From the Gut to the Brain:
Hormonal Regulation of Brain Dopamine Homeostasis and Cocaine Reward

By

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Dedicated to my parents, Colleen and Jay,

and my husband, Daniel, for all their love and support.

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LIST OF ABBREVIATIONS

2-AG	2-arachidonylglycerol
3-MT	3-Methoxytyramine
5-HT	Serotonin
6-OHDA	6-hydroxydopamine
AA	Arachidonic Acid
AADC	Aromatic amino acid decarboxylase
ACSF	Artificial cerebrospinal fluid
ADHD	Attention Deficit Hyperactivity Disorder
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	AMPA receptor
AMPH	Amphetamine
ANOVA	Analysis of variance
ARH	Arcuate nucleus of the hypothalamus
AUC	Area under the curve
AUD	Alcohol use disorder
AUDIT	Alcohol Use Disorders Identification Test
BAC	Bacterial artificial chromosome
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CB1R	Cannabinoid 1 receptor
CBT	Cognitive behavioral therapy
CCK	Cholecystokinin
CNS	Central nervous system
COMT	Catechol-O-methyl transferase

CPP	Conditioned place preference
CREB	cAMP response element binding protein
D1R	Type I dopamine receptor
D2R	Type II dopamine receptor
DA	Dopamine
DAG	Diacylglycerol
DAGL	DAG lipase
DAT	Dopamine transporter
dDAT	drosophila DAT
hDAT	human DAT
DIO	Diet-induced obesity
DMEM	Dulbecco's modified Eagle's medium
DMH	Dorsomedial nucleus of the hypothalamus
DMSO	Dimethyl sulfoxide
DOPA	3,4-Dihydroxyphenylalanine
DOPAC	3,4-Dihydroxyphenylacetic acid
DPP4	Dipeptidyl peptidase-4
DS	Dorsal striatum
DSM	Diagnostic and Statistical Manual of Mental Disorders
ECL	Enhanced chemiluminescence
EPSC	Excitatory postsynaptic current
ERK	Extracellular signal-regulated kinase
Ex-4	Exendin-4
Ex-9	Exendin-(9-39) amide
FDA	U.S. Food and Drug Administration
fMRI	Functional magnetic resonance imaging

GABA	γ-aminobutyric acid
GB-D	Gallbladder to duodenum diversion
GB-IL	Gallbladder to ileum diversion
GFP	Green fluorescent protein
GLP-1	Glucagon-like peptide 1
GLP-1R	GLP-1 receptor
GPCR	G protein-coupled receptor
H2DCFDA	2.7-dihydrodichlorofluorescein diacetate
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HVA	Homovanillic acid
HWM	Hidden water maze
ICSS	Intracranial self-stimulation
IHC	Immunohistochemistry
i.p.	Intraperitoneal
ISH	In-situ hybridization
IsoK	Isoketal
KA	Ketoaldehyde
KH	Krebs-Henseleit buffer
K_M	Michaelis constant
LH	Lateral hypothalamus
LS	Lateral septum
LTD	Long term depression
LTP	Long term potentiation
MAGL	Monoacylglycerol lipase
MAO	Monoamine oxidase

MFB	Medial forebrain bundle
MSN	Medium spiny neuron
NAc	Nucleus accumbens
NE	Norepinephrine
NEDD4-2	neural precursor cell expressed, developmentally downregulated 4-2
NMDA	N-methyl-D-aspartic acid
NMDAR	NMDA receptor
NO	Nitric oxide
NOS	NO synthase
NTS	Nucleus of the tractus solitaries
OCA	Obeticholic acid
OF	Open field
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
PF	Pair-fed
PI3K	Phosphoinositide 3-kinase
PICK1	Protein that interacts with C-kinase 1
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PFC	Prefrontal cortex
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
p.o.	By mouth
PP1	Protein phosphatase 1
PVDF	Polyvinylidene difluoride

PVN	Paraventricular nucleus of the hypothalamus
PYY	Peptide YY
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RYGB	Roux-en-Y gastric bypass
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SN	Substantia nigra
TGR5/Gpbar1	Takeda g-protein-coupled receptor/g protein-coupled bile acid receptor 1
TH	Tyrosine hydroxylase
THC	Tetrahydrocannabinol
TST	Tail suspension test
VMAT	Vesicular monoamine transporter
Vmax	Maximal velocity
VMH	Ventromedial nucleus of the hypothalamus
VNTR	Variable number tandem repeat
VTA	Ventral tegmental area

CHAPTER I

INTRODUCTION

Preface

The following introduction will attempt to provide the necessary foundational knowledge with which to interpret the original research described herein. It is not meant to be a comprehensive review of all mentioned topics. Rather, it attempts to highlight concepts that underlie our hypotheses and experimental design and inform interpretations of our data. Important details relevant to the interpretation of data, if not discussed here, are described within the introductions to each of the results sections.

The topics I have chosen to discuss here include the processing of dopamine (DA) in the brain and its role in the brain's reward circuitry. I pay special attention to describing the DA transporter (DAT) and its regulation, as many of our studies focus on the transporter as a major player in regulating DA reward circuitries and the response to cocaine. From here, I present a high-level view of reward and addiction, focusing in more detail on psychostimulants and food reward as well as the overlap between food and drug reward. Finally, I discuss how hormones associated with regulating feeding behavior and metabolism have targets within the brain's reward system and present evidence supporting the idea that these hormones may modulate reward for drugs of abuse.

Introduction to dopamine (DA)

Neurotransmitter systems in the brain allow for chemically-mediated communication between neurons and encode diverse neuronal and behavioral responses. DA (3-hydroxytyramine) is a neurotransmitter responsible for regulating movement, mood, cognition, and motivated behaviors. Dysfunctions in DA signaling have been implicated in Parkinson's disease, major depressive disorder, schizophrenia, obesity, and addiction, among other disorders.[1-5] DA is produced primarily in midbrain neurons whose cell bodies are located in the substantia nigra pars compacta (SN) and ventral tegmental area (VTA), while a smaller amount of DA is produced by the hypothalamus. This process involves, first, the conversion of tyrosine to L-DOPA by the enzyme tyrosine hydroxylase (TH) (**Figure 1**). This is the rate-limiting step in DA synthesis. L-DOPA is then converted to DA by aromatic L-amino acid decarboxylase (AADC). Of note, DA can be metabolized to norepinephrine (NE) by DA β-hydroxylase. Together, DA and NE make up a class of neurotransmitters called catecholamines.

DA produced by the cells residing in the SN is released from terminals located in the forebrain dorsal striatum (DS). This pathway makes up the nigrostriatal circuit, and is primarily involved in the control of movement, but notably also plays roles in motivated feeding behavior and habit formation.[6,7] DA produced by neurons originating in the VTA is released at terminals in both the ventral striatum, also known as the nucleus accumbens (NAc), as well as the prefrontal cortex (PFC). These projections are known as the mesolimbic and mesocortical systems, respectively. The final DA projection consists of the tuberoinfundibular system which projects from the hypothalamus to the pituitary and regulates prolactin secretion. A summary of the DA circuitry can be found in **Figure 2** (tuberoinfudibular pathway not shown).

DA is generated by enzymatic processes occurring in the cell's distal terminals. Here, DA is taken up into vesicles by the vesicular monoamine transporter (VMAT), primarily the

VMAT2 isoform in brain (Figure 1). This transporter actively concentrates DA in the vesicle using energy harnessed from moving protons from the acidified vesicle to the more basic cytosol. Increasing evidence also suggests that the neurotransmitters glutamate[8] and γ-aminobutyric acid (GABA)[9] may be co-released from midbrain DA-producing neurons. The functional implications of this finding are still being investigated, but this suggests that neurotransmission *via* DA neurons may possess more complex signaling modalities than previously believed.

Following the accumulation of DA into vesicles, release of DA may occur with appropriate neuronal excitation. Neuronal depolarization following an action potential drives the activation of voltage-gated calcium channels in neuronal terminals. These channels allow for calcium ion influx into the terminals. Calcium ions, in turn, trigger the release of vesicles containing DA into the extracellular space.

DA release is known to occur by either tonic or phasic stimulation. Under tonic stimulation, DA is continuously released at low levels. Tonic DA release sets the background level of DA in the brain, which is believed to be an important contributor to a number of traits and disorders, including reward sensitivity, impulsivity, schizophrenia, and depression.[10-12] Tonic DA release is driven primarily by internal pacemaker currents.[13] On the other hand, highly relevant external stimuli may drive trains of action potentials *via* stimulation by presynaptic afferents coming from cortical and subcortical areas.[14-16] This results in phasic firing patterns which acutely elevate levels of synaptic DA. Phasic DA levels are responsible for immediate behavioral reactions to salient environmental stimuli.

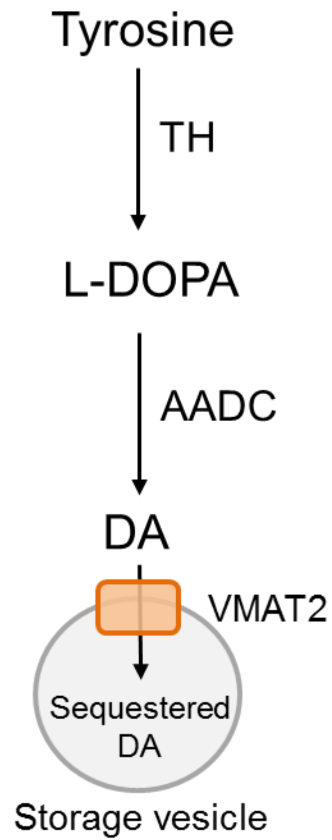

Figure 1. *The DA synthesis and storage pathway*. The rate-limiting step in DA synthesis is the conversion of tyrosine to L-DOPA using the enzyme TH. L-DOPA is subsequently converted to DA by the enzyme AADC. DA is packaged into vesicles by VMAT2. Adapted from [17].

