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Metabolic Regulation of Neuronal Activity and Affective Behavior in the Basolateral Amygdala

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Abstract

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By Brendan Murrihy O'Flaherty

The prevalence of major depressive disorder (MDD), one of the most common disorders in the United States, is doubled in patients with type 2 diabetes mellitus (T2DM). Emerging evidence suggests that MDD and T2DM may share a common etiology: dysregulated metabolism. Dysregulated metabolism could disrupt neuronal activity in key limbic areas, contributing to MDD. The "master" metabolic regulator AMP-activated protein kinase (AMPK) may mediate this relationship. AMPK has been shown to regulate neuronal activity in the hypothalamus. AMPK may also regulate BLA activity and mediate the relationship between metabolic dysfunction and depression. However, AMPK's role in the control and "depressed" BLA remains unknown. The central hypothesis of this study was that the metabolic regulatory molecule AMPK regulates neuronal excitability in the control BLA, and that AMPK becomes dysregulated in rats fed a high-fructose diet (HFrD; a model of metabolic syndrome) leading to BLA hyperexcitability and anxiety-/depression-like behavior.

Firstly, we characterized the effects of a HFrD in male Sprague Dawley rats. We found that as previously reported in Wistar rats, a HFrD increased body fat mass in Sprague Dawley rats, but we did not replicate the reported anxiety-/depression-like phenotype. We also did not observe increased BLA excitability HFrD-fed rats. We then repeated these experiments in a cohort of Wistar rats. Once again we found that a HFrD increased body fat mass, but did not increase anxiety- or depression-like behavior in male or female Wistar rats. Although limited by low statistical power, our experiments in Wistar rats suggest that environmental differences between laboratories best explain the discrepancy between our results and previous studies. These data show that researchers should be extremely careful to control for environmental variables when designing experiments. We also characterized the role of AMPK regulating neuronal excitability in the control BLA. We found that the AMPK inactivator compound C increased BLA excitability, but the AMPK activator AICAR had no effect. We also found that the antidepressant rolipram increased AMPK excitability in an AMPK-dependent manner, suggesting that rolipram's antidepressant effects might also be AMPK-dependent. These experiments identified AMPK as a regulator of BLA excitability and a possible antidepressant target. Collectively, these data lay the groundwork for understanding the relationship between metabolism and mood in both health and disease.

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Chapter 1 General Introduction

1.1 Major Depressive Disorder

Major depressive disorder (MDD) is one of the most common diseases in the United States, with a lifetime prevalence of about 17% [1, 2]. The Diagnostic and Statistical Manual of Mental Disorders (DSM–5) [3] defines MDD as a period of two weeks or longer during which a patient experiences at least one of two core symptoms:

1. Depressed mood.
2. Loss of interest or pleasure (anhedonia).

In addition, a patient must show at least four of the following symptoms indicating a change in their baseline activity:

3. Unexplained loss or gain of weight and/or appetite.
4. A slowing down of thought or physical movement.
5. Repeated feelings of fatigue or loss of energy.
6. Repeated feelings of worthlessness or guilt.
7. Diminished ability to concentrate or make decisions
8. Recurrent thoughts of death, suicidal ideation without a specific plan, a specific plan for committing suicide, or a suicide attempt.

Approximately 7% of U.S. adults reported having at least one depressive episode in 2017 [4]. According to the World Health Organization, almost 800,000 people die through suicide every year, and is the 2nd leading cause of death in 15-29 year olds

[5]. Despite depression's widespread and increasing prevalence [6], the etiology of depression remains largely unknown.

MDD's unknown etiology complicates the development of new treatments. The "monoamine hypothesis" of depression has been the most common hypothesis of MDD pathophysiology for several decades, and posits that depression is caused by alteration in the levels of the monoamine neurotransmitters (serotonin, norepinephrine, and dopamine). Support for this hypothesis rests mainly on the clinical efficacy of selective serotonin reuptake inhibitors (SSRIs) and selective norepinephrine reuptake inhibitors (SNRIs) in many patients. Depressed patients have also been found to have lower levels of serotonin metabolites than controls [7]. However, SSRIs and SNRIs generally take 2-4 weeks to become efficacious, despite rapidly elevating monoamine levels. Additionally, current pharmacotherapies are ineffective in a sizable treatment-resistant subpopulation consisting of $\approx 30\%$ of MDD patients [8, 9]. Thus while it appears monoamine neurotransmission plays a role in MDD, the ultimate neural basis of depression needs further investigation.

Several other hypotheses of MDD have been developed to address the inconsistencies of the monoamine hypothesis. For example, the newer "neuroplasticity hypothesis" of MDD posits that MDD is caused by disrupted neuronal plasticity in key limbic areas. Specifically, the neuroplasticity hypothesis suggests that long-term potentiation (LTP) is impaired in the "dorsal executive system" (consisting of the hippocampus, prefrontal cortex, and other cortical areas), but is enhanced in the "ventral emotional system" comprised of the amygdala.

Indeed, a hyperplastic amygdala may thus contribute to many of the symptoms in MDD. The amygdala is a key area of the limbic system, and is involved in both emotional learning and controlling the stress response. Dysregulation of amygdala activity could thus underlie MDD's emotional symptoms such as depressed mood, anhedonia, and suicidal ideation (thinking about, considering, or planning suicide).

The "metabolic hypothesis" of depression has also been developed to address the problems of the monoaminergic hypothesis. The metabolic hypothesis proposes that neuronal metabolic processes become dysregulated in key limbic regions, leading to abnormal neuronal activity and MDD. Patients with type-2 diabetes are more than twice as likely to be diagnosed with MDD than the general population [10]. Indeed, patients with MDD have been shown to metabolize glucose abnormally in many brain areas, including the amygdala [11, 12].

The "stress hypothesis" of depression posits that MDD occurs after prolonged hyperactivation of the body's stress response, specifically the hypothalamic-pituitary-adrenal (HPA) axis. Indeed, prolonged stress is the most common causal factor in depression [13]. Elevated glucocorticoid levels and a failure of HPA axis negative feedback mechanisms are key biomarkers of depression [14, 15], indicating these processes may play key roles in MDD pathophysiology.

The metabolic molecule AMP-activated protein kinase (AMPK) stands at the crossroads of the monoaminergic, neuroplasticity, metabolic, and stress hypotheses of depression. Serving as the master metabolic regulator of almost all eukaryotic cells, AMPK receives input from a huge number of upstream signaling cascades (including serotonergic and stress hormone signaling), and acts to control the energy production/expenditure of cells (including excitability/plasticity in neurons). AMPK is thus poised to unite elements of the monoaminergic, neuroplasticity, metabolic, and stress hypotheses of depression into a unified hypothesis of MDD pathophysiology. However, little is known about AMPK's potential role in MDD etiology or treatment, or AMPK's role in regulating neuronal activity in the BLC. Understanding AMPK's role in limbic circuits, both in healthy and diseased conditions, is therefore necessary for understanding MDD's pathophysiology.

1.2 The Amygdala in Major Depressive Disorder

As the neuroplasticity hypothesis suggests, human studies have found the amygdala is hyperactive during MDD, which normalizes with successful pharmacotherapy [16]. Indeed, studies report that greater depression severity is associated with increased amygdala activity when viewing emotional stimuli [17] and during emotional regulation [18]. The amygdala also shows increased glucose metabolism in MDD [19], further suggesting pathological hyperexcitability in MDD.

However, a more detailed examination of the amygdala is necessary to elucidate the specific neuronal mechanisms underlying MDD. The amygdala is not a single nucleus, but instead is comprised of at least 13 sub-regions that can be differentiated by their cytoarchitecture, connectivity, and/or neurotransmitter content [20–23]. In addition, each nucleus contains functionally distinct heterogeneous cell-types, any one of which could contribute to amygdala dysfunction in depression. Despite the complexity of the amygdala, three functionally distinct systems have been identified within the amygdala: the basolateral complex (BLC), the centromedial nuclei, and the superficial nuclei [23]. The basolateral complex is of particular interest because studies suggest neuroplasticity in this area is disrupted in MDD.

1.2.1 The Basolateral Complex of the Amygdala

The BLC serves as the primary site of sensory input into the amygdala, and serves as an "emotional valence detector" as part of the greater limbic network. The BLC is critical for fear learning, extinction, consolidation, and reconsolidation [22, 24], and is the classical site of Pavlovian fear conditioning [21] although it also plays a key role in reward processing [25]. The BLC functions abnormally in MDD, as patients with MDD show both enhanced fear learning [26] and enhanced fear extinction [27] in some studies. Because of the BLC's important role in the larger limbic network, this disrupted activity in the BLC is likely of high clinical importance in MDD.

Microcircuitry of the Basolateral Complex

The BLC is a "cortical-like" structure [28] that is composed of the lateral (LA), basal (BA), and accessory basal (AB) nuclei. Together, the basal and accessory basal nuclei are referred to as the basolateral amygdala (BLA) [29, 30]. The BLA has been further split into the magnocellular (anterior) and parvocellular (posterior) subnuclei. All three nuclei of the BLC share common cell-types, a canonical intrinsic neural circuitry, and are reciprocally connected (see Figure 1). The BLA is the main focus of this dissertation, however the following chapter will discuss the BLA within the context of the greater BLC.

Sensory information enters the BLC through a variety of pathways, including glutamatergic thalamic and cortical inputs [32, 33]. Importantly, information flow into the amygdala is topographically organized and consists of unimodal and multimodal sensory input from all exteroceptive and interoceptive modalities [23, 34]. The BLC also receives cortical input thought to represent higher-order cognitive processing [35]. Hence, the topographic organization of BLA inputs would suggest that the different subnuclei of the BLC play different roles in fear memory formation and extinction [23, 30, 34, 36–39]. For example, while the LA receives the densest auditory afferents, the AB receives the densest olfactory input, suggesting divergent roles in auditory and olfactory fear conditioning respectively [40]. Consistent with this premise, results from fear conditioning studies suggest that the BLC allows both serial and parallel processing of sensory information to initiate unique aspects of fear behavior [41]. Indeed, efferents of each of the BLC sub-regions innervate many non-overlapping target regions, further supporting the idea of functional specialization within the nuclei of the BLC [42].

Within the amygdala, information flow occurs through a series of highly ordered connections that are conserved across species [43]. In general, information flows from more posterior regions to anterior regions within each nucleus of the BLC [30].

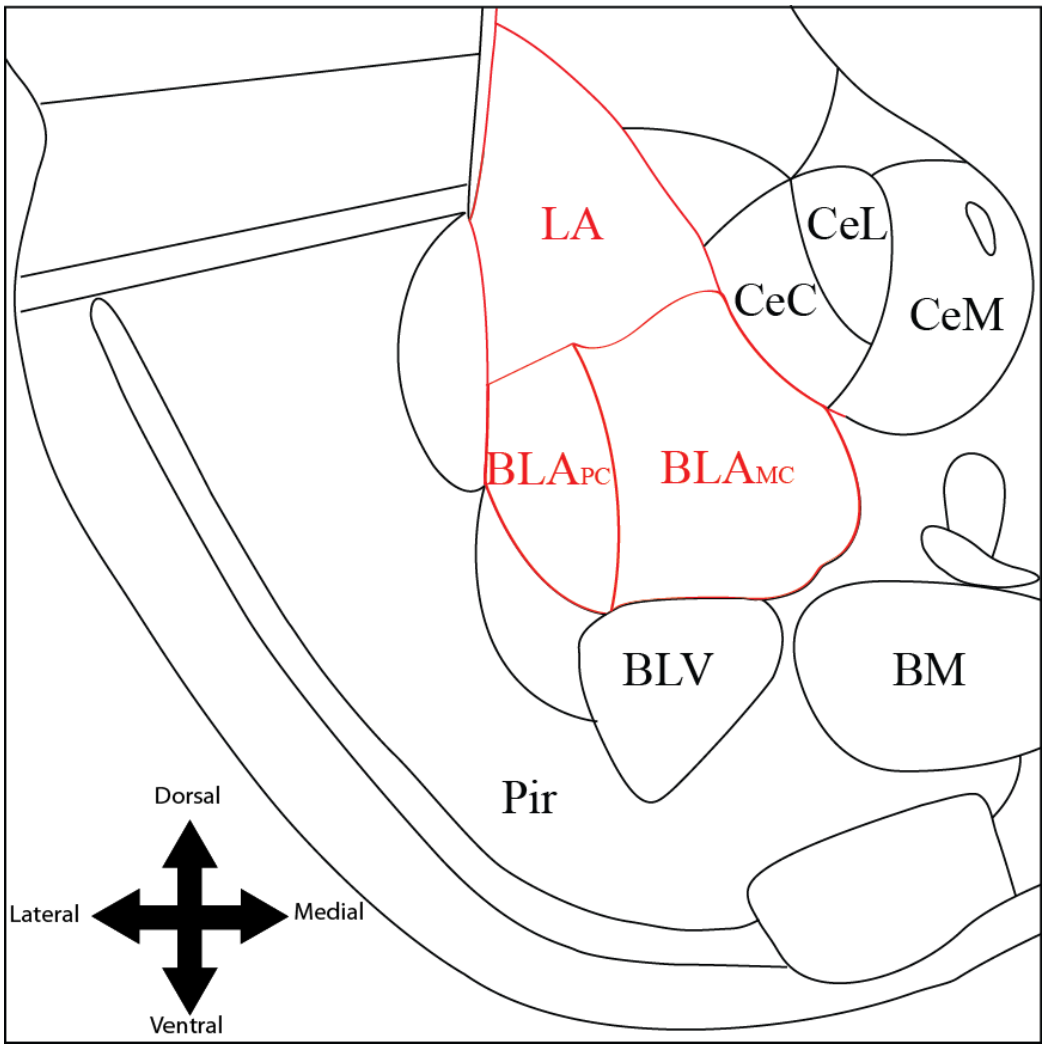

Figure 1.1: Anatomical structure of the basolateral complex. Subdivisions of the basolateral complex are shown in grey. Abbreviations: LA, lateral amygdala; BLA_{PC}, basolateral amygdala parvocellular division; BLA_{MC}, basolateral amygdala magnocellular division; BLV, basolateral amygdala ventral nucleus; BM, basomedial amygdaloid nucleus; CeC, central amygdala, capsular division; CeL, central amygdala, lateral division; CeM, central amygdala, medial division; Pir, piriform cortex. BLA_{MC} and BLA_{PC} divisions correspond to the anterior and posterior divisions of the BLA respectively. Adapted from [31].

However, additional subtlety of information processing is achieved via more lateral connections. For example, the magnocellular and parvocellular subdivisions of the BA are reciprocally connected, whereas the magnocellular and parvocellular subdivisions of the AB are not. Interdivisional connections are precisely targeted and mostly non-overlapping indicating that information flow through the amygdala is split into multiple parallel and serial pathways, perhaps representing unique stimulus qualities. While the intrinsic circuitry of the BLC most likely reflects modality-specific information processing, it probably also reflects the need to separate those pathways regulating aversive conditioning from those contributing to appetitive conditioning in which the BLC is also implicated [44–46]. Topographically organized input into the BLC thus suggests that the BLC can be split into functionally distinct sub-networks. Notably, the functional sub-specialization suggested from animal studies is also observed in the human amygdala [47].

Neuronal Diversity in the BLC

The BLC consists of two broad classes of neurons: excitatory glutamatergic principal neurons, and inhibitory GABAergic local circuit interneurons. Excitatory principal neurons compose the primary output neurons of the BLC, and make up approximately 80-85% of the BLC's neuronal population [48]. Alterations in the firing patterns of these neurons most likely represent the engram of fear learning [49, 50]. Although early cytochemical studies hinted at the existence of multiple classes of BLC principal neurons (e.g. magnocellular vs parvocellular), many of the original immunohistochemical and electrophysiological studies of BLC principal neurons found evidence for only one, or at most, two subclasses [34, 51–54].

However, more recent studies using viral gene transfection and optogenetics are beginning to tell a more complex story. For example, two distinct subpopulations of BLC principal neurons are reported to fire selectively during either fear learning or extinction, respectively [55, 56], suggesting that specialized subnetworks of non-

overlapping principal neurons may exist that encode different aspects of affective memory formation [57]. Consistent with this premise, in the Thy-1-H transgenic mouse line [58] only about 60% of BLC principal neurons express EYFP driven by the Thy-1 promoter. Optogenetic stimulation of these Thy-1 EYFP-expressing BLC neurons had no effect on fear memory acquisition, but enhanced extinction of fear memory [59], supporting the idea that a sub-network of BLC principal neurons is explicitly involved in extinction memory formation. Additionally, these BLC sub-networks may have different efferent targets, as BLC principal neurons that project to the prelimbic PFC activate during times of fear learning, while BLC principal neurons targeting the infralimbic PFC activate during extinction learning [60].

It is thus becoming increasingly apparent that BLC principal neurons are an anatomically, and functionally heterogeneous population, sub-networks of which most likely differentially regulate appetitive and aversive memory formation and extinction. Similar heterogeneity in genetic, morphological, and functional diversity of CA1 principal neurons in the hippocampus have been reported [61–63]. Understanding the role of these different BLC neuronal subpopulations is thus a key step in understanding the BLC's role in MDD.

The remaining 15 -20% of BLC neurons are GABAergic interneurons, which can be roughly divided into four mutually exclusive subgroups: (1) parvalbumin immunoreactive interneurons making up approximately 40% of the total population (PV) [64–66], and which can be further subdivided into at least two subtypes based on electrophysiological properties [67, 68]; (2) somatostatin interneurons making up 20% of this neuronal population (SOM) [69–71]; (3) cholecystokinin interneurons representing 20% of BLC interneurons (CCK) [72, 73]; and (4) calretinin and vasoactive intestinal peptide interneurons that represent the remaining 20% (CR/VIP) [74–76]. Interneurons control the output of principal neurons at all levels in the BLC, and form both feedforward and feedback inhibitory circuits. Interneurons control global

BLC activity in at least to two ways: (1) by controlling the net excitatory tone of the BLC; and (2) by playing a key role in precisely organizing the firing pattern of BLC principal neurons. Classically, GABAergic interneurons were seen as modulators of overall BLC excitation. For example, early studies showed that infusion of the $GABA_A$ receptor agonist muscimol into the amygdala prevented fear memory acquisition by inhibiting BLC principal neurons [77, 78]. However, the view that interneurons only inhibit net BLC activity is overly simplistic. Different subtypes of interneurons have different functional roles within the BLC: for example, while most PV interneurons inhibit BLC principal neurons, CR/VIP interneurons (as well as a small population of PV interneurons) selectively inhibit other interneurons [79]. When activated, CR/VIP interneurons would thus disinhibit BLC principal neurons, increasing their activity and promoting fear and appetitive learning. Nevertheless it is clear that dysregulation of inhibitory interneurons alters the net excitatory tone of BLC principal neurons.

Recently GABAergic interneurons have also been shown to play a number of critical organizational roles in the BLC, such as gating LTP at thalamo- and cortico-BLC synapses, and determining the time window during which inputs are able to generate action potentials in principal neurons [80]. Notably, individual PV interneurons innervate multiple principal neurons [67, 81], and physiologically distinct PV interneurons are electrically coupled by gap junctions to form discrete functional syncytia [81]. PV interneurons thus coordinate the firing of large numbers of BLC principal neurons through rhythmic inhibition. Moreover, PV interneurons have also been shown to control spike timing precision in BLC principal neurons, thus generating synchronized oscillations of the BLC, which play an important role in fear learning [82]. Additionally, in the hippocampus a subpopulation of PV interneurons preferentially innervate amygdala projecting CA1 principal neurons and receive preferential input from CA1 principal neurons that project to the mPFC [83], suggesting that functional

sub-networks of principal neurons and PV interneurons segregate different streams of information processing. Consequently, stress-induced dysregulation of BLC inhibition would be predicted to have a deleterious effect on BLC function, producing not only hyperexcitability but also disorganizing information within the BLC.

Although the nuclei of the BLC receive topographically organized sensory input as described above, principal neurons and interneurons are organized in a more-or-less common intrinsic circuitry throughout the BLC. For example, principal cells project mostly to other principal cells, but also to interneurons. Most interneurons are predominantly driven by input from BLC principal cells, and form feedback and feedforward connections with both principal neurons and other inhibitory neurons. Despite the fact that BLC principal neurons project heavily to other principal neurons, paradoxically BLC principal neurons have limited excitatory drive [84]. This finding may be due to the fact that BLC principal neurons project preferentially to proximal interneurons and distal principal neurons, activating feed-forward inhibitory mechanisms that prevent local runaway excitation while allowing associative interactions [85]. Importantly, the common circuitry found throughout the BLC is in no way at odds with the idea of specialized BLC sub-networks, as identical neural circuits can be adapted for different purposes by altering the incoming information.

Synaptic Plasticity in the BLC

The BLC has traditionally been thought of as the region where associative fear learning and extinction learning take place [21, 86, 87]. The most widely accepted cellular substrates for associative learning are long-term potentiation (LTP) and long-term depression (LTD) [88, 89]. Fear learning studies have laid the groundwork for our understanding of the BLC's microcircuitry and role of the BLC in the wider limbic network, and continue to be the most accepted model of amygdala-dependent synaptic plasticity today. However, the BLC is not exclusively involved in fear learning. The BLC acts as the main "emotional valence detector" of the limbic system, assigning

both positive or negative emotional valences to significant life events [90].

Importantly, amygdala activity is strongest early in fear conditioning when the association between the CS and US isn't yet known, and after trials in which the CS is not followed by the US (i.e. during extinction learning). In other words, synaptic plasticity in the BLC is most important early in associative learning and extinction, when there is a large degree of uncertainty as to the CS-US relationship. BLC activity therefore appears to encode a "predictive error", i.e. an insufficient ability of the brain's existing associations to predict the new CS-US relationship. Activity in the BLC is therefore thought to draw attention to new, uncertain CS-US associations in order to accelerate learning [91].

Although the BLC's role is not limited to fear learning, most studies of BLC synaptic plasticity have focused on Pavlovian fear conditioning in rodents [92–95]. Such studies can be considered general probes of synaptic plasticity in the BLC, with implications for other forms of associative learning as well [27]. In Pavlovian fear learning, a noxious unconditioned stimulus (US), usually a shock, is repeatedly paired with a normally innocuous conditioned stimulus (CS), e.g. a tone, until the CS takes on the affective salience of the US. Specifically, the "cellular hypothesis" of fear learning postulates that fear learning occurs through associative LTP at BLC principal neurons [96, 97]. The cellular hypothesis specifically postulates that when subcortical afferent synapses carrying information about the CS fire concurrently with thalamic afferent synapses carrying information about the US, the subcortical CS synapses are strengthened such that the CS can now recruit downstream target structures previously only recruited by the US [98].

The BLC is also involved in extinction learning, i.e. learning when the CS no longer predicts the US. While LTP is often thought to underlie aspects of fear learning, LTD and/or depotentiation has classically been considered as a substrate for fear extinction [99, 100]. Indeed, recent evidence suggests that induction of LTP

at thalamic synapses to LA neurons, representing memory consolidation, can be deconsolidated or reversed, potentially weakening the fear memory trace [101, 102]. However, extinction does not appear to be an erasure of existing fear memory. Instead, extinction is thought to involve learned inhibition of fear pathways. However, it is likely that both LTP and LTD play a key role in fear memory formation and extinction [103].

The "neuroplasticity hypothesis" of depression postulates that enhanced synaptic plasticity in the amygdala may lead to hyperexcitability and contribute to MDD development. This hypothesis therefore predicts that patients with MDD may have abnormal associative learning / extinction due to abnormal plasticity in the BLC. Consistent with this hypothesis, depressed patients assign a more negative emotional valence to ambiguous or aversive stimuli [104–107]. Depressed patients have also been shown to have enhanced fear conditioning relative to nondepressed controls, specifically in their ability to discriminate between a conditioned stimulus (CS+) and non-conditioned stimulus (CS-) as measured by skin conductance response (a measurement of the sympathetic response to a stressor) [26]. Furthermore, studies have found depressed patients have enhanced extinction learning relative to healthy controls [27, 108], consistent with the premise of a hyperplastic amygdala. Interestingly, the strength of the enhanced extinction correlates with the total illness duration [27], suggesting a close relationship between BLC hyperplasticity and MDD. In MDD, the BLC could therefore form stronger-than-normal associations between environmental cues (CS) and negative stimuli (US), contributing to BLC hyperexcitability.

As mentioned above, BLC activity appears to encode a "predictive error", responding most strongly when a new CS-US pairing is presented. In other words, the BLC responds most strongly when the US is unpredictable. This may play be of importance in MDD, as the amygdala is hyperactive at baseline and responds more strongly to negative life events (a form of US) in MDD patients than in healthy

controls [16, 104–107]. This stronger-than-normal BLC response suggests that the depressed amygdala responds to all negative stimuli *as if they were unpredictable*. Because unpredictable negative life events are more stressful than predictable negative life events, this erroneous "unpredictable" signal originating from the BLC might result in a greater-than-normal sympathetic response when confronted with minor stressors [91]. When confronted by a series of "unpredictable" stressors, the brain may be unable to generate a successful coping strategy to deal with the stressor, generating a feeling of hopelessness over time.

The BLC and Pathological Stress: Mechanisms of Hyperexcitability

As outlined above, depressed patients appear to have abnormal synaptic plasticity in the BLC. BLC appears to be hyperactive in depressed patients, both at baseline and when exposed to negative stimuli [16, 104–107]. However, the mechanism of BLC hyperexcitability in MDD remains unknown. There are several possible mechanisms by which the BLC may become hyperexcitable in depression.

Many studies have focused on the effect of repeated or severe stress in rodents on BLC hyperexcitability. These studies are relevant to our discussion of MDD, as stress is the most common causal factor in the development of MDD [13, 109, 110]. However, it is worth noting that extreme stress in humans often results in post-traumatic stress disorder (PTSD) instead of depression. Although frequently comorbid with depression [111], PTSD features many symptoms not seen in MDD such as flashbacks, fear generalization, and hypervigilance [3], suggesting that the changes in BLC excitability after traumatic stress far exceed those seen in MDD. However, the general mechanisms by which stress causes BLC hyperexcitability could be of clinical relevance for MDD as well. Three general mechanisms account for stress-induced BLC hyper-excitability:

1. Increased net excitatory drive within the BLC.

2. An increase in the intrinsic excitability of BLC principal neurons.

3. A reduction in net inhibitory drive.

A growing body of evidence suggests that all three mechanisms may contribute to BLC hyper-excitability after pathological stress, indicating a series of complex interactions.

Firstly, studies have shown that pathological stress results in an increase in excitatory drive to BLC neurons. Regular fear conditioning is reported to increase the magnitude of excitatory synaptic potentials at cortico-amygdala synapses [112], as well as lower the stimulation threshold required for LTP induction in BLC principal cells (Figure 1.2) [113]. It is thus possible that in MDD, chronic stress results in an inappropriate magnitude of LTP in the BLC, or an abnormally low threshold for LTP induction, potentially resulting in the abnormal fear / associative learning described above.

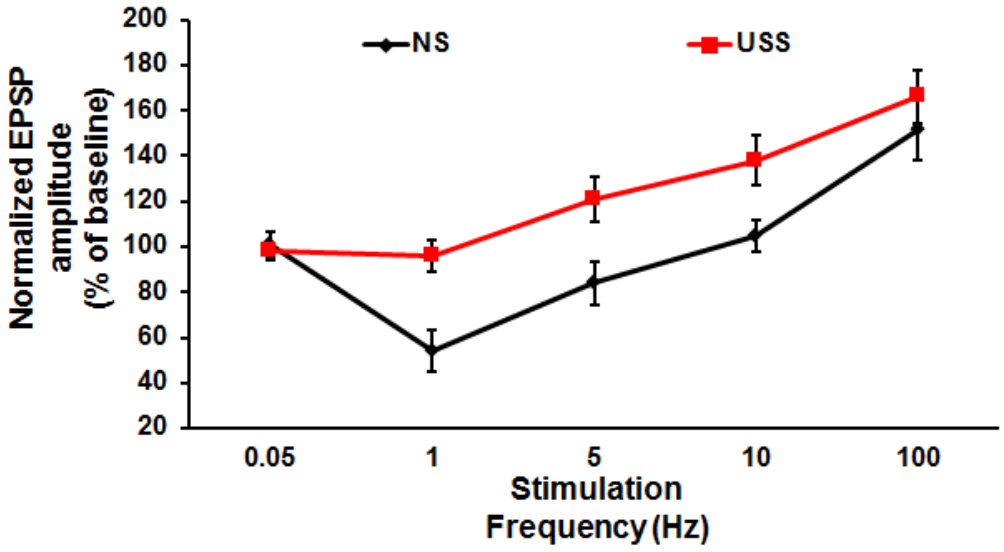

Figure 1.2: Synaptic plasticity in the BLC is regulated by prior stress exposure. Repeated stress alters the frequency- response curve for the induction of LTD or LTP, leading to increased BLC excitability. In control rats (NS, black line), frequencies below 10 Hz induce LTD, while frequencies above 10 Hz induce LTP. In rats exposed to repeated unpredictable shock stress (USS, red line), frequencies below 5 Hz induce LTD, while frequencies above 5 Hz induce LTP. Note that 0.05 Hz (the stimulation frequency used to monitor the strength of synaptic transmission throughout the experiments) had no effect on synaptic transmission in both NS and USS animals. [113]

Numerous studies have also described an increase in the frequency [114–116] or amplitude [112, 117] of excitatory synaptic events recorded in BLC principal neurons following stress manipulations, suggesting increased excitatory input may also contribute to BLC hyperexcitability after stress.

