960). The LH is an integrator of sensory information, satiety signals, reward incentives, and nutritional states (Håkansson et al., 1998; Harris et al., 2005; Ono et al., 1981; Oomura et al., 1974), while the AcbSh processes motivation and reward (Baldo and Kelley, 2007; Hernandez and Hoebel, 1988; Wirtshafter and Stratford, 2010). Individually, these regions appear to be essential for the motivation and feeding.

However, these regions have additional roles in defensive behaviors, disliking of foods, and reductions in food intake. Inhibition of the caudal AcbSh with muscimol produces all of these behaviors (Reynolds and Berridge, 2001). Excitation of certain LH regions has been shown to produce aggressive responses (Halász et al., 2002). Also, activation of leptin-receptive or VGluT2-containing populations of LH neuron population decreases food intake, with the latter population also producing aversion (Jennings et al., 2013; Laque et al., 2013). Further, these regions are anatomically connected. Retrograde tracer injections into the AcbSh, predominantly the caudal half, label neurons in the vlLH (Brog et al., 1993; Phillipson and Griffiths, 1985), while anterograde tracer injection this LH subregion has been reported to label axons/terminals in the accumbens (Goto et al., 2005). These data suggest that the LH and the AcbSh may
communicate in the regulation of aversive behaviors and food intake reduction.

Some evidence describes the neurotransmitter phenotypes of LH neurons projecting to the AcbSh. LH neurons containing orexin and melanin concentrating hormone (MCH), send fibers to the dorsomedial AcbSh (Bittencourt et al., 1992; Fadel and Deutch, 2002), and receptors for these transmitters have also been detected on AcbSh neurons (Saito et al., 2001; Trivedi et al., 1998). Further, a study using pseudo-rabies virus injection into the AcbSh showed the retrograde label co-localizing with MCH or orexin in some LH neurons (Kampe et al., 2009). However, other neurotransmitters and their markers have not received as much attention. For instance, the anorectic peptide transmitters cocaine and amphetamine regulated transcript (CART) and nesfatin are located in within the LH (Broberger, 1999; Foo et al., 2008). Also of importance are LH leptin-receptive (LepRb) neurons, which have been categorized as a separate population of neurons from orexin and MCH cells that serves to regulate food intake (Laque et al., 2013). Most importantly, the role of the primary amino acid neurotransmitters glutamate and GABA in LH to AcbSh projections has not been characterized. Although their co-transmission with some of the aforementioned peptides has been demonstrated (Elias et al., 2008; Henny et al., 2010), populations of AcbSh-projecting LH neurons that transmit glutamate or GABA without these peptides have not been characterized.

Three main aspects of the LH to AcbSh circuit remain unclear: 1. the specific distribution of LH neurons projecting to the AcbSh, 2. whether or not the LH preferentially innervates the rostral or posterior AcbSh, and 3. what neurotransmitters these LH neurons may utilize other than orexin or MCH. In this study, we examined these issues by injecting retrograde tracers into different AcbSh subregions and using immunohistochemical staining to determine co-localization of retrograde label with neurotransmitter markers.
Methods

Subjects

The 33 adult male Sprague Dawley rats used in this study weighed 350-450 grams at the time of surgery and were individually housed in wire mesh cages in a temperature-controlled vivarium at 21 °C with a 12 hour light-dark schedule and food and water ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California – Riverside.

Antibody Characteristics

Antibodies used in this study were all purchased from commercial sources and have been tested by other labs previously. We used the following antibodies:

1. Polyclonal goat anti-orexin A antiserum (Santa Cruz Biotechnology, Santa Cruz, CA). Staining patterns matched those previously reported; somatic labeling only in the lateral, perifornical, and dorsal hypothalamic areas, with moderate fiber labeling radiating outward from these regions (Baldo et al., 2003). Diluted to 1:5000.

2. Polyclonal rabbit anti-melanin concentrating hormone (MCH) antiserum (Phoenix Pharmaceuticals, Burlingame, CA). Staining patterns matched those previously reported; somatic labeling only in the lateral, perifornical, and dorsal hypothalamic areas and in the zona incerta (Bittencourt et al., 1992). Modest fiber labeling was seen radiating outward from these regions as previously shown. Registered with JCN antibody database. Diluted to 1:10,000.

3. Polyclonal goat anti-cocaine and amphetamine regulated transcript (CART) antiserum (Santa Cruz Biotechnology, Santa Cruz, CA). Distribution of labeled somata was similar to that reported by others and partly matched that of MCH neuron distribution, as MCH
is shown to be an occasional co-transmitter (Broberger, 1999). Diluted to 1:2000.