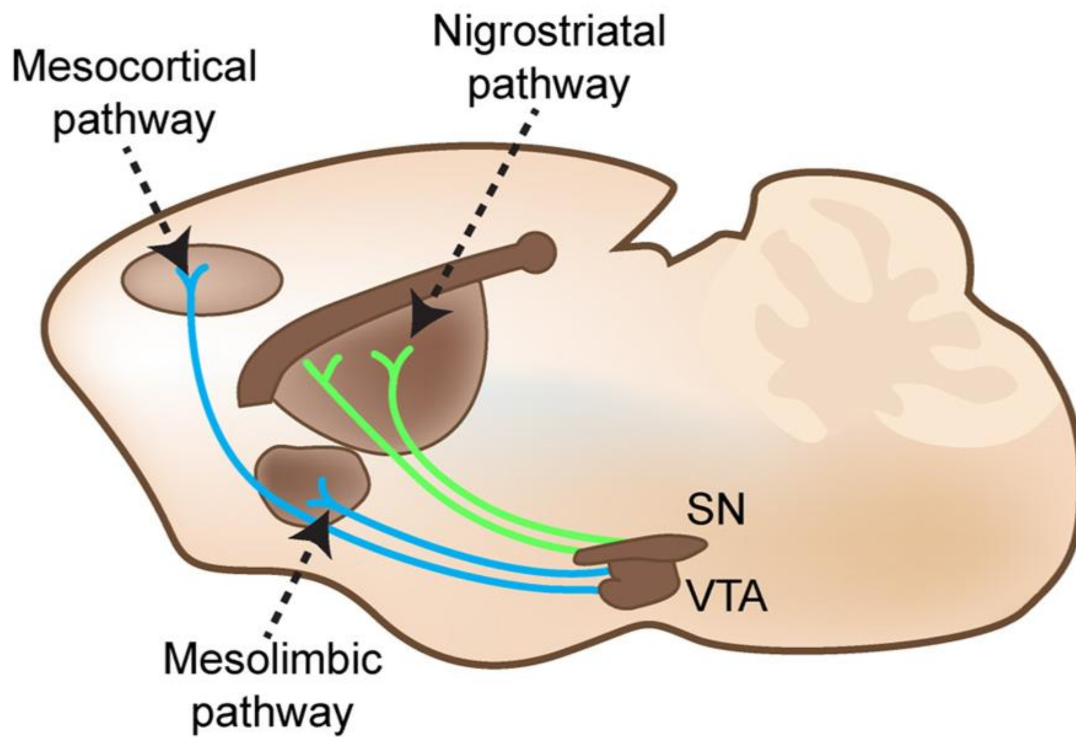

Figure 2. *Sagittal depiction of nigrostriatal, mesocortical, and mesolimbic pathways in the adult mouse brain*. Projections from the SN (pars compacta) to the DS make up the nigrostriatal pathway (green). The mesolimbic pathway and mesocortical pathway (blue) consist of DAergic projections from the VTA to the NAc and medial frontal cortex, respectively. Tuberoinfundibular pathway not shown. Adapted from [18].

Introduction to DA receptors

DA signals through two classes of g protein-coupled receptors (GPCRs): D1-like DA receptors and D2-like DA receptors. D1-like receptors include the D1 and the D5 receptors, with D1 being the most abundant in the central nervous system (CNS). D1-like receptors are primarily Gs-coupled GPCRs, meaning that they activate stimulatory g proteins that allow for the upregulation of cyclic adenosine monophosphate (cAMP) signaling in the cell. D2-like receptors, on the other hand, include receptors D2, D3, and D4, and couple to the inhibitory g protein, Gi, and inhibit the formation of cAMP. D2 receptors are the most abundant of the D2-like DA receptors and can be found on the postsynaptic membrane as well as on presynaptic terminals. These presynaptic autoreceptors are a shorter isoform of the D2 receptor and act to provide feedback regarding extracellular DA levels and regulate DA production and release.

Importantly, because DA signals through metabotropic g protein cascades rather than ligand-gated ion channels, DA neurotransmission is slow and insufficient to drive postsynaptic neuronal firing on its own. Rather, DA is thought of as a modulatory neurotransmitter because it modulates inputs from fast ionotropic GABAergic and glutamatergic inputs.

The DA transporter (DAT)

Extracellular, synaptic DA levels and DA neurotransmission are regulated not only by vesicular release, but also by mechanisms meant to terminate the DA signal. DA is degraded by two enzymes: monoamine oxidase (MAO) and catechol-o-methyltransferase (COMT). MAO is located intracellularly within the presynaptic neuron, while COMT is found within the synaptic cleft. Despite the presence of degrading enzymes, the primary means for DA clearance at the synapse is by active reuptake through the DA transporter (DAT; *SLC6A3*). DAT protein is functionally expressed on the cell surface in the perisynaptic space where it primarily recycles

DA back into the presynaptic cytosol, although the DAT possesses some affinity for NE and 5-HT as well. This process allows the cell to repackage DA into vesicles for later release and, importantly, regulates the duration of extracellular DA signaling.

Recently, DAT from *Drosophila melanogaster* (dDAT) was crystallized in an outward-open conformation bound to the tricyclic antidepressant, nortriptyline, providing greater insight into DAT three dimensional conformations and functional domains.[19] The DAT is a twelve transmembrane domain protein belonging to the *SLC6* family of Na^+/Cl^--dependent transporters. Its N- and C-termini both extend intracellularly and are important sites for regulating DAT function. Helical transmembrane domains 1 and 6 form the core of the transporter; residues in these domains interact with DAT substrates and ions and promote movement of the helical domains during the transport cycle.

DA reuptake is an active process. This is made thermodynamically favorable by the co-transport of Na+ and Cl- ions with their concentration gradient. The rate of reuptake by the transporter is approximately one molecule of DA per second per transporter,[20] a process which is limited by the time to bind DA, 2 Na^+ ions, and Cl^- and then transition from an outward- to an inward-facing conformation. On a larger scale, the efficiency with which a DA signal is terminated is determined by the amount of functional DAT expressed on the plasma membrane. Several factors have been identified as prominent regulators of DA reuptake, including the expression of DAT at the cell surface, enzymatic modification of the DAT, protein-protein or protein-lipid interactions involving the DAT, and finally, the presence of DAT substrates or inhibitors.[21] A summary of some of the mechanisms regulating DAT surface expression can be found in **Figure 3** and these mechanisms are described in greater detail below.

DA uptake is regulated in part by DAT cell surface expression

Of the possible mechanisms for DAT regulation, its surface expression and trafficking is of particular interest. The DAT traffics rapidly between the cell surface and intracellular recycling vesicles, allowing for the tight regulation of extracellular DA levels.[22] This process is highly regulated; the DAT constitutively traffics to and from the cell surface and its surface expression is also responsive to DAT substrates and a number of intracellular signaling kinases.

Newly synthesized DAT is generated in the endoplasmic reticulum. From here, the DAT moves on to Golgi vesicles where it gets glycosylated at several sites on the eventual extracellular face of the transporter.[23,24] Glycosylation is an important feature of DAT processing that allows the DAT to be expressed at the cell surface, although it does not appear to regulate uptake once expressed.[25] Following initial expression, the DAT is constitutively trafficked from the cell surface and may be recycled back to the membrane. Its surface half-life is approximately 13 minutes[26,27] and the basal surface DAT endocytic rate is 3-5% per minute.[22] The constitutive internalization of the DAT is believed to be a clathrin- and dynamin-dependent phenomenon.[28] The DAT is internalized into small-GTPase-expressing vesicles, including Rab-5 and Rab-11,[29,30] which mark a subset of recycling endosomes. DAT protein can be held within these vesicles to rapidly insert into the membrane as needed.