In addition to influencing the strength and/or the number of synaptic inputs onto BLC principal neurons, stress can also alter the intrinsic properties of principal neurons (Mechanism 2 above). BLC principal neurons in rodents are reported to show increased firing rates in response to depolarizing current injection following chronic stress [118]. Repeated stress has been shown to influence the excitability of LA and BA neurons in adolescent rats through modulation of post-spike afterhyperpolarizing potentials (AHPs), although the effect was only seen in LA neurons of adult rats [114]. It may thus be possible to modulate the intrinsic excitability of BLC neurons through the calcium-activated potassium channels responsible for the AHP. This would not only directly regulate neuronal excitability but also influence key intracellular signaling cascades in BLC neurons by altering Ca^{2+} influx.

Thirdly, reduction in BLC GABAergic inhibition could also underlie hyperexcitability of the BLC. Increased GABAergic activity has been negatively correlated with an anxiety-like phenotype [119], which is relevant to MDD due to the frequent comorbidity of anxiety and MDD [120]. Most BLC principal neurons express a robust and tonic GABA current, affecting their excitability [121], and it is possible to induce an anxiety-like phenotype by decreasing GABAergic tone [122, 123]. Repeated administration of CRF, an anxiogenic neuropeptide, can also reduce phasic inhibition in the BLC, leading to an anxiety-like phenotype [122]. Many studies have reported that chronic stress decreases tonic GABA inhibition in the BLC, thus disinhibiting BLC principal neurons and contributing to the anxiety-like phenotype [124–126].

GABAergic interneurons are also subject to morphological changes as a result of chronic stress. For example, in mice subjected to repeated restraint stress, BLC in-

terneurons showed reduced dendritic arborization, dendritic spine density, and synaptic input. These changes would reduce excitatory drive onto inhibitory interneurons [127]. GABAergic inhibition also suppresses LTP in BLC principal neurons [80]. The loss of interneuron excitatory drive could thus disorganize the activity of the BLC, as well as lead to amygdala hyperexcitability by increasing LTP.

1.2.2 Inter-Regional Connectivity of the BLC

Besides serving as the main processing center for sensory input in the amygdala, the BLC also projects to multiple brain regions that are implicated in the etiology of MDD, including the hippocampus and medial prefrontal cortex [128, 129]. As a growing body of evidence suggests that aberrant BLC activity and plasticity is involved in the etiology of MDD, the question arises: how do changes in BLC activity affect downstream target structures? In this section, we will review recent findings concerning BLC regulation of two downstream targets involved in MDD.

Hippocampus

The hippocampus has repeatedly been implicated in the pathophysiology of MDD. Depressed patients have reduced hippocampal volume relative to control [130] and decreased hippocampal activity may underlie the cognitive impairments in MDD such as decreased memory and attention. The BLC may play a role in hippocampal dysfunction in MDD, as the BLC sends strong excitatory projections to the hippocampus. Specifically, the BLC projects to the entorhinal cortex (EC), CA1, and CA3 regions of the hippocampal formation and has been shown to play a critical role in hippocampal plasticity and emotional memory [131–133]. For example, lesions or pharmacological inactivation of BLC can impair hippocampal LTP and emotional memory consolidation [134–136]. Intra-BLC infusion of a β-adrenergic receptor agonist showed enhanced arousal emotional memory and the expression of Arc (a plasticity-related molecular marker) in the dorsal hippocampus [137]. Consistent with this observation,

Bass and colleagues demonstrated that brief bursts of 50 Hz electrical stimulation applied to the BLC enhanced hippocampal-dependent episodic memory [138]. Furthermore, inactivation of the BLC suppresses proliferation and activation of newborn neurons that are associated with fear memory formation in the dentate gyrus (DG), suggesting that the BLC directly modulates hippocampal neurogenesis [139]. Activity in the BLC therefore has the potential to affect hippocampal LTP and memory. The BLC hyperactivity seen in MDD also could impair hippocampal LTP and memory, possibly leading to the cognitive deficits seen in depression [140]. Studies have shown that different BLC stimulation parameters can either impair, facilitate, or have no effect on DG or CA1 LTP [141–144], suggesting that the timing, intensity, and frequency of BLC stimulation are critical factors in regulating DG and CA1 LTP. It is therefore possible that hyperactivity in the BLC (as seen in MDD) can impair hippocampal function as well.

One caveat to the above experiments is that electrical stimulation is non-specific and cannot discriminate between direct local activation/inactivation of different cell-types or fibers of passage. With the development of cell-type-specific optogenetics we can functionally dissect specific neural circuits throughout the CNS [145]. Optogenetic inhibition of inputs from BLC projection neurons to the ventral hippocampus (vHPC) decreases anxiety-like behavior in the elevated plus maze and open field test, whereas optogenetic activation of BLC inputs to vHPC increases anxiety-like behavior, as well as decreases feeding during a novelty-suppressed feeding test [146]. Together, these findings further suggest that hyper-activation of BLC circuits specifically in MDD could have profound effects on hippocampal circuits.

Medial prefrontal cortex

The medial prefrontal cortex (mPFC) is another brain region that has been heavily implicated in the pathophysiology of MDD. The mPFC has been implicated in "self-referential processing" in healthy subjects: the ability to monitor the body's

overall internal state [147]. One key feature of MDD is increased self-focus, which is also qualitatively different than normal [148]. Self-focus in MDD is characterized by abstract, analytical cognition ("thinking about" experience) rather than a concrete, intuitive awareness of moment-to-moment experience. However, the role of the mPFC in depression remains somewhat unclear, as studies have reported both mPFC hyper- and hypoactivity in MDD [149], likely due to differences in experimental design [150]. However, the mPFC is highly affected by stress [151] in both human and rodent models [152, 153], and therefore remains an important player in the pathophysiology of depression.

The mPFC is also reciprocally connected with the BLC. BLC projection neurons innervate layer 2 and 5 projection neurons of the mPFC which in turn project to cortical, autonomic, and subcortical regions including the BLC [154–156]. Intriguingly, the BLC input also selectively innervates a sub-population of parvalbumin-containing interneurons in the mPFC [157]. Hence, depending on the pattern of activation, BLC output neurons can recruit either feed-forward activation and/or inhibition in the mPFC [154, 158, 159].

BLC inputs also preferentially activate mPFC principal neurons that project back to the BLC [156], suggesting that cross-talk between the BLC and the mPFC is an essential component in regulating affective state. Like BLC projections to the mPFC, layer 2 and 5 mPFC projection neurons innervate both principal neurons and PV interneurons in the BLC [156, 160], suggesting that mPFC inputs to the BLC can regulate BLC activity in the same way that BLC activity can regulate activity in the mPFC [161].

It is clear that because of their shared reciprocal connectivity, hyperactivity in the BLC in MDD could dysregulate activity in the mPFC and vice versa. However, given the complex nature of the BLC-mPFC circuit, involving both feedforward activation and inhibition, it is not yet clear specifically how BLC hyperreactivity is related to

mPFC function in MDD. This is further complicated in that neither the BLC nor the mPFC are homologous units. As mentioned above (Section 1.2.2) BLC principal neurons can be separated into two neuronal subpopulations involved in associative learning and extinction respectively. The mPFC can similarly be divided into the prelimbic (PL) and infralimbic (IL) cortices, which have roles in fear expression and extinction respectively [60, 162, 163]. PL- or IL-projecting BLC neurons (which are intermingled within the BLC) also have distinct roles in fear expression and extinction [60]. It therefore appears two parallel but intermingled circuits exist between the mPFC and BLC, complicating the potential relationship between these brain regions in MDD. However, the strong reciprocal connectivity between the BLC and mPFC makes a vicious cycle possible in MDD, with dysregulated activity in one area disrupting the other and vice versa.

1.2.3 Role of Monoamines in MDD and BLC Excitability

In addition to influencing other brain regions through extrinsic connections, the BLC receives neuromodulatory input from many different areas of the brain. These neuromodulators can have multiple effects on each of the different BLC cell-types due to the heterogeneous expression of their cognate receptors. As a result, each neuromodulator has the ability to differentially affect BLC activity due to specific activation and inhibition of different cell-types.

The following section is an overview of monoaminergic neuromodulation of the BLC. Although the monoaminergic hypothesis is an inadequate model of MDD pathophysiology, MDD likely has an important monoaminergic component. Indeed, the most widely prescribed antidepressant drugs target monoaminergic pathways. Understanding the role of monoaminergic neurotransmission in the BLC is therefore vital to understanding why SSRIs and SNRIs are effective antidepressant drugs. Understanding how current antidepressant drugs work is vital for uncovering targets for

more effective and rapidly-acting antidepressants.

While this section individually discusses the activity of different neuromodulators, it should be remembered that their activity is oftentimes concurrent and synergistic. Hence, it is important to appreciate the complexity, and state-dependent nature, of the response of BLC principal neurons due to such an intricate regulatory mechanism.

Serotonin

Serotonin (5-HT) is a particularly important neurotransmitter in MDD pathophysiology. The "serotonergic hypothesis" of MDD has been the main hypothesis of the pathophysiology of depression for decades and posits that MDD is caused by diminished serotonergic activity in the brain. Indeed the most common antidepressant drugs, selective serotonin reuptake inhibitors (SSRIs) target the serotonergic system. However, the serotonergic hypothesis of MDD has not been clearly substantiated by recent studies.

The clearest evidence that serotonin plays a causal role in MDD comes from tryptophan depletion studies, in which an acute dietary manipulation is used to deplete serotonin's precursor amino acid. Although healthy controls show no changes in mood, formerly depressed patients show acute depressive symptoms [164]. Low tryptophan levels can also be observed in the blood of patients with severe depression [165]. Increased serotonergic activity can also result in increased BDNF signaling, which in turn increases hippocampal neurogenesis. As decreased hippocampal neurogenesis has been implicated in the pathophysiology of MDD as well, this mechanism could also potentially underlie the antidepressant efficacy of SSRIs [166].

Modulation of serotonergic input into the amygdala may underlie the antidepressant efficacy of SSRIs. In both healthy and depressed patients, SSRI administration also leads to positive shifts in the way the brain processes emotionally-valenced information. This effect seems to be mediated through changes in the serotonergic input to the limbic system, especially the amygdala [167]. As the BLC serves as

the amygdala's main emotional valence detector, these findings likely implicate the BLC specifically. Serotonin thus appears not to improve mood directly, but instead influence the way the brain responds to acute emotional stimuli over time.

The major source of serotonin input to the amygdala primarily comes from specific subdivisions of the dorsal raphe nucleus [168–170]. Anterograde and retrograde tracing studies have revealed clearly labeled 5-HT neurons in the dorsal and caudal DRN [168, 171]. Significantly, experimental manipulations of these connections lead to altered stress-related behavior [172–174], and it can be seen that 5-HT levels in the BLC increase during psychological and physiological stressors [175, 176]. In control animals, 5-HT release in the BLC generally decreases the excitability of principal neurons through three main mechanisms:

1. Hyperpolarization of a subpopulation of BLC projection neurons through activation of 5-HT_{1A} receptors [177].

2. Depolarization of GABAergic PARV interneurons through activation of 5-HT_{2A} receptors, resulting in increased inhibitory drive onto principal neurons [178].

3. Presynaptic inhibition of glutamate release through activation of 5-$HT_{1A/B}$ receptors [179, 180].

Decreased BLC excitation in response to 5-HT can be blocked by prior application of a $GABA_A$ receptor antagonist, suggesting that activation of PARV interneurons is the predominant response to 5-HT release under non-stressful conditions [177, 181]. Nevertheless, selective activation of 5-HT_{1A} receptors in the BLC has also been shown to reduce anxiety-like behavior, suggesting serotonin also acts directly on principal neurons as well [182, 183]. Consequently, 5-HT release in the BLC has been viewed as contributing to an inhibitory feedback-loop that may act to terminate an ongoing stress response as part of a coping mechanism [177, 184, 185]. Consistent, with this hypothesis, selective depletion of 5-HT in the BLC facilitates fear-potentiated startle

[186], and local infusion of the SSRI citalopram decreases fear conditioning [187, 188]. Reduced 5-HT signaling may thus interfere with this coping mechanism, thus enhancing the negative emotional reaction to stressful events, ultimately contributing to MDD.

Interestingly, while serotonin normally inhibits overall BLC activity, after stress serotonin appears to *increase* BLC excitability. Animals that have undergone prolonged uncontrollable stress show increased excitability of BLC principal neurons in response to 5-HT, as opposed to decreased excitability in controls [182]. Stress-induced changes in the expression of serotonergic receptors in the BLC are thought to contribute to the alteration in the response to 5-HT input. For example, inescapable stress has been reported to downregulate 5-HT_{2A} receptor expression in GABAergic interneurons, increasing the excitability of BLC principal neurons [178]. A down-regulation of 5-HT_{1A} receptors may also be a mechanism behind stress-induced anxiety-like behavior [189]. Because stress is the most common causal factor underlying MDD, the loss of serotonin's normally inhibitory influence may also contribute to amygdala hyperexcitability in MDD.

There are also 5-HT receptors that normally act to increase BLC excitability and produce anxiety-like behavior, such as 5-HT_{2C} receptors [187, 190]. Hence, the behavioral consequences of activation of 5-HT_{2C} receptors may be unmasked by the down-regulation of other 5-HT receptors by stress. Consistent with this premise, local infusion of the selective 5-HT_{2C} receptor antagonist, SB 242,084, into the BLC prevented the enhancement of anxiety-like behavior in response to uncontrollable stress [182]. It is therefore possible that the loss of serotonin's normally inhibitory influence after stress contributes to BLC hyperexcitability in MDD.

Norepinephrine

Like serotonin, norepinephrine has also been implicated in the pathophysiology of depression. Selective norepinephrine reuptake inhibitors (SNRIs) are used as clinically

effective antidepressants. Indeed, norepinephrine depletion after successful treatment with norepinephrine-specific antidepressants results in acute depression [191, 192]. However, there is less evidence that alteration of norepinephrine systems in the amygdala contributes to MDD etiology. Increased norepinephrine expression has been found in the frontal cortex of depressed patients, but no literature reports changes in the amygdala [193, 194]. However, norepinephrine nevertheless plays an important role in modulating BLC circuitry, and therefore remains a potential mechanism of both MDD pathophysiology and antidepressant efficacy.

Afferents from the locus coeruleus (LC) are the main source of norepinephrine (NE) input into the BLC [195], and NE release in the BLC has been shown to be critical for the consolidation of memory, particularly emotional memory [196–201]. Consistent with this observation, projections from the LC target both BLC principal neurons and interneurons [202], and NE levels increase in the amygdala during stress. However, despite the growing body of evidence supporting a role for NE in memory consolidation, the cellular mechanism/s underlying this response are complex and involve multiple receptor subtypes and the concurrent release of other neurotransmitters.

NE release in the BLC has been reported to result in overall inhibition of BLC firing activity [195, 203]. However, different subpopulations of BLC neurons were shown to either decrease or increase their firing rates, suggesting that NE may differentially affect principal neurons and interneurons depending on their NE receptor expression patterns [203]. Indeed α1A receptor activation increases GABAergic inhibition in the BLC through a direct postsynaptic membrane depolarization of BLC interneurons [204, 205]. In contrast, β receptors are thought to be preferentially expressed in principal neurons and are reported to cause an increase in neuronal firing activity in response to local NE release [203, 206]. However, another study found that BLC principal neurons had no direct response to NE [207]. The disparity between

these two observations most likely results from the use of NE versus the selective β-adrenergic receptor agonist, isoproterenol, to examine the response to β receptor activation. In control animals, NE therefore appears in inhibit BLC activity.

Similar to serotonin, NE causes an overall increase in BLC activity in animals that have undergone chronic stress, despite being normally inhibitory [208]. Recordings from BLC principal neurons of stressed animals showed no significant NE-induced GABAergic activity compared to controls [204]. As serotonin exhibits a similar reversal after chronic stress, this suggests NE may play a role in a general stress-induced dysregulation of monoaminergic neuromodulation in the BLC.

A proposed mechanism for the alteration in the NE response is a stress-induced decrease in α1A receptor activity (though desensitization, downregulation, or internalization of the receptor) or a change in its intracellular signaling pathways [204]. Another possibility could include an increase in the response to β receptor activation. The response of BLC circuits to NE release may depend on the timing of its release in relation to corticosteroid release [196]. Hence, the concurrent release of corticosteroids and NE might act synergistically to enhance emotional memory formation and facilitate LTP induction in the BLC [209], potentially leading to hyperexcitability in MDD. The stress-induced facilitation of LTP was attenuated by prior application of corticosteroid- or β receptor antagonists. Moreover, if corticosteroid release occurs asynchronously it can prevent or suppress the effects of β receptor activation, thus allowing for a bidirectional modulation of the NE response in the BLC. A change in corticosteroid release could therefore indirectly dysregulate the NE system independently of NE receptor expression. NE therefore remains a potential monoaminergic mechanism of MDD pathophysiology, consistent with the observation that NE reuptake antagonists are clinically effective antidepressants.

Dopamine

Dopamine (DA) has also been implicated in the etiology of depression. In MDD, the dopaminergic system throughout the brain is hypoactive. This dopaminergic hypoactivity is thought to underlie anhedonia (an inability to feel interest or pleasure), which is one of the two core symptoms of MDD [210]. The dopaminergic system in the BLC also appears to be hypoactive in MDD patients [211], suggesting that a lack of DA signaling in the BLC contributes to MDD psychopathology.

In the BLC, the function of DA is to facilitate the transmission of exteroceptive information (information about the outside environment) into the BLC. Reduced DA activity therefore reduces the BLA's ability to process exteroceptive information, leading to increased interoceptive (information about the body's internal state) processing. This increased interoceptive processing could contribute to the ruminative, brooding behavior often seen in MDD [212].

Most dopamine input into the BLC arrives via afferents arising from the ventral tegmental area (VTA) and substantia nigra (SN) [213–215]. In the BLC, the function of dopamine is to:

1. Facilitate transmission of salient sensory input.

2. Enhance synaptic plasticity.

3. Synchronize the firing activity of ensembles of principal neurons.

Firstly, DA facilitates pathway-specific sensory input into the BLC [216]. Early studies reported that dopamine acts to reduce the efficacy of synaptic input from the mPFC to the BLC, while at the same time enhancing sensory cortical input [216, 217]. DA also facilitates sensory input by reducing feed-forward inhibitory drive onto BLC principal neurons [218, 219]. DA release therefore facilitates the presynaptic entry of exteroceptive information into the BLC.

DA also facilitates synaptic plasticity in the BLC in a pathway-specific manner. D1 receptors in the BLC are co-distributed with NMDA receptors on the spines of principal neurons. As spines are the primary site of afferent innervation of principal neurons, locally released DA is well-positioned to modulate NMDA currents and influence LTP induction in sensory pathways [220, 221]. Indeed, local DA release onto BLC principal neurons is a pre-requisite for LTP induction [222], specifically depending on D1-mediated activation of the adenylate cyclase cascade.

Activation of the adenylate cyclase cascade via D1 receptors is also required for DA's third mechanism (above): synchronizing BLC principal neuron firing. Adenylate cyclase activation amplifies an intrinsic sub-threshold membrane potential oscillation (SMPO) in BLC principal neurons [82], which facilitates the rhythmic and synchronized spiking of BLC principal neurons. This synchronized spiking in the BLC would functionally increase BLC output to other brain regions. Critically, the adenylate cyclase signaling cascade in turn feeds into a number of other pathways, including the metabolic AMP-activated protein kinase cascade (AMPK). The implications of this relationship will be covered in much greater detail later in this dissertation.

The function of dopamine is therefore to facilitate the entry of exteroceptive sensory input into the BLC and to facilitate synaptic plasticity and BLC principal neuron firing based on this sensory information. The function of DA is therefore excitatory in the BLC. However, a hypoactive dopamine system in depressed patients is not inconsistent with the general amygdala hyperexcitability seen in MDD. As outlined above, the BLC is not a homogenous nucleus, and instead contains many parallel circuits with often opposing functions. Dopamine predominately excites BLC circuits related to exteroceptive processing and reward learning, both of which are reduced in MDD [223, 224]. If activity in reward / exteroceptive circuits is reduced, this might cause a comparatively increased activity in aversive / interoceptive circuits. This could in turn lead to an increased stress response, decreased responsiveness to reward

(anhedonia), and a shift to brooding and interoception in MDD patients [225].

1.2.4 Role of Stress Hormones in MDD and BLC Excitability

Like monoaminergic neurotransmitters, stress hormones also have an effect on BLC excitability and have also been implicated in the etiology of depression. Specifically, many studies have shown that MDD involves dysregulation of the hormones regulating the hypothalamic-pituitary-adrenal (HPA) axis.

The HPA axis regulates the neuroendocrine portion of the stress response. Specifically, the paraventricular nucleus (PVN) of the hypothalamus releases two hormones: corticotrophin-releasing factor (CRF) and arginine vasopressin (AVP) into the bloodstream. Blood vessels carry these hormones directly to the pituitary gland, where they cause the release of adrenocorticotropic hormone (ACTH) into general circulation. Upon reaching the adrenal glands, ACTH causes the synthesis and release of glucocorticoids (mainly cortisol in humans and corticosterone in rodents). Glucocorticoids then bind to two main intracellular receptors: the high-affinity mineralocorticoid (MR or Type I) receptor, or the low-affinity glucocorticoid (GR or Type II) receptor. As the MR binds to glucocorticoids with higher affinity than the GR, MRs are bound to glucocorticoid during basal conditions, while GRs become bound during periods of high glucocorticoid release (i.e. during stressful events). When bound to glucocorticoids, these receptors (especially GRs) then act as transcription factors, promoting and inhibiting protein synthesis in such a way as to help the organism respond to a stressful event.

Critically, in healthy organisms the HPA axis is regulated by a negative feedback cycle: glucocorticoids inhibit the release of CRF and AVP in the hypothalamus and ACTH in the pituitary. This negative feedback cycle helps prevent overactivation of the HPA axis, which can lead to negative health effects.

Stress hormones also play a major role in the BLC. Specifically, glucocorticoids

and CRF have major effects on BLC excitability. The following sections will outline what role these hormones play in the BLC and how they are dysregulated in MDD.

Glucocorticoids

Glucocorticoids play a major role in regulating BLC excitability. Firstly, GR signaling enhances the excitability of BLC principal neurons, specifically through a direct depolarization and decreased amplitude of inhibitory $GABA_A$ potentials [226]. Consistent with this finding, glucocorticoid signaling enhances memory consolidation in the BLC, which can be blocked by inhibiting noradrenergic signaling [227]. Glucocorticoids therefore act to increase BLC excitability, enhancing the BLC's ability to form associations during stressful life experiences.

As an important regulator of BLC excitability in healthy conditions, glucocorticoids are another mechanism of BLC hyperexcitability in MDD. Elevated glucocorticoid levels are among the most robust biological findings seen in MDD patients [14, 15]. Chronic administration of corticosterone in rats also produces robust depression-like behavior [228]. Additionally, experiments have shown that glucocorticoid-mediated negative feedback of the HPA axis is reduced in MDD patients [229, 230]. Increased glucocorticoid release and failure of the negative-feedback system could result in chronically elevated glucocorticoid levels, placing the BLC into a long-term state of hyperexcitability.

Corticotrophin Releasing Factor

As described earlier, corticotrophin-releasing factor (CRF) is released by the PVN to regulate the release of ACTH and glucocorticoids. However, CRF is also expressed in the BLC [231], where it acts as an excitatory neuropeptide through activation of two G-protein coupled receptors: CRF_1 and CRF_2. Notably, CRF is not the only endogenous ligand that binds to the CRF_1 and CRF_2 receptors; the related peptides urocortin I, urocortin II, and urocortin III also activate CRF receptors. CRF binds preferentially to CRF_1, while urocortin II and III bind preferentially to

CRF_2 (urocortin I binds to both receptor subtypes).

There is ample evidence suggesting that the CRF system is dysregulated during depression. In human MDD patients, CRF levels are elevated in cerebrospinal fluid relative to healthy controls [232, 233], which is thought to reflect enhanced CRF release from extrahypothalamic brain areas (including the amygdala) [234]. This CRF elevation is normalized upon successful antidepressant treatment [235]. Despite being a reliable biomarker for MDD, clinical trials for a CRF_1 antagonist antidepressant medication ended in failure [236], suggesting CRF is not the ultimate cause of MDD. However, examining the underlying biology of CRF may still illuminate possible mechanisms of MDD.

CRF also plays a critical role in regulating stress in rodents. In mice, ventricular infusion of CRF_1 agonists into the brain increases anxiety-like behavior [237], while CRF_1 knockout reduces anxiety-like behavior. These effects occur independently of their effects on the HPA axis [238]. Intraventricular CRF infusions also induce anxiety-like responses in the social interaction test, acoustic startle test, and elevated plus maze [239–241].

Dysregulated CRF in the BLC could contribute to BLC hyperexcitability and MDD symptoms in depressed patients. The main receptor expressed in the BLC is CRF_1. CRF infused into the BLC increases activity in BLC principal neurons [242], specifically by reducing the slow afterhyperpolarization currents [243]. CRF_1 is also expressed in BLC GABAergic interneurons, specifically in most parvalbumin- and half of cholecystokinin-expressing cells, [244]. Although expressed in both principal neurons and interneurons, infusion of urocortin into the BLC has been shown to reduce spontaneous and evoked inhibitory potentials in the BLC, suggesting CRF reduces net inhibition [122]. Overall BLC hyperexcitability through principal neuron activation and GABAergic inhibition is consistent with the anxiety-like phenotype seen in urocortin- and CRF-infused animals [239–241, 245].

1.2.5 Monoamine and Stress Hormone Signaling Converge onto the AMP-Activated Protein Kinase Pathway

As outlined above, monoaminergic and stress hormone signaling in the BLC are complex, and vary according to neurotransmitter, neuronal subpopulation, and receptor expression pattern. It is also clear that serotonin, norepinephrine, dopamine, glucocorticoids, and CRF all likely contribute to dysregulation of the BLC in MDD. However as outlined above, the monoaminergic hypothesis of MDD is an insufficient explanation of MDD pathophysiology. Antidepressants targeting monoaminergic neurotransmission are often (but not always) efficacious, however they take two weeks to have antidepressant effects despite rapidly elevating monoamine levels. Similarly, stress hormones do not provide a complete explanation for the pathophysiology of depression. One possible explanation for this is that rather than acting directly, altered monoaminergic and stress hormone signaling during antidepressant therapy results in altered intracellular signaling cascades, indirectly causing the antidepressant phenotype. Uncovering the "final common pathway" of MDD pathophysiology may therefore provide a comprehensive explanation of MDD etiology, and uncover more direct, effective, and rapid targets for future antidepressant drugs [246].

One such downstream pathway is the AMP-activated protein kinase (AMPK) signaling pathway, which we hypothesize reduces excitability in the BLC and which will be explored in detail in the next section. However, at this point it is important to note that the monoamines and stress hormones described above converge onto the AMPK pathway. The AMPK pathway is thus a candidate "final common pathway" of MDD.

Glucocorticoids and AMPK

Glucocorticoid signaling influences (and is influenced by) AMPK activity. For example, chronic exposure to glucocorticoids reduces LKB1 (one of AMPK's major upstream activators) expression in cultured rat astrocytes [247] and skeletal muscle

[248]. AMPK activity is also critical for the GR-mediated downregulation of GR expression, one of the main mechanisms of HPA axis negative feedback (which is reduced in MDD) [247]. Reduced AMPK signaling could therefore reduce HPA axis negative feedback, potentially leading to the elevated glucocorticoid levels seen in MDD.

Additionally, glucocorticoids and AMPK affect many of the same downstream metabolic processes, which will be covered in detail in the next section. Glucocorticoid signaling regulates appetite in CNS, glucose uptake in the heart and skeletal muscles, gluconeogenesis in the liver, insulin release from the pancreas, and fat accumulation/synthesis in adipose tissue, all of which are also regulated by AMPK [249, 250]. Indeed, many of the metabolic effects of glucocorticoid signaling appear to be regulated by AMPK, as AMPK phosphorylates GRs through the p38/MAPK signaling cascade, affecting GR-mediated transcriptional processes [251]. AMPK activity is therefore not only influenced by glucocorticoid levels, but also plays a complex role mediating the metabolic effects of GR signaling. It is therefore probable that AMPK plays a major role in MDD by interacting with the glucocorticoid system.