4. Polyclonal rabbit anti-nesfatin-1 antiserum (Phoenix Pharmaceuticals, Burlingame, CA). Recognizes rat nesfatin-1 (1-82) peptide. A recent study (Vas et al., 2013) revealed far more cells in the LH than amounts shown in prior studies, due to the use of staining amplification substances. We observed even more LH cells with nesfatin-IR, so we performed our own control on the antibody. We tested the antibody's specificity by adding rat nesfatin-1 (1-82) peptide (also from Phoenix Pharmaceuticals) at a 2 mM concentration in a batch of anti-nesfatin primary solution. Using this peptide + antibody solution in the same staining procedure that we used the antibody-only solution, a complete loss of somatic staining occurred; no labeled cells were evident. Thus, we suggest that the antibody is indeed specific, but the number of nesfatin-IR cells revealed may vary significantly depending on parameters of tissue fixation, antibody staining, and section imaging. Diluted to 1:4000.

5. Polyclonal goat anti-vesticular GABA transporter (VGAT) antiserum (cat. #sc-49574; Santa Cruz Biotechnology, Santa Cruz, CA). Antibody was raised against the N-terminus of human VGAT. The manufacturer tested it with Western Blot, and the antibody recognized a 57 kDa peptide in human whole cell lysates, which corresponds to the size of VGAT protein. Elution of the primary antibody with the control peptide at a 0.5 mM concentration. This antibody has been used in another study (Wang et al., 2013). Diluted to 1:5000.

6. Polyclonal rabbit anti-glutamic acid decarboxylase 67 (GAD67) antiserum (Santa Cruz Biotechnology, Santa Cruz, CA). Raised against human GAD67 amino acids 1-101. Tested by manufacturer in Western Blot; this antibody recognized a 67 kDa protein in whole cell lysates, corresponding to the molecular weight of GAD67. This antibody was
used in another study (Bubar et al., 2011). Diluted to 1:200.

7. Monoclonal mouse anti-vesicular glutamate transporter 2 (VGlut2) antiserum
   (NeuroMAb facility at UC Davis, Davis, CA). Recognizes amino acids 501-582 of rat

8. Monoclonal mouse anti-vesicular glutamate transporter 3 (VGlut3) antiserum
   (NeuroMAb facility at UC Davis, Davis, CA). Recognizes amino acids 546-588 of rat
   VGlut3 protein. This antibody was used by others (Fyk-Kolodziej et al., 2011). Diluted
   to 1:2000.

9. Monoclonal rabbit anti-phosphorylated signal transducer and activator of transcription 3
   (pSTAT3) antiserum (cat. #: 9145; Cell Signaling, Danvers, MA). Recognizes STAT3
   only when tyrosine 705 is phosphorylated. Preliminary stains showed that the antibody
   produced nuclear-like staining in brain sections from rats that had received leptin I.C.V.
   Stained very few cells in rats that did not receive leptin treatment. Diluted to 1:1000.

10. Polyclonal rabbit anti-phaseolus vulgaris leucoagglutinin antiserum (cat. #AS-2300,
    Vector Labs, Burlingame, CA) was used at 1:5000. This recognizes the PHA-L protein
    and does not stain brain tissue that has not been injected with PHA-L.

Biotinylated donkey anti-mouse, anti-rabbit, and anti-donkey secondary antibodies and the
streptavidin-AlexaFluor647 conjugate were purchased from Jackson ImmunoResearch (West
Grove, PA).

Tract Tracing Chemicals

The retrograde tracer Fluorogold (FG; 2-3%) and the anterograde tracer phaseolus
vulgaris leucoagglutinin (PHA-L; 2.5%) were used in this study. Tracers were dissolved in a
heat-sterilized artificial cerebrospinal fluid vehicle, containing 147 mM Na⁺, 154 mM Cl⁻, 3 mM
K⁺, 1.2 mM Ca²⁺, and 0.9 mM Mg²⁺. Tracers were forward-filled into glass micropipettes with 20-30 µm tip sizes via suction on the back of the micropipette with polyethylene tubing connected to a 1 mL syringe. The pipettes were backfilled with sterilized artificial cerebrospinal fluid.