Once at the plasma membrane, DAT expression can be stabilized or destabilized by a number of protein-protein interactions. These proteins include PICK1 (protein that interacts with C-kinase),[31] Hic-5,[32] α-synuclein,[33] flotillin,[34] and even DAT itself, allowing for the formation of DAT oligomers.[25] PICK1, α-synuclein, and DAT oligomerization stabilize DAT at the cell surface, while Hic-5, a potential scaffolding protein, decreases DAT on the plasma membrane when overexpressed. Interestingly, flotillin is a protein which stabilizes the DAT within particular microdomains in the plasma membrane, but also appears to be necessary for PKC-induced

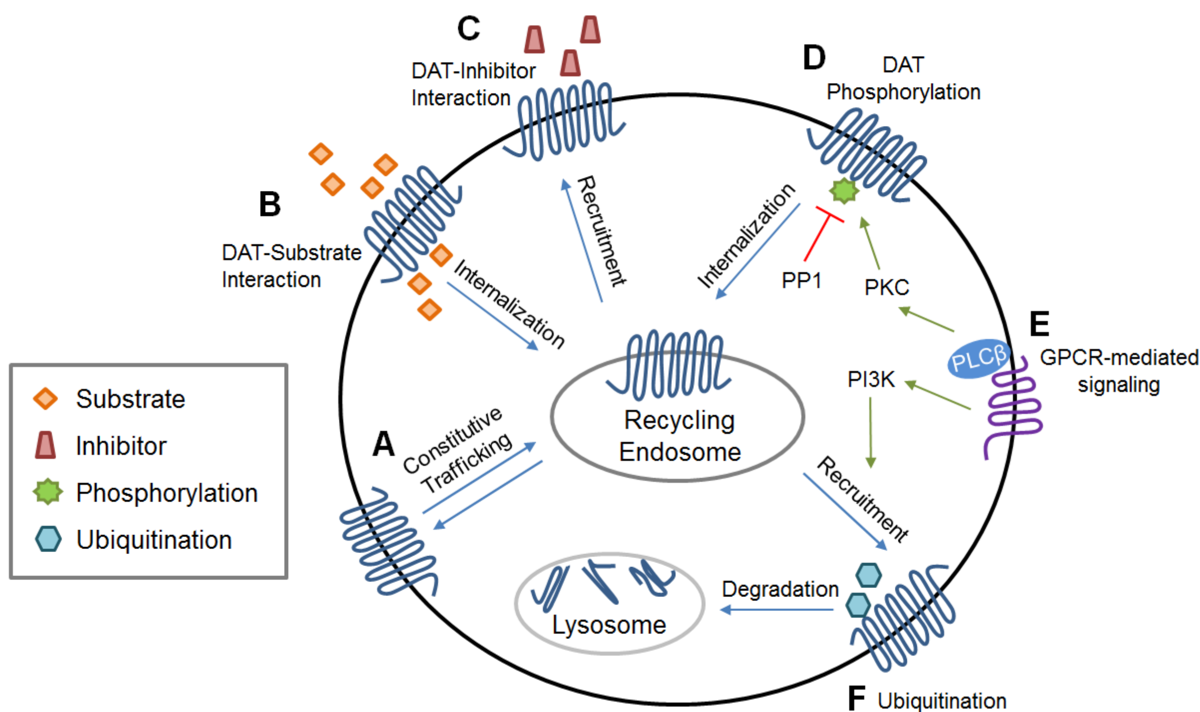

Figure 3. *Factors affecting DAT trafficking and membrane expression.* As described in the text, a number of factors regulate DAT trafficking and expression, including (A) constitutive trafficking, (B) DAT-substrate interactions, as with amphetamine, (C) interactions with cocaine-like inhibitors, (D) the phosphorylation state of DAT, (E) GPCR-induced intracellular signaling cascades, and (F) the ubiquitination state of DAT. Adapted from [21].

DAT internalization, thus playing roles in both the stabilization and destabilization of the DAT under varying conditions.[34] The stability of DAT expression at the cell surface may have powerful implications for disease processes; for example, it has been suggested that PICK1 and α-synuclein could promote toxicity from substrate-induced oxidative stress, leading to conditions such as Parkinson's disease.[33,35,36]

The DAT is also trafficked to and away from the cell surface in response to substrates and inhibitors. The response to substrates is thought to be biphasic in that brief exposure to substrate promotes DAT surface expression while prolonged exposure results in DAT internalization.[22] In heterologous cells and rat striatal synaptosomes, a 30 second to 2 minute exposure to DAT substrates DA or amphetamine rapidly increases DAT surface expression as well as uptake function.[37] Cocaine, a DAT blocker, inhibits this form of substrate-induced trafficking to the plasma membrane. Given that the half-life of DA clearance in the rat striatum is 26-57 seconds,[38] this rapid upregulation of DAT fits within a physiologic time frame for enhanced clearance.

The early increase in DAT surface expression and function may be a response to initially deal with increased substrate burden. However, prolonged exposure of cells to substrate may be toxic, as high levels of cytosolic DA and amphetamine are known to induce oxidative stress.[39] Amphetamine is also able to deplete intracellular DA stores.[40] Thus it is perhaps not surprising that DAT surface expression is downregulated in response to prolonged exposure to substrates, an effect which is also blocked by cocaine.[40,41] Interestingly, cocaine itself increases surface DAT when cells are exposed to it for long periods of time.[41,42] These findings *in vitro* may be paralleled by human positron emission tomography (PET) studies which show that DAT binding is increased in brains from cocaine addicts[43] and decreased in chronic methamphetamine users.[44]

This process of DAT internalization and recycling is, importantly, regulated by posttranslational modifications such that the cell can respond to changing environmental conditions requiring more or less reuptake. These posttranslational modifications include phosphylation and ubiquitination. Kinases regulating DAT trafficking include protein kinase C-β (PKC-β), protein kinase B (PKB, also known as Akt), phosphatidylinositol 3 kinase (PI3K), Ca2+/calmodulin kinase (CAMKII), and extracellular signal-regulated kinases (ERK1/2). PKC-β has been particularly implicated in constitutive trafficking processes. Protein phosphatase 1 (PP1) regulates DAT phosphorylation state and trafficking in the opposite direction.[45] Major sites for DAT regulatory phosphorylation are found on the intracellular face of the transporter, and includes five important serine residues on the N-terminal domain.[46] However, it should be noted that phosphorylation at some of these sites regulate other DAT-mediated processes (e.g. DA efflux) rather than DAT trafficking.[47]

A change in phosphorylation state that ultimately regulates DAT surface expression and function may be induced through signaling cascades initiated by a number of different receptors. The D2 autoreceptor has been well-studied in this regard. D2/D3 receptor agonists increase DAT surface expression and DA uptake, an effect which is g protein-, PI3K-, and ERK1/2-dependent.[48,49] Interestingly, the D2 receptor may interact directly with the DAT.[50] The functional significance of this interaction, however, is unclear since downstream signaling cascades appear to be necessary to affect trafficking. Other receptors have also been shown to influence trafficking. The insulin receptor is a receptor tyrosine kinase expressed throughout the brain, including regions expressing the DAT. Insulin signaling increases DAT surface expression by activating Akt and PI3K. Of note, insulin impairs amphetamine-induced DAT internalization and amphetamine behaviors in hypoinsulinemic rats.[51,52]

The DAT also contains several ubiquitination sites. These are lysine residues targeted by the protein NEDD4-2 (neural precursor cell expressed, developmentally downregulated 4-2),

which contains an E3 ubiquitin ligase.[53] Interestingly, ubiquitination sites on three key N-terminal lysine residues on the DAT may be necessary for PKC-induced internalization.[54] Ubiquitination, or polyubiquitination, likely serves to target the DAT for lysosomal degradation, which may be an additional source of regulated DAT turnover.

Additional mechanisms regulating DA reuptake

There are a number of factors regulating DA uptake function for which DAT surface expression changes have not yet been identified. These include the molecules arachidonic acid (AA) and nitric oxide (NO). AA is a highly diffusible molecule produced through the metabolism of lipid membranes in neurons and other cells. It has been shown to inhibit DA transport and impair binding of a cocaine analog in heterologous cells.[55] Furthermore, inhibitors of AA metabolism inhibit DA uptake while compounds that raise levels of endogenous AA inhibit DA transport.[56] The mechanism behind this effect has been unclear. However, studies have shown that AA can induce a novel cation conductance through the DAT (which is blocked by cocaine)[57] and that AA inhibition of DAT is not PKC-dependent.[55] NO is another molecule produced by neurons that can act as a neurotransmitter. NO exists as a gas and can therefore act across membranes and across synapses. It has been shown that inhibition of the production of NO by NO synthase (NOS) reduces DA levels in the striatum[58] and that NO itself inhibits DAT-mediated DA uptake.[59] These findings support the idea that NO increases tonic DA levels in the striatum by exerting inhibitory influence over the DAT. That diffusible compounds like AA and NO can affect DAT function further suggests that the DAT is highly susceptible to its local microenvironment.

The importance of DAT

The importance of DAT in regulating synaptic DA levels is highlighted by the fact that dysfunctional DAT has been strongly linked to a number of human diseases.[60] Deficiency of functional DAT can be caused by rare mutations at particular sites on the DAT.[61] In humans, these mutations can lead to a syndrome called DAT deficiency syndrome, also known as infantile parkinsonism-dystonia, characterized by generalized muscle dystonia, bradykinesia, and postural instability, among other motor deficits.[62] DAT mutations have also been linked to bipolar disorder, autism, and attention-deficit hyperactivity disorder (ADHD) in humans,[63,64] pointing to DAT's relevance to a number of diverse neuropsychiatric disorders for which tight DA regulation is important.