G-Protein-Coupled Receptor Signaling

Serotonin, dopamine, and norepinephrine also influence AMPK activity via G-protein-coupled receptors (GPCRs). GPCRs are seven-transmembrane domain receptors on the cell membrane. Ligands binding to a GPCR cause a conformational change in the GPCR, which then releases an intracellular second-messenger protein to carry the signal to downstream effector proteins. There are three primary types of GPCR second messengers: G_q, $G_{i/o}$, and G_s.

Once unbound from the GPCR, the function of the G_q protein is to activate the protein phospholipase, which then results in the formation of inositol trisphosphate (IP3). IP3 then binds to a receptor on the endoplasmic reticulum, causing Ca^{2+} to be released into the cell. G_q signaling also causes the formation of diacylglycerol, which

then activates protein kinase C.

$G_{i/o}$ and G_s proteins act to decrease and increase the activity of adenylyl cyclase respectively. The function of adenylyl cyclase is to convert ATP to cyclic AMP (cAMP). Therefore, $G_{i/o}$ signaling decreases cAMP levels, and G_s signaling increases cAMP levels. cAMP then has a number of downstream effects, including activation of protein kinase A and activation of cAMP-gated ion channels.

In the BLC, serotonin, norepinephrine, dopamine, and CRF all signal through these pathways. Below is an overview of the monoamine / CRF receptors present in the BLC and their associated G-protein second messengers:

Table 1.1: Monoaminergic and CRF signaling in the BLC act through G-protein coupled receptors. Serotonin, norepinephrine, dopamine, and CRF signaling are mediated through G_q-, $G_{i/o}$-, and G_s-coupled receptors.

	Serotonin (5-HT)	**Norepinephrine**	**Dopamine**	**CRF**
G_q	5-HT_{2A}, 5-HT_{2B}	α1A		
$G_{i/o}$	5-HT_{1A}, 5-HT_{1B}		D2, D3	
G_s		β	D1	CRF_1

GPCR Signaling Converges on the AMPK Cascade

The G-protein signaling cascades described above all converge onto the AMPK signaling cascade. AMPK is a protein kinase that is activated two primary ways: (1) through binding of AMP and (2) through phosphorylation by two upstream kinases, LKB1 and CamKKβ. Both of these can be affected by GPCR signaling.

Firstly, $G_{i/o}$ and G_s signaling pathways feed into the AMPK pathway by modulating cAMP levels. Specifically, in neurons cAMP is converted to AMP by the protein phosphodiesterase 4 (PDE4). Increased cAMP levels therefore lead directly to increased AMP levels, activating AMPK.

Secondly, the G_q pathway increases intracellular calcium levels. One of AMPK's upstream kinases: CamKKβ, activates in response to increases in intracellular calcium. Increased G_q signaling therefore increases intracellular calcium levels, activating AMPK through CamKKβ.

The signaling pathways of the monoamines and CRF therefore converge onto AMPK via G-protein mediated signaling. This raises the intriguing possibility that AMPK is the "final common pathway" of depression. Disruptions to any of the above upstream signaling cascades could therefore dysregulate AMPK activity, leading to hyperexcitability in important limbic brain regions like the BLC.

It is also important to note at this point that non-monoaminergic antidepressant drugs also converge onto the AMPK pathway. One such drug: rolipram, is a rapidly-acting antidepressant that failed clinical trials due to emetic (causing vomiting) side effects. However, its rapidly-acting antidepressant properties suggest that rolipram more directly affects MDD's "final common pathway" than monoaminergic medications. Critically, rolipram is a PDE4 inhibitor, directly feeding into the AMPK pathway as described above. These concepts will be explored in greater detail in the next section after a review of the structure and function of AMPK.

Intriguingly, the convergence of many upstream pathways onto AMPK could help explain the physiological heterogeneity of MDD. Disruption of *any* pathway upstream of AMPK could potentially dysregulate AMPK activity. Two patients could therefore experience the same disrupted AMPK activity (and therefore MDD) through dysregulation of entirely different upstream pathways. This could also explain why some MDD patients are resistant to traditional antidepressants. For example, a patient with disrupted AMPK activity via dysregulated monoaminergic signaling might respond to an SSRI, whereas a patient with disrupted AMPK activity via dysregulated CRF signaling would not respond. The "AMPK hypothesis" of depression could therefore explain the heterogeneity seen in MDD patients.

1.3 AMP-Activated Protein Kinase

The protein AMP-activated protein kinase (AMPK) serves as the master regulator of cellular energy balance in almost all eukaryotic cells. When activated by

increased AMP levels (generated by increased cellular ATP use), AMPK phosphorylates many downstream effector proteins, ultimately increasing cellular catabolic (ATP-generating) processes and decreasing anabolic (ATP-utilizing) processes [252]. In neurons, AMPK activation could thus have a huge impact on neuronal activity, as action potential generation is highly energy-intensive, and neurons have no reserve energy source (being entirely dependent on circulating glucose) [253, 254]. Indeed, AMPK has been shown to play an important role in regulating neuronal activity in the hypothalamus, regulating whole-body energy intake [255–258].

However, AMPK's role outside the hypothalamus is largely unknown, despite being expressed by all neurons. If dysregulated in key limbic circuits, AMPK could severely dysregulate neuronal activity (i.e. energy expenditure), contributing to disorders such as MDD. The following section will serve as a brief review of AMPKs cellular role, specific role in the brain, and relationship with several key antidepressant signaling cascades.

1.3.1 The Cellular Role of AMP-Activated Protein Kinase

AMP-activated protein kinase (AMPK) performs its energy-sensing role by monitoring the intracellular AMP/ATP ratio and adjusting its activity accordingly. AMPK activity is increased by AMP binding and decreased by ATP binding. During periods of high ATP use (i.e. high energy demand), large amounts of AMP are generated as a byproduct of ATP hydrolysis and the adenylate kinase reaction (2 ADP $\leftrightarrow$ ATP + AMP). When AMP binds to the γ subunit of AMPK, AMPK becomes activated and phosphorylates many downstream effector proteins. Overall, AMPK acts to increase the rate of catabolic (ATP-generating) pathways and decrease the rate of anabolic (ATP-utilizing) pathways [252]. The net effect of AMPK activation is thus to increase energy production and decrease energy use during times of high energy demand, restoring energy homeostasis.

AMPK consists of three heterotrimeric subunits: a catalytic α subunit, and regulatory β and γ subunits. The α subunit of AMPK contains the catalytic domain with which AMPK phosphorylates downstream effector proteins. It also contains the threonine 172 (Thr172) amino acid site, phosphorylation of which is required for maximum AMPK activity. The β subunit plays an important role in tethering the α and γ subunits together [259]. The β subunit also contains a glycogen-binding domain, potentially allowing AMPK to sense intracellular glycogen levels, however additional studies are required to test this idea [260]. Finally, the γ subunit contains the AMP/ADP/ATP-binding site that allows AMPK to sense intracellular energy levels. This site can bind up to three adenosine nucleotides.

These AMPK subunits have different isoforms (two α, two β, and three γ), allowing for 12 different heterotrimers to be expressed. Depending on which subunits are expressed, AMPK can therefore potentially have a number of cell-type-specific functions. The biological role of these subunit combinations is largely unexplored, however there is preliminary evidence that different AMPK subunit isoforms have different biological functions [250], including tissue-specific expression [261, 262], subcellular localization [263], cross-talk with the AKT pathway [264], and response to AMP binding [265]. The differential roles of AMPK isoforms are beyond the scope of this dissertation but are nevertheless important considerations for future research.

Regulation of AMPK activity

There are two primary ways AMPK activity is regulated. Firstly, AMPK can be phosphorylated by a number of upstream kinases, specifically at the Thr172 site of the α subunit. The two main such kinases are the tumor suppressor LKB1 [266] and the Ca^{2+}/calmodulin-dependent CamKKβ [267]. LKB1 is thought to be constitutively active [268], while CamKKβ activates in response to increased intracellular Ca^{2+}. Besides these two main kinases, AMPK can also be phosphorylated by TGF β-activated kinase 1 (TAK1) [269]. Phosphorylation of the Thr172 site increases the

kinase activity of the AMPK α subunit by >100-fold [270].

Secondly, AMPK is activated by AMP binding to the γ subunit of AMPK. AMP-binding increases AMPK activity via three mechanisms:

1. By increasing AMPK phosphorylation by LKB1 [271].

2. Causing a conformational change that inhibits dephosphorylation of Thr172 by protein phosphatases (such as PP2Cα) [272].

3. Causing allosteric activation of the kinase, resulting in >10-fold kinase activity [271]

ADP and ATP can also bind to the same site of the AMPK γ subunit. ATP-binding results in inhibition of all three mechanisms described above. ADP-binding can cause the same conformational change as AMP preventing dephosphorylation of Thr172 (Mechanism 2 above), though this effect requires 10x greater concentration of ADP than AMP. As physiological ADP concentrations are ≈ 10x greater than AMP, both molecules may contribute to this effect. However, AMP remains the primary physiological activator of AMPK, because ADP binding does not induce allosteric activation of AMPK (Mechanism 3 above), and because intracellular AMP levels change far more than ADP or ATP levels in response to high metabolic demand [271].

Because AMPK activity can be controlled through both AMP-binding and phosphorylation, AMPK activity has a wide dynamic range. Thr172 phosphorylation increases AMPK activity >100-fold, while AMP-binding increases AMPK activity >10-fold [273, 274]. Theoretically, the combination of these events allows for a net increase in AMPK activity >1000-fold [274]. AMPK is therefore uniquely situated to respond to large variations in cellular energy balance. Such variations occur naturally in neurons firing action potentials, as ≈ 50-80% of a neuron's energy can be directed towards neuronal activity [275].

Metabolic effects of AMPK activation

AMPK activation regulates a large number of downstream pathways, the full range of which is beyond the scope of this dissertation. However, this section will serve as a brief overview of the metabolic effects of AMPK activation.

As mentioned above, AMPK activation restores ATP levels during periods of high metabolic demand by inhibiting ATP-consuming pathways and activating ATP-producing pathways. To accomplish this, AMPK regulates pathways related to glucose and lipid metabolism, protein metabolism, autophagy, mitophagy, mitochondrial fission, and rRNA synthesis [276].

Firstly, AMPK activation influences glucose and lipid metabolism. AMPK activation increases the translocation of GLUT4 and GLUT1 glucose transporters into the membrane via phosphorylation of TBC1D1 and TXNIP, promoting glucose uptake [277, 278]. AMPK also controls overall lipid metabolism by phosphorylating ACC1 and ACC2, suppressing fatty acid synthesis and promoting fatty acid oxidation. AMPK also inhibits sterol synthesis by phosphorylating and inhibiting HMGCR, and promotes lipid absorption and release by phosphorylating the lipases HSL and ATGL [279, 280]. AMPK activation therefore promotes glucose uptake, suppresses fatty acid synthesis, and promotes fatty acid oxidation.

Secondly, AMPK activation inhibits protein synthesis. AMPK directly inhibits the mTORC1 complex, which is the master regulator of the mTOR pathway. The function of the mTOR pathway is to promote cell growth by stimulating a number of metabolic pathways, especially concerning protein translation. AMPK and mTOR thus act as antithetical regulators of protein metabolism at many different levels. AMPK also blocks protein synthesis by preventing the transcription of RNA-polymerase I [281], and prevents protein elongation by activating eEF2K (an elongation inhibitor) [282]. AMPK therefore inhibits processes such as mTOR signaling to inhibit protein synthesis.

In addition to directly regulating glucose, lipid, and protein metabolism directly, AMPK phosphorylates several transcription factors and co-factors that are themselves master regulators of cellular metabolism. For example, AMPK inhibits gluconeogenesis (the de novo synthesis of glucose: an energy-consuming process) by phosphorylating and inhibiting the transcriptional co-factors CRTC2 and class II histone de-acetylases, preventing the transcription of genes necessary for gluconeogenesis [283, 284]. AMPK also phosphorylates and inhibits transcription factors such as SREBP1, HNF4α, and ChREBP which regulate glycolytic and lipogenic transcriptional programs [285–287].

Thirdly, AMPK activation stimulates autophagy, the process by which proteins, organelles, and other molecules are degraded in the lysosomes. One way AMPK promotes autophagy is by phosphorylating and activating ULK1, a kinase which triggers the autophagic cascade (and is inhibited by mTOR signaling) [288]. AMPK also suppresses nonessential vesicle trafficking in favor of membrane trafficking in the autophagy pathway [289]. AMPK also promotes autophagy through transcriptional mechanisms, especially indirect (via mTOR) inhibition of TFEB, a master transcriptional regulator of lysosomal genes and autophagy [290].

AMPK activity also stimulates mitophagy, the process by which defective mitochondria are degraded. Indeed, ULK1 activation appears to be necessary for proper mitophagy to occur [291]. An important component of mitophagy is called mitochondrial fission, in which damaged mitochondrial fragments are separated and concentrated for turnover. AMPK also induces mitochondrial fission by phosphorylating a number of regulatory proteins [292]. In addition to increasing mitophagy, AMPK activation has also been reported to increase the biogenesis of new mitochondria [293, 294]. AMPK therefore serves as an important regulator of mitochondrial quality.

1.3.2 Dietary Fructose and AMPK

Having outlined the cellular role of AMPK in normal conditions, it is also important to understand how the dysregulation of AMPK contributes to metabolic disease. AMPK has a well-characterized role in metabolic disorders such as diabetes, obesity, and metabolic syndrome. One of the major risk factors for these metabolic disorders is fructose consumption. Indeed, increased consumption of high-fructose corn syrup correlates alarmingly with the recent "epidemic" of metabolic disorders. These metabolic disorders in turn are risk factors for MDD. Because we use a high-fructose diet (HFrD) paradigm in our studies (Chapters 2 and 3), it is important to understand the relationship between dietary fructose, disrupted metabolism, AMPK, and MDD. The following section will serve as a general overview of these concepts.

As described above, AMPK plays a key role in maintaining metabolic homeostasis. It is therefore not surprising that in diabetic patients, AMPK is less active than normal in skeletal muscle [295] and adipose tissue [296]. Moreover, reduced skeletal muscle AMPK activity has also been seen in animals models of diabetes [297]. Reversing this deficit in AMPK activity helps to treat diabetes. The most common drug prescribed for diabetes, metformin, acts as an indirect AMPK activator [298].

Dietary fructose also has been strongly linked to metabolic disorders. The metabolism of fructose is not regulated, as it bypasses the rate-limiting step in glycolysis. Because the rate of fructose metabolism is not regulated, and because the early steps of fructose metabolism require ATP, unregulated metabolism of large amounts of fructose depletes ATP levels, leading to activation of AMP deaminase and generation of uric acid which is harmful at the cellular level [299]. The consumption of high-fructose corn syrup increases the risk of developing metabolic disorders such as obesity, metabolic syndrome, and diabetes in humans [300–302]. The increasing presence of high fructose corn syrup in the food supply [303] correlates alarmingly with rising rates of metabolic diseases [304–306]. Fructose is therefore a risk factor for

metabolic diseases such as diabetes, which is linked to AMPK as described above.

In rat models, consumption of a HFrD produces insulin resistance, hypertension, and dyslipidemia [307, 308]: the symptoms of metabolic syndrome [309]. Similar symptoms are also seen in humans who consume fructose [310, 311]. The HFrD-fed rat is therefore often used as a model for metabolic syndrome [312]. Interestingly, studies have also reported that HFrD-fed rats display anxiety- or depressive-like phenotypes [313, 314]. Diabetes, metabolic syndrome, and obesity are also risk factors for developing MDD and vice versa [10, 315–317]. The links between fructose, AMPK, metabolic disorders, and MDD suggest that just as peripheral AMPK dysregulation contributes to metabolic disorders, central AMPK dysregulation may contribute to MDD. For this reason, we used a HFrD-fed rat as a model of depression in our experiments (Chapters 2 and 3). The role of AMPK in the brain and the potential role of AMPK in MDD will be explored in greater detail below.

1.3.3 AMP-Activated Protein Kinase in the Brain

Although the cellular functions of AMPK have been well characterized in multiple tissue types, the roles of AMPK in neurons remain largely unexplored. Despite this, AMPK likely plays a hugely important role in regulating neuronal energy expenditure. Neurons consume huge amounts of energy, utilizing over 50% of circulating glucose [318]. Indeed, the brain's ATP consumption is roughly comparable to a leg muscle's during a marathon, accounting for roughly 20% of the body's resting metabolism despite composing $\approx$ 2% of the body's weight [253, 254]. Firing action potentials (and the resulting activity of the Na^+/K^+ ATPase) consumes about 25-50% of this energy consumption, and synaptic transmission consumes most of the remainder [253, 319]. Despite this huge energy consumption, the brain lacks any medium- or long-term energy storage, relying on circulating glucose for energy [320]. To control this huge amount of energy expenditure, the brain must therefore utilize some sort of energy

regulatory mechanism. As the master energy regulator in eukaryotic cells, AMPK is the most likely candidate. However, AMPK's role in the brain is not limited to reducing neuronal energy expenditure during energy-restricted conditions. In the hypothalamus, AMPK activation *increases*, rather than decreases, neuronal activity in a select population of feeding-related neurons in response to low-energy conditions. AMPK is therefore not only a homeostatic regulator of energy use, but also allows neurons to respond dynamically to metabolic state in a cell-type-specific manner.

AMPK in the Hypothalamus

Despite the largely unexplored role of AMPK in most neurons, AMPK has been shown to regulate feeding behavior by influencing neuronal activity in the hypothalamus. AMPK subunits are highly expressed in multiple hypothalamic nuclei, and play a critical role in controlling whole-body energy balance through feeding behavior. AMPK $\alpha 1$, $\alpha 2$, $\beta 1$, and $\gamma 1$ subunits are highly expressed in the arcuate (ARC), paraventricular (PVN), ventromedial (VMH), and latero-anterior (LHA) hypothalamic nuclei, which all play key roles in the regulation of feeding behavior and whole-body energy balance [255–258]. AMPK activity levels correlate with food intake, as fasting increases hypothalamic AMPK activity and feeding inhibits it [255, 321, 322]. Furthermore, altering AMPK activity also alters feeding behavior in rodents. Expressing a constitutively active form of AMPK increases feeding and body weight gain, while inhibition of hypothalamic AMPK (by expressing a dominant-negative form of AMPK) promotes anorexia and decreases body weight [255, 321, 323].

Hypothalamic AMPK serves to integrate many peripheral signals of energy status. Specifically, molecules that signal energy deficit increase hypothalamic AMPK activity, and molecules signaling energy surplus decrease hypothalamic AMPK activity. Orexigenic (feeding promoting) signals that increase AMPK activity include hypoglycemia, glucocorticoids, adiponectin, and ghrelin [321, 324–326]. Anorexigenic (feeding-inhibiting) signals such as glucose, leptin, insulin, and α-melanocyte-

stimulating hormone conversely decrease AMPK activity [255, 327–329].

AMPK also plays an important role in maintaining long-term energy balance in the arcuate nucleus of the hypothalamus (ARC). The ARC serves as the master regulator of feeding behavior in the brain and is composed of two neuronal populations that receive nutritional signals: (1) orexigenic neurons expressing agouti-related protein (AgRP) / neuropeptide Y (NPY), and (2) the anorexigenic neurons expressing pro-opiomelanocortin (POMC). These neurons project to other hypothalamic nuclei, and collectively control feeding behavior. For example, when energy intake surpasses expenditure, orexigenic neuropeptides (such as AgRP/NPY) are expressed less, while anorexigenic neuropeptides such as POMC are expressed more [330, 331].

Some studies suggest AMPK plays a role in both orexigenic AgRP neurons and anorexigenic POMC neurons. In mice, knocking out AMPK α2 in POMC neurons led to mild obesity and a decreased metabolic rate. Conversely, mice without AMPK α2 in AGRP neurons were leaner than wild type [258].

However, other studies have found that AMPK activators primarily act by *increasing* AgRP neuronal activity in response to low-energy conditions. One study found that the AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) increased intracellular Ca^{2+} levels in AgRP neurons, but not POMC neurons [332]. However, AICAR does not appear to act postsynaptically, but instead presynaptically increases the excitatory synaptic input onto AgRP neurons by acting on axon terminals [333]. The authors found that AMPK activation causes Ca^{2+} release from ryanodine-sensitive internal Ca^{2+} stores in presynaptic terminals. This Ca^{2+} release both increases the probability of release from the presynaptic terminal onto AgRP neurons and causes increased CamKKβ activity resulting in a positive feedback loop.

Critically, this mechanism increases neuronal activity in ARC AgRP neurons in response to AMPK activation. In almost every other tissue, AMPK activation has been shown to decrease energy use and increase energy production. In a neuronal

system, this principle would suggest that neurons would decrease their neuronal activity in response to AMPK activation, as neuronal activity has a high energy cost. This appears to happen in other neuronal systems (see below). However, studies of hypothalamic AMPK suggest that the AMPK signaling cascade can bidirectionally influence neuronal activity, depending on which downstream effectors are activated by AMPK. AMPK is therefore more than a homeostatic regulator of neuronal energy use but allows neurons to dynamically alter their firing in a cell-type-specific manner in response to metabolic state.

AMPK's Effect on Membrane Excitability

Despite the emerging evidence of AMPK's widespread and varied role in the brain, little is known about the role AMPK could be playing in healthy neurons outside the hypothalamus. However, AMPK directly or indirectly controls the activity a number of ion channels [334], thereby regulating cellular membrane voltage in neurons and other cells [335]. Therefore, AMPK may play a direct role in regulating the membrane voltage and activity of many different neuronal cell-types and circuits. Interestingly, AMPK activation can either increase or decrease neuronal activity in a region-specific manner.

In the hypothalamus, AMPK activation has been shown to activate AgRP neurons in response to low-energy states, despite inhibiting energy-use in most other tissue types. However, AMPK activation can also suppress neuronal activity in other brain regions, which is more consistent with AMPK's usual role as an overall energy-use inhibitor. For example, perfusion of activated AMPK into cultured rat hippocampal neurons also caused a decrease in the number of action potentials evoked by a current pulse [336]. In the nucleus accumbens (NAc) core, activating AMPK also appears to reduce neuronal activity and vice versa. Increasing NAc AMPK activity with AICAR or constitutively active AMPK reduced cue-induced cocaine-seeking behavior, correlating with reduced NAc activity [337]. Conversely, inactivating AMPK with

Compound C or kinase-dead AMPK increased cue-induced cocaine-seeking behavior, correlating with increased NaC core activity. AMPK also phosphorylates the $GABA_B$ receptor, enhancing $GABA_B$ receptor activation of inhibitory GIRK channels [338]. A recent study also showed that in HEK293 cells, AMPK activity caused a membrane hyperpolarization by phosphorylating and activating Kv2.1 potassium channels [336]. Kv2.1 channels have an inhibitory effect on action potential generation and make up a large portion of the delayed rectifier K^+ current occurring in many CNS neurons [339]. Furthermore, AMPK activity has been shown to increase ligand-gated K^+-ATP channel currents in the substantia nigra [340] and the subthalamic nucleus (STN) of the thalamus [341], both of which would similarly dampen neuronal excitability.

Despite increasing neuronal activity in hypothalamic AgRP neurons, AMPK therefore reduces neuronal activity in other neuronal populations. Collectively, these findings suggest that AMPK can bidirectionally regulate neuronal activity in a cell-type-specific manner. However AMPK's role in regulating the activity of BLA principal neurons has yet to be explored. In the following studies, we hypothesized that AMPK was a key regulator of the membrane excitability of BLA principal neurons.

1.3.4 The Potential Role of AMP-Activated Protein Kinase in Major Depressive Disorder

As covered briefly above, AMPK is a strong candidate for the "final common pathway of depression". As the "master metabolic regulator" in neurons, AMPK receives input from a wide variety of upstream pathways and profoundly influences both protein activity and gene transcription (including proteins and genes considered "master regulators" themselves). Disruptions to the AMPK pathway could therefore cause severe cellular dysfunction. The most common antidiabetic drug: metformin, achieves its anti-diabetic effect by activating AMPK [342]. If a similar disruption of AMPK occurred in the neurons of key limbic areas (such as the BLC), the energy expenditure (and therefore neuronal activity) of these areas could be severely affected.

Supporting this hypothesis, there is a strong correlation between metabolic diseases and depression. The lifetime risk of MDD is more than doubled in patients with type-2 diabetes [10]. Similarly, obesity and metabolic syndrome increase the risk of developing MDD [316, 317]. Indeed in rats, a high-fructose diet (a common model of metabolic syndrome) was reported to induce depressive-like behavior in male Wistar rats [313]. In addition to being positioned to unite many pathways implicated in MDD pathophysiology, AMPK is therefore also positioned to mediate the relationship between metabolic disorders and MDD. In our studies, we hypothesized that rats fed a high-fructose diet would have lower AMPK activity in the BLA, leading to BLA hyperexcitability and anxiety-/depression-like behavior. This hypothesis will be explored in more detail below.

Indeed, many of the most common antidepressant therapies produce cellular changes that converge on the AMPK pathway. As mentioned previously, the AMPK pathway receives upstream input from serotonin, norepinephrine, and dopamine GPCRs. The most widely prescribed antidepressants (SSRIs and SNRIs) increase signaling in these pathways rapidly, but still take two weeks to become efficacious. One possible explanation for this delay is that despite rapidly elevated monoaminergic signaling, it takes two weeks for altered GPCR signaling to influence the AMPK cascade.

The AMPK pathway also receives input from several non-monoaminergic antidepressant signaling cascades. Three of the most notable are the antidepressant drugs ketamine, metformin, and rolipram. These antidepressants are rapidly-acting, suggesting that they act more directly on the key signaling cascades causing MDD than monoaminergic drugs to produce their antidepressant effect. All three drugs influence AMPK activity. For example, ketamine appears to increase AMPK activity [343], possibly modulating its antidepressant effect. One study found that ketamine's antidepressant effect was dependent on increased AMPK activity in the hippocampus [344], though this finding raises the possibility that ketamine acts to modulate AMPK

activity in other brain regions as well. Similarly, the antidiabetic drug metformin has antidepressant properties [345, 346] and also increases AMPK activity [342].

Finally, rolipram is a rapidly acting antidepressant that failed clinical trials due to its emetic side effects [347–349]. Rolipram also affects AMPK activity, raising the possibility that AMPK mediates rolipram's antidepressant effects. However, its rapidly-acting antidepressant efficacy is interesting given that rolipram is a PDE4 inhibitor. PDE4 serves to convert cAMP to AMP and therefore is an important regulator of AMP levels / AMPK activity. Inhibiting PDE4 with rolipram inhibits the conversion of cAMP to AMP, increasing cAMP levels and decreasing AMP levels. A rolipram-induced reduction in AMP levels would therefore decrease AMPK activity (Figure 1.3).

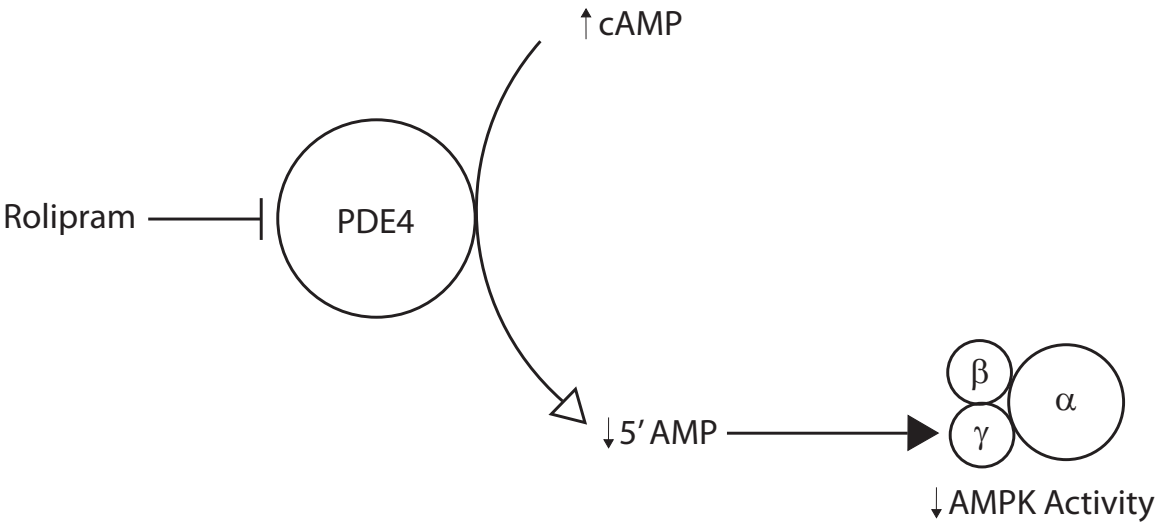

Figure 1.3: Effect of rolipram on AMPK signaling. Rolipram inhibits the enzyme PDE4, preventing the conversion of cAMP into 5' linear AMP. Reduced AMP levels would in turn decrease AMPK activity.