**Tracer Injection Procedure**

Subjects were anesthetized with sodium pentobarbital (50 mg/kg), given atropine (0.27 mg/kg) to prevent respiratory difficulties, and placed into a stereotax. After an initial incision and trephination of the skull above the injection site, tracer-filled micropipettes were lowered to the desired injection sites. FG was injected into the posterior half of the AcbSh (n = 18) aimed, with respect to the bregma reference point, at 1.2 mm anterior, 1.0 mm lateral, and 7.2 mm ventral or into the anterior half of the AcbSh (n = 8) at 2.0 mm anterior, 0.8 mm lateral, and 7.0 mm ventral. Further, the anterograde tracer PHA-L was injected into the LH, relative to bregma, at 2.5 mm posterior, 2.0 mm lateral, and 9.2 mm ventral (n = 3) or at 3.5 mm posterior, 2.0 mm lateral, and 9.0 mm ventral (n = 4) to corroborate the retrograde labeling from the AcbSh. Tracers were injected from the pipette iontophoretically. A cathode wire was lowered into the saline in the back of the micropipette, and an anode wire was inserted into the open wound on the scalp. Tracers were injected with +6 µA current, pulsed 7s on/7s off, for 15 minutes. A -5 µA retention current was applied while inserting or retracting the micropipette from the tissue to prevent tracer leakage. The wound was closed with surgical staples, and after recovery the animal was returned to its home cage.

**Ventricular Injections**

In one subset of subjects that received a posterior AcbSh FG injection (n = 8), colchicine (Sigma-Aldrich) was used to increase somatic localization of peptides, allowing for clearer neuronal
labeling. During surgery and immediately following tracer injection, rats were stereotaxically implanted with a 18 mm length, 26 gauge stainless steel guide cannula into the lateral ventricle (AP -0.6, ML +1.8, DV -2.7 in mm from bregma) ipsilateral to the tracer injection site. Cold cure dental acrylic, anchored to the skull by stainless steel screws, secured the cannulas in place. A plastic guard was embedded in the dental cement to protect the cannula from bending, and 33 gauge stainless steel obturators were inserted into the cannulas to maintain patency. Colchicine (100 µg in 10 µL) was injected in artificial cerebrospinal fluid volume of 10 µL via 33 gauge injectors protruding 1 mm beyond the end of the guide cannula over the course of 5 minutes. Injections were administered at least five days after tracer injections to minimize interference with intracellular tracer transport and two days prior sacrifice to ensure thorough somatic localization of peptides.

In another set of subjects that received a posterior AcbSh FG injection (n = 6), recombinant rat leptin obtained from Dr. A.F. Parlow (National Hormone & Peptide Program at Harbor-UCLA Medical Center, Torrance, CA) was similarly injected over 5 minutes into the lateral ventricle to activate leptin-receptive neurons. Leptin dosage used was 20 µg/kg body weight in 10 µL PBS, pH 8.1. One hour later, these subjects were sacrificed for pSTAT3 staining. (Münzberg et al., 2004) showed that central leptin administration is an effective method for activating leptin receptors, and that such activation induces pSTAT3. pSTAT3 can then be immunohistochemically stained in order to identify leptin-activated neurons. The dose for leptin was determined from the i.c.v. dose (scaled for body weight) used in mice that maximally induces pSTAT3 at one hour in non-arcuate hypothalamic regions (Faouzi et al., 2007).

Histological Preparation

Subjects were sacrificed seven days after tracer injection. Subjects were fatally
anesthetized with sodium pentobarbital (390 mg/kg) and transcardially perfused with 0.9% saline for 2 minutes, then with phosphate-buffered 4% formaldehyde solution for 15 minutes, with both solutions at pH 7.4. Brains were extracted, post-fixed overnight in the same formaldehyde solution, and were saturated in a 20% sucrose solution until sinking. Brains were frozen with dry ice and cut into 40-50 µm sections with a microtome, then these sections were used for antibody staining. Whole brains or brain sections that were not used immediately were maintained in cryoprotectant solution (30% ethylene glycol, 50% 0.05 M KPBS, and 20% glycerol) for long term storage at either +4 or -20 °C. Whole brains maintained in cryoprotectant were transferred and soaked in sucrose for two days prior to sectioning to wash out cryoprotectant residues in the brain tissue. Sections were rinsed multiple times in KPBS before antibody staining.

Immunohistochemistry

Antibody information can be found in Table 2. All antibody serums were diluted in KPBS with 3% normal donkey serum, 0.3% Triton X-100, and 0.05% sodium azide (though solutions with only streptavidin conjugates did not include normal donkey serum). After sectioning or recovery from cryoprotectant, tissue sections were rinsed in 3 exchanges of KPBS over 15 minutes, blocked in KPBS with 3% normal donkey serum, 0.3% Triton X-100, and 0.05% sodium azide for 30 minutes, and incubated in a primary antibody solution overnight at 4 °C. After primary antibody incubation, sections were rinsed, incubated in a biotinylated secondary antibody solution for 2-3 hours at RT, rinsed, incubated in streptavidin-AlexaFluor 647 for 1.5 hours at RT, rinsed, and mounted on glass microscopy slides. Slides were coverslipped with 1:1 mixture of glycerol and 0.4 M potassium bicarbonate solution and sealed with colored nail polish.