Rodent models of DAT dysfunction also clearly demonstrate the importance of DAT and its tight regulation under normal conditions. DAT knockout mice, for instance, have reduced DA tissue levels but high extracellular DA, reduced DA release, and lower levels of DA receptors compared to wild-types.[65] This results in increased basal locomotor activity in these mice. Interestingly, conditioned place preference (CPP) for psychostimulants directly targeting the DAT, including amphetamine and cocaine, remains intact in DAT knockouts. This is widely believed to be the result of molecular adaptations due to knockout from birth. However, if the DAT is made insensitive to cocaine, cocaine CPP is no longer observed, suggesting that DAT is required for cocaine reward and locomotor stimulation.[66] Importantly, a number of DAT knockdown models have provided additional insights into DAT's behavioral relevance.[67-69] These models have the advantage of avoiding some of the adaptations that derive from a complete deletion of DAT from birth. Some of these models express ~10% of wild-type DAT.[67,68,70,71] These mice predictably exhibit chronically elevated levels of extracellular DA. Behaviorally, they show novelty-induced hyperactivity, impaired response habituation, greater

incentive performance for sweet rewards in a runway task, enhanced progressive ratio operant responses for food rewards, and enhanced cocaine-induced locomotion and CPP.[67,70,71]

Importantly, psychomotor stimulants, or psychostimulants, are a class of reinforcing drugs that act on the DAT. This class of drugs includes cocaine, amphetamine, and their derivatives. Amphetamine acts as a substrate for the DAT in that it gets taken up by the DAT, thus competing with DA for uptake. Amphetamine additionally inhibits function of VMAT2 in the brain and promotes reverse transport (i.e. efflux) of DA by the DAT. Cocaine, on the other hand, increases extracellular DA by inhibiting reuptake of DA by the DAT. In rodents, these drugs are known for their ability to acutely increase motor activity, reduce food intake, and decrease thresholds for intracranial self-stimulation (ICSS).[72,73] Importantly, psychostimulants are powerful reinforcers; animals will respond in an operant fashion to self-administer them and can also be trained to respond to conditioned stimuli associated with psychostimulants.[74,75]

The DA reward system: historical perspective and new revelations

DA is the neurotransmitter that allows us to interact with the outside world; it gives us the ability to move and motivates us to seek out those things that will keep us moving. If provided with all possible resources for survival and procreation before us, we would yet cease to survive and procreate without the motivating influence of DA. This underlies the essential nature of DA and the brain's reward system, a system which evolved to compel us to seek out valuable resources with survival and reproductive advantage.

Reward is often defined as pleasure derived from any salient environmental stimulus. Reward can be experienced from ingested substances, such as food or certain drugs, but it can also be experienced through social interactions with others or activities (e.g. gambling, exercise). Reinforcement is any event that increases the probability of a response, and is

related to reward because rewarding stimuli typically enhance reward-seeking behaviors.[76] Thus rewarding stimuli themselves are said to be "reinforcing."

That DA is a key neurotransmitter in reward and reinforcement has been well recognized. Prior to Arvid Carlsson's 1957 Nobel Prize-winning discovery that DA is a neurotransmitter in the brain, it was believed that DA was simply a precursor to NE.[77] Carlsson found that rabbits made catatonic through treatment with reserpine could be made mobile and active again by treatment with the DA precursor, L-DOPA.[78] Further, the brains of these rabbits contained high levels of DA - visible by spectrophotofluorimetry - but not NE. We now know that reserpine blocks the activity of VMAT leading to DA depletion and induces a Parkinson's-like phenotype *in vivo*. The discovery that DA could indeed act as a neurotransmitter with important biological functions opened the way for further exploration of DA's roles within the CNS.[79]

In the 1960s, a "catecholamine hypothesis" of reward began to gain traction among scientists.[80] This hypothesis stated that catecholamine-containing neurons drive reward behaviors, such as ICSS observed in rodents by Olds and Milner in their classic 1954 study.[81] Prior to the catecholamine hypothesis and the work of Olds and Milner, the prevailing view was that reward-seeking was governed by general mechanisms and circuitry related to learning and memory, rather than a dedicated system for reward. The catecholamine hypothesis emerged from early studies showing that psychostimulant drug actions are impaired by treatment with reserpine and restored by L-DOPA[82] and was supported somewhat later through studies showing that DA receptor blockade reduces ICSS and lever-pressing for food,[83,84] among other evidence.[80] While controversial at first, the idea that catecholamines, particularly DA, drive motivated behavior has overwhelmingly dominated the study of reward for the past several decades.

A number of brain regions have been linked to reward and motivated behavior. Among them, the ventral striatum, or NAc, emerged early as a major site of reward, although its particular role in reward and reinforcement has been debated and refined over the years. It was first noted that rats electrically self-stimulate the NAc, as well as the upstream VTA, a phenomenon which could be blocked by accumbal injection of a DA receptor antagonist.[85,86] Furthermore, rats will self-administer drugs that directly or indirectly enhance DA signaling in the NAc.[87,88]

To understand the NAc's functional properties, an understanding of its structure and circuitry is required. The NAc, like the rest of the striatum, is composed of approximately 95% medium spiny neurons (MSNs). These are GABAergic projection neurons. The other 5% of neurons are interneurons which employ acetylcholine or GABA as well as NO, somatostatin, neuropeptide-Y, and a number of other neurotransmitters. An important property of striatal MSNs is their expression of DA receptors. Approximately half of striatal MSNs express D1 DA receptors while the other half express D2 DA receptors. These receptors receive input from DA-producing neurons whose cells bodies are localized to the VTA. D1 receptor-expressing neurons are thought to promote reward, while D2 receptor-expressing MSNs promote aversion.[89-91]

The NAc is further subdivided into NAc shell and core.[92] The shell surrounds the core and has been implicated in limbic processing and motivational salience.[93] Drugs of abuse promote a larger release of DA in the shell than the core.[94] Neurons in this subregion project to the ventral pallidum, amygdala, hypothalamus, and back to the VTA.[95] The core, on the other hand, is involved in the motor aspects of reward seeking.[96] Core neurons contain greater dendritic branching and are more susceptible to plastic changes, especially in response to rewarding stimuli.[97-99] The core projects primarily to the globus pallidus and SN.[95]

Despite near consensus that mesolimbic DA is important for reward, its exact role in encoding reward and determining resultant behavioral responses has been surprisingly difficult to establish and has been a topic of great debate. Several questions have emerged: Is DA responsible for the subjective enjoyment of a reward? Does DA allow us to learn about the value of a reward? Is DA simply directing motor patterns in pursuit of reward? Does DA play a role in the anticipatory phase or cravings associated with reward? These questions highlight the inadequacy of the term "reward" to describe these various phenomena which are likely distinct not only in concept but also in biology.

Defining the role of mesolimbic DA depends first on understanding the stimuli that elicit mesolimbic DA and the patterns or modes of DA modulation in response to different stimuli. Most importantly, virtually all rewarding stimuli have been shown to increase DA release in the NAc. This includes so-called "natural rewards" such as social reinforcers, sex, and palatable food, but also includes highly addictive or pleasurable activities (e.g. gambling), direct brain stimulation in specific areas, and drugs of abuse. Interestingly, all abused drugs increase accumbal DA levels. This is despite the fact that abused drugs may directly target other neurotransmitter systems (e.g. opiates target opioid receptors/signaling), suggesting that mesolimbic DA makes up a final common pathway for drug reward.[100] DA release for rewarding stimuli is typically greater upon initial exposure or consumption and lessens with repeated exposures.[101] On the other hand, secondary reinforcers or cues associated with rewarding stimuli can elicit DA release on their own.[101,102] Cues can include unconditioned stimuli (e.g. the smell of a cheeseburger), or conditioned stimuli from repeated association between the reward and cue (e.g. the sight of drug paraphernalia). These cues may even dominate the DA response as DA neurons cease to respond the primary rewarding stimulus and fire increasingly in response to secondary reinforcers.[103]

The context in which a reward is received is extremely important and may affect the amount and timing of DA release. These variables likely encode additional information relevant to reward processing. One of the most influential theories on DA and reward was proposed by Wolfram Schultz in 1997.[104] His theory, known as "reward prediction error," states that DA encodes the discrepancy between actual reward received and reward expectancy. A "positive prediction error," for example, means that reward received was unexpected or larger than anticipated. This results in an increase in DA release. When a reward occurs as predicted, no DA release occurs. Finally, a "negative prediction error" elicits an inhibition of tonic DA release when an expected reward is omitted. These different modes of DA signaling were experimentally confirmed through high resolution *in vivo* electrophysiologic recordings of DA release when reward-associated stimuli were presented or omitted and the reward itself was presented or omitted. More recent work from Schultz and others have refined the reward prediction error model.[105,106] In the most recent iteration of the model, DA serves to unselectively identify potential rewards or reward-predicting stimuli through an initial short-latency, short-duration phasic response followed by a second slower graded component which signals the value of the reward. Thus the process is sequential and serves to alert an animal to the *salience* of a stimulus, as well as a value judgment.

Whether an individual or animal administers a drug to themselves or is provided with drug by an experimenter in a non-response contingent manner also affects DA release mechanisms. Indeed, "yoked" rats exhibit substantially smaller increases in DA in response to cocaine injection than rats who were able to self-administer the drug.[107] This not only demonstrates the idea that DA neurotransmission involves contextual information, but also shows that even the pharmacology of abused drugs that act on the DA system relies on this contextual information.