Work in our lab has shown that rolipram administration into the BLC increases BLC activity in control rats, decreasing the threshold for LTP induction and increasing the baseline startle response [113]. This suggests that in the control BLC, decreasing AMPK activity with rolipram increases BLC excitability (i.e. the normal role of AMPK in the BLC is inhibitory). However, because increased BLC excitability

is associated with MDD, it is unclear why then rolipram is an effective antidepressant in depressed humans. There are at least two potential solutions to this paradox: (1) Rolipram's antidepressant efficacy depends on increasing activity of non-BLC brain regions that are hypoactive in MDD (such as the dorsolateral prefrontal cortex [350–352]) by reducing AMPK activity, or (2) in MDD, AMPK inhibition with rolipram reduces BLC excitability instead of increasing it. The purpose of the following studies was in part to shed light on the "rolipram paradox". Because one potential mechanism of rolipram's antidepressant effect is through AMPK, our goal was to characterize the role of AMPK regulating BLC excitability in control conditions and metabolically-challenged/depressed conditions (HFrD) in rats.

1.4 Experimental Rationale

As detailed above, the AMPK signaling cascade is a strong candidate for the "final common pathway" of depression. Not only do multiple signaling pathways implicated in MDD converge on AMPK, but AMPK can influence neuronal excitability at multiple levels. Indeed, AMPK has been shown to influence the excitability of hypothalamic neurons in relation to whole-body energy state. However, AMPK's role outside the hypothalamus is largely unknown, and its role in the BLC has never been studied. In addition, it is unclear how the AMPK signaling cascade may become dysregulated in metabolically abnormal or depressive states.

The following experiments were designed to test the hypothesis that in control conditions, AMPK in the BLC influences principal neuron excitability and therefore affective behavior. Furthermore, we hypothesized that in metabolically dysregulated / depressed conditions [313], AMPK activity would be reduced (increasing BLA activity) and that subsequent AMPK activation would rescue the depressed phenotype. Finally, we hypothesized that rolipram influences BLA excitability in an AMPK-dependent fashion. To test our hypotheses, we used a high-fructose diet (HFrD) to

dysregulate the metabolism of both Sprague Dawley and Wistar rats. The next two chapters (Chapters 2 and 3) will discuss the results of our HFrD studies. Chapter 4 will examine AMPK/rolipram's role in regulating excitability in control BLC principal neurons.

Chapter 2 High-fructose Diet Initiated During Adolescence does not Affect Basolateral Amygdala Excitability or Affective-like Behavior in Sprague Dawley Rats

Content presented in Chapter 2 previously published in:

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Abstract

Patients with type-2 diabetes, obesity, and metabolic syndrome have a significantly increased risk of developing depression. Dysregulated metabolism may contribute to the etiology of depression by affecting neuronal activity in key limbic areas. The basolateral amygdala (BLA) acts as a critical emotional valence detector in the brain's limbic circuit, and shows hyperactivity and abnormal glucose metabolism in depressed patients. Furthermore, studies have shown administering a periadolescent high-fructose diet (HFrD; a model of metabolic syndrome) to male Wistar rats increases anxiety- and depressive-like behavior. Repeated shock stress in Sprague Dawley rats similarly increases anxiety-like behavior and increases BLA excitability. We therefore investigated whether a metabolic stressor (HFrD) would have similar effects as shock stress on BLA excitability in Sprague Dawley rats. We found that a HFrD did not affect the intrinsic excitability of BLA neurons. Fructose-fed Sprague Dawley rats had elevated body fat mass, but did not show increases in metabolic efficiency and fasting blood glucose relative to control. Finally unlike Wistar rats, fructose-fed Sprague Dawley rats did not show increased anxiety- and depressive-like behavior. These results suggest that genetic differences between rat strains may affect susceptibility to a metabolic insult. Collectively, these data show that a periadolescent HFrD disrupts metabolism, but does not change affective behavior or BLA excitability in Sprague Dawley rats.

2.1 Introduction

Major depressive disorder (MDD) is one of the most common psychiatric illnesses, with a lifetime prevalence of 17% [1, 2]. However, the etiology of depression remains largely unknown. Recent evidence suggests dysregulated metabolism plays a role in MDD development. For example, patients with type 2 diabetes are twice as likely to develop MDD than the general population [10, 315]. Obesity and metabolic syndrome similarly increase the risk of developing MDD [316, 317]. Dietary high-fructose corn syrup in turn increases the risk of developing metabolic disorders such as obesity, metabolic syndrome, and diabetes [300–302]. Indeed, increased consumption of high-fructose corn syrup [303] correlates alarmingly with rising rates of both metabolic diseases [304–306] and MDD [353, 354]. This correlation further increases the need to establish the nature of the relationship between fructose, dysregulated metabolism, and mood disorders. Some studies suggest dysregulated metabolism contributes to MDD development by dysregulating the neuronal activity in key limbic regions. One such region is the basolateral amygdala (BLA), which serves as a critical emotional valence detector in the brain's limbic circuit [355]. BLA principal neurons form reciprocal connections with brain regions involved in cognition, motivation, and stress responses, the dysregulation of which are core symptoms of MDD [356]. Furthermore the BLA is hyperactive during MDD, which then normalizes with successful pharmacotherapy [16]. This hyperactivity correlates with abnormal glucose metabolism in depressed patients [11, 12, 19]. Dysregulated metabolism may therefore contribute to MDD by increasing the excitability of BLA principal neurons.

In this study, we examined the relationship between dysregulated metabolism and BLA hyperexcitability in rats. Administration of a high-fructose diet (HFrD) in rats is a common model of metabolic challenge in rats. HFrD-fed rats develop abdominal obesity, insulin resistance, and dyslipidemia, and are commonly used as a model of metabolic syndrome [307, 308]. In male Wistar rats administration of a HFrD during

adolescence and adulthood also increases anxiety- and depressive-like behavior [313]. The behavioral effects of the HFrD are similar to those of repeated shock stress, which in Sprague Dawley rats causes increased anxiety-like behavior and increased BLA excitability [113]. We therefore hypothesized a metabolic challenge (HFrD) would also cause increased BLA excitability in Sprague Dawley rats. After HFrD administration we tested the excitability of BLA principal neurons and assessed the impact of HFrD on anxiety- and depressive-like behavior and metabolism in Sprague Dawley rats.

We hypothesized HFrD administration to Sprague Dawley rats would mimic the effects of HFrD administration to Wistar rats. Specifically, we hypothesized a HFrD would increase body fat mass independently of body weight, increase metabolic efficiency, increase fasting blood glucose, and increase anxiety- and depressive-like behaviors. Finally, we hypothesized HFrD administration would also increase intrinsic BLA principal neuronal excitability, which has been shown to increase after chronic adolescent stress [114, 357]. We found that HFrD administration increases fat mass, but not metabolic efficiency or fasting blood glucose. We also found HFrD administration did not increase the intrinsic excitability of BLA principal neurons. We also did not observe increased anxiety- and depressive-like behaviors in the HFrD group. These data suggest that the effects of a HFrD are influenced by genetic strain differences between Sprague Dawley and Wistar rats, or by environmental variables.

2.2 Methods

2.2.1 General Housing

All animal experiments followed the guidelines described in the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023). Animals were housed in the Yerkes National Primate Research Center vivarium at Emory University. A total of 48 male Sprague Dawley rats were used in the following experiments.

Animals were ordered from Charles River (Wilmington, MA). Rats arrived in the vivarium on postnatal day (P) 25, and were immediately randomly assigned to either a control or high-fructose diet (HFrD, described below). Rats were kept on an artificial regular 12:12-h light cycle, and were housed in ventilated cages kept at 22° C. Rats were either housed in groups of four (n=32) or pair housed (n=16). All experimental protocols conformed to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Emory University.

Three cohorts of animals (16 rats each, 8 per diet) were used during this study. Metabolic efficiency, body weight, fasting blood glucose were measured in the first cohort. In addition to these measurements, the second and third cohorts (32 animals total) underwent behavioral testing as described below, and then were euthanized for assessment of body fat (Cohorts 1+2) and electrophysiology (Cohort 3) measurements respectively. Unless otherwise specified, no cohort differences were detected and cohorts were combined for appropriate analyses.

2.2.2 Diet

Consistent with previous work in Wistar rats [313, 358], we used a HFrD to induce metabolic challenge in Sprague Dawley rats. Sprague Dawley rats were randomly assigned to either a "control" diet (Lab Rodent Diet 5001, 0.30% kcal from fructose) or a HFrD (Research Diets D05111802, 55% kcal from fructose). Both diets contained sufficient vitamins and minerals to maintain rodent health. Food consumption per cage was measured daily, and was used to calculate the total weekly caloric consumption per cage. Total weekly caloric consumption per cage was divided by the number of rats per cage to determine average weekly caloric intake per rat, which was used to determine metabolic efficiency. Once a week, animals were subjected to an overnight fast before fasting blood glucose measurements (described in Section 2.2.4). Food was

removed 1-2 hours before the onset of the dark cycle, and returned after blood glucose measurements approximately 16 hours later. Except for weekly overnight fasts, animals had *ad libitum* access to food and always had *ad libitum* access to water.

2.2.3 Behavior

Rats used for behavior in this study underwent three behavioral tasks over three days in order of least to most stressful: the open field maze task, elevated O maze task, and forced swim task. Starting on P83, animals were habituated to the behavioral room for 7 days (n=16 per group). On habituation days, each animal was individually handled for 10 minutes each. All behavior started approximately three hours after the beginning of the light cycle, and ended three hours before the end of the dark cycle. Control and HFrD rats were alternated throughout the day to control for circadian effects. All behavioral apparatuses were cleaned thoroughly with Quatricide (PRL Pharmacal, Naugatuck, CT) before and after each behavioral task. Animals were subjected to the following behavioral tasks:

Open Field Maze

The open field maze allows for assessment of both motor behavior and provides a metric of anxiety-like behavior [359]. Rats were placed in the center of the open field maze illuminated by red light, and were free to move throughout the open field for six minutes during the task. Control and HFrD rats were alternated throughout the day to control for circadian effects. An overhead video camera captured the animal's movement within the open field maze to measure baseline motor activity. Videos were analyzed using CleverSys Topscan software (Reston, VA). The open field apparatus used in this experiment was composed of 91x91 cm black plexiglass floor, surrounded by 28 cm walls.

Elevated O Maze

The elevated zero (O) maze is a widely validated test of anxiety-like behavior [360], with slight advantages over the elevated plus maze while retaining comparable results [361]. Rats were subjected to an elevated O maze task to assess anxiety-like behavior under red light illumination. Rats were free to move between the open and closed arms of the maze for 6 minutes during the task. An overhead video camera was used to track the animals' movement for later analysis. Using CleverSys Topscan software (Reston, VA), we measured the time the rat spent in the open arm, closed arm, and "stretch attend" position of the maze (consisting of the first 3" of the open arm immediately outside the closed arm). The specifications for the elevated O maze are as follows: 46.5" diameter, 4" wide track, 19.5" height (open arms), 30" height (closed arms).

Forced Swim Task

The forced swim task is a well-characterized antidepressant screen, and has also been used to study the neurological underpinnings of depression [362, 363]. We utilized the one-day forced swim task, which is an effective antidepressant screen [364, 365], has been used to validate rodent models of depression such as the Flinders Sensitive Strain [366–369], and captures similar immobility behavior compared to the two-day forced swim task [370]. Rats underwent the forced swim task on P93. Animals were placed in clear plexiglass cylinders (20.5" tall, 8.5" diameter) containing 30 cm of 25° C water for six minutes. Two animals (one from each group) underwent behavior simultaneously in two adjacent plexiglass containers separated by an opaque plastic barrier. The location of each group (left or right) was alternated throughout the day to avoid bias. The animals' movement was captured using a video camera and analyzed using CleverSys ForcedSwimScan software (Reston, VA). Immobility was defined as the animals' hind limbs remaining motionless for over two seconds. After the six-minute task, animals were immediately removed from the water, thor-

oughly dried using paper towels, and moved to recovery cages containing food, water, and heating pads. Animals recovered for at least an hour before being moved back to general housing.

2.2.4 Metabolic Outcomes

Fasting blood glucose and body weight were measured weekly after an overnight fast. Fasting blood glucose was measured via tail prick using a Freestyle glucometer (Abbot Laboratories, Chicago, IL). Fasting blood glucose was not collected during week 9 to avoid stressing the animals prior to behavioral testing. The weekly metabolic efficiency of each rat was determined by dividing the individual weight gained by the average weekly caloric intake per rat. After 10 weeks on the diet, animals were anesthetized using open-drop exposure to isoflurane. After 90-120 seconds of isoflurane exposure, rats entered a deep plane of anesthesia as verified by decreased respiratory rate and loss of the righting reflex. Once the loss of spinal reflexes was verified using a toe-pinch, rats were sacrificed via decapitation. In cohorts 1 and 2 (16 rats per group total), the epidydimal and perigonadal fat pads were extracted post- euthanasia and immediately weighed to measure fat pad mass. Rats in cohort 3 (8 rats per group) were instead used for electrophysiology as described below.

2.2.5 Slice Electrophysiology

Electrophysiological recordings started seven days after the forced swim task (P100) to allow rats to recover after the forced swim tasks. Rats (n=8 per group) were decapitated under isoflurane anesthesia, and prepared for slice electrophysiology. One to two rats were euthanized per recording day over the course of the next 21 days. Rats remained on the assigned diet during the time period. The date of recording did not affect the observed electrophysiological properties (see Section 2.3.4).

The rats' brains were removed and processed for slice electrophysiology as described previously [113]. Brains were immersed in ice-cold "cutting solution" consist-

ing of (in mM): NaCl (130), $NaHCO_3$ (30), KCl (3.5), KH_2PO_4 (1.1), $MgCl_2$ (6.0), $CaCl_2$ (1.0), glucose (10), ascorbate (0.4), thiourea (0.8), sodium pyruvate (2.0), and kynurenic acid (2). Coronal sections (350 μM thick) were obtained using a Leica VTS-1000 vibrating-blade microtome. Slices were left to incubate in cutting solution for an hour, before being transferred to room-temperature "regular artificial cerebrospinal fluid" (aCSF) consisting of (in mM): NaCl (130), $NaHCO_3$ (30), KCl (3.5), KH_2PO_4 (1.1), $MgCl_2$ (1.3), $CaCl_2$ (2.5), glucose (10), ascorbate (0.4), thiourea (0.8) and sodium pyruvate (2). Cutting solution and aCSF were perfused with a 95% oxygen / 5% carbon dioxide gas mixture.

Individual slices were transferred to a recording chamber and were visualized using a Leica DM6000 FS microscope (Leica Microsystems Inc., Bannockburn, IL) as described previously [113]. Slices were continuously perfused by gravity-fed oxygenated 32° C aCSF at a flow rate of 2–3 ml/ min. Thin-walled borosilicate glass patch electrodes (WPI, Sarasota, FL) were then used to acquire whole-cell patch-clamp recordings of projection neurons in the basolateral amygdala (BLA). Electrodes had a resistance of 4–6 MΩ, were filled with a "patch solution", containing (in mM): K+-gluconate (130), KCl (2), HEPES (10), $MgCl_2$ (3), K-ATP (2), Na-GTP (0.2), and phosphocreatine (5), adjusted to pH 7.3 with KOH, and having an osmolarity of 280–290 mOsm. BLA principal neurons were identified according to their characteristic size, shape, and electrophysiological properties [52, 371].

Data acquisition and analysis were performed using a MultiClamp700B amplifier in conjunction with pClamp 10.2 software and a DigiData 1320A AD/DA interface (Molecular Devices, Sunnyvale, CA). Whole-cell patch-clamp recordings were obtained and recorded voltages were low-pass filtered at 5 kHz and digitized at 10–20 kHz. Pipette offset and capacitance were automatically compensated for using Multiclamp software. Series resistance was compensated for manually, and recordings with series resistances of >30 MΩ were discarded.

2.2.6 Statistical Analysis

Measurements were analyzed using Graphpad Prism 8 (La Jolla, CA). Unpaired two-tailed Student's t-tests or 1-way and 2-way repeated measures Analysis of Variance (ANOVA) tests were performed using α=0.05. Tukey and Holm-Sidak post-hoc testing were performed when appropriate. Two levels of the independent factor "diet" (control and HFrD) were used for both 1- and 2-way ANOVAs. For 2-way ANOVAs, eight levels of the dependent factor "Time" (Weeks 2-9) were used. We also calculated simple linear regression to predict BLA electrophysiological properties (membrane resistance, spike threshold, and number of action potentials per pA of positive current) based on recording date. All values are reported as mean +/- standard deviation.

2.3 Results

2.3.1 Peri-Adolescent High-Fructose Diet Increases Visceral Fat Mass but not Metabolic Efficiency

Prior to the initiation of the dietary intervention, body weight was equivalent between the control (58.81 g +/- 6.35) and HFrD rats (59.94 g +/- 8.04; t_{30}=0.44, p=0.66). The high fructose diet did not alter body weight and weights remained similar between groups throughout the study ($F_{1,46}$=2.4, p=0.13, Figure 2.1-A). Although body weight was similar, HFrD rats had a greater body fat percentage relative to control, defined as the weight of the epididymal (t_{30}=4.4, p=0.0001; Figure 2.1-B) and perigonadal fat pads (t_{30}=4.1, p=0.0003; Figure 2.1-C), divided by final total body weight. This effect cannot be explained by alterations in metabolic efficiency as HFrD rats were no more metabolically efficient (weight gained /average cage kCal consumed) than control rats ($F_{1,46}$=1.387, p=0.25, Figure 2.1-D). However, we did find a main effect of cohort on metabolic efficiency for both control ($F_{2,21}$=9.1, p=0.0014) and HFrD rats ($F_{2,21}$=10.87, p=0.0006) consistent across diet, explaining 3.2% and 6.6% of variation respectively. The cohort effect was not explained by the number of

rats per home cage, as pair housed rats were not significantly different from group-housed rats for either control ($F_{1,22}$=0.08, p=0.77) or HFrD ($F_{1,22}$=0.87, p=0.36) groups.

2.3.2 HFrD Decreases Fasting Blood Glucose

At the start of the experiment, fasting blood glucose was equivalent between the groups of rats that would be assigned to standard chow (73.13 mg/dl +/- 14.65) or HFrD (64.44 mg/dl +/- 11.15; t_{30}=1.87, p=0.07). After administration of the diet, HFrD rats had lower fasting blood glucose values relative to control-fed rats ($F_{1,\ 46}$= 25.41, p<0.0001, Figure 2.1-E). In addition, fasting blood glucose was influenced by time (defined as number of weeks on the diet, $F_{7,\ 322}$= 4.201, p=0.0002), but no interaction between diet and time ($F_{7,\ 322}$= 0.9881, p=0.4397). Therefore, this effect was likely driven by effects of developmental age of the rats or repeated blood sampling.

2.3.3 HFrD Did Not Alter Motor Behavior or Affective-like Behaviors

Control and HFrD groups traveled the same distance in the open field maze (t_{30}=.69, p=0.50; Figure 2.2-A), and both average velocity (Control: 60.39 mm/s +/- 7.56, HFrD: 63.40 mm/s +/- 6.77, $F_{1,30}$=0.44, p=0.51) and maximum velocity (Control: 260.6 mm/s +/- 8.27, HFrD: 269.7 mm/s +/- 12.75, $F_{1,30}$=0.27, p=0.61) were similar between the groups. In addition, the zones of the open field in which the rats spent time were equivalent between the control and HFrD groups: outer (t_{30}=0.63, p=0.78), middle (t_{30}=0.44, p=0.78), and center (t_{30}=1.46, p=0.40; Holm-Sidak method, α=0.05, Figure 2.2-B).

Control and HFrD rats displayed the same number of crossings between the open arm, "stretch-attend", and closed arm zones, suggesting similar motor activity (t_{30}=0.37, p=0.72, Figure 2.2-C). Further, a HFrD did not alter time spent in the

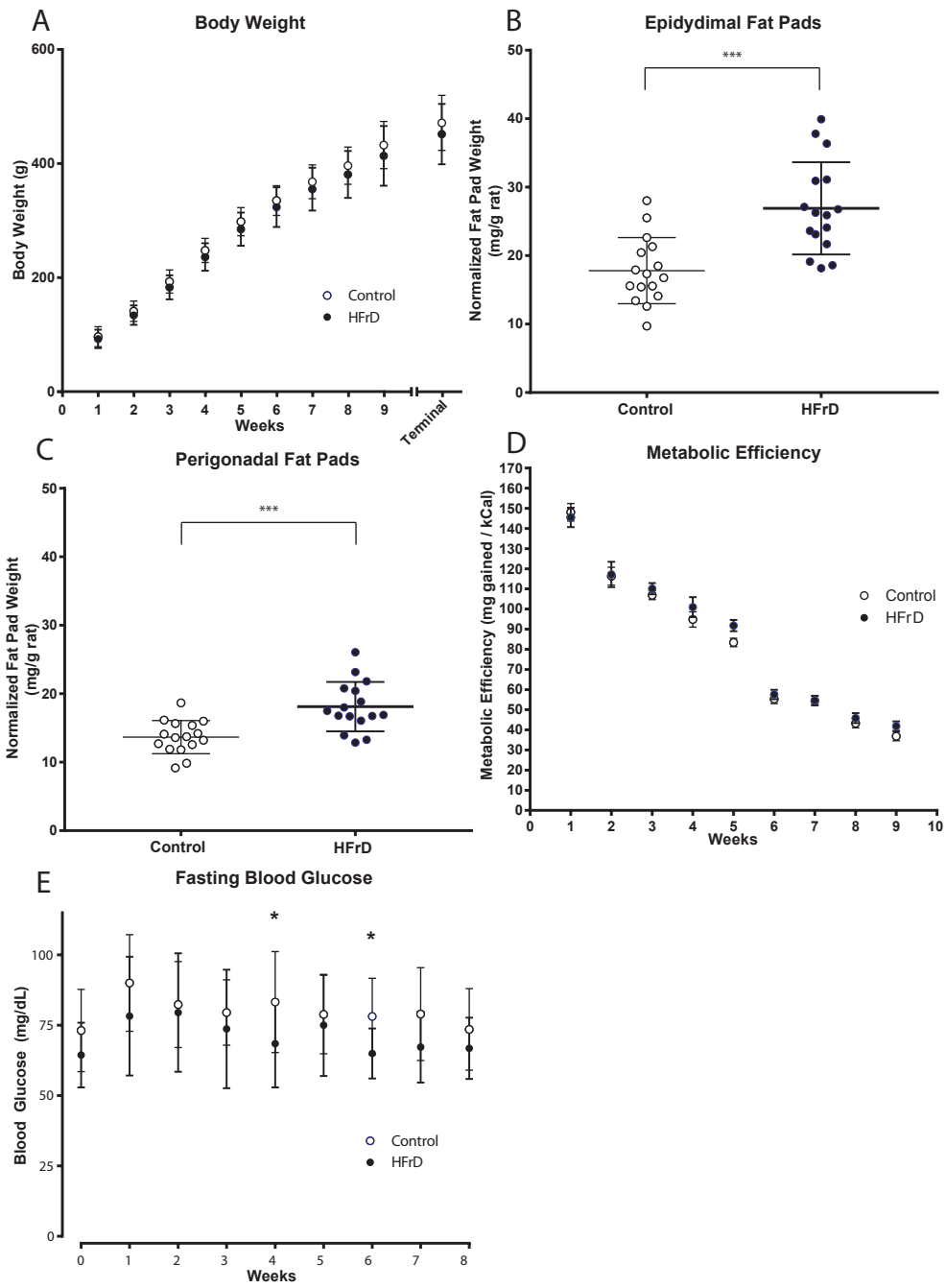

Figure 2.1: Metabolic effects of a HFrD in Sprague Dawley rats. A) Body weight of control and HFrD rats (Weeks 1-9, n=24 per group. Terminal, n=16 per group). B) Normalized epididymal fat pad weight (mg fat / g rat body weight) for control and HFrD rats (n=16 per group). C) Normalized perigonadal fat pad weight (mg fat / g rat body weight) for control and HFrD rats (n=16 per group). D) Metabolic efficiency (mg rat body weight / average cage kcal consumed) of control and HFrD rats over nine weeks (Week 1, n = 16 per group; Weeks 2-9, n=24 per group). E) Fasting blood glucose of control and HFrD rats over eight weeks (n=24 per group). *: $p<0.05$, ***: $p<0.001$

open arms (t_{46}=0.46, p=0.88), closed arms (t_{46}=0.14, p=0.89), or "stretch attend" zone (t_{46}=0.79, P=0.82) of the elevated O maze, as compared to behavior of the control diet rats (Holm-Sidak method, α=0.05, Figure 2.2-D).

HFrD did not alter time spent struggling in the forced swim test (t_{30}=0.008, p=0.99, Figure 2.3-A). In addition, neither latency to the first $\geq$ 2 sec immobility event (t_{30}=0.08, p=0.94, Figure 2.3-B), or total time spent immobile (t_{30}=0.13, p=0.90, Figure 2.3-C) were impacted by consumption of the high fructose diet.

2.3.4 HFrD Did Not Affect Membrane Excitability of BLA Principal Neurons

High fructose diet (Control, n=20; HFrD, n=19) did not alter membrane resistance (t_{37}=1.14, p=0.26, Figure 2.4-A) or spike threshold (t_{37}=1.01, p=0.32, Figure 2.4-B) of BLA principal neurons. Additionally, there was no difference between control and HFrD groups in the evoked firing response: the average number of action potentials fired per pA of depolarizing current (t_{39}=0.65, p=0.52, Figure 2.4-C). Recording date did not influence membrane resistance (Control: $F_{1,18}$=0.06, p=0.8, R^2<0.01; HFrD: $F_{1,17}$=0.86, p=0.37, R^2=0.05), spike threshold (Control: $F_{1,18}$=1.208, p=0.29, R^2=0.06; HFrD: $F_{1,17}$=0.27, p=0.61, R^2=0.02) or the average number of action potential fired per pA of depolarizing current (Control: $F_{1,18}$<0.01, p=0.98, R^2<0.01; HFrD: $F_{1,17}$=4.39, p=0.051, R^2=0.21).

2.4 Discussion

Collectively, the data presented here demonstrate that although there are metabolic implications of a high fructose diet initiated in adolescence in male Sprague Dawley rats, neither affective-like behaviors nor BLA excitability were altered by HFrD consumption. The effects of a HFrD on fat pad mass are consistent with previous work [313], and are indicative that the HFrD disrupted metabolism. However, we did not observe the behavioral changes previously reported in Wistar rats fed a HFrD. We

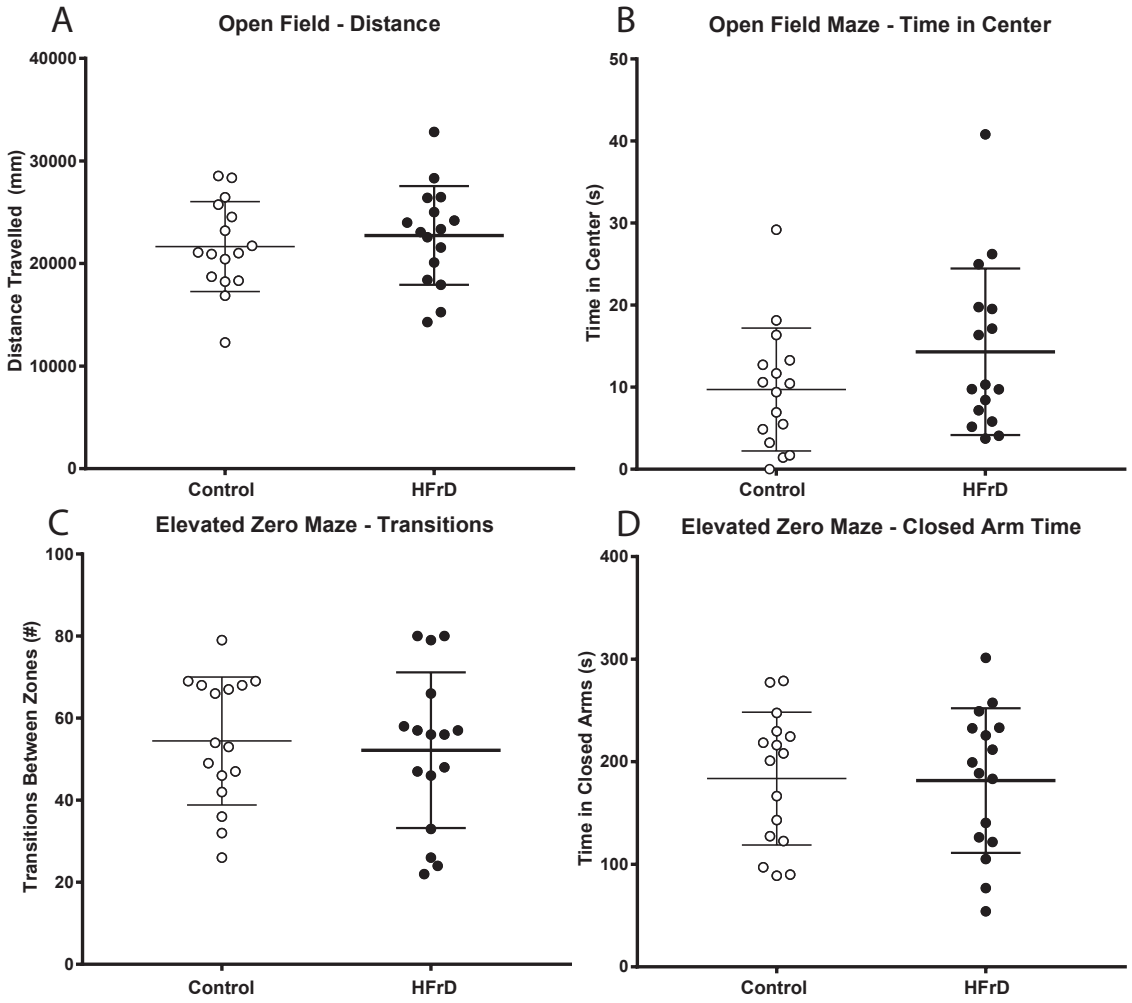

Figure 2.2: HFrD does not affect behavior in the open field or elevated zero mazes. A) Distance traveled (in mm) during the six-minute open field maze task for control and HFrD rats (n= 16 per group). B) Time (in sec) control and HFrD rats spent in the center (25% total maze area) of the open field maze (n=16 per group). C) Number of transitions (defined as a crossing between the closed arm, "stretch-attend", and open arm zones) during the Elevated Zero Maze task for control and HFrD rats (n=16 per group). D) Time (in seconds) spent in the closed arm during the six-minute Elevated Zero Maze task for control and HFrD rats (n=16 per group).