Staining procedures differed for VGluT3, VGAT, and pSTAT3 staining. For these three
antigens cases, proteolytic antigen retrieval was used. Sections stained for these antigens were rinsed briefly in distilled water, incubated in 0.05% pepsin in 0.2 N HCl at 37 °C for 10 min, and rinsed. Sections stained for pSTAT3 were further incubated in 0.03% SDS in distilled water for 10 minutes and then rinsed. All sections stained for these antigens were incubated in 1% H2O2 for 15 min to block endogenous peroxidase activity, rinsed, blocked in normal donkey serum as mentioned above, and incubated in primary antibody overnight at 4 °C (except the rabbit anti-pSTAT3 incubation, which was for 24 hours at RT). Then, sections were rinsed, incubated in a biotinylated secondary for 2 hours, rinsed, incubated with streptavidin-horseradish peroxidase, rinsed, incubated in biotin tyramide at 1:1000 with 0.003% H2O2 for 5 minutes, rinsed, incubated in streptavidin-AlexaFluor647, rinsed, mounted, and coverslipped as described above. The pSTAT3 staining protocol used here was partly adapted from another study (Cui et al., 2012).

**Analysis with Confocal Microscopy**

Slides were analyzed under a Leica TCS SP5 scanning laser confocal microscope. A 405 nm violet diode was used to excite Fluorogold directly, and a 633 nm helium-neon laser was used to excite AlexaFluor 647. Adjustable bandpass emission filters were set to ranges of 570-610 nm for FG and 650-700 for AlexaFluor 647. Images and Z-stacks were recorded as separate channels that were later merged and color balanced using ImageJ NIH software. LH subregions were determined by shape of and distances from the fornix, optic tract, internal capsule, and median eminence. Abbreviations for all brain regions described in this study can be found in Table 5.1. FG-labeled neuron distribution was traced camera lucida using the GIMP image editing program.
Results

Injection Sites and Distribution of Labeled Cells or Fibers

FG injections into the AcbSh were directly beneath the lateral ventricle, mostly within the dorsomedial AcbSh (Fig 5.1B-D). In brains that received FG injection into the posterior medial AcbSh, there were many moderate to strongly-labeled FG cells in the LH, with infrequent cell labeling in PeFLH. As coronal sections went from anterior to posterior, the most numerous group of labeled cells swept from lateral to ventral of the fornix (Fig 5.2C-H). In anterior sections, FG-labeled neurons were most concentrated directly lateral to the fornix, while in posterior sections, labeled cells were most concentrated below the fornix. Labeled cells in the tuberal LH were in an intermediate area – ventrolateral of the fornix. FG injections into anterior AcbSh areas resulted in substantially fewer labeled cells in all hypothalamic areas.

To corroborate the retrograde labeling observed in the LH, PHAL was administered to the same regions of the LH and the AcbSh was analyzed for anterogradely-labeled fibers. PHAL injections were successfully deposited in the ventrolateral LH (Fig 5.3A-C). Injections in this ventrolateral region, especially slightly anterior of tuberal LH (about 2.7 mm behind bregma), demonstrated modest fiber labeling predominantly in the caudal AcbSh (Fig 5.3D & 5.3E), and little to no fiber labeling in more anterior AcbSh areas. Numerous PHAL-labeled fibers were seen in the nearby lateral septum, while PHAL-labeled fibers were almost never seen in the AcbC.

Distribution of Peptide Markers in LH Subregions

Cells stained for orexin A were most dense in the PeFLH with fewer seen in the dLH, vILH and sFLH (Fig 4A). MCH-positive neurons were mostly localized to the dLH and PeFLH areas, with few cells seen within the vILH and sFLH (Fig 5.4A'). CART-stained cells had overlapping distribution with most MCH cells (Fig 5.5A). However, a few CART-positive cells
were seen in the vlLH and sfLH. Neurons intensely labeled for nesfatin exhibited similar
distribution to MCH and CART neurons (Fig 5.5A’). Interestingly, there were different
populations of nesfatin-positive neurons in the LH – those that stained intensely and matched
distributions described in initial studies of the peptide, and a larger number that stained weakly
but still noticeably. The strongly stained nesfatin neurons were mostly in the dLH and PeFLH
while the weakly-stained nesfatin cells distributed broadly throughout all LH subregions. Cellular
staining patterns for Orexin, MCH, and CART (Fig. 5.4B, 5.4B’, and 5.5B) revealed somatic
labeling and axonal labeling with no nuclear labeling. Nesfatin staining was entirely somatic,
with no nuclear labeling and no axonal labeling (Fig. 5.5B’).