The behavioral responses that are generated from directly manipulating modes of DA signaling tell us whether DA signaling is necessary and sufficient to drive different components of reward. To this end, early studies revealed that DA receptor agonists and antagonists could modulate reward in ways that are unlikely to be accounted for by altered sensorimotor function alone.[108-110] Additionally, lesions to accumbal DA terminals using the neurotoxin 6-hydroxydopamine (6-OHDA) reduce the consumption of food, drug, and brain stimulation rewards.[84,111,112] Rats will fail to consume food to the point of starvation despite sufficient motor function to consume readily available foods.[113] A similar aphagic phenotype is seen in genetically DA-deficient mice which lack the enzyme TH in DA neurons.[114] Supplementing with L-DOPA or replacement of TH in the striatum is sufficient to rescue feeding behavior. Still, the results have been challenging to interpret given that DA is crucial for directing sensorimotor function. Supplementing DA-deficient mice with caffeine, however, allows the mice to regain some degree of motor function. These mice are, interestingly, able to learn the location of rewards and can form a conditioned place preference, but they remain aphagic. This finding suggests that DA may not be necessary for reward learning, or the recognition that something is indeed rewarding, but rather underlies motivational processes necessary to actually acquire a reward.

The idea that DA supports an anticipatory or motivational component to reward has been espoused by the research of Kent Berridge who has supported a model of "incentive salience."[110,115] Under this model, DA designates a stimulus as both perceptually salient and attractive or motivating. DA thus drives a subjective "wanting" as well as a behavioral response in the form of reward seeking. Berridge has been a strong proponent of the separation of this "wanting" from "liking" which is the hedonic reaction to a rewarding stimulus, typically during the consummatory phase.[116] In this manner, a reward can be wanted even if it does not produce a subjective state of enjoyment or euphoria. Berridge's ideas have been supported by studies

seeking to distinguish “liking” and “wanting” at neurobiological and behavioral levels. Hedonia or “liking” can be observed for food using a taste reactivity assay which relies on evolutionarily conserved and observable affective reactions to a sugary solution (e.g. tongue protrusions).[117] Interestingly, blocking mesolimbic DA signaling through DA receptor antagonism or 6-OHDA lesions does not impact hedonic reactions to sweet stimuli,[118] although such lesions may impair seeking behaviors.[119,120]

Importantly, DA cannot play any role in reward without post-synaptic receptors and neurons to drive responses to DA. The interplay between direct and indirect pathway neurons in the striatum and how each drives DA reward responses is a complex topic. This fact derives in part from messy DA receptor pharmacology and the measurement of likely non-physiologic responses. However, with the emergence of new techniques like optogenetics, researchers have been able to better isolate specific signaling through D1- and D2-type neurons. Using cocaine as a reinforcer, multiple groups have been able to show that optogenetic activation of D1-type neurons promotes drug seeking and reward-context associations while D2-type neurons cause aversion to cocaine.[89,90] Overexpression of the transcription factor ∆FosB, a transcription factor stably upregulated following repeated cocaine administration, in D1 direct but not D2 indirect MSNs also increased behavioral responses to cocaine.[121] Furthermore, D1-type populations of MSNs are the primary cell type activated by entry into a cocaine-paired environment, as measured by *in vivo* calcium imaging during a CPP task.[91] Despite these findings supporting D1-driven responses, there is still a great deal of evidence that D2 receptor signaling supports motivated behavior. For instance, Jonathan Javitch and colleagues showed that viral overexpression of the D2 receptor in the NAc induces higher levels of operant responding when work requirements to achieve a palatable food reward were increased, while increased D2 receptor expression did not affect sensitivity to reward devaluation.[122]

Taken together, the literature points toward a role for DA in motivational reward seeking. This understanding serves to better inform our interpretation of the alterations in both DA homeostasis and behavior following pharmacologic and genetic manipulations. It further allows us to interpret changes in DA signaling in relation to pathological states of reward impairment (e.g. drug addiction, obesity; described in detail below).

Central reward: beyond mesolimbic DA

While mesolimbic DA signaling has been at the forefront of the research into reward and addiction, several other brain regions and neurotransmitter systems have been implicated in both natural and drug-mediated reward. These neurotransmitters and regions deserve recognition because they participate in aspects of reward not directly regulated by the mesolimbic DA system. Furthermore, many of these regions modulate signaling within the mesolimbic system with the ability to shift behavioral responses.

In addition to the mesolimbic system, both the lateral hypothalamus (LH) and the lateral septum (LS) may be "primary" reward centers in that electrical self-stimulation in these regions is considered rewarding. In now classic studies, Olds and Milner (1954) discovered that rats would press a lever to electrically stimulate sites along the medial forebrain bundle (MFB), a bundle of axons which includes projections to and from the LH, VTA, NAc, and LS.[81] While less attention has been devoted to the LH and LS, these regions nevertheless play important roles in reward processes.

The LH has long been known as both a feeding center and reward center.[123] Electrical stimulation of the LH appears to be rewarding in itself,[81] and also induces an immediate hyperphagic response in satiated animals.[124] Mechanisms underlying this effect were, until somewhat recently, unknown. Interestingly, LH neurons expressing neuropeptides known to be

involved in feeding regulation, including melanin-concentrating hormone and orexin, project to mesolimbic circuitry.[125-127] Based on this anatomical observation, Dileone and colleagues proposed that these neuropeptide-expressing LH neurons might support reward processes and even addiction.[127] Indeed, recent studies support this notion. One study found that activation of LH orexin neurons is strongly linked to preferences for cues associated with food and drug reward and that direct administration of orexin into the VTA was sufficient to reinstate extinguished drug-seeking behavior.[128] In addition to neuropeptides, direct GABA and glutamate stimulation from the LH onto VTA DA and GABA neurons modulates compulsive sucrose consumption.[129] Through an elegant set of studies, these authors describe a loop circuit by which LH-VTA neuron encode learned reward-seeking behavior while a reciprocal VTA-LH circuit encode reward expectancy. Thus the LH appears to regulate important aspects of reward-seeking behavior through direct manipulation of the VTA.

The LS is another region which is highly rewarding by ICSS and may regulate outflow from mesolimbic reward circuitry. The LS is a multifunctional brain region; it is most known for regulating affective states including mood and fear, and could impact processes related to schizophrenia and a number of other neuropsychiatric disorders.[130] It is composed primarily of GABAergic projection neurons which innervate regions involved in reward and affective state, including the VTA, NAc, LH, amygdala, and raphe nuclei. It receives projections from many different areas, most notably glutamatergic innervations from the PFC, hippocampus, and hypothalamus, and DAergic projections from the VTA. The major LS input and output areas are summarized in **Figure 4**.

Thus the LS sits at a node where it is poised to regulate a number of neuropsychiatric processes. But what about its role in motivation? Is there evidence that it interfaces with the mesolimbic DA system? Indeed, exposure to stimuli that have previously been paired with positive reinforcers (food, cocaine) increases neuronal activity in the LS.[130] Lesioning the MFB

axons leading from the LS to the LH and VTA prevents ICSS.[131] Moreover, electrical stimulation of the LS regulates the firing rates of VTA neurons,[132] suggesting that the LS provides top-down regulation of the mesolimbic system. In support of this notion, application of tetrodotoxin to the LS enhances DA release in the NAc as measured by *in vivo* voltammetry.[133] As the LS receives DAergic input from the VTA as well, this interaction is likely reciprocal allowing for circuit-level integration and feedback.[130] Accordingly, the LS expresses both D1-like and D2-like DA receptors. The exact role of DA in the LS is unclear, likely due to varying conditions required to activate D1 vs D2 signaling. Like the striatum, the interplay between D1-type and D2-type signaling is complex making it difficult to say how DAergic innervation from the VTA influences septal function. Nevertheless, it is clear that the LS exhibits behavioral relevance to reward and regulates the rewarding properties of the DA-enhancing drug, cocaine. Notably, applying baclofen, a GABA receptor agonist, to the LS is able to block cocaine CPP in rats.[134] The authors of this study suggested that this occurs through inhibiting activation of LH orexin cells which, as described above, modulate outputs from mesolimbic circuitry related to reward-context associations. Another study by this same group used tracers to define a circuit connecting the CA3 region of the hippocampus through the LS to the VTA.[135] As one might expect, this circuit is important for context-reward associations; chemically inactivating components of this circuit was sufficient to block context-induced reinstatement of cocaine seeking in rats. These results point to a role for the LS in integrating components of reward from different areas to be able to account for not only the rewarding effects of the drug itself, but also the cues or context associated with it. That cocaine CPP or reinstatement does not exist without septal influence highlights the essential nature of the LS.