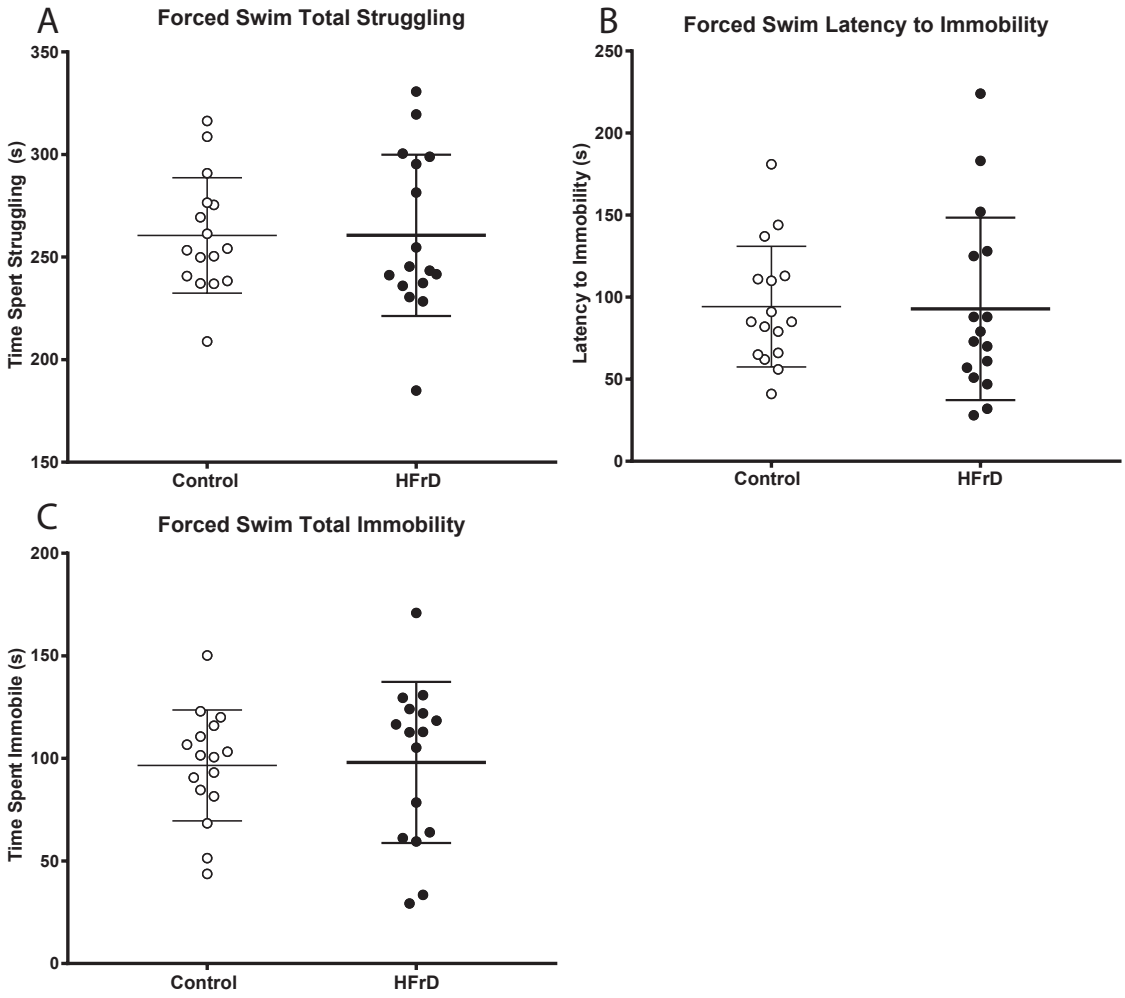

Figure 2.3: HFrD does not affect behavior in the forced swim task. A) Total time (in sec) spent struggling during the six-minute Forced Swim Task for control and HFrD rats (n=16 per group). B) Latency to a 2-second immobility event (in sec) during the six-minute Forced Swim Task for control and HFrD rats (n=16 per group). C) Total time (in sec) spent immobile during the six-minute Forced Swim Task for control and HFrD rats (n=16 per group).

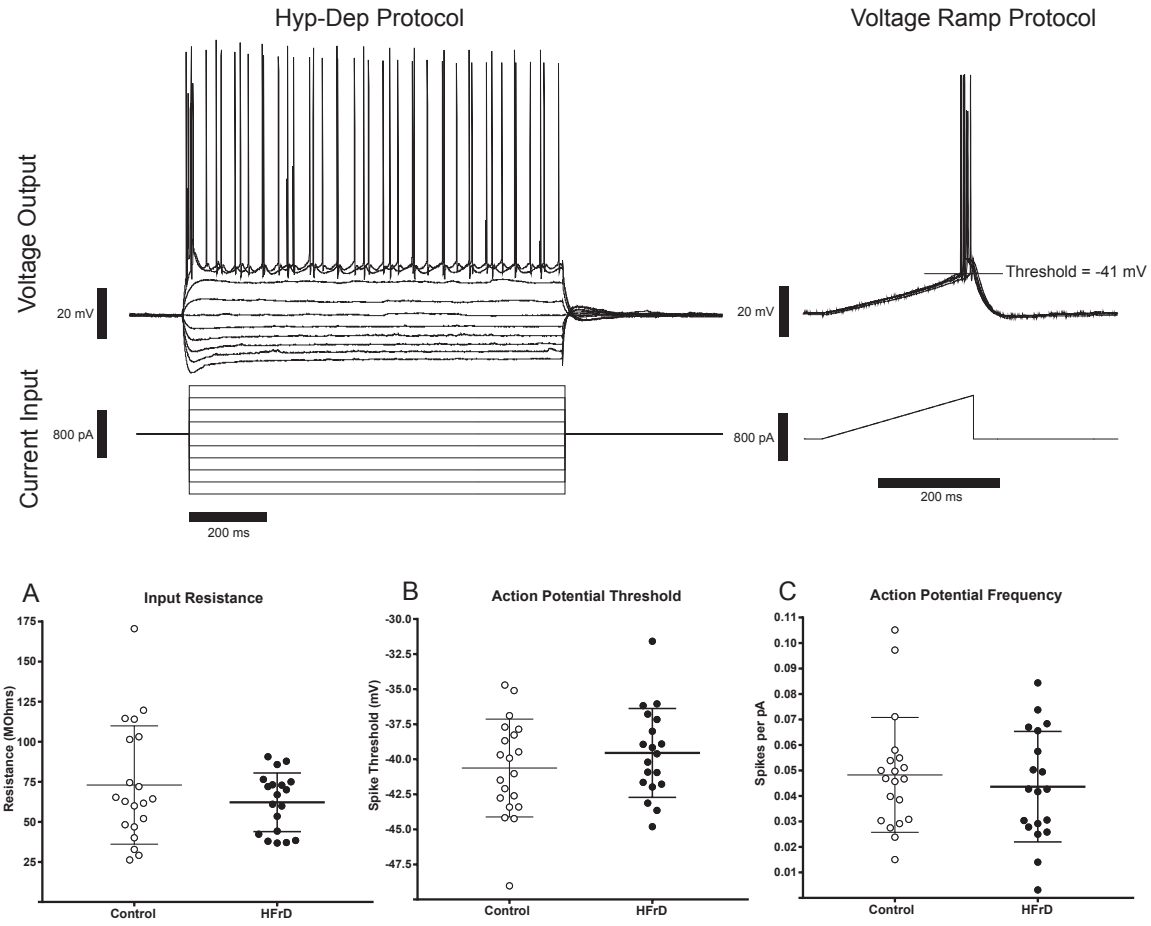

Figure 2.4: HFrD does not affect the excitability of BLA principal neurons. TOP: Example recordings from a control BLA principal neuron. LEFT: Hypdep protocol, used to calculate membrane resistance / action potential frequency. RIGHT: Voltage Ramp protocol, used to calculate action potential threshold. BOTTOM: A) Input resistance of BLA principal neurons from control and HFrD rats (n=20 control; n=19 HFrD). B) Action potential threshold (in mV) of BLA principal neurons from control and HFrD rats (n=20 control; n=19 HFrD). C). The average number of action potentials fired per pA of depolarizing current in control and HFrD animals (n=20 control; n=19 HFrD).

also did not observe changes in BLA physiology, correlating with our behavioral results. The physiological/behavioral response to dietary fructose may therefore be influenced by either strain-dependent genetic variation or environmental influences. Future studies might utilize the strain-dependent effects of a HFrD to investigate the mechanisms by which a HFrD can elicit neural and behavioral effects.

2.4.1 Genetic Strain Differences Could Affect Response to Dietary Fructose

Consumption of a HFrD has been shown to increase body fat mass, impair insulin sensitivity, and increase plasma leptin and triglyceride levels in rats [307, 308, 372–374]. However, the physiological outcomes of HFrD administration may be strain-dependent. Differences between Sprague Dawley and Wistar rats could be a major source of variation between this and previous studies. At baseline Sprague Dawley rats are more sensitive to insulin than Wistar rats [375], possibly ameliorating fructose-induced insulin resistance. Higher baseline corticosterone levels have also been reported in Wistar rats compared to Sprague Dawley [376], possibly affecting both fructose-induced hepatic gluconeogenesis [377] and affective behavior via HPA axis activation [313, 378]. Cultured hepatocytes from Sprague Dawley rats also exhibit faster glucose and amino acid metabolism than in Wistar rats [379], suggesting Sprague Dawley and Wistar rats may metabolize fructose in the liver at different rates.

Strain differences also affect the outcomes of diet administration. Wistar rats fed a high-fat diet had more pronounced weight gain, obesity, hyperinsulinemia, and hyperleptinemia than Sprague Dawley rats [380, 381]. Conversely, Sprague Dawley rats fed a 90-day HFrD failed to develop fructose-induced insulin resistance or glucose tolerance [382]. Wistar rats could thus be more susceptible than Sprague Dawley rats to the physiological effects of a metabolic insult. Genetic strain differences may have also influenced the anxiety- and depressive-like behavior observed in this study. Wis-

tar and Sprague-Dawley rats exhibit similar behavior in most tests [383]. However, some studies have reported increased baseline motor [383] and anxiety-like behavior [384] in Wistar relative to Sprague Dawley rats. Studies have even reported significantly different anxiety-like behavior between Wistar rats from different vendors, highlighting the importance of small genetic variations [385, 386].

This genetic variation may be of use in future studies investigating genetic susceptibility to a metabolic insult. Indeed, contrasting control and disease-model genomes using QTL mapping has identified genetic loci associated with alcohol-seeking behavior [387], anxiety- and depression-like behavior [388, 389], and diabetes [390] in various rodent models. Contrasting Sprague Dawley and Wistar rats in a similar manner may identify genetic loci associated with pathological responses to dietary fructose.

2.4.2 Laboratory Environment Could Affect Response to Dietary Fructose

Seemingly benign environmental differences between laboratories can also affect both physiology and behavior in rats. For example, bedding material and the frequency of bedding replacement affects body weight and inflammatory markers [391]. Similarly, the level of environmental enrichment can affect stress tolerance [392] and depression-like behavior [393, 394]. Controlling these variables can be difficult, as identical mouse strains exhibited different behavior when tested in different laboratories despite rigorous efforts to standardize protocols and environmental variables [395]. A follow-up study revealed interaction effects between laboratory environment and mouse strain, with the potential to alter "moderate genetic effects" [396]. Similarly, laboratory environment could interact with between- and within-strain genetic variability, influencing the anxiety- and depression-like behavior.

It is possible rats in this study were exposed to more environmental stress than in previous studies. Unlike a similar study [313], rats were kept on a 12:12h light

cycle, which has been shown to increase anxiety- and depressive-like behavior [397]. Moreover, rats were shipped during adolescence and subjected to a weekly blood glucose test. However, Harrell et al. [313] found no interaction effect between stress and response to dietary fructose in the open field, elevated plus, and forced swim task. Furthermore, control rats in this study exhibited similar anxiety- and depressive-behavior as control groups in previous studies. It is therefore unlikely that environmental stress strongly affected the behavior observed in this study.

2.4.3 HFrD does not change BLA electrophysiology

HFrD administration did not change the intrinsic excitability of BLA principal neurons (Figure 2.4). This finding correlates with the lack of behavioral changes observed during this study. The membrane properties of BLA principal neurons observed in this study were comparable to those described previously in control Sprague Dawley rats [371]. It is possible that a HFrD changed more subtle properties of BLA excitability not assessed in this study, such as synaptic plasticity. It is also possible any differences in BLA principal neuron excitability between the HFrD and control groups was lost during the slice procedure, but given our previous experience and success with these techniques, this is an unlikely alternative explanation. We can conclude that under the conditions used in this study, BLA principal neuron excitability was not altered by exposure to a high fructose diet initiated during adolescence.

2.4.4 A HFrD Changes Fat Pad Mass, but not Fasting Blood Glucose or Metabolic Efficiency in Sprague Dawley Rats

The increased body fat percentage in the HFrD group confirms that the HFrD disrupted metabolism in Sprague Dawley rats. This result is consistent with multiple studies reporting similar HFrD-induced increases in body fat percentage without increases in body weight [313]. We did not observe the increased metabolic efficiency reported in other studies using Wistar and Sprague Dawley rats [313, 398]. How-

ever, as adiposity is one of the core symptoms of metabolic syndrome, the increased fat pad mass observed in this study is sufficient confirmation that a HFrD disrupted metabolism in Sprague Dawley rats. However, we did not measure other metabolic outcomes disrupted by HFrD administration such as insulin resistance, hyperleptinemia, dyslipidemia, or liver steatosis [307, 308, 372–374]. The Sprague Dawley rats in this study could therefore be less susceptible to these fructose-induced metabolic effects, possibly affecting both behavioral and electrophysiological outcomes. Additional study is needed to fully assess the physiological outcomes of fructose administration in Sprague Dawley rats.

We found a significant effect of cohort on metabolic efficiency. This effect was not explained by whether rats were pair- or group-housed. As Sprague Dawley rats are an outbred strain, genetic variation between cohorts may explain the observed cohort effect. Indeed studies have reported phenotypic differences in Sprague Dawley rats originating from different vendors and from different colonies within vendors [399, 400]. Despite the effect of cohort on metabolic efficiency, there was no interaction effect between cohort and diet on metabolic efficiency. Thus, cohort did not influence the physiological response to dietary fructose.

We observed that HFrD administration significantly decreased fasting blood glucose. Most studies report that HFrD increases fasting blood glucose in Wistar [313, 401, 402] and Sprague Dawley rats [403]. However, some studies have found no HFrD-induced change in fasting blood glucose in either Wistar or Sprague Dawley rats [382, 404]. One possible factor is before the administration of the diet, the HFrD group had an almost significant ($p = 0.07$) reduction in baseline fasting blood glucose relative to control. Despite efforts to randomize rats between diet groups, this finding also suggests animals used in this study were genetically heterogeneous.

2.4.5 Overall Conclusions

Administration of a HFrD from adolescence into adulthood causes elevated fat pad mass in Sprague Dawley rats, which is indicative of disrupted metabolism. However unlike Wistar rats [313], HFrD administration does not affect motor, anxiety-like, or depressive-like behavior in Sprague Dawley rats. HFrD administration also does not change the intrinsic excitability of BLA principal neurons, correlating with our behavioral findings. These findings suggest that the physiological and behavioral effects of HFrD administration are strain-dependent, although environmental variables may also play a role. This study highlights the importance of accounting for strain and subtle environmental variables in diet studies. Furthermore, comparing Sprague Dawley and Wistar strains may offer a strategy to elucidate the mechanisms by which dietary fructose can alter neural endpoints and behavior. In conclusion, HFrD administration changes body fat, but not affective behavior or BLA excitability in male Sprague Dawley rats.

Chapter 3 High-fructose Diet Initiated During Adolescence does not Change Affective-like Behavior in Wistar Rats

Abstract

In the previous chapter, we concluded that unlike previous studies in Wistar rats, Sprague Dawley rats fed a high-fructose diet (HFrD) did not display increased anxiety- or depressive-like behavior. We concluded that this could be due to genetic differences between rat strains, environmental differences between laboratories, or a combination of both. We therefore repeated the experiments described in Chapter 2, feeding a periadolescent HFrD to male and female Wistar rats. Unlike previous experiments, rats were born in-house, and the experimental protocol was modified slightly to reduce stress in rats undergoing behavior. Although our results are limited by low statistical power, we found that similar to our results in Sprague Dawley rats, Wistar rats fed a HFrD displayed higher fat pad mass, but did not show elevated fasting blood glucose or metabolic efficiency. Furthermore HFrD-fed Wistar rats did not show increased anxiety- or depressive-like behavior relative to control. These results suggest that the discrepancies between our experiments and previous studies are likely due to environmental differences between laboratories. Collectively, these data show that a HFrD did not induce an anxiety- or depressive-like phenotype in male or female Wistar rats.

3.1 Introduction

In the last chapter, we concluded that a high-fructose diet (HFrD) increased fat pad mass in Sprague Dawley rats, but did not change affective-like behavior or basolateral amygdala (BLA) excitability. We were able to replicate the HFrD-increased fat pad mass, but not the anxiety-/depressive-like behavior seen in previous studies [313]. The metabolic, behavioral, and electrophysiological response to dietary fructose in rodents may therefore be subject to environmental factors, genetic differences between Sprague Dawley and Wistar rats, or a combination of both. To further explore the association between dietary fructose and affective-like behavior, we repeated the experiments described in Chapter 2 using Wistar rats. We also redesigned the study to control for some of the potential confounds in the previous experimental design.

Unlike the Sprague Dawley rats used in the Chapter 2 experiments, Wistar rats used in this study were born in-house rather than shipped during their early adolescence. Studies have shown that transportation disrupts rodent behavior, corticosterone levels, and body weight for at least three days [405–407]. It was possible that transportation stress during the earliest days of the diet perturbation (and potentially the time period when rats are most vulnerable to fructose) may have had long-term effects on the experiments in Chapter 2.

In addition, animals undergoing behavioral testing also did not undergo weekly fasting. Instead, weekly fasting blood glucose was measured in a parallel cohort of littermates. This approach has two advantages: (1) rats undergoing behavior will have complete *ad libitum* access to the HFrD, maximizing the HFrD's potential effects and (2) the rats undergoing behavioral testing were spared a weekly stressor (a tail prick) that could potentially influence performance in behavioral tasks. Firstly, fasting has been shown to lessen the fructose-induced disruption of gene expression in rat adipose tissue [408]. A 24-hour fast also lessened levels of non-esterified fatty acids, triglycerides and microsomal triglyceride transfer protein mRNA in rat liver

after long-term fructose administration, suggesting even relatively short-term fasts can alter metabolism in rats. By eliminating fasting, we ensured that the HFrD would have the maximum possible effect on rats undergoing behavior in the following experiments. Secondly, experiencing a weekly unavoidable stressor could have affected stress levels during behavioral testing [409]. Although our control rats in Chapter 2 showed similar behavior to control rats in other studies, it is nevertheless possible that the weekly tail-prick stressor had an effect on affective-like behavior.

In this study, we examined the effect of a periadolescent HFrD on metabolism and affective behavior in male and female Wistar rats. Based on previous work [313], we hypothesized that Wistar rats fed a HFrD would show increased body fat mass independent of body weight, increased metabolic efficiency, increased fasting blood glucose, and increased anxiety- and depressive-like behaviors. We found that a HFrD increased body fat, but did not affect fasting blood glucose or metabolic efficiency. We also did not observe increased anxiety- and depressive-like behaviors in the HFrD group. These data suggest that in our hands, the effects of a HFrD are similar for Sprague Dawley and Wistar rats. Genetic strain effects thus do not alter the behavioral response to a HFrD. For this reason, we interpret the discrepancy between our studies and that of others [313] is due to environmental differences between laboratories, rather than strain differences. Researchers should therefore pay close attention to laboratory environment as a potential confounding variable when designing experiments.

3.2 Methods

3.2.1 General Housing

General housing for rodents in the following experiments was identical to conditions described in Section 2.2.1 except for the following:

Four time-mated females were ordered from Charles River (Wilmington, MA) ap-

proximately one week before giving birth. Forty pups (20 male, 20 female) were then born in-house at Yerkes National Primate Research Center vivarium at Emory University, and used for the experiments below. Animals were weaned on postnatal day (P) 21, and separated into "Behavioral" and "Metabolic" cohorts (Figure 3.1). Rats in the behavioral cohort were housed in groups of three, while rats in the metabolic cohort were housed in groups of two. For both males and females, cagemates from two litters were assigned to the control diet, while the other two litters were assigned to the HFrD. However, the female metabolic cohort consisted of four rats from only one litter.

Rats in the behavioral cohort underwent weekly body weight measurements, and then underwent behavioral testing as described below. Rats in the metabolic cohort underwent weekly body weight and fasting blood glucose measurements. These rats were then euthanized for assessment of body fat (described below). Unless otherwise specified, no cohort differences were detected and cohorts were combined for appropriate analyses.

Like previous experiments, all animal experiments followed the guidelines described in the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023). All experimental protocols conformed to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Emory University.

3.2.2 Diet

The diet paradigm used in the following experiments was identical to the paradigm described in Section 2.2.2. Two days post-weaning (P23) animals in both the behavioral and metabolic cohorts were assigned to either a "control" diet (Lab Rodent Diet 5001, 0.30% kcal from fructose) or a HFrD (Research Diets D05111802, 55% kcal from fructose). Food consumption per cage was measured daily, and was used to calculate

the total weekly caloric consumption per cage. Total weekly caloric consumption per cage was divided by the number of rats per cage to determine average weekly caloric intake per rat, which was used to determine metabolic efficiency.

Once a week, animals in the metabolic cohort were subjected to an overnight fast before fasting blood glucose measurements (described in Section 2.2.4). Food was removed 1-2 hours before the onset of the dark cycle, and returned after blood glucose measurements approximately 16 hours later. Except for weekly overnight fasts, animals in the metabolic cohort had *ad libitum* access to food and always had *ad libitum* access to water. Animals in the behavioral cohort did not undergo weekly fasts, and had constant *ad libitum* access to both food and water.

3.2.3 Behavior

Rats in the behavioral cohort underwent habituation and behavioral testing in the Open Field maze, Elevated O maze, and Forced Swim task as described in Section 2.2.3. Rats in the metabolic cohort did not undergo behavioral testing. Males underwent Open Field, Elevated O, and Forced Swim testing (in order) from P91-93, while females underwent the tasks (in order) from P94-96.

3.2.4 Metabolic Outcomes

For rats in the metabolic cohort, fasting blood glucose and body weight were measured weekly after an overnight fast. Rats in the behavioral cohort only underwent weekly body weight measurements, and were not subjected to fasting. Fasting blood glucose measurements were obtained using the same protocol described in Section 2.2.4. Four rats from each group (control and HFrD, 2 rats per litter) in the metabolic cohort were also anesthetized and euthanized for fat-pad measurements as described in Section 2.2.4.

3.2.5 Statistical Analysis

Measurements were analyzed using Graphpad Prism 8 (La Jolla, CA). Unpaired two-tailed Student's or Welch's t-tests or 1-way and 2-way repeated measures Analysis of Variance (ANOVA) tests were performed using α=0.05. Tukey and Holm-Sidak post-hoc testing were performed when appropriate. Data points from littermates were averaged into one data point Two levels of the independent factor "diet" (control and HFrD) were used for both 1- and 2-way ANOVAs. For 2-way ANOVAs, eight levels of the dependent factor "Time" (Weeks 2-9) were used. All values are reported as mean +/- standard deviation.

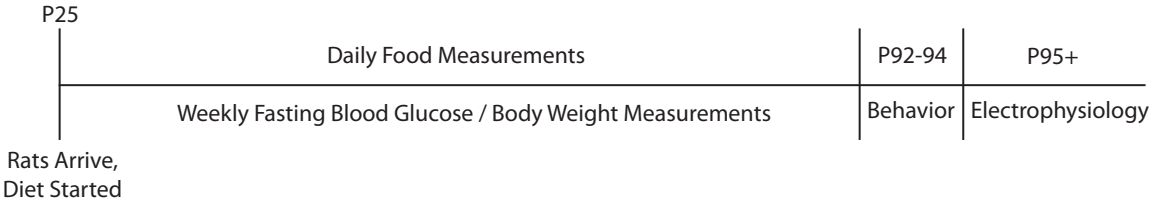

Figure 3.1: Experimental paradigm used for Wistar rats. Rats were born in-house at the Yerkes National Primate Center Vivarium, and weaned on P21, when they were separated into the "Metabolic" and "Behavioral" cohorts. On P23, two litters each were assigned to the control and HFrD diets in both cohorts. The female "metabolic" cohort only consisted of rats from one litter; all other cohorts (male "metabolic", male/female "behavioral") consisted of rats from two litters. Littermates were averaged into a single data point for analysis.

3.3 Results

3.3.1 Peri-Adolescent High-Fructose Diet Increases Visceral Fat Mass, but does not Change Metabolic Efficiency

In the behavioral cohort, prior to the initiation of the dietary intervention body weight was equivalent between the male rats that would receive the control diet (62.83 g +/- 0.71, n=2) and the HFrD (66.67 g +/- 9.43, n=2; $t_{1.01}$=0.57, p=0.67), as well as the female rats that would receive the control diet (63.50 g +/- 6.36, n=2) and HFrD (60.17 g +/- 3.54, n=2; $t_{1.56}$=0.65, p=0.60). The high fructose diet did not

alter body weight, which remained similar between groups throughout the study for both male ($F_{1,2}$=0.39, p=0.59, Figure 3.2-B) and female ($F_{1,2}$=13.69, p=0.07, Figure 3.2-D) rats. Body fat was not measured in the behavioral cohort. Metabolic efficiency did not differ between male control and HFrD rats ($F_{1,2}$=2.09, p=0.28 Figure 3.4-B), or female control and HFrD rats ($F_{1,2}$=9.26, p=0.09, Figure 3.4-D).

In the metabolic cohort, the female control rats and HFrD rats were not compared statistically, as the female HFrD group consisted of only one litter. Prior to the initiation of the dietary intervention body weight was equivalent between the male rats that would receive the control diet (57.33 g +/- 0.94, n=2) and the HFrD (65.50 g +/- 12.02, n=2; $t_{1.01}$=0.96, p=0.51). The female rats that would receive the control diet weighed 53.13 g +/- 3.0 (n=2) prior the dietary intervention, and the rats that would receive the HFrD weighed 52.0 g (n=1, Figure 3.2-C). The HFrD did not alter body weight, which remained similar between groups throughout the study for male rats ($F_{1,2}$=0.41, p=0.59, Figure 3.2-A). Furthermore, for male rats in the metabolic cohort, normalized epididymal body fat ($t_{1.5}$=6.27, p<0.05, Figure 3.3-A) in the HFrD group was significantly higher relative to control, but perigonadal body fat ($t_{1.19}$=1.9, p=0.28, Figure 3.3-B) was not significantly different. Metabolic efficiency also did not differ between male control and HFrD rats ($F_{1,2}$=0.33, p=0.62, Figure 3.4-A).