GAD67-positive neurons were numerous and were distributed throughout all LH
subregions (Fig. 5.6A). VGAT-positive neurons were most concentrated in medial hypothalamic
areas, but were still in large numbers in lateral hypothalamic subregions. They did not stain as
clearly as GAD67-containing neurons (Fig. 5.6A’). Neurons positive for pSTAT3 had the
expected distribution around the fornix, being mostly confined to the semi-posterior PeFLH and
appearing sparsely in more lateral LH subregions (Fig. 5.7A). Unlike prior studies, our staining
approach revealed processes extending from stained nuclei/somata (Fig 5.7B). Despite inclusion
of antigen retrieval, multi-step stain amplification, and colchicine treatment both separately and
combined, very few or no VGluT2-labeled cells were seen in the LH subregions observed.
Instead, only diffuse staining was observed. However, somatic staining was seen within the
thalamus in the same coronal sections (Fig. 5.8A). VgluT3-containing neurons were thoroughly
distributed throughout the LH and were most concentrated in the ventromedial hypothalamus
(Fig. 5.7A’). In most preparations that underwent pepsin treatment and tyramide signal
amplification, the staining pattern of individual cells was “speckled” and did not fill the entire
perikarya of neurons (Fig. 5.7B’). In the same sections, cells in other regions such as the
hippocampus exhibited more homogenous staining throughout the perikarya (Fig. 5.8B).

**Assessment of Peptide and FG Co-Distribution and Cellular Co-localization**

The distribution of hypothalamic FG-labeled cells strongly overlapped with some neurotransmitter markers examined in this study and rarely with others. Specifically, FG-labeled neurons rarely co-mingled with orexin (Fig. 5.4A), MCH (Fig. 5.4A'), or CART (Fig 5.5A) neurons. Orexin, MCH, and CART were mainly confined to the PeFLH and ZI as described in other studies, while FG labeling was largely constricted to the vILH. Even in areas where these peptides had overlapping distribution to FG-labeled cells, orexin and MCH almost never co-localized with FG labeling in the same cells (Fig. 5.4D & 5.4D') and only occasionally co-localized with CART (Fig 5.5D). Nesfatin-positive cells possessed complete overlapping distribution with FG-labeled neurons, as nesfatin-labeled cells were relatively ubiquitous throughout the LH (Fig 5.5A'). However, FG was not typically localized to cells that stained strongly for nesfatin, but instead almost always co-localized with weakly-stained nesfatin neurons in the vILH (Fig. 5.5D').

As VGAT and GAD67 were thoroughly distributed within the LH, their distributions overlapped with FG labeling distribution completely (Fig. 5.6A & 5.6A'). All FG-labeled neurons possessed GAD67, and a majority also possessed VGAT (Fig. 5.6D & 5.6D'). Neurons labeled for pSTAT3 only had overlapping distribution with FG-containing neurons in the vLH beneath the fornix (Fig. 5.7A). FG neurons did not possess pSTAT3 staining in this region (Fig. 7D). VGluT3 staining co-distributed with FG-labeled neurons and FG-labeled cells stained for VGluT3 (Fig. 5.7A' & 5.7D').
Discussion

In this study we have highlighted a specific population of neurons in the LH, spanning through its rostrocaudal extent and contained within mostly ventrolateral subregions, that project specifically to the posterior medial AcbSh. The AcbSh-projecting vILH and sFLH neurons appeared to rarely contain orexin A or MCH and did not appear to be leptin-receptive. However, these neurons on some occasion contained CART and more frequently contained GAD67, VGAT, and nesfatin, suggesting the use of GABA and nesfatin as their primary neurotransmitters. Many FG-labeled neurons were labeled with VGluT3, as well. Previous evidence has demonstrated that the LH sends projections containing orexin or MCH to the dorsomedial AcbSh (Baldo et al., 2003; Bittencourt et al., 1992). We confirm that such projections do exist in more dorsal subregions such as the dLH and PeFLH. However, we observed greater numbers of labeled cells in the vILH and sFLH, and it appears that a majority of these AcbSh-projecting LH neurons utilize GABA, nesfatin, and/or glutamate.