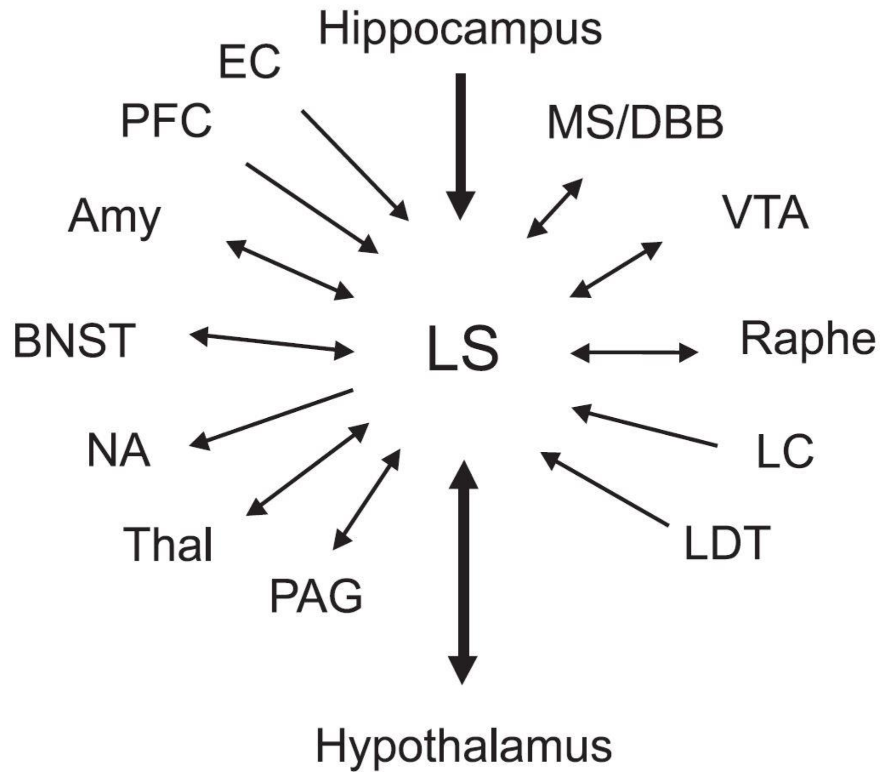

Figure 4. *A simplified summary diagram of the afferent and efferent connections of the LS.* The LS receives strong glutamatergic inputs from the hippocampus and reciprocal inputs from the hypothalamus, including LH. It also receives input from the VTA and sends projections to the NAc. Amy, amygdala; BNST, bed nucleus of the stria terminalis; EC: entorhinal cortex; LC, locus coeruleus; LDT, laterodorsal tegmentum; MS/DBB, medial septum/diagonal band of Broca; NA, nucleus accumbens; PAG, periaqueductal gray; PFC, prefrontal cortex; Thal, thalamus; VTA, ventral tegmental area. Adapted from [130].

While the ventral striatum garners much of the attention in regards to reward, the DS also plays a distinct but important role. The DS is typically associated with motor components of reward seeking because the DS, with its direct and indirect motor circuits, forms the central circuitry of the basal ganglia. Whereas the NAc is implicated in responses to conditioned and unconditioned reward stimuli, the DS controls the physical ability to respond to rewarding stimuli. Lesions of the DS, thus, affect behaviors such as response initiation and reaction time to acquire rewards.[136,137] As in the NAc, DA is critical to these functions.[138-140] Importantly, the DS has been implicated in the compulsive aspects of reward seeking.[75] These compulsions develop over time as motor patterns of reward seeking become engrained. Thus the DS is often associated with "habitual" components of reward seeking.

The glutamate system also plays a very important role in reward. Glutamate inputs to the VTA promote DA neuron firing; moreover, DA would have little effect on its downstream targets without a source of excitation as MSNs are quiescent without excitatory input.[141] Glutamate interacts with the mesolimbic DA system through projections to the VTA from the hippocampus, the amygdala and extended amygdala, and the PFC, and a similar set of inputs to NAc MSNs.[142,143] The hippocampus and amygdala modulate the reward system by providing information about context and emotional valence, while the PFC provides top-down regulation of the reward system through impulse control and conscious decision-making. Interestingly, in degenerative disorders of the PFC such as frontotemporal dementia, patients exhibit hyperphagia as they are no longer able to make appropriate decisions regarding palatable food consumption.[144]

Importantly, afferent glutamatergic projections to the VTA and NAc drive learning processes including long-term depression (LTD) and long-term potentiation (LTP). Learning is an essential component of reward because it allows the reward system to recognize rewarding stimuli it has encountered previously. It is particularly important for establishing associations

with conditioned cues. Fast glutamatergic synaptic signaling is mediated by ionotropic glutamate receptors, AMPAR and NMDAR. These receptors bind glutamate and, under the right conditions, become permeable to cationic currents. These currents are known as excitatory postsynaptic currents (EPSCs) and can be measured through slice electrophysiology. Notably, AMPAR- and NMDAR-mediated EPSCs have little to do with the acute effects of rewarding stimuli. However, the expression of AMPARs and NMDARs and the development of their currents are crucial to behavioral adaptation and synaptic adaptations, particularly those that develop from prolonged drug use.[145] These concepts are discussed further in the next section in the context of addiction.

If DA primarily directs motivation and incentive salience, what neurotransmitter(s) is responsible for the hedonic properties of rewards? Opioid peptides and their receptors are one clear candidate. Opiates are drugs which target opioid receptors and are known for their pain-relieving properties as well as for being highly addictive. Interestingly, while DA receptor agonists and antagonists do not seem to modify the hedonic properties of sucrose in a taste reactivity assay, opioid receptor agonists and antagonists do.[146] Furthermore, "hedonic hotspots" have been identified within the NAc shell and ventral pallidum which are highly responsive to the opioid receptor agonist, DAMGO.[147] In regards to drugs of abuse, there may be substantial crosstalk between opioid systems and drugs of abuse as opioid receptor antagonists (e.g. naltrexone) are able to modify drug-seeking behavior, even for drugs whose primary targets are thought to lie outside the opioid system (e.g. alcohol).[148]

Another candidate for hedonia is the endocannabinoid system. Endocannabinoids are a class of neuroactive lipids which include primarily anandamide and 2-arachidonylglycerol (2-AG). These molecules are generated upon membrane depolarization by the enzymatic remodeling of membrane phospholipids as described in **Figure 5**. Given their hydrophobic composition, endocannabinoids are able to cross neuronal cell membranes easily within a small

diffusible area (**Figure 6**).[149] This allows them to interact with presynaptic receptors, primarily the CB1 endocannabinoid receptor (CB1R) which is highly expressed in the CNS. They can also be degraded into AA and ethanolamine or glycerol within presynaptic neurons or glial cells by the enzymes fatty acid amide hydrolase and monoacylglycerol lipase (MAGL). The endocannabinoids have been implicated in mood, memory, and appetite. Importantly, endocannabinoids have been strongly linked to reward. Endocannabinoid signaling has been shown to increase feeding behavior[150] and is also the target of tetrahydrocannabinol (THC)-based drugs which exhibit rewarding properties in humans and other animals.[151] Compared to some other drugs, THC-based drugs are not thought to be strongly reinforcing. Yet, importantly, pharmacological blockade of CB1Rs reduces the seeking for palatable food and drugs of abuse, suggesting that endocannabinoids do indeed regulate reward processes in the brain.[152] Furthermore, recent reports have shown that another drug of abuse, namely cocaine, is able to mobilize 2-AG within the mesolimbic system,[153] while THC and other cannabinoid receptor agonists increase DA levels in the striatum.[154] Thus there is likely a great deal of cross-talk between DA and endocannabinoid systems, although they may possess unique functional niches (i.e. hedonia vs motivated behavior).

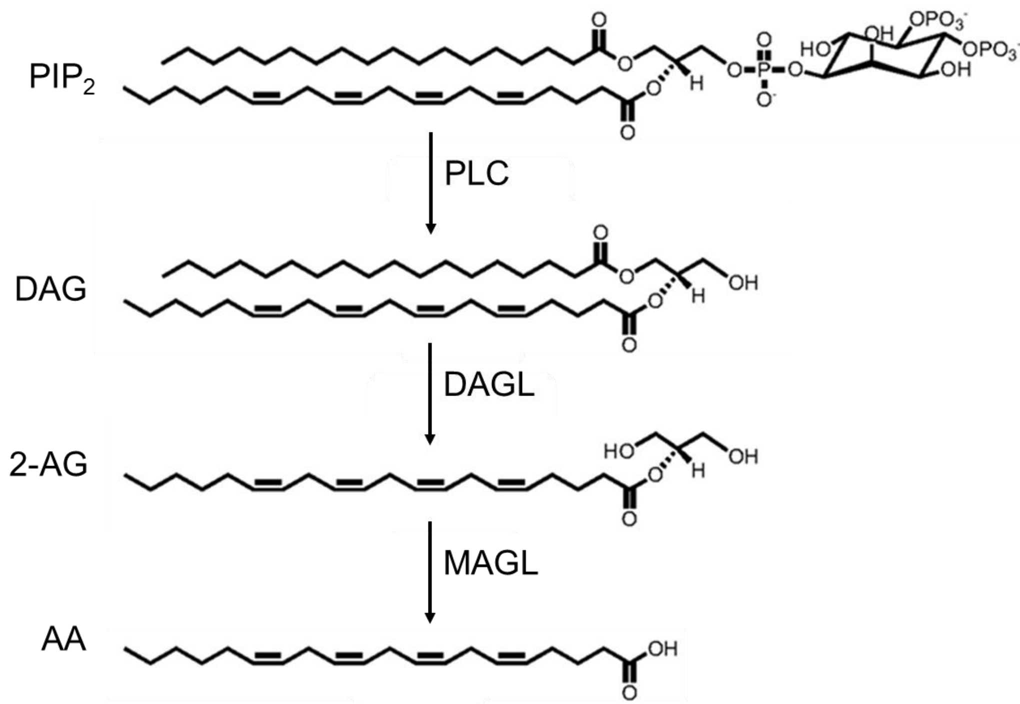

Figure 5. *Major biosynthetic and degradative pathway of 2-AG*. AA, arachidonic acid; 2-AG, 2-arachidonoylglycerol; DAG, diacylglycerol; DAGL, diacylglycerol lipase; MAGL, monoacylglycerol lipase; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C. Adapted from [155].