3.3.2 HFrD Did Not Alter Fasting Blood Glucose

All measurements of fasting blood glucose were measured using the metabolic cohort of animals. Once again, the female control rats and HFrD rats were not compared statistically, as the female HFrD group consisted of only one litter (Figure 3.5-B). At the start of the experiment, fasting blood glucose was equivalent between the groups of male rats that would be assigned to standard chow (81.00 mg/dL +/- 19.80) or HFrD chow (74.50 mg/dL +/- 0.07; t_1=0.46, p=0.72). HFrD rats did not have significantly different blood glucose relative to control rats throughout the

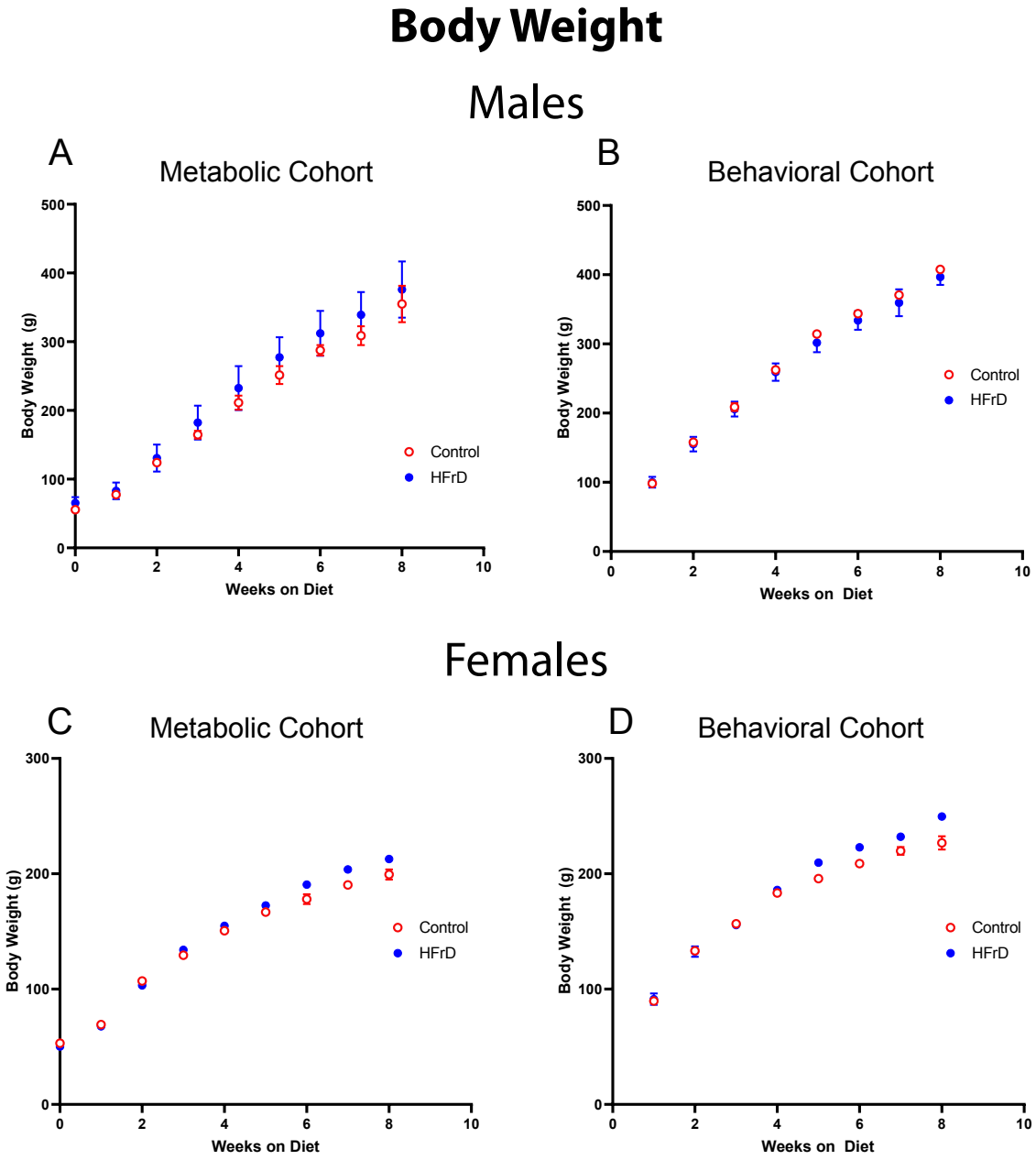

Figure 3.2: HFrD does not affect body weight in male or female Wistar rats. Littermates were averaged into a single data point for analysis. A) Body weight of male control and HFrD Wistar rats in the Metabolic Cohort (n=2 litters per group). B) Body weight of male control and HFrD Wistar rats in the Behavioral Cohort (n=2 litters per group). C) Body weight of female control and HFrD Wistar rats in the Metabolic Cohort (Control: n=2 litters, HFrD: n=1 litter). D) Body weight of female control and HFrD Wistar rats in the Behavioral Cohort (n=2 litters per group).

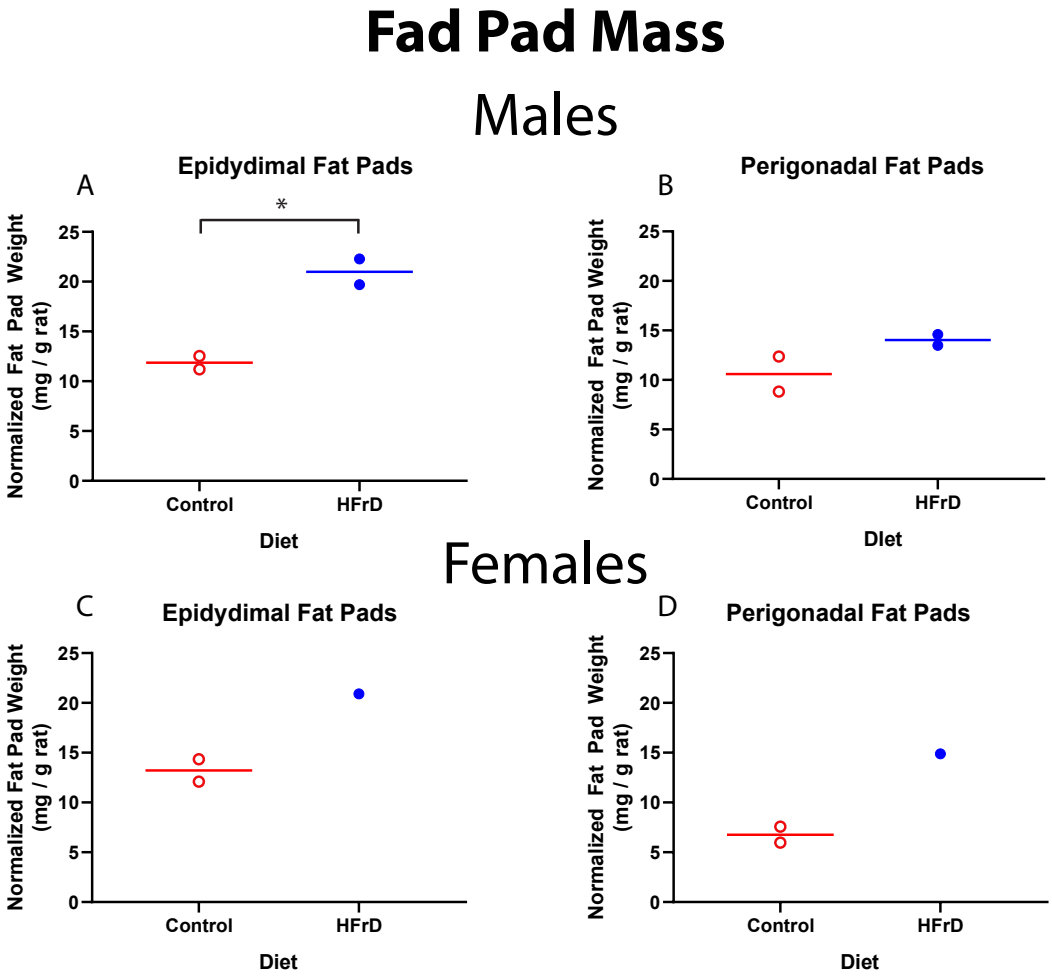

Figure 3.3: HFrD increases fat pad mass in male Wistar rats. A) Normalized epidydimal fat pad mass and B) normalized perigonadal fat pad mass in male Wistar rats from the Metabolic cohort (n=2 litters per group). C) Normalized epidydimal fat pad mass and D) normalized perigonadal fat pad mass in female Wistar rats from the Metabolic cohort (Control: n=2 litters, HFrD: n=1 litter). *: $p<0.05$

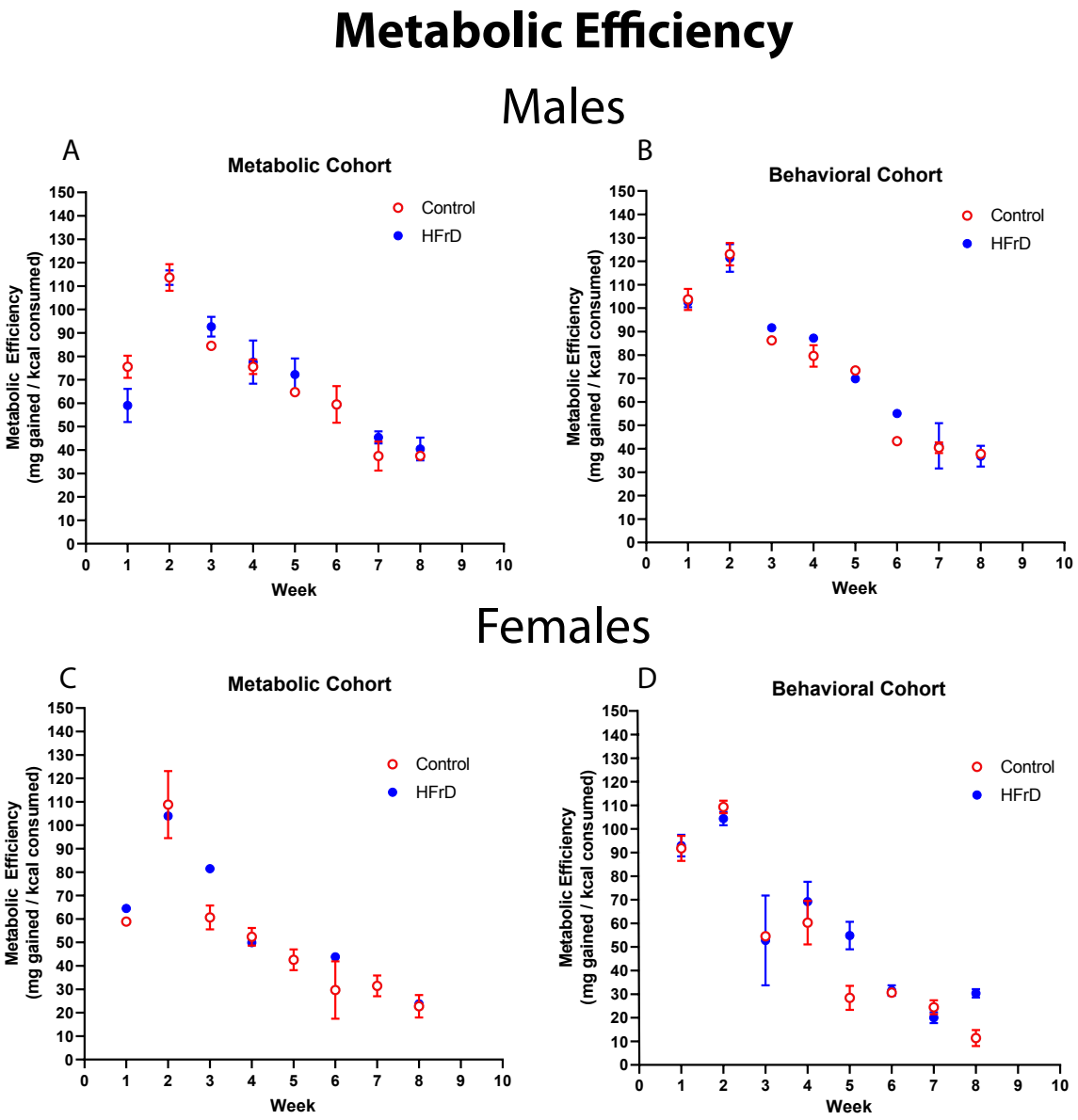

Figure 3.4: HFrD does not affect metabolic efficiency in male or female Wistar rats. Metabolic efficiency of: A) male Wistar rats in the Metabolic cohort (n=2 litters per group). B) male Wistar rats in the Behavioral cohort (n=2 litters per group). C) female Wistar rats in the Metabolic cohort (Control: n=2 litters, HFrD: n=1 litter). D) female Wistar rats in the Behavioral cohort (n=2 litters per group).

study ($F_{1,2}$=13.69, p=0.07, Figure 3.5-A). In addition, fasting blood glucose was not influenced by time ($F_{1.3,2.7}$=7.2, p=0.08) and there was no interaction between diet and time ($F_{6,12}$=1.81, p=0.18).

3.3.3 HFrD Did Not Alter Motor or Affective-like Behaviors

Control and HFrD groups traveled the same distance in the open field maze for both males ($t_{1.27}$=0.57, p=0.65, Figure 3.6-A) and females (t_1=1.40, p=0.39, Figure 3.6-C). Average velocity was also the same for control and HFrD male rats (Control: 83.91 mm/s +/- 18.64, HFrD: 93.98 mm/s +/- 14.24, $F_{1,2}$=0.86, p=0.45) and control and HFrD female rats (Control: 91.22 mm/s +/- 16.86 , HFrD: 101.6 mm/s +/- 19.20, $F_{1,2}$=0.68, p=0.49), as was maximum velocity for males (Control: 241.5 mm/s +/- 32.00, HFrD: 286.2 mm/s +/- 36.53, $F_{1,2}$=2.8, p=0.24) and females (Control: 284.1 mm/s +/- 50.73, HFrD: 304.5 mm/s +/- 44.28, $F_{1,2}$=1.33, p=0.37). This suggested that there was similar motor activity in the different diet groups. In addition, the zones of the open field maze in which the rats spent time were equivalent between the control and HFrD groups for males: outer (t_6=0.33, p=0.75), middle (t_6=2.70, p=0.07), and center (t_6=3.04, p=0.07; Holm-Sidak method, α=0.05, Figure 3.6-B) and females: outer (t_6=0.45, p=0.69) middle (t_6=0.83, p=0.69) and center (t_6=1.27, p=0.58; Holm-Sidak method, α=0.05, Figure 3.6-D).

In the elevated zero maze, control and HFrD rats displayed the same number of crossings between the open arm, "stretch-attend", and closed arm zones, and this was true for both males (t_2=3.301, p=0.08, Figure 3.7-A) and females (t_2=3.015, p=0.10, Figure 3.7-C). This also suggested that there was similar motor activity in the different diet groups. Furthermore, a HFrD did not alter the time spent in the open arms (Males: t_6=0.17, p=0.87 Females: t_6=0.71, p=0.88), "stretch-attend" zone (Males: t_6=1.82, p=0.53, Females: t_6=0.19, p=0.88) or closed arms (Males: t_6=1.35, p=0.53, Figure 3.7-B, Females: t_6=0.52, p=0.88, Figure 3.7-D) of the elevated O maze

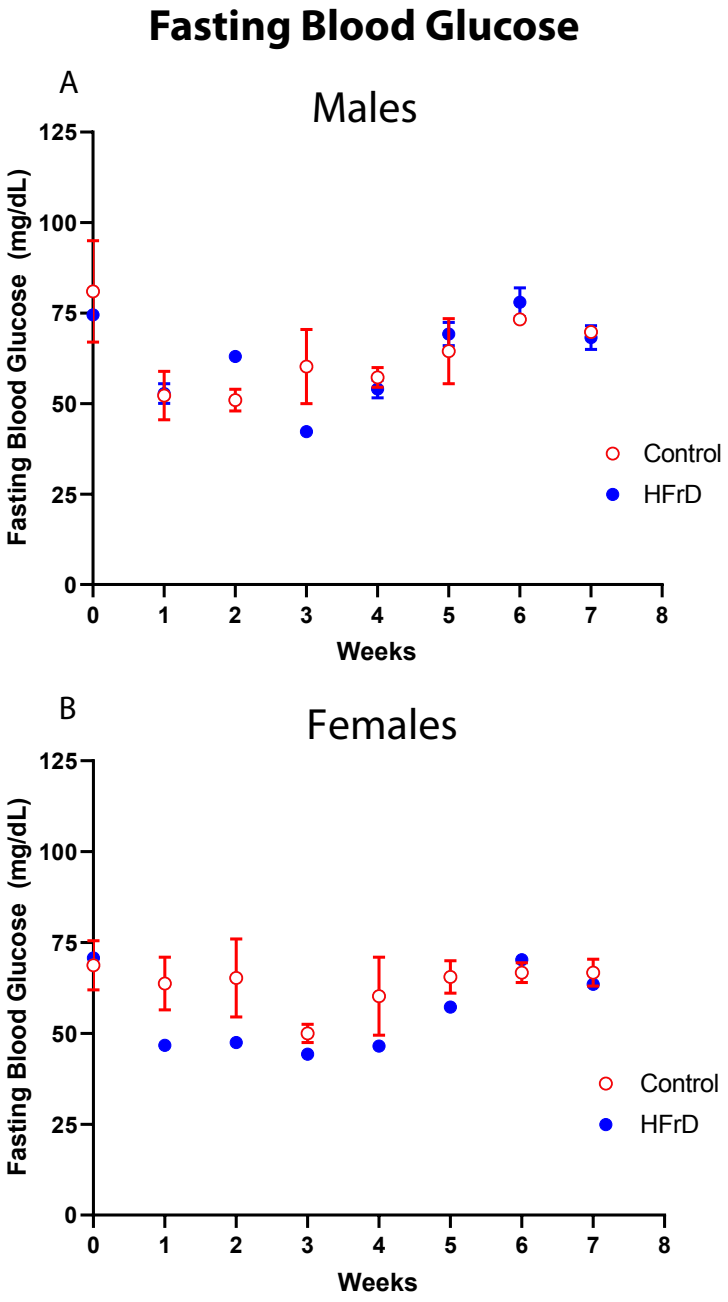

Figure 3.5: HFrD does not affect fasting blood glucose in male or female Wistar rats. Fasting blood glucose in A) male rats from the Metabolic cohort (n=2 litters per group). B) female Wistar rats in the Metabolic cohort (Control: n=2 litters, HFrD: n=1 litter).

compared to the behavior of the control diet rats (Holm-Sidak method, α=0.05).

Finally HFrD did not alter time spent struggling in the forced swim test in males (t_2=0.72, p=0.55, Figure 3.8-A) or females (t_2=1.52, p=0.27, Figure 3.8-D). In addition, a HFrd did not change latency to the first $\geq$ 2 sec immobility event for males (t_2=0.11, p=0.93, Figure 3.8-B) or females (t_2=1.80, p=0.21, Figure 3.8-E). Finally, a HFrD did not change total time spent immobile for males (t_2=0.72, p=0.55, Figure 3.8-C) or females (t_2=1.52, p=0.27, Figure 3.8-F).

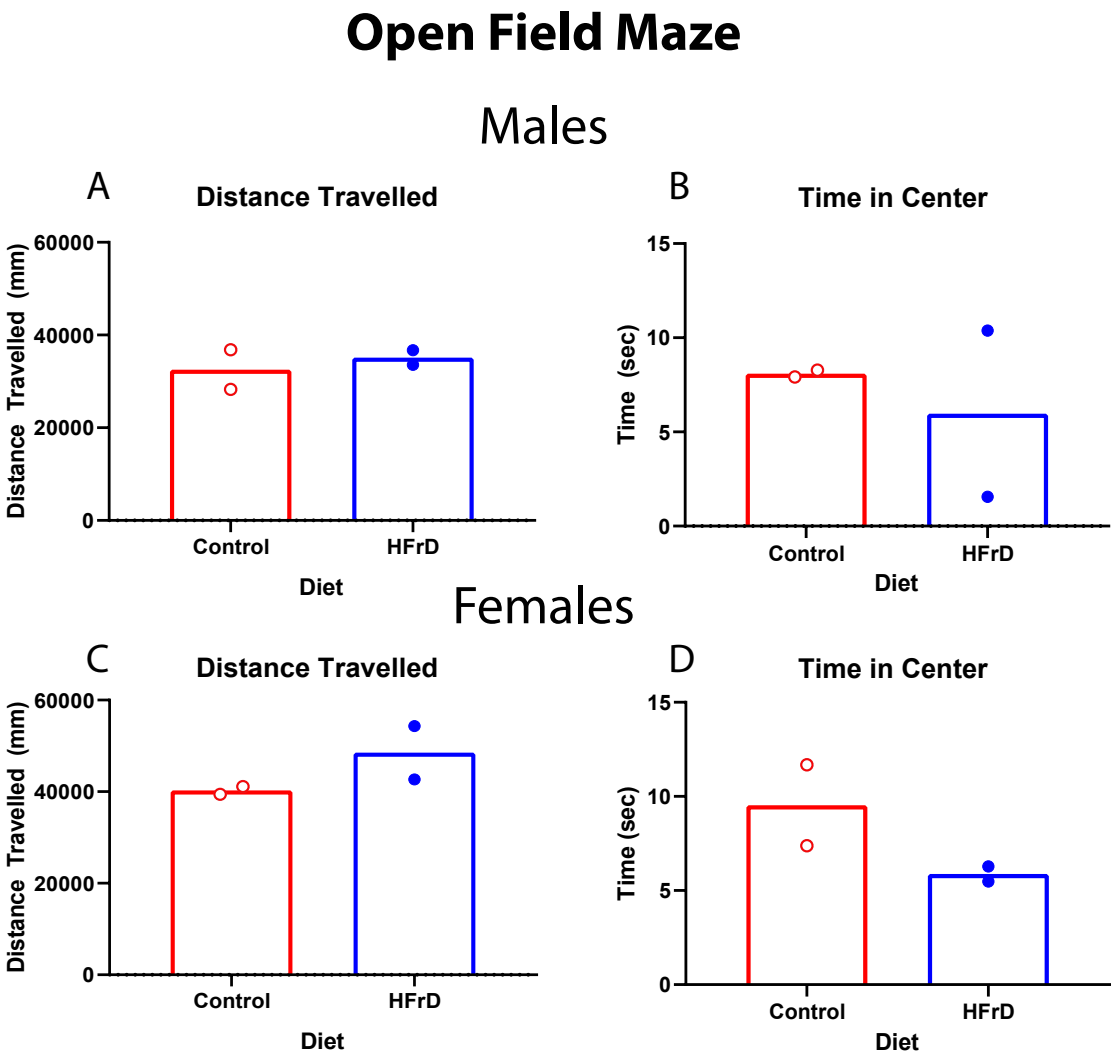

Figure 3.6: HFrD does not affect behavior in the open field maze in male or female Wistar rats. Only rats in the "behavioral" cohort underwent these experiments. A) Total distance traveled in the open field maze & B) in time spent in the center of the open field maze in male Wistar rats (n=2 litters per group). C) Total distance traveled in the open field maze & D) in time spent in the center of the open field maze in female Wistar rats from the Behavioral cohort (n=2 litters per group).

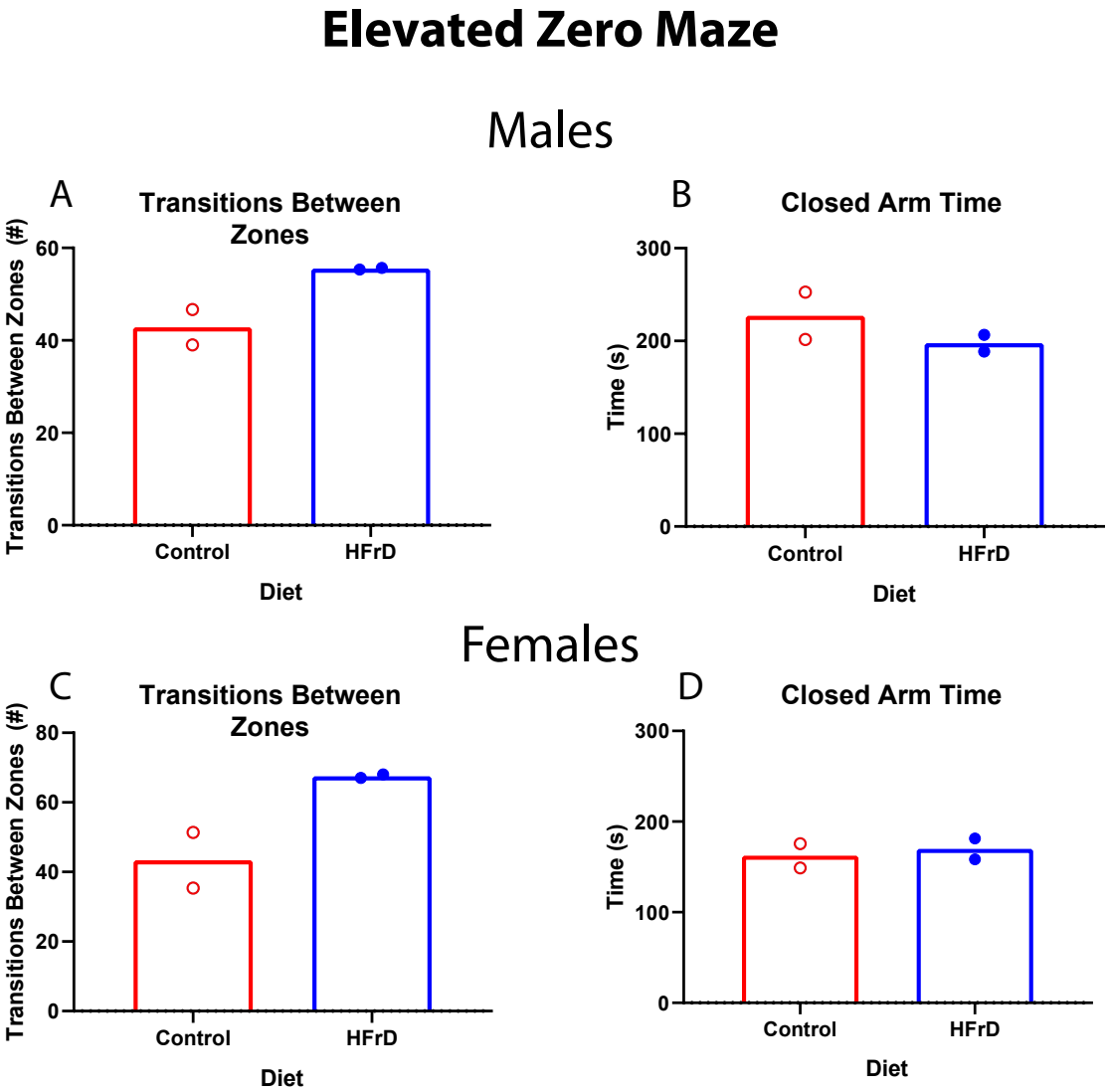

Figure 3.7: HFrD does not affect behavior in the elevated zero maze in male or female Wistar rats. Only rats in the "behavioral" cohort underwent these experiments. A) Total transitions between zones of the elevated zero maze & B) time spent in the closed arm of the elevated zero maze in male Wistar rats (n=2 litters per group). C) Total transitions between zones of the elevated zero maze & D) time spent in the closed arm of the elevated zero maze in female Wistar rats (n=2 litters per group).

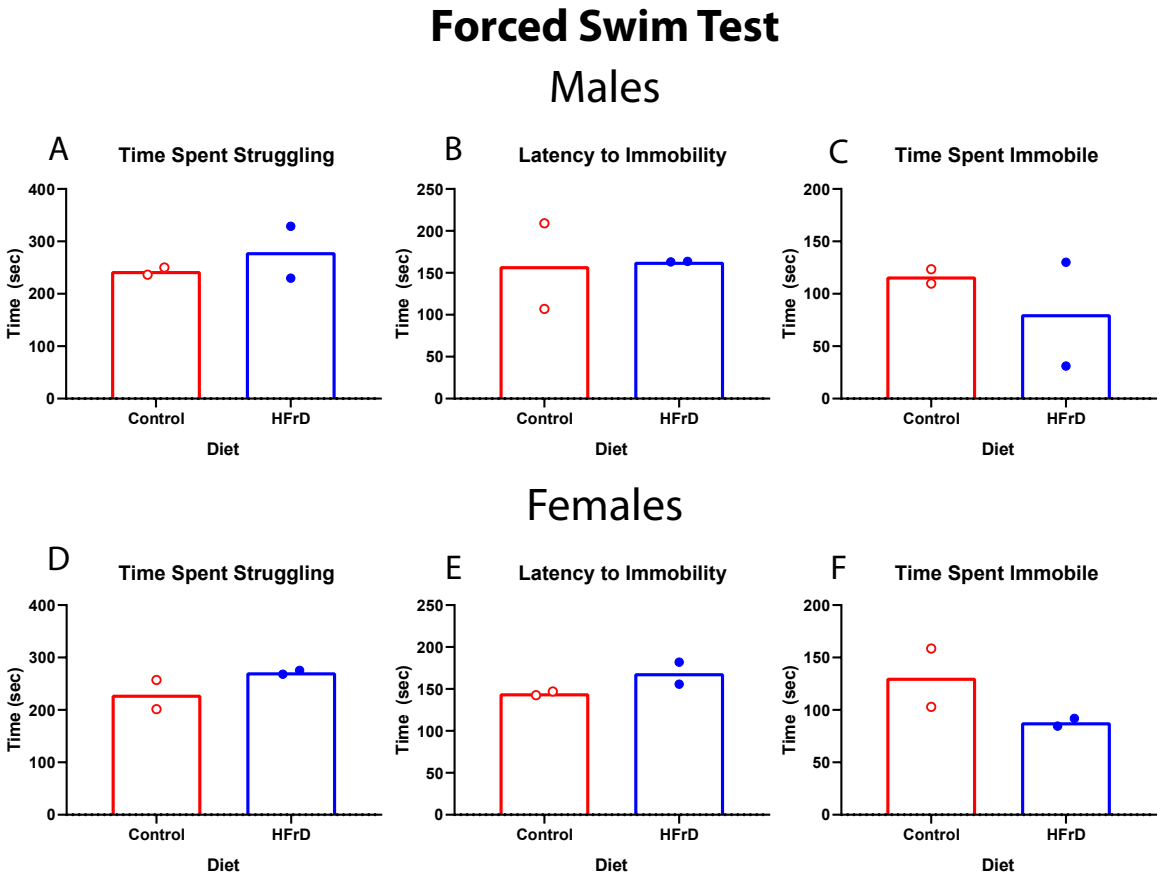

Figure 3.8: HFrD does not affect behavior in the forced swim task in male or female Wistar rats. Only rats in the "behavioral" cohort underwent these experiments. A) Time spent struggling in the forced swim task in male Wistar rats (n=2 litters per group). B) Latency to >2 second immobility event in male Wistar rats (n=2 litters per group). C) Time spent immobile in male Wistar rats (n=2 litters per group. D) Time spent struggling in the forced swim task in female Wistar rats (n=2 litters per group). E) Latency to >2 second immobility event in female Wistar rats (n=2 litters per group). F) Time spent immobile in female Wistar rats (n=2 litters per group).