A study using pseudo-rabies virus (PRV) into the AcbSh suggested that a moderate number of lateral hypothalamic neurons projecting to the AcbSh express orexin or MCH (Kampe et al., 2009). This study, however, showed many more labeled neurons in the PeFLH and dLH than amounts seen in our study or in others (Brog et al., 1993; Phillipson and Griffiths, 1985). The moderate number of neurons double-labeled for PRV and orexin or MCH may be due to more retrograde labeling of neurons in the PeFLH and dLH subregions where orexin and MCH neurons are most prominent. In our study, the retrograde labeling was mostly outside the areas in which orexin and MCH neurons reside. This discrepancy may be due to a difference in uptake of FG versus PRV in certain neuron types. Alternatively, orexin and MCH neurons may have taken up FG only to below detectable levels, while PRV uptake was detectable as the virus propagates itself and increases labeling.
Nesfatin was recently characterized as an anorexigenic neurotransmitter created from nucleobindin2 (Foo et al., 2008). Nesfatin immunoreactivity has been in neurons in several brain areas, including both the accumbens and several hypothalamic areas. LH nesfatin neuron distribution appeared overlapped with that seen from LH neurons labeled from AcbSh FG injections (Foo et al., 2008). Thus, we had aimed to assess whether these AcbSh-projecting LH neurons utilized nesfatin. Further, LH CART neurons usually co-transmit nesfatin (Foo et al., 2008). Here, we have shown that some caudal AcbSh-projecting LH neurons transmit CART, and most transmit nesfatin. As nesfatin decreases food intake when injected ICV or into the LH (Chen et al., 2012; Goebel et al., 2011) and CART injection decreases food intake when injected into the AcbSh (Yang et al., 2005), these LH neuron types projecting to the caudal AcbSh may serve to decrease food intake, as caudal AcbSh inhibition via muscimol does decrease intake (Reynolds and Berridge, 2001).

In this study, we did not observe VGluT2-positive neurons specifically within the LH, though such neurons are known to exist within the LH. VGluT1, VGluT2, or VGluT3-containing neurons have been identified previously in the LH using IHC and/or ISH (Herzog et al., 2004; Vong et al., 2011; Ziegler et al., 2002). Orexin fibers projecting to the locus coeruleus have been shown to contain VGluT2, suggesting glutamatergic co-transmission though these fibers did not possess VGluT1 or VGluT3 (Henny et al., 2010). However, another study had shown that orexin neurons contained VGluT1 or VGluT2 mRNA (Rosin et al., 2003), suggesting that orexin-VGluT1 neurons may target areas other than the locus coeruleus. Further, fluorescent protein expression in LH VGluT2 neurons reveals that such neurons are plentiful in at least the dorsal and perifornical LH (Jennings et al., 2013). Despite showing that the majority of AcbSh-projecting LH neurons are GABAergic, these cells may yet be additionally glutamatergic. For example, VGluT2 and GAD65 mRNA co-localize within arcuate proopiomelanocortin neurons.
(Jarvie and Hentges, 2012), demonstrating that neurons in other hypothalamic regions co-transmit glutamate and GABA. Elsewhere, VGlut2 and VGAT or VGlut3 and VGAT are associated to the same synaptic vesicles (Stensrud et al., 2013; Zander et al., 2010). Considering that a majority of FG-labeled LH neurons shown in this study were VGlut3-positive and a majority were either VGAT- or GAD67-positive, these neurons may possess both of these vesicular transporters. Glutamate and GABA, co-released from these neurons, may serve to fine-tune each other in regard to LH to AcbSh signaling.

A subpopulation of GABAergic leptin-activated neurons, which do not contain orexin or MCH, has been characterized in the LH, and these neurons utilize GABA and galanin (Laque et al., 2013). However, whether these neurons projected to the AcbSh had not yet been assessed. Here, we demonstrate that these specific leptin-receptive neurons are not a predominant population of LH neurons projecting to the AcbSh. Another LH neuron population implicated in metabolism and energy balance, neurons receptive to alpha-melanocyte stimulating hormone and agouti-related peptide, do not appear to project to the AcbSh (Cui et al., 2012). The apparent absence of a direct LH to AcbSh connection that is directly modulated by energy state signaling suggests that metabolic information may not be relayed by this circuit.

Different behavioral effects are exhibited from inhibiting the rostral versus caudal AcbSh. Muscimol infusion into the rostral AcbSh produces appetitive responses such as increased food intake and orofacial responses indicating liking of foods, while in the caudal AcbSh muscimol infusion produces or potentiates negative affective behaviors such as defensive treading and disgust (Faure et al., 2010; Reynolds and Berridge, 2001). The functional segregation between rostral and caudal AcbSh subregions suggests that these areas may receive different input patterns. Indeed, the caudal AcbSh is the predominant recipient of LH innervation, whereas the rostral AcbSh has less LH innervation. Further, defensive and aversive responses seen in the
caudal AcbSh may be partly governed by the sFLH. This notion is supported by data showing that
electrical stimulation of the sFLH results in certain defensive and aggressive behaviors (Kruk et
al., 1983). This same LH area has been shown to innervate and activate the lateral septum, a
region also involved in aggressive behaviors (Halász et al., 2002; Poulain, 1983). Thus, LH
GABAergic inputs (and their peptide co-transmitters) to the posterior mAcbSh may serve to elicit
or potentiate defensive reactions to and disliking of stimuli.