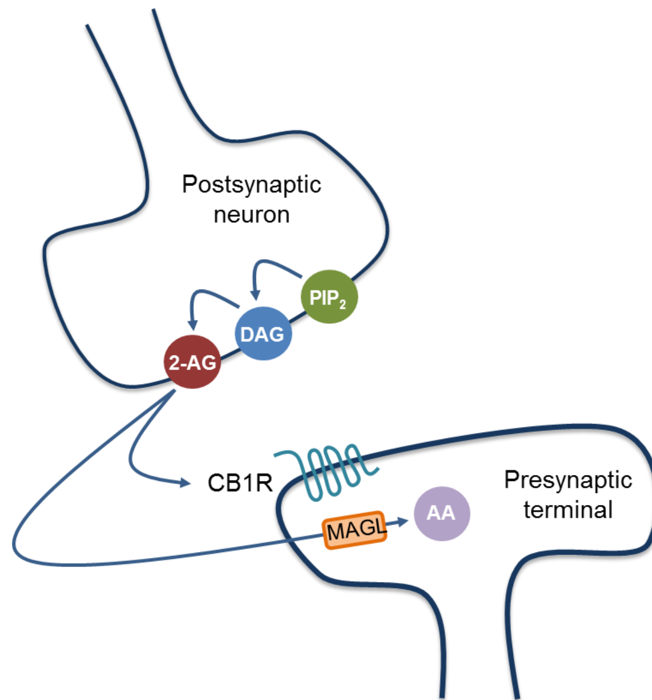

Figure 6. *Model of retrograde shuttling and metabolism of 2-AG and AA.* 2-AG is generated from phospholipids in the postsynaptic neuron. It can bind diffuse and bind to the CB1 cannabinoid receptor (CB1R), activating a Gi-coupled inhibitory signaling cascade. 2-AG can also diffuse into the presynaptic terminal where it is degraded to AA by MAGL. AA, arachidonic acid; DAG, diacylglycerol; MAGL, monoacylglycerol lipase; PIP2, phosphatidylinositol 4,5-bisphosphate. Adapted from [156].

An introduction to drugs of abuse and addiction

Brain reward systems evolved to compel us to interact with others and our environment in a manner which would increase our reproductive fitness, particularly in times of scarcity of reproductive partners or food. For example, the reward system encourages consumption of energy-dense foods with less need for foraging. These qualities of the reward system conferred fitness benefits for the vast majority of evolutionary time. However, with the increased societal availability of natural rewards like calorie-dense foods and the emergence of drugs of abuse which hijack brain reward systems, pathological states have emerged within the reward system. These pathological states include drug addiction, but a number of other addictions may exist including addictions to sex, food, gambling, and even the internet.[157] Of these, drug addiction is likely the best understood and also one of the most problematic in terms of public health ramifications.

Drug addiction is the compulsive use of a drug despite negative consequences, such as health problems, disability, and failure to meet major responsibilities at work, school, or home. In the Diagnostic and Statistical Manual of Mental Disorders (DSM V), an individual is considered to have a substance use disorder if there is evidence of impaired control, social impairment, risky use, and pharmacological criteria specific to a particular drug class. The DSM V recognizes substance use disorders for the following classes of drugs: tobacco, alcohol, caffeine, stimulants, opioids, hallucinogens, cannabis, hypnotics, anxiolytics, inhalants, sedatives, and certain prescription drugs.

According to the U.S. Department of Health and Human Services, in 2013 an estimated 21.6 million people aged 12 and older (8.2% of the U.S. population) were classified with substance dependence or abuse based on the DSM IV.[158] Substance use disorders confer wide-ranging societal costs from lost work productivity to crime and healthcare costs. Addiction

has an estimated cost burden of $700 billion annually in the U.S. alone.[159] Furthermore, the large demand for drugs has led to a countless number of deaths and incarcerations associated with illegal drug trades.

Treatment options for drug addiction are fairly limited and, depending on the drug class, range from behavioral therapy only to combined behavioral and pharmacotherapy. Behavioral therapy may consist of psychiatric individual or group cognitive behavioral therapy (CBT), motivational interviewing, motivational incentives, multidimensional family therapy for youths,[160] as well as community support groups (e.g. Alcoholics Anonymous). CBT has been shown to have low to moderate efficacy, depending on the substance.[161] Combining CBT with pharmacotherapies may have some additional benefit for certain drug classes;[162] however the benefits do not appear to be additive.[161]

Medication-based therapies primarily address withdrawal symptoms, prevent relapse, and treat co-occurring diseases (**Table 1**). Most FDA-approved pharmacotherapies target the same proteins acutely targeted by the drugs themselves (**Figure 7**). For instance, for opiate abuse we use opioid receptor agonists (methadone) or partial agonists (buprenorphine) to ease the transition into a withdrawal period by lessening withdrawal symptoms, while antagonists (naltrexone) block the effects of opioids at the opioid receptor. As is clear from table 1, there are very few options for patients with addictions. Indeed, there are no effective pharmacotherapies to address addiction to psychostimulants. The lack of options for patients with addiction presents a major challenge to their treatment. Furthermore, the treatment of addiction is often complicated by comorbid diseases that tend to accompany addiction, including mood and anxiety disorders, cardiovascular disease, and chronic viral illnesses (hepatitis C, human immunodeficiency virus).[159,163]

Table 1. *Pharmacotherapies targeted at several major classes of abused drugs.*

Drug class	Pharmacologic treatment options
Tobacco (nicotine)	Nicotine replacement (patch, gum, etc.) Bupropion Varenicline
Alcohol	Disulfiram Naltrexone Acamprosate
Opiates (heroin & pain relievers)	Methadone Buprenorphine Naltrexone
Psychostimulants (cocaine, amphetamine)	None
Cannabis (marijuana)	None

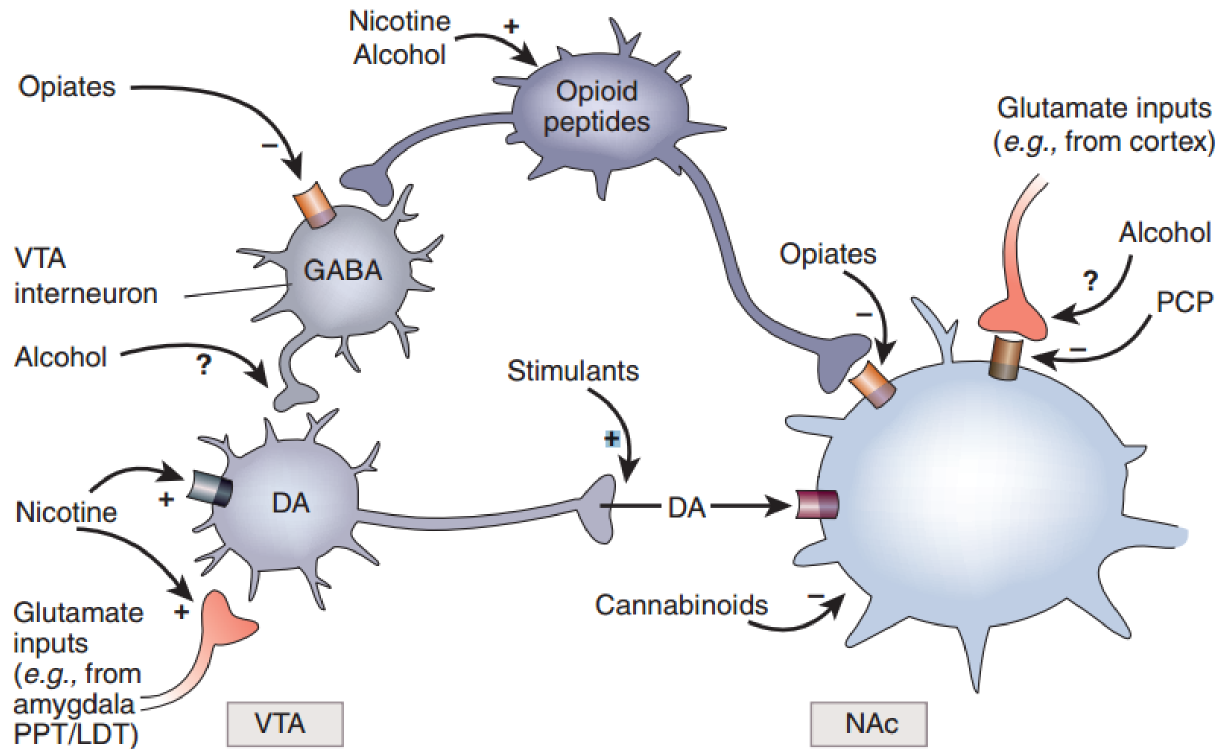

Figure 7. *Simplified scheme of converging acute actions of drugs of abuse on the VTA-NAc*. Drugs of abuse promote the elevation of DA in the NAc, despite differing acute targets. Proposed sites of action are depicted here. PPT/LDT, peduncular pontine tegmentum/lateral dorsal tegmentum. Adapted from [164].