3.4 Discussion

Collectively, the data presented here demonstrate that the effects of a periadolescent high-fructose diet in male / female Wistar rats are similar to those seen in male Sprague Dawley rats (described in Chapter 2). The effects of a HFrD on fat pad mass seen in both Wistar and Sprague Dawley rats in our studies are consistent with previous work [313], and indicate that the HFrD did clearly disrupt metabolism. However, we did not observe the behavioral changes previously reported in Wistar rats [313]. Indeed, our behavioral results in Wistar rats are consistent with our behavioral results in Sprague Dawley rats (Chapter 2). These results suggest that environmental variables, more so than genetic variability between rat strains, affects the behavioral response to a high-fructose diet. However, this study is limited by low statistical power. Future studies investigating the relationship between fructose and behavior should be extremely careful to minimize environmental variability between laboratories, including seemingly benign differences.

3.4.1 Litter effects and Statistical Power

One major limitation of these experiments is statistical power. To control for litter effects, littermates were averaged into a single data point, instead of treating littermates as independent data points. Because two litters were assigned to each diet, our functional n equals 2 despite our higher animal numbers. Indeed, the female metabolic cohort consisted of four littermates for an n=1, making a statistical comparison to the control group impossible. The relatively low statistical power of these experiments therefore increases the chance of making a type II (false negative) error, i.e. failing to observe an effect caused by the HFrD. For this reason, we cannot conclusively say that the HFrD does not produce a behavioral phenotype in Wistar rats based on this data. However these results influence how we interpret our Chapter 2 data, as described below.

Treating littermates as independent data points would have increased the statistical power of our analysis, however this approach would have introduced additional sources of variation into our study. For example, naturally occurring variations in the quality of maternal care can influence the stress reactivity [410], fearfulness [411], and depressive-like symptoms [412] of pups later in life. As littermates all experience approximately the same quality of maternal care, they cannot be treated as fully independent data points.

3.4.2 Effects of a HFrD on Metabolism and Behavior are Similar for Sprague Dawley and Wistar Rats

Despite being underpowered, our results in Wistar rats were largely similar to those seen in our Sprague Dawley rats (Chapter 2). A HFrD significantly increased epidydimal body fat in the male metabolic cohort relative to control, and although not significant perigonadal fat was trending in the same direction. Statistical comparisons for the female metabolic cohort are not possible, but the data also showed a similar trend (Figure 3.3). We interpret this as proof that a HFrD indeed disrupts metabolism in Wistar rats, as adiposity is one of the core symptoms of metabolic syndrome. Furthermore, the increased body fat in our Wistar rats is consistent both with our findings in Sprague Dawley rats (Chapter 2) and other studies in Wistar rats [313]. This indicates that some of the metabolic effects of HFrD administration are robust and consistent across both rat strains and laboratories.

In addition, a HFrD did not influence fasting blood glucose in either male or female Wistar rats, which is inconsistent with previous research showing elevated fasting blood glucose [313, 413, 414]. However, our results in Sprague Dawley rats were also inconsistent with these studies, and indeed showed significantly lower blood glucose at some time points. Other studies also report that a HFrD does not change fasting blood glucose in either Wistar or Sprague Dawley rats [382, 404]. Unlike our body fat findings (above), it appears HFrD-induced hyperglycemia is a less robust

effect of a HFrD, and is subject to inter-laboratory variation.

Finally, a HFrD did not significantly influence motor, anxiety-like, or depressive-like behavior in male or female Wistar rats (Figures 3.6-3.8). These findings are also consistent with our findings in Sprague Dawley rats (Chapter 2), but inconsistent with previous findings in Wistar rats [313]. Although our Chapter 3 results are underpowered, they alter our interpretation of our Chapter 2 results as explained below.

Firstly, it is unlikely that stressors in our Chapter 2 experimental design (such as being shipped to the vivarium immediately post-weaning) impeded our ability to measure increased anxiety- and depressive-like behavior in the HFrD group. Our Wistar rats were not subject to such stressors (as they were born in-house), yet neither our male or female HFrD groups showed (or trended towards) greater anxiety- or depressive-like behavior in the elevated O maze or forced swim task respectively. It is similarly unlikely that subjecting our Sprague Dawley rats to a weekly fast / tail prick affected the behavioral outcomes observed in Chapter 2. Once again our Wistar rats were not subjected to these stressors, as our behavioral cohort was never fasted (and was thus subjected to the "full force" of the HFrD) and only underwent weekly body weight measurements. Getting rid of these stressors in our Wistar rats did not affect our general behavioral conclusions, suggesting they were not of importance in our Chapter 2 experiments.

Secondly, our Chapter 3 results suggest that in our hands, rat strain does not affect the response to dietary fructose. If Wistar rats were indeed significantly more vulnerable than Sprague Dawley rats to a HFrD due to genetic strain differences, we expected to see significantly increased metabolic disruption and/or increased anxiety-/depressive-like behavior in our Wistar rats (Chapter 3) relative to our Sprague Dawley rats (Chapter 2). Instead, our Wistar rats showed the same basic metabolic response to a HFrD (increased body fat, no increased fasting blood glucose) and rel-

atively similar behavior as our Sprague Dawley rats in all three behavioral tasks in both control and HFrD groups. In our laboratory, rat strain (Sprague Dawley vs Wistar) therefore does not appear to influence the behavioral response to a HFrD, although this interpretation is limited by low statistical power as stated above.

We favor the interpretation that differences in laboratory environments underlie the discrepancy between our findings and previous studies [313]. For example, all rats used in our experiments were on a potentially stressful regular 12h:12h light cycle [397], while Harrell and colleagues used a 14h:10h reverse light cycle. Similarly, we housed our rats in ventilated cages while Harrell and colleagues used open-top cages, which has been shown to alter food intake (but not body weight) [415]. Another possibility is that the biological sex of the experimenter influenced the behavioral results. A *Nature Methods* study found that exposure to the scent of male but not female experimenters causes stress in rodents [416]. As our experiments were done by a male experimenter and Harrell's experiments were carried out by a female experimenter, it is possible the HFrD-induced anxiety- and depressive-like behaviors are only visible if a female experimenter carries out the experiment. It was not obvious to us when designing these experiments that such environmental differences could have such a profound effect on our behavioral results. Nonetheless, our results suggest that any researcher attempting to replicate an experiment must pay strict attention to such considerations.

3.4.3 Overall Conclusions

Collectively, these data show that administering a HFrD to male and female Wistar rats increased body fat percentage, but did not increase fasting blood glucose, motor behavior, or anxiety-/depressive-like behavior. Overall, these results are consistent with our results in Sprague Dawley rats, although our experiments in Wistar rats are limited by low statistical power. Overall, these data suggest that the variabil-

ity between our and previous studies [313] is due to environmental differences between labs, and not due to genetic strain differences between Sprague Dawley and Wistar rats. Researchers attempting to replicate an experiment (especially one involving animal behavior) should therefore design their experiments extremely carefully, paying close attention to laboratory environment as a potential confounding variable.

Chapter 4 The Role of AMP-Activated Protein Kinase Regulating the Excitability of Control Basolateral Amygdala Neurons

Abstract

The brain is the most metabolically demanding organ in the body, but because it lacks long-term energy reserves it relies on circulating glucose for energy. The brain's energy use and production therefore must be strictly regulated, however the mechanism of this regulation is unknown. As the master energy regulator in eukaryotic cells, AMP-activated protein kinase (AMPK) is the most likely candidate, and indeed has been shown to control hypothalamic neuronal activity in accordance with energy state. However, AMPK's role outside the hypothalamus is poorly understood, and its role in the BLA is completely unknown. Recent experiments suggest that AMPK regulates the activity of BLA neurons, as the antidepressant rolipram (which indirectly inhibits AMPK) has been shown to influence BLA activity. The goal of the following experiments was to test the effect of AICAR (an AMPK activator) and compound C (an AMPK inactivator) on BLA excitability in control rats. We also tested if rolipram's effect on BLA excitability was dependent on AMPK activity. We hypothesized that rolipram would increase BLA excitability, and compound C would mimic the effects of rolipram. Furthermore, we hypothesized AICAR would reduce BLA excitability and that administering AICAR together with rolipram would block the effect of rolipram alone. As we hypothesized, we found both rolipram and Compound C increased BLA excitability. AICAR alone had no effect on BLA excitability, however AICAR blocked rolipram's effect when the drugs were administered together. Together, these data suggest that AMPK does influence BLA excitability and that rolipram increases BLA excitability by inactivating AMPK.

4.1 Introduction

In the previous chapters, we wanted to test the idea that a high-fructose diet (HFrD) induced anxiety- and depressive-like behavior in rats, specifically by decreasing AMP-activated protein kinase (AMPK) activity in the BLA. To test this, we used a high-fructose diet to disrupt metabolism in both Sprague Dawley and Wistar rats, and collected metabolic, electrophysiological, and behavioral data. However, we could not replicate anxiety- or depressive-like phenotype seen in previous studies [313], nor could we induce BLA hyperexcitability in HFrD-fed rats. We therefore could not examine the potential role of BLA AMPK activity on anxiety- / depressive-like behavior. However, AMPK may still be regulating neuronal excitability in the healthy amygdala. In these experiments, we tested the effect of AMPK activators and inactivators on the evoked network excitability of the BLA.

Emerging evidence suggests that AMPK is an important regulator of neuronal excitability throughout the CNS. The brain lacks long-term energy stores, and relies on circulating glucose for energy [320]. Despite this the brain is the most metabolically demanding organ in the body, weighing only two pounds but consuming 20% of total energy and 50% of circulating glucose [253, 254, 318]. A similarly large percentage of the brain's total energy usage (50%) is directed towards generating neuronal activity, especially action potential generation and Na^+/K^+ ATPase activity [275]. Given this huge energy utilization, ATP generation and expenditure must be tightly regulated in the brain, however the mechanism underlying this regulation remains unknown.

The most likely candidate controlling the brain's energy regulation is AMPK. AMPK is the "master metabolic regulator" in all eukaryotic cells. When activated during times of high metabolic demand (i.e. a high AMP/ATP ratio), AMPK phosphorylates many downstream effector proteins, limiting energy use and facilitating energy production in most tissues. Indeed, there is evidence that AMPK activation in the brain limits energy-intensive neuronal activity: AMPK reduces excitability

in cultured hippocampal neurons [336] and the nucleus accumbens core [337], and increases the inhibitory effects of $GABA_B$ receptors [338] and potassium currents [336, 340, 341]. However, AMPK activation can also increase neuronal excitability. AMPK's most well-characterized role is in the arcuate nucleus of the hypothalamus, where AMPK activation causes the release of intracellular Ca^{2+} in presynaptic terminals, increasing the excitatory drive onto AgRP neurons and activating the brain's feeding circuit. AMPK can therefore bidirectionally modulate neuronal activity in response to energy state, and is likely an important regulator of neuronal activity throughout the brain. Metabolic state can also influence activity in limbic circuits, as acute hypoglycemia (which increases hypothalamic AMPK expression [257]) can cause anxiety [417] which likely correlates with disrupted amygdala activity [418]. However, AMPK's role controlling neuronal excitability in most brain regions including the BLA remains unknown.

Recent experiments involving the antidepressant rolipram indeed suggest that AMPK is an important regulator of BLA activity. Rolipram (a fast-acting antidepressant that failed clinical trials due to side effects) acts as a PDE4 inhibitor, preventing the conversion of cAMP into 5' AMP and reducing AMPK activity (Figure 4.1). Previous work in our lab indeed showed that rolipram both increased BLA excitability and induced an anxiety-like phenotype in control rats, possibly by modulating AMPK activity [113]. We therefore hypothesized that rolipram would increase BLC activity by reducing AMPK activity.

Interestingly, our experiments suggest that rolipram increases BLA excitability in control conditions [113]. However, the BLA is hyperactive during depression, and this hyperactivity normalized with successful pharmacotherapy [16]. It is therefore unclear why rolipram is an effective antidepressant, as it appears rolipram would increase the excitability of an already hyperexcitable BLA. There are two solutions to this apparent paradox: (1) rolipram acts on non-BLA regions that are hypoactive

during depression to achieve its antidepressant effect, or (2) in depressed patients reducing AMPK activity with rolipram *reduces* BLA excitability (unlike in controls). In order to understand how rolipram affects the BLA in depressed patients, it is first necessary to understand specifically how rolipram affects the control BLA. The goal of these experiments was to therefore to examine the role of AMPK in the control amygdala.

We hypothesized that rolipram would increase the excitability of the BLC by reducing AMPK activity. We also hypothesized that compound C (an AMPK inactivator) would also increase BLA excitability, while AICAR (an AMPK activator) would both reduce BLA excitability and block rolipram's excitatory effect (Figure 4.1). We found that as expected, rolipram increased BLA excitability, which was mimicked by Compound C and blocked by AICAR.

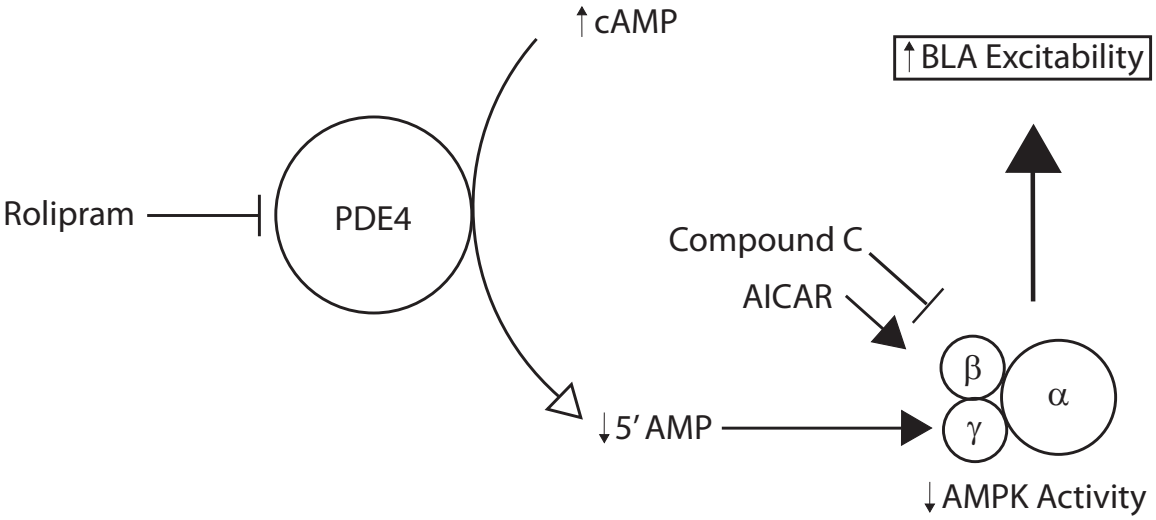

Figure 4.1: The hypothesized mechanism of rolipram, compound C, and AICAR on BLA excitability. Rolipram inhibits the enzyme PDE4, which in turn prevents the conversion of cAMP into linear AMP. We hypothesized decreased AMP levels would lead to reduced AMPK activity and increased BLA excitability. We also hypothesized that AICAR (which is converted to the AMP mimetic ZMP) would increase AMPK activity and decrease BLA excitability. Finally we hypothesized the AMPK inactivator compound C would increase BLA excitability, mimicking rolipram.

4.2 Methods

4.2.1 General Housing

All animal experiments followed the guidelines described in the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023). Animals were housed in the Yerkes National Primate Research Center vivarium at Emory University.

A total of 27 male Sprague Dawley rats were used in the following experiments. Animals were ordered from Charles River (Wilmington, MA). Rats arrived in the vivarium at four weeks of age, and allowed to habituate to the vivarium for at least a week before being used in the following experiments. Rats were given *ad libitum* access to the same "control" diet described in Chapters 2 and 3 (Lab Rodent Diet 5001). Rats were kept on an artificial regular 12:12-h light cycle, and were housed in ventilated cages kept at 22° C.

Rats were housed in groups of four. All experimental protocols conformed to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Emory University. All animals were used for slice electrophysiology as described below.

4.2.2 Slice Electrophysiology

Rats were decapitated under isoflurane anesthesia, and prepared for slice electrophysiology. The rats' brains were removed and processed for slice electrophysiology as described previously [113]. Brains were immersed in ice-cold "cutting solution" consisting of (in mM): NaCl (130), $NaHCO_3$ (30), KCl (3.5), KH_2PO_4 (1.1), $MgCl_2$ (6), $CaCl_2$ (1), glucose (10), ascorbate (0.4), thiourea (0.8), sodium pyruvate (2), and kynurenic acid (2). Coronal sections (350 μM thick) were obtained using a Leica VTS-1000 vibrating-blade microtome. Slices were left to incubate in cutting solution for an hour, before being transferred to room-temperature "regular artificial cere-

brospinal fluid" (aCSF) consisting of (in mM): NaCl (130), $NaHCO_3$ (30), KCl (3.5), KH_2PO_4 (1.1), $MgCl_2$ (1.3), $CaCl_2$ (2.5), glucose (10), ascorbate (0.4), thiourea (0.8) and sodium pyruvate (2). Cutting solution and aCSF were perfused with a 95% oxygen / 5% carbon dioxide gas mixture.

Suction Electrode Recordings

We collected extracellular recordings using the suction electrode technique (see below) from 24 rats in order to measure the overall network excitability of the BLA. Suction electrode recordings are primarily composed of the summed population potentials from the axons and cell bodies within the electrode (for description see methods in [419]). Suction electrodes were made by hand-pulling thin-walled borosilicate glass pipettes over a Bunsen burner. Suitable electrodes were then broken to a suitable diameter, and polished by hand using a sharpening stone.

Individual slices were transferred to a recording chamber and were visualized using a Leica DM6000 FS microscope (Leica Microsystems Inc., Bannockburn, IL) as described previously [113]. Slices were continuously perfused by gravity-fed oxygenated 32° C aCSF at a flow rate of 2–3 ml/ min. The BLA was visually identified, and drawn into the suction electrode using gentle negative pressure. A tungsten unipolar stimulating electrode was also lowered into place on the external capsule. After five to ten minutes, the negative pressure in the suction electrode was completely released, and a small amount of negative pressure was reintroduced to keep the recording stable.

Data acquisition and analysis were performed using an A-M Systems Inc. Model 3000 AC/DC differential amplifier (Sequim, WA) in conjunction with pClamp 10.2 software and a DigiData 1020A AD/DA interface (Molecular Devices, Sunnyvale, CA). The external capsule was stimulated 5 times (3 mA, 150 μs pulse width) at 0.2 Hz every five minutes, and the evoked response was recorded (and averaged into a single data point per five minute interval). A stable baseline recording lasting 30-60 minutes was obtained before adding drugs to the bath. A low-pass filter was set at

20 kHz, and no high-pass filter was used. Recordings were digitized at 10–20 kHz.

Patch-Clamp

In addition to suction electrode experiments, we collected six intracellular recordings from three animals, using an identical stimulation protocol as described above. These recordings allowed us to correlate our evoked extracellular suction electrode recordings with their intracellular correlates.

For patch-clamp experiments, individual slices were transferred to a recording chamber and were visualized using a Leica DM6000 FS microscope (Leica Microsystems Inc., Bannockburn, IL) as described previously [113]. Slices were continuously perfused by gravity-fed oxygenated 32° C aCSF at a flow rate of 2–3 ml/ min. Thin-walled borosilicate glass patch electrodes (WPI, Sarasota, FL) were then used to acquire whole-cell patch-clamp recordings of projection neurons in the BLA. Electrodes had a resistance of 4–6 MΩ, were filled with a "patch solution", containing (in mM): K+-gluconate (130), KCl (2), HEPES (10), $MgCl_2$ (3), K-ATP (2), Na-GTP (0.2), and phosphocreatine (5), adjusted to pH 7.3 with KOH, and having an osmolarity of 280–290 mOsm. BLA principal neurons were identified according to their characteristic size, shape, and electrophysiological properties [52, 371]. We then performed an identical stimulation protocol as described above, using a tungsten stimulation electrode on the external capsule for at least 20 minutes.

Data acquisition and analysis were performed using a MultiClamp700B amplifier in conjunction with pClamp 10.2 software and a DigiData 1320A AD/DA interface (Molecular Devices, Sunnyvale, CA). Whole-cell patch-clamp recordings were obtained and recorded voltages were low-pass filtered at 5 kHz and digitized at 10–20 kHz. Pipette offset and capacitance were automatically compensated for using Multiclamp software. Series resistance was compensated for manually, and recordings with series resistances of >30 MΩ were discarded.

4.2.3 Pharmacology

The drugs rolipram (100 μM), 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR, 50 μM), and compound C (5 μM) were used in this study. All drugs were perfused through the aCSF bath, and were therefore applied extracellularly to the BLA. Rolipram was dissolved in DMSO, while other drugs were dissolved in H_2O. DMSO was also added to the aCSF bath (1.33 μL/mL, equivalent to the rolipram condition) during control recordings.

4.2.4 Statistical Analysis

Data were analyzed using a combination of Clampfit 10.3 software (Molecular Devices, Sunnyvale, CA), Graphpad Prism 8 (La Jolla, CA), and custom-written Matlab script (Mathworks, Natick, MA). For all suction electrode recordings, the pre-drug recording (or pre-DMSO recording for control experiments) was subtracted from all post-drug recordings (Figure 4.2-A). The resulting subtraction trace therefore represented the change in the raw signal relative to the pre-drug recording.

After subtraction, a custom Matlab script calculated the total area under the curve (negative or positive) between 7-17 ms after stimulation (Figure 4.2-B). The interval 7-17 ms post-stimulation was selected after visual inspection of the data revealed an effect of drug on this specific period. The total area under the curve from 17-27 ms was also quantified, and served as a "control" interval to test the temporal specificity of the drugs. Finally, these values were analyzed in Graphpad Prism 8 using a combination of one-sample T tests and mixed-effects analyses. For mixed effect analyses, five levels of the factor "Drug" were used (representing Control, Rolipram, Compound C, AICAR, and Rolipram+AICAR), and ten levels of the factor "Time" were used (representing 5-minute intervals after adding drug or DMSO).

4.3 Results

4.3.1 Rolipram Increases Stimulation-Evoked Neuronal Activity

Firstly, the addition of DMSO ("Control") did not alter the area of evoked neuronal activity from 7-17 ms post-stimulation, as control evoked area was not significantly different from zero (t_9=1.09, p=0.30, One Sample T-test). There was a main effect of Drug treatment ($F_{4,19}$=3.30, p=0.03) and Time ($F_{1.92,\ 33.91}$=6.40, p=0.005), but no interaction effect (Drug x Time, $F_{36,159}$=1.41, p=0.08, mixed effects analysis) on evoked activity from 7-17 ms. There was no effect of either Drug ($F_{4,19}$=1.36, p=0.28) or Time ($F_{1.62,\ 28.67}$=2.93, p=0.08, mixed effects analysis) on the area of evoked activity from 17-27 ms post-stimulation, suggesting the effect of Drug specifically affected the neuronal response between 7-17 ms. All further analysis concerns the period from 7-17 ms post-stimulation.

The addition of rolipram (100 μM) also significantly enhanced the negative component of the evoked response relative to control (p<0.0001, Tukey multiple comparisons test, Figure 4.2-A / Figure 4.4-A).

4.3.2 Rolipram-Enhanced Negative Peak Correlates with Evoked Action Potential Firing

To better interpret our extracellular recordings, we collected intracellular patch-clamp recordings from BLA principal neurons. We stimulated the external capsule at the same intensity as above to evoke action potential firing, and compared the timing of intracellular events to the timing of our extracellular evoked responses.

The timing of this negative component relative to stimulation onset correlates with action potential firing in BLA principal neurons (Figure 4.3). We therefore interpret the rolipram-induced enhancement of the negative evoked response as an increase in BLA principal neuron excitability, possibly driven by increased BLA principal

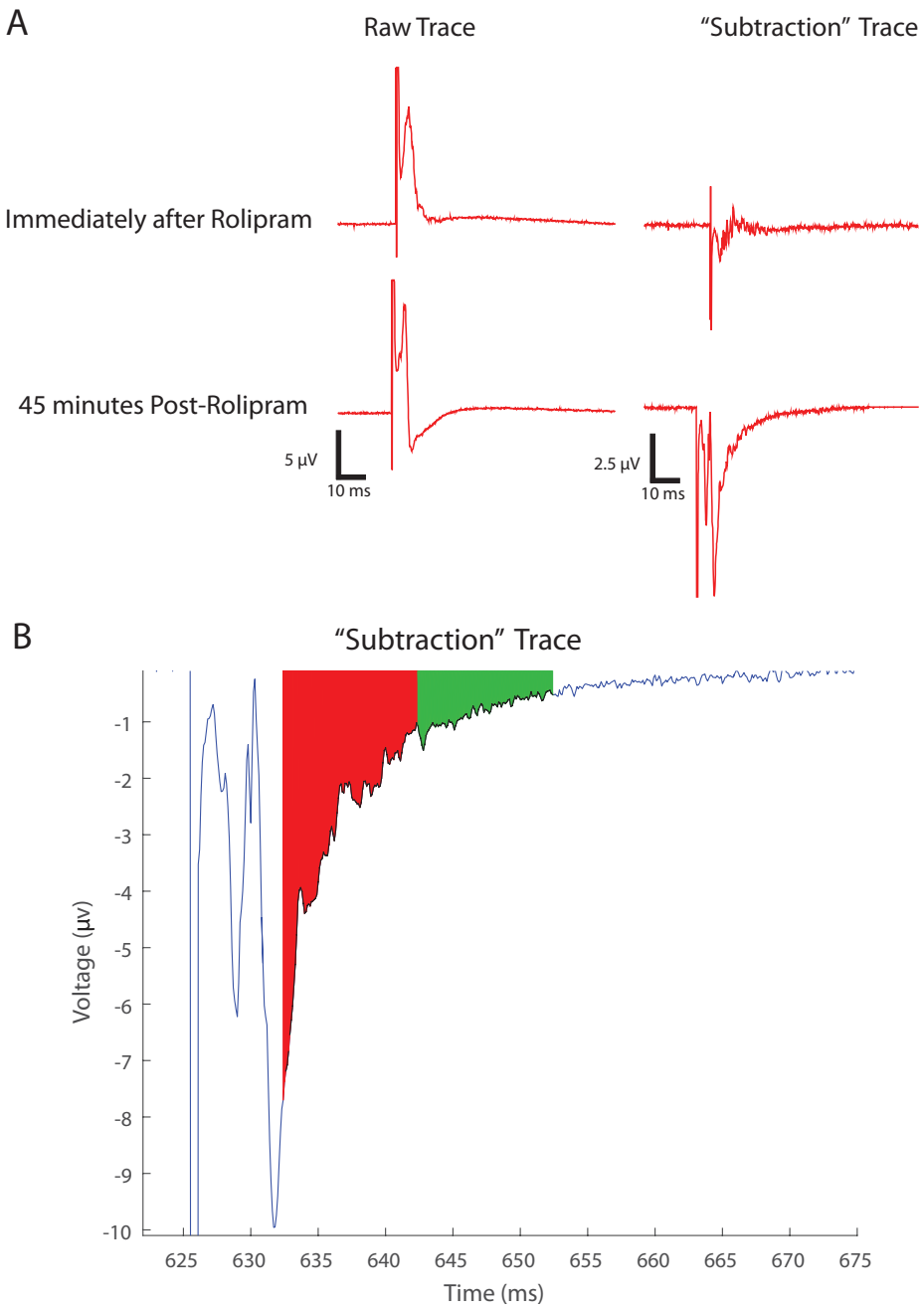

Figure 4.2: Rolipram Increases BLA Evoked Network Activity - Analysis. A) Rolipram (n=6, representative trace shown) enhances a negative peak in the evoked BLA network response. A "control" file (recorded immediately before applying drug to the slice) was subtracted from the raw file (left) to generate a "subtraction" file (right). The subtraction file therefore shows the change in the signal relative to the pre-drug trace. B) Custom Matlab code analyzed each "Subtraction" trace, measuring the area above the curve from 7-17 ms post-stimulation (shown in red). The area above the curve from 17-27 ms post-stimulation was also measured as a control (shown in green).

neuronal recruitment to an excitatory input or increased excitability of individual BLA principal neurons.