This study and others (Brog et al., 1993; Goto et al., 2005) emphasize an ascending
projection from LH to AcbSh, which compliments the descending AcbSh to LH connection. This
descending connection is indicated by AcbSh tracer injections labeling fibers in the PeFLH/dLH
(Usuda et al., 1998) and unilateral AcbSh inhibition activating neurons in the same area
(Stratford, 2005). Interestingly, these pathways appear non-overlapping – the AcbSh to LH
pathway utilizes signaling in the dorsal LH, while the LH to AcbSh pathway utilizes signaling in
the ventral LH. The AcbSh to dLH pathway is involved with feeding behavior, while the
vILH/sFLH to AcbSh pathway may be involved with defensive behavior as mentioned above.
Though these circuits may appear to be separate, the PeFLH and dLH receiving AcbSh input
projects onward to the vILH and sFLH (Hahn and Swanson, 2010). This intra-LH connection may
be a means of activating a feedback circuit through which the LH can arrest appetitive behaviors.

Our study has demonstrated a connection from the LH to the AcbSh that may utilize a
combination of inhibitory and excitatory neurotransmitters. This circuit may regulate negative
affective responses to aversive stimuli, or otherwise may act as a feedback circuit to halt food
intake. Further research may yet demonstrate how the LH to AcbSh pathway regulates such
behaviors, and how the circuit may interact with the traditional role of the LH in regulating food
intake.
References


Table 5.1
Brain Region Abbreviations
ac – anterior commissure
AcbSh – nucleus accumbens shell
AcbC – nucleus accumbens core
dLH – dorsal lateral hypothalamus
fx – fornix
ic – internal capsule
LV – lateral ventricle
LS – lateral septum
opt – optic tract
PeFLH – perifornical lateral hypothalamus
sfLH – subfornical lateral hypothalamus
vlLH – ventrolateral lateral hypothalamus
Figure 5.1: FG injection into the medial AcbSh. Two diagrams (A) adapted from a brain atlas (Paxinos and Watson, 2005) show the general areas, designated by blue boxes, that FG injections were targeted to in the anterior (left) and posterior (right) AcbSh. An example injection is shown in B. Injections into the anterior (C) or posterior (D) AcbSh were traced. The pink filled-in trace in D is the injection site shown in B. Blue tracers represent subjects that received LV leptin injections. Scale bar in B is 200 µm.
Figure 5.2: Distribution of FG-labeled neurons in the LH. An example coronal section diagram, adapted from a brain atlas (Paxinos and Watson, 2005), shows one analyzed region designated by a blue box (A) and its respective photomicrograph (B). Tracers of several coronal sections, anterior to posterior, demonstrate numerous FG labeled cells in ventral LH subregions (C-H). Scale bars are 200 µm.
Figure 5.3: Anterograde labeling from the LH to the AcbSh. PHAL was injected into the vILH (A) at rostral (B) and caudal (C) tuberal vILH sites. The filled-in pink region in B is shown in the photomicrograph in A. These LH PHAL injections labeled fibers and terminals in the medial AcbSh (D), primarily the posterior half (E). Scale bars are 200 µm.
Figure 5.4: Orexin (A-D) and MCH (A'-D') labeling in the LH. Orexin co-distributes with FG labeling (A) as does MCH (A'). Boxed regions represent the areas analyzed at higher magnification in subsequent photomicrographs. Orexin neurons (B) intermingle with FG neurons (C) but does not co-localize in the same neurons (D). MCH neurons (B') intermingle with FG neurons (C') but do not co-localize (D'). Scale bars are 200 µm.
Figure 5.5: CART (A-D) and Nesfatin (A'-D') labeling in the LH. CART (A) and Nesfatin (A') co-distribute with FG labeling. CART neurons (B) intermingle with FG neurons (C) and sometimes colocalize (D). Nesfatin neurons (B') intermingle with FG neurons (C'). Most FG neurons contain nesfatin (D'). Scale bars are 200 µm. Arrows designate co-localization of peptide and retrograde labeling. Arrow heads indicate CART neurons that are not FG-labeled.
Figure 5.6: GAD67 (A-D) and VGAT (A'-D') labeling in the LH. GAD67 (A) and VGAT (A') co-distribute with FG labeling. GAD67 neurons (B) intermingle with FG neurons (C). Nearly all FG neurons are GAD67-positive (D). VGAT neurons (B') intermingle with FG neurons (C'). A majority of FG neurons are VGAT-positive (D'). Scale bars are 200 µm. Arrows designate co-localization of peptide and retrograde labeling.
Figure 5.7: pSTAT3 (A-D) and VGluT3 (A'-D') labeling in the LH. pSTAT3 (A) and VGluT3 (A') co-distribute with FG labeling. pSTAT3 neurons (B) intermingle with FG neurons (C) but do not co-localize (D). VGluT3 neurons (B') intermingle with FG neurons (C'). Many FG neurons are VGluT3-positive (D'). Scale bars are 200 µm. Arrows designate co-localization of peptide and retrograde labeling.
Figure 5.8: VGluT2- positive neurons in the thalamus (A) and VgluT3-positive neurons in the hippocampus. Note that in both cases, partial or complete staining of the soma is seen. Arrows indicate such somata. Scale bars are 100 µm.
Chapter 6 – Conclusion

The studies shown in this dissertation propose a case for a feeding-specific descending AcbSh to LH circuit that is regulated by the primary amino acid neurotransmitters glutamate and GABA. These studies demonstrate that this descending connection is ipsilateral and likely not contralateral. The anatomical evidence highlights a reciprocal ascending connection from the LH to the AcbSh that primarily uses GABA and some inhibitory peptide neurotransmitters. Together, these studies provide an image of a striato-hypothalamic circuit that regulates feeding (Fig. 6.1).

Some discrepancies and unaddressed issues still remain. First, the behavioral functions of the ascending ventrolateral LH to caudal AcbSh projection need to identified. At first glance, the feedback connection, by using GABA neurotransmission to the caudal AcbSh, makes sense. Caudal AcbSh GABA$A_R$ activation prevents feeding (Reynolds and Berridge, 2001, 2002). However, the same study demonstrates that caudal AcbSh inhibition may do so by increasing defensive behaviors and negative affect. This effect would then not be specific to feeding, but would only secondarily suppress intake as a product of overriding behaviors. Indeed, states of stress hinder AcbSh-mediated feeding (Reynolds and Berridge, 2008). Having defensive behavior circuitry active may coincide with stress-like behaviors, this suppressing feeding non-specifically. However, this issue has yet to be resolved.

Second, the sources and destinations of AcbSh projections into the LH do not particularly overlap with those of the ascending LH to AcbSh connection. AcbSh neurons projecting to the LH are distributed throughout the rostrocaudal extent of the AcbSh (Duva et al., 2005), and these neurons project primarily to the perifornical and dorsolateral LH throughout their rostrocaudal extents (Usuda et al., 1998). Indeed, AcbSh inhibition activates orexin neurons, which are known to distribute throughout these LH areas (Baldo et al., 2004). The ascending LH projections to the AcbSh are far more topographically conserved. I and others (Brog et al., 1993; Goto et al., 2005)
have shown that LH neurons projecting the AcbSh are largely located within the ventrolateral LH throughout its rostrocaudal extent, and these projections terminate selectively to the caudal medial AcbSh. Thus, these circuits appear anatomically separate. It was proposed in the Chapter 5 Discussion that interconnectivity between LH subregions may allow for these two pathways to communicate, but this has yet to be determined. However, this may indeed occur – AcbSh inhibition that activates perifornical and dorsolateral LH orexin neurons also effectively activates some orexin and numerous non-orexin neurons in the ventrolateral LH while exposure to a novel environment does not (Baldo et al., 2004). Although these two circuits may be connected in some manner, they may govern separate behaviors.

Despite these uncertainties, the current evidence suggests that the AcbSh projects directly to the LH to regulate feeding, and that the LH likely projects back to the AcbSh to also regulate feeding in some manner. Aside from these circuits, other downstream/upstream brain regions such as the ventral pallidum, VTA, and PFC can modulate AcbSh-LH signaling. The AcbSh and the LH, in concert with these other brain regions, form a circuit by which feeding, reward, and affect can be regulated. Further research on this collection of circuits can serve to hasten the development of anti-obesity treatments. These treatments can be aimed at reducing the issue of overconsumption of foods caused by dysregulation of central hedonic or energy status regulators.
References


Figure 6.1: A diagram of a cropped sagittal section of the rat brain. The rostral and caudal AcbSh shell possess AMPARs (dark green triangles) and GABA_A Rs (red triangles). These AcbSh regions project to the perifornical and dorsolateral LH subregions with GABAergic projections (red lines). These hypothalamic regions, as well as the ventrolateral LH, possess NMDARs (bright green triangles) and GABA_A Rs. Only the ventrolateral LH projects back to the caudal AcbSh, and does so with GABAergic, glutamatergic (green lines), and/or peptidergic (blue lines) projections.