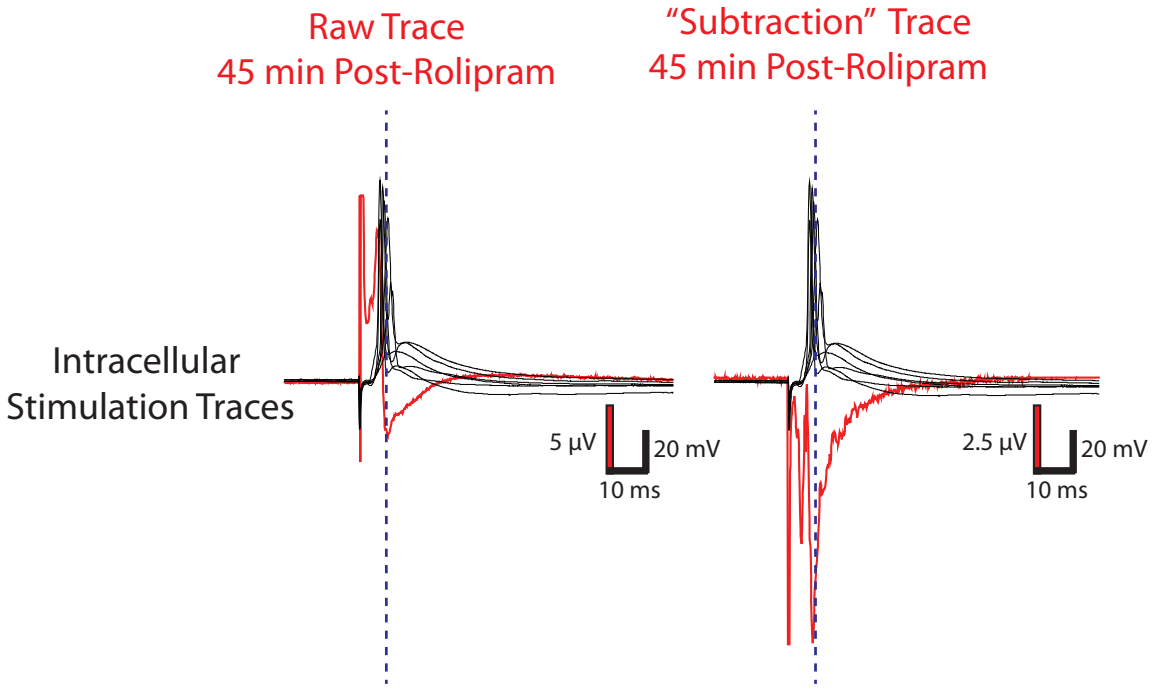

Figure 4.3: Rolipram-enhanced negative peak correlates with action potential firing in BLA principal neurons. Rolipram enhances a negative peak in the suction electrode recordings (red) that correlates with action potential firing in BLA principal neurons during intracellular recordings (black, n=6 cells from 3 animals).

4.3.3 Compound C Mimics Rolipram-Induced Increase in Evoked Stimulation Response

Similar to rolipram, the AMPK inactivator Compound C (5 µM) negatively enhanced the evoked stimulation response relative to control (p=0.05, Tukey multiple comparisons test, Figure 4.4-B). Furthermore compound C's effect did not differ significantly compared to rolipram's (p=0.17). We therefore conclude that compound C produces a similar increase in BLA principal neuron excitability as Rolipram.

4.3.4 AICAR Blocks Rolipram-Induced Increase in Evoked Stimulation Response

AICAR (50 μM) did not significantly change the evoked stimulation response relative to control (p=0.12, Tukey's multiple comparisons test, Figure 4.4-B) and was significantly different relative to rolipram (p<0.001). However, AICAR administered in conjunction with rolipram blocked the effect of rolipram alone on evoked stimulation response (Rolipram+AICAR vs Control, p=0.61; rolipram+AICAR vs rolipram, p<0.001, Figure 4.4-C). Rolipram+AICAR's effect also differed from that of AICAR alone (p=0.006).

4.4 Discussion

4.4.1 Rolipram Increases BLA Excitability

Firstly, our data suggest that rolipram increases BLA excitability in control Sprague-Dawley rats. Rolipram specifically enhanced a negative peak in our suction electrode recordings at 7-17 ms after stimulation, which is the same time post-stimulation that BLA principal neurons fire action potentials (Figure 4.3). We thus interpret this rolipram-enhanced negative peak as increased evoked BLA network excitability. This finding is consistent with previous observations in our lab that rolipram decreases the threshold required for LTP, and increases the startle-response in awake/behaving Sprague Dawley rats (an anxiety-like phenotype linked to BLA hyperexcitability) [113].

We interpret our extracellular recordings and predominately measuring BLA principal neuron activity. Suction electrode recordings are primarily composed of the summed population potentials from the axons and cell bodies within the electrode [419]. Principal neurons make up 80-85% of neurons in the BLA, compose the BLA's only output, and are much larger than GABAergic interneurons [48], making it likely that principal neuron activity would overwhelm GABAergic interneuron activity in

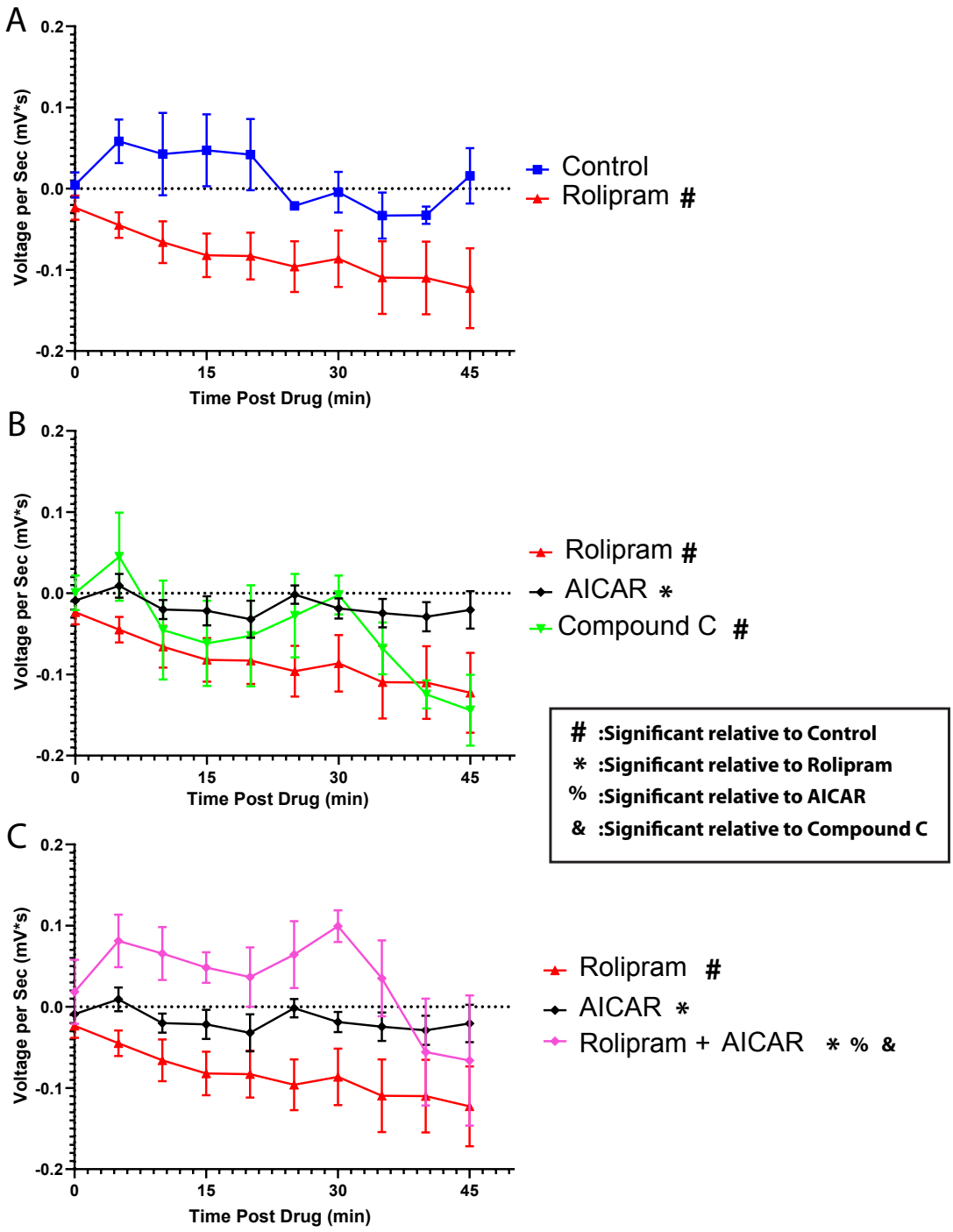

Figure 4.4: Rolipram increases evoked BLA network excitability in an AMPK-dependent fashion. A) Rolipram (n=6) induces a significant increase in negative evoked peak area relative to control (n=3). B) Rolipram's effect is mimicked by Compound C (n=5), but not by AICAR (n=5). C) Rolipram's effect is blocked by simultaneous administration of Rolipram+AICAR (n=5). The effect of Rolipram+AICAR is also significantly different relative to AICAR alone.

our recordings. However, as we recorded extracellularly from a large portion of the BLA, our experiments measure overall network excitability and do not directly distinguish between principal neurons and GABAergic interneurons. It is possible that in addition to affecting principal neurons, Rolipram is also influencing GABAergic interneuronal activity.

Unlike our previous findings [113], our experimental protocol did not use high-frequency stimulation to induce synaptic plasticity in the BLA. Despite this, we still observed increased excitability in the BLA network. This suggests that rolipram also increases BLA excitability through LTP-independent mechanisms. Possible mechanisms include a decrease in AMPK-dependent Kv2.1 potassium currents [336] and ligand-gated K^+-ATP channels [340]. By indirectly inhibiting AMPK, rolipram could decrease these potassium currents, having an excitatory effect on BLA principal neuron activity. Another possibility is that rolipram increases BLA principal neuron recruitment in response to an excitatory stimulus. More detailed experimentation is necessary to identify rolipram's specific mechanism of action in the BLA.

4.4.2 Compound C Mimics Rolipram's Effect

Similar to rolipram, compound C also increases evoked BLA excitability. As compound C is an AMPK inactivator, these findings support the idea that inhibiting AMPK increases the evoked network excitability of the BLC. Furthermore, the finding that compound C and rolipram have similar effects is consistent with the idea that rolipram acts to increase BLA excitability by indirectly inhibiting AMPK activity.

However, the results concerning compound C should be interpreted with caution. Compound C does not specifically inhibit AMPK activity, and in fact inactivates several receptor tyrosine kinases at similar or greater potencies than AMPK [420]. Therefore while our compound C results are consistent with our hypothesis that rolipram acts by inhibiting AMPK activity, we cannot rule out the possibility that

there are contributions from AMPK-independent mechanisms for rolipram and compound C. The interpretation of our compound C data is therefore dependent on the other experiments done in this study (see below).

4.4.3 AICAR Blocks Rolipram's Effect

We hypothesized that AICAR (which is converted to ZMP, an AMP analog) would increase AMPK activity and decrease BLA excitability. Contrary to our hypothesis, administration of AICAR alone did not decrease BLA excitability. We also hypothesized that administering AICAR simultaneously with rolipram would block the rolipram-induced increase in BLA excitability. Consistent with our hypothesis, simultaneous administration of rolipram+AICAR did not increase BLA excitability. In fact, rolipram+AICAR administration trended in the opposite direction as rolipram alone, although this trend was not significant.

Together, our AICAR and compound C experiments present an interesting puzzle: compound C (an AMPK inactivator) increases BLA excitability, but AICAR (an AMPK activator) has no effect. There are two possible explanations for these findings. Firstly, baseline AMPK activity in the BLA could be relatively high. High baseline AMPK activity would limit the effect of AMPK activators (AICAR), but leave the effect of AMPK inactivators (compound C) intact. Although BLA principal neurons *in vitro* display relatively little activity, AMPK activity could still be high considering the large metabolic demand of neurons in general.

The second interpretation is that AMPK activity has no effect on BLA excitability in control states. As mentioned above, compound C affects the activity of many different kinases. If we assume that compound C's effect on BLA excitability was due to "off-target" effects, then the finding that AICAR does not affect BLA excitability might suggest that AMPK was not an important regulator of neuronal excitability.

However, our results concerning simultaneous rolipram+AICAR administration

make the first possibility (high baseline AMPK activity) more likely than the second (that AMPK does not affect BLA activity). AICAR is widely used as an AMPK activator, and has fewer reported off-target effects than compound C. One study did identify that AICAR suppresses the mTOR pathway independently of AMPK, however the same study found that compound C had the same effect, which does not explain why AICAR and compound C behaved differently in this study [421]. It is therefore likely that AICAR blocked rolipram's effect by increasing AMPK activity, suggesting that rolipram also acts through AMPK. Because compound C mimicked rolipram, it is also likely that compound C's effect on BLA excitability was AMPK-dependent.

4.4.4 Overall Conclusions

Collectively, the findings of this study are as follows:

- Rolipram increases BLA evoked network activity
- Compound C similarly increases BLA activity
- AICAR alone has no effect on BLA activity
- AICAR blocks Rolipram-induced increases in BLA activity

The most parsimonious explanation of these findings is that (1) Rolipram and compound C inhibit AMPK activity, thus increasing BLA excitability; (2) AICAR blocks rolipram's effect by increasing AMPK activity; and (3) AICAR alone has no effect on BLA excitability because AMPK activity is normally high in the control amygdala.

Overall, these data suggest that rolipram increases BLA excitability in control Sprague Dawley rats by inhibiting AMPK. To our knowledge, this is the first study identifying AMPK as an important regulator of BLA activity. These data are consistent with previous observations that in control rats, rolipram decreases LTP threshold

in the BLA (increasing excitability) and increases anxiety-like behavior [113]. However, in humans rolipram is a rapidly acting antidepressant [347–349], and successful treatment with most antidepressants result in *decreased* BLA excitability [16]. It is possible that the AMPK system is dysregulated in depressed patients such that rolipram has a different effect in the depressed BLA relative to control. Indeed, AMPK activation in the hypothalamus increases AgRP neuron activity, suggesting it is possible for AMPK's role to switch from inhibitory to excitatory. If such a "switch" occurred in MDD, reducing AMPK activity with rolipram might indeed normalize BLA hyperexcitability. This idea will be explored in greater detail in Chapter 5. Overall, these data collectively implicate AMPK as an important regulator of BLA excitability, and suggest manipulating AMPK activity could be an effective strategy for future rapidly-acting antidepressants.

Chapter 5 General Discussion and Future Directions

The above experiments set out to test the role of AMPK regulating BLA excitability in control rats and in a rat model of metabolic syndrome / depression (a high-fructose diet; HFrD). Firstly, we set out to replicate the anxiety- and depressive-like behavior in rats fed a HFrD [313]. Once we replicated the HFrD-induced behavioral phenotype, we planned on manipulating the BLA AMPK signaling cascade in both control and HFrD rats and characterizing any differences.

We found in Chapter 2 that contrary to previous studies, a high-fructose diet (HFrD) did not induce anxiety- or depressive-like behavior in Sprague Dawley rats. In Chapter 3, we found that a HFrD also did not induce anxiety- or depressive-like behavior in Wistar rats, suggesting that the variability in the HFrD-induced phenotype was due to environmental differences instead of strain differences (although these results were statistically underpowered). Because we could not replicate the depressive-like behavioral phenotype, we could not use the HFrD as a model of depression as planned, and we never tested the effect of manipulating the AMPK signaling cascade in HFrD-fed rats. The results described in Chapter 2 were published in the journal *Brain Behavioural Research*, and the contribution of those experiments to the field will be discussed below.

Although we could not characterize the relationship between a HFrD, AMPK, and a depressive-like phenotype, we did examine the role of AMPK in the control BLA. In Chapter 4, we tested the effect of the antidepressant rolipram on BLA excitability. We observed that rolipram increased BLA excitability, which was consistent with previous studies in our lab using rolipram in control rats [113]. Furthermore, we tested whether rolipram's effect on BLA excitability was mediated through AMPK

activity. We mimicked rolipram's effect with the AMPK inactivator compound C, and blocked rolipram's effect with the AMPK activator AICAR. These experiments therefore identified AMPK as an important regulator of neuronal excitability in the BLA, and implicated AMPK as a potential antidepressant target. The importance of the experiments in Chapter 4 and the implications of this dissertation as a whole will also be discussed below.

5.1 The Effects of a High-Fructose Diet are Variable

Unlike previous studies [313] we did not observe an anxiety- or depressive-like phenotype in either Sprague Dawley or Wistar rats. There are several possibilities that could explain our results. Firstly as outlined in Chapter 2, the discrepancy between our results and previous studies could be explained by a genetic strain difference between Sprague Dawley and Wistar rats. There is evidence of metabolic differences between the two strains which could reduce Sprague Dawley rats' susceptibility to a HFrD relative to Wistar rats [375, 376, 378, 379]. Another factor that could explain the discrepancy between our and previous results is an environmental difference between laboratories, which also has the potential to profoundly affect behavior [392–394]. The experiments described in Chapter 2 alone do not provide sufficient evidence to support one explanation over the other.

However, we also examined the effects of a HFrD using Wistar rats in Chapter 3, and still did not observe any behavioral differences between control and HFrD groups. If Wistar rats were more vulnerable than Sprague Dawley rats to a HFrD, we expected our HFrD-fed Wistar rats to show increased anxiety- and depressive-like behavior as previously reported [313]. However, our HFrD-fed Wistar rats once again showed equal anxiety- and depressive-like behavior as control rats. These results suggest that environmental differences between laboratories, rather than genetic strain differences,

were responsible for our inability to replicate the expected HFrD-induced anxiety- / depressive-like phenotype. However we must interpret our results in Chapter 3 with caution, as only two litters of rats were assigned to each diet, for an n=2. The Chapter 3 results are therefore underpowered, and there is a chance we are committing a type II (false-negative) error.

It is also possible that the effects of a HFrD are variable between individual Sprague Dawley and/or Wistar rats. For example, some individual rats may consume larger amounts of the HFrD than others, and therefore be subjected to a larger metabolic challenge. One limitation of our studies in Chapters 2 and 3 is that we did not control for this potential individual variation. We measured the total food consumed per cage per day, and therefore did not keep track of calories consumed by individual rats. Our studies therefore do not assess how the "dose" of HFrD affects behavior or BLA electrophysiology. Future studies should keep this potential source of variability in mind.

Despite our difficulties replicating the results of previous studies, we nevertheless published our results described in Chapter 2. In doing so, we hope to contribute to a much larger discussion about replicability and reproducibility in science. A 2016 survey in *Nature* found that more than 75% of biological researchers had tried and failed to reproduce another scientist's experiments, and 60% had failed to reproduce their own experiments [422]. However most of these scientists did not go onto publish their negative data, having little incentive to do so. The same survey found that only 13% of scientists had successfully published a failed replication, and 10% of survey participants were unable to publish a failed replication even after repeated attempts.

Negative results are critical to scientific research. Without publishing negative results, scientists will inevitably spend unnecessary time and money performing experiments others have already attempted. Additionally, negative results can be scientifically relevant in and of themselves. Understanding when a HFrD *will not* influence

behavior is equally important as understanding when it will. Only by understanding both positive and negative aspects of HFrD administration can we hope to understand the underlying biology and eventually inform clinical approaches. The importance of negative results extends far beyond biology, affecting every field of science. Encouragingly, scientists increasingly realize the importance of publishing negative results and journals are increasingly willing to publish them [423–425].

5.2 The Role of AMPK in the Basolateral Amygdala

As described in Chapter 4, administering compound C increased BLA excitability in control rats. Administration of AICAR alone did not affect BLA excitability, however AICAR blocked rolipram's effect on BLA excitability. The most parsimonious explanation for these findings is as follows: (1) Rolipram increases BLA excitability by indirectly inhibiting AMPK activity, (2) Compound C mimics rolipram by inhibiting AMPK, (3) AICAR blocks rolipram's effect by activating AMPK, and (4) AICAR does not reduce BLA excitability, due to naturally high baseline AMPK activity.

To our knowledge, this is the first study examining the role of AMPK in the amygdala, and one of only a few examining the role of central AMPK in control conditions outside the hypothalamus. Although well characterized as a regulator of neuronal excitability in the hypothalamus, elucidating AMPK's role in the BLA not only allows deeper exploration the relationship between energy state and normal/diseased BLA function, but also calls attention to the likely importance of AMPK in other brain structures.

Although compound C did increase BLA excitability, the effect was smaller than anticipated, given AMPK's profoundly influential role on cellular metabolism. Furthermore, AICAR did not affect BLA excitability when administered alone. It is likely that AMPK is only one regulator of neuronal activity among many. Lacking

long-term energy reserves, the brain must be able to dynamically adjust its activity in response to energy availability. However, it would be seriously maladaptive if the BLA's activity (or the activity of other brain areas) was overly suppressed by AMPK during low energy states, impeding an organisms ability to learn and behave. Instead of shutting down entire networks, AMPK's function in the brain during low energy states is likely to more subtly influence network activity (and therefore behavior) by prioritizing feeding and food-acquisition behaviors. AMPK's ability to integrate large amounts of metabolic information and influence feeding behavior via hypothalamic activity has already been well characterized [258], and it would make sense if AMPK acted similarly in other brain regions such as the BLA.

Supporting this hypothesis, energy state has also been shown to affect BLA-dependent reward learning. A recent *Nature Neuroscience* study [426] found that at baseline (having eaten only 4 hours before), rats would engage in a low level of lever pressing in exchange for a sucrose reward. However the next day the rats were reexposed to sucrose either when "sated" (after 4 hours of food deprivation) or when "hungry" (after 20 hours of food deprivation). The following day, the rats were placed back into the operant chambers for a probe test while "sated". Rats who had been reexposed to sucrose while "hungry" showed significantly elevated lever pressing compared to rats reexposed to sucrose while "sated", despite both groups of rats being sated during the probe test itself. The "hungry" group therefore assigned a higher reward value to sucrose than the "sated" group. Furthermore, the authors show that both encoding and retrieving the memory of sucrose's "reward value" was BLA-dependent. The BLA therefore assigns a higher reward value to sucrose when hungry, i.e. during the same low-energy conditions that activate AMPK in the hypothalamus [258]. The potential role of AMPK in this energy state-dependent reward learning remains unknown, however AMPK is one possible mechanism by which energy state could influence BLA excitability during the above experiments.

In conclusion, our experiments suggest that AMPK is an important regulator of neuronal activity in the BLA. However, AMPK's influence is not so powerful as to dysregulate the entire BLA network. Instead, it is more likely that AMPK acts to integrate metabolic information into the BLA's associative learning process by subtly adjusting synaptic plasticity [113] and intrinsic neuronal excitability.

5.3 Potential Solutions to the "Rolipram Paradox"

The effects of rolipram (a fast-acting antidepressant) on BLA excitability were blocked by administration of AICAR (an AMPK activator) and mimicked by compound C (an AMPK inactivator). Together, these are strong indications that rolipram's enhancement of BLA excitability was mediated (at least in part) through decreased AMPK activity.

These findings support the idea that AMPK is a potential target for rapidly-acting antidepressants. As outlined in Section 1.3.3, traditional antidepressants such as SSRIs and SNRIs indirectly influence the AMPK signaling cascade, whereas rapidly-acting antidepressant drugs like ketamine and rolipram have more direct effects on AMPK activity. Our findings are therefore consistent with the hypothesis that AMPK is the "final common pathway" of MDD.

However, rolipram's effect on BLA excitability appears paradoxical. The BLA is hyperexcitable in MDD patients, and this hyperexcitability normalizes with successful pharmacotherapy [16]. As an effective and rapidly-acting (albeit badly tolerated) antidepressant, rolipram would be expected to decrease BLA excitability. However in the above experiments, rolipram increases BLA excitability in control animals. This finding is consistent with previous work in our lab showing that administration of rolipram into the BLA decreased the threshold required for LTP (increasing excitability) and enhanced the startle response in rats (an anxiety-like phenotype) [113].

As mentioned previously, there are at least two possible solutions to the "rolipram paradox": (1) Rolipram's antidepressant effect is dependent on increasing the activity of brain areas hypoactive in MDD (such as the dorsolateral prefrontal cortex [350–352]) or (2) The AMPK system is dysregulated in depressed patients such that rolipram has a different effect on the depressed BLA relative to control.

Having characterized rolipram's effect in control conditions, the next step of resolving the "rolipram paradox" is to characterize rolipram's effect on BLA excitability in the "depressed" condition. The model of depression we selected for use in these experiments was the high-fructose diet (HFrD) rat model. However, because we did not observe an anxiety- or depressive-like phenotype in our HFrD-fed rats, we were unable to explore the AMPK system in the "depressed" condition in this set of experiments. One possible direction for future studies is to build on our results by exploring rolipram's effect on BLA excitability and behavior in other rat models of depression, such as the chronic mild stress model or social defeat model [427].

Rolipram's antidepressant efficacy might also rely on increased cAMP signaling. Rolipram is a PDE4 inhibitor, and the function of PDE4 is to convert cAMP to AMP. Rolipram thus increases levels of cAMP and decreases levels of AMP (indirectly inactivating AMPK). We blocked rolipram's effect on BLA excitability by activating AMPK, suggesting that AMPK inhibition is necessary for rolipram to increase BLA excitability. However, it is still possible that increased cAMP activity is necessary (but not sufficient) for rolipram's effect on BLA excitability. It is also possible that brain regions outside the BLA respond predominately to rolipram's effect on cAMP signaling rather than AMPK signaling.

5.4 Future Directions

To extend the findings described in this dissertation, future researchers should focus on three major directions: (1) To characterize AMPK's role in the "control" BLA

in greater detail; (2) To examine how metabolic stress and/or depression could dysregulate the AMPK system, and (3) To elucidate AMPK's role in regulating neuronal activity in other non-hypothalamic brain regions.

Much of AMPK's role in the control BLA remains to be characterized. Firstly, it is unknown what role AMPK plays in different BLA cell-types. We interpret our suction electrode recordings in Chapter 4 as being mostly composed of currents from BLA principal neurons. However, these recordings do not distinguish between principal neurons and interneurons, and so the respective roles of rolipram and AMPK activation/inactivation in these different cell populations remain unknown. Future studies could use genetic manipulations to express either constitutively active or kinase-dead AMPK in either principal neurons or interneurons, examining the role of AMPK activity in either population. Intracellular patch-clamp recordings could also be done to characterize AMPK's role in different cell-types. Secondly, studies should examine the role of different AMPK isoforms in the BLA and other brain regions. There are 12 possible isoforms of AMPK based on its α, β, and γ subunits. These combinations differ in their cell-type expression [263], subcellular localization [263, 428, 429], cross-talk with other signaling pathways [264], and critically their response to AMP binding [265]. AMPK isoforms might also differ in their sensitivity to direct AMPK activators and inactivators such as compound C and AICAR, and also respond differently to rolipram-induced changes in AMP levels. Future studies should take this important source of variation into account.

Next, future studies should examine the AMPK system in the "depressed" condition. We attempted to examine the role of BLA AMPK activation/inactivation in "depressed" rats using a HFrD paradigm. However, we did not observe an anxiety- or depressive-like phenotype in our HFrD rats, and so we were unable to complete these experiments. Future studies could examine the role of AMPK activation/inactivation in other rodent models of depression. Additionally, the effect of rolipram on BLA

excitability in a rodent model of depression should be characterized, in order to shed light on the "rolipram paradox" and determine rolipram's antidepressant mechanism. These experiments could identify novel antidepressant targets (such as AMPK) for future antidepressant therapies.

Finally, future studies should also examine AMPK's role in regulating the activity of other brain regions. AMPK is expressed widely throughout the brain, and there is no reason to suspect AMPK's role in regulating neuronal activity is restricted to only the hypothalamus and BLA. Furthermore as AMPK's role in other brain regions becomes increasingly clear, it is important to characterize the AMPK system in both healthy and diseased states.

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