Part of developing therapies to address addiction is to understand that addiction develops in stages and that each of these stages may require different behavioral or medical interventions.[103] The first step in the development of an addiction is experimentation, during which an individual tries the drug for the first time and may feel some of its euphoric sensations. An individual may progress to the binge/intoxication phase in which they take the drug on multiple occasions, but the intake remains subchronic and lacks a compulsive nature; rather, this early phase tends to rely on impulsive behavior and the positively reinforcing qualities of the drug. Over the first few drug administrations, an individual may sensitize to the acutely rewarding effects of the drug. However, over longer periods of repeated drug administration, an individual will develop tolerance to the drug, leading to dose escalation. Indeed, dose escalation is commonly observed in animal models of contingent drug administration.[165,166] This effect is likely due to potent neuronal adaptations within the brain reward system. For example, DA receptors may be downregulated in response to repeated drug administration such that greater amounts of drug are necessary to induce a state of internal satisfaction in response to the drug.[103] Furthermore, these adaptations alter the basal neurochemical balance within the brain, which may promote drug cravings and altered affective state. Taken together, these adaptations resulting from chronic use are believed to promote greater compulsive intake. The molecular bases for adaptations in response to drugs of abuse are discussed in further detail in the next section.

With escalating intake, an individual may begin to experience some of the negative consequences of drug abuse such as the deterioration of important relationships, poor work performance, and the development of risky behaviors associated with taking or acquiring the drug. An individual may attempt to reduce intake following negative consequences, but with many drugs of abuse this can produce withdrawal symptoms which can range from intense drug cravings to negative affect to physical distress. These immediate adverse consequences of

abstaining from drug-taking may, by themselves, support drug-seeking to satisfy drug cravings and alleviate withdrawal symptoms. Thus drug-taking shifts from being driven by positive reinforcement to negative.

At this point, the individual is no longer in control of his or her drug use. Herein lays the core component of addiction: the inability to stop abusing drugs despite negative life consequences. If an individual is able to stop taking the drug for an extended period of time, they may be able to get past the initial stage of withdrawal symptoms. However, a common finding is that addicts exhibit a high rate of relapse. Relapse tends to occur when an addict encounters cues, people, or environments associated with prior drug-taking. Additionally, negative life events (e.g. the death of a loved one) or negative affective states (e.g. high stress, depression) can trigger a relapse event in which the addict consumes the drug once again. In these events, the drug-taking behavior again becomes a chronic situation.

Drug-induced adaptations and predisposing factors to addiction

Each of these steps in the development of an addiction to drugs comes with molecular adaptations in the reward system that drive continued use and relapse. Human imaging studies point to some of these chronic adaptations and, in the absence of well-controlled, longitudinal interventions in humans, animal models have provided greater information regarding the causality of molecular changes in addiction. As DA is clearly an important mediator of at least some component of normal reward processes, it comes as little surprise that DA plays a role in addiction. As has been previously mentioned, all drugs of abuse acutely elevate mesolimbic DA levels, mimicking phasic DA cell firing, despite differing direct mechanisms of action. This suggests a final common pathway for addiction which has been espoused by Eric Nestler and others (Figure 7).[164] In the early stages of addiction, drugs may sensitize the DA system such

that DA transmission increases progressively with repeated administration. This effect is thought to underlie, at least in part, the increase in ambulatory activity that is observed in rodents following repeated administration of many abused drugs. Similarly, DA responses to drug-associated cues increase with repeated exposures.

Despite these early increases in DA transmission, a prevailing hypothesis on addiction states that chronic drug use leads to a state of DA deficiency.[103] This hypothesis suggests that chronic drug use characterized by high acute levels of DA promotes chronic DA system fatigue and the downregulation of mechanisms of DA neurotransmission. This may include reduced DA production and release, altered DA reuptake, and receptor downregulation. Indeed, chronic drug administration tends to decrease TH expression in NAc terminals (although it may increase TH in the VTA) and reduce D2 receptor expression in striatum.[11,164,167] Reduced DA neurotransmission in response to a drug may act to paradoxically increase motivation for drugs of abuse due to a lack of tonic stimulation. This is thought to contribute, at least in part, to escalating drug intake. Interestingly, conditioning triggered by drugs has been shown to promote *enhanced* DA signaling in response to conditioned cues. Thus, while tonic DA stimulation may be low in addiction, phasic DA in response to conditioned cues may be higher and drive cravings.

One of the most replicated findings in this regard is a reduction in D2 receptor expression in the striatum (including ventral striatum) in addicts. Using human PET imaging techniques, Nora Volkow and colleagues found that addicts for virtually any abused drug exhibited reduced D2 receptor binding.[11] Whether this is an “adaptation” or a pre-existing condition is of some debate. Do high levels of DA neurotransmission from chronic drug abuse cause a downregulation of DA receptors? Or, are persons vulnerable to addiction more likely to have lower D2 receptor expression, thus driving future seeking behavior? These questions have not been fully answered yet, but there is evidence to support both scenarios, which are not

necessarily mutually exclusive. In support of low pre-existing D2 receptor availability, high D2 receptor availability in the striatum is linked to resilience against the development of addiction,[168] as well as optimal goal-directed behaviors[169] and decision-making based on effort expenditure.[170] Furthermore, in non-drug-abusing individuals, low levels of D2 expression predicted higher self-reports of "drug-liking" in response to the cocaine-like substance, methylphenidate.[168] In support of drug-mediated D2 receptor changes, non-human primates treated chronically with cocaine exhibit long-lasting decreases in D2 receptor availability by PET,[171] and a response to treatment for cocaine addiction in humans parallels an increase in signaling through the D2 receptor.[172] Longitudinal studies in humans will be necessary to better document whether low D2 receptor availability precedes addiction or is the result of chronic drug use.

The glutamate system has also been strongly implicated in chronic neuroadaptations linked to addiction. As reward utilizes mechanisms of synaptic plasticity, so addiction engages similar mechanisms to strengthen motivational and motor circuits within the striatum. The types of adaptations which occur depend greatly on the length of exposure to drug (acute vs chronic) and the time since last exposure.[173] Acute exposure to cocaine, for example, has little effect on striatal glutamate receptor expression and function. Repeated chronic exposure to cocaine, however, engages NMDARs on silent synapses (synapses lacking AMPAR expression). Silent synapses are highly plastic and may make the reward system vulnerable to the formation of memory traces associated with the drug. Appropriately, these changes in glutamate receptor expression coincide with an increased dendritic arborization and spine density in NAc MSNs in response to chronic administration of several different classes of drugs.[174] The morphological changes occurring within the NAc reflect altered electrophysiologic properties of MSNs. These include synaptic LTD and LTP. While these are opposing processes, they have both been observed following chronic drug administration and they likely both contribute to aspects of

drug-seeking behavior. This is a complex topic which is outside the scope of this dissertation; however, there are some salient points worth mentioning in regards to neuroadaptations. First, repeated cocaine injections in mice result in a long-lasting depression of excitatory synaptic transmission in the NAc 24h after a challenge injection.[175] In contrast, there is an increase in synaptic strength following abstinence that is depotentiated following re-exposure. In contrast, LTP is inducible in slices from rats treated repeatedly with cocaine when no withdrawal period is given.[176] Interestingly, administration of drugs, such as cocaine, can support molecular processes associated with changes in LTD and LTP. For example, cocaine interferes with endocannabinoid-mediated LTD.[177] Cocaine has also been shown to enhance accumbal EPSCs through GluA2-lacking AMPARs, particularly after a withdrawal period.[178] GluA2-lacking AMPA receptors have greater calcium ion permeability and thus enhanced AMPAR synaptic currents, which may support cravings and drug-seeking behavior during periods of abstinence.[179]

A number of the molecular changes described above may be induced by activation of the transcription factor, CREB (cAMP response element binding protein), which is activated in the VTA and NAc following repeated administration of cocaine, amphetamine, or opiates.[180-182] CREB activation is thought to drive changes in gene expression related to addiction (e.g. TH, GluR1).[183] ΔFosB is another transcription factor that gets induced and accumulates in the NAc in response to chronic drug exposure and has been implicated in some of the morphological changes following chronic drug exposure.[184] Importantly, overexpression of ΔFosB increases behavioral reactions to cocaine and opiates.[185,186]

Later in the course of addiction, other brain regions beyond the ventral striatum may affected by pathological adaptations. Indeed, the DS becomes recruited as drug-seeking takes on a more compulsive, habitual nature. Similar to the ventral striatum, there is increased spine density in the putamens of non-human primates.[187] Furthermore, DA neurotransmission in the DS appears to be necessary for drug-seeking.[7] Interestingly, a number of studies have also

demonstrated deficits within cortical regions, namely hypofrontality following chronic drug exposure. For instance, human imaging studies reveal impaired metabolic function of the orbitofrontal cortex, cingulate gyrus, and dorsolateral PFC in addicted subjects.[188] These changes are believed to underlie impaired decision-making abilities along with increased impulsivity and compulsivity observed in addiction.

It is important here to recognize that the brains of addicts may be different from non-addicts even before the addict was ever exposed to drug. Indeed some of the aforementioned "adaptations" may not be adaptations at all, but rather, predisposing factors (although they are not necessarily mutually exclusive). Like many other highly prevalent disorders, addiction is a highly multifactorial disease involving a constellation of genetic and environmental factors. We know that an individual who tries a drug will not necessarily become addicted. In fact, according to the Institute of Medicine of the National Academy of Science, depending on the drug, only 10-30% of people who try a drug will go on to become addicted. Of course, understanding biological underpinnings of vulnerability to drugs may help us discover who is at greatest risk and what we can do to prevent addiction or intervene in ongoing addictions.

Some known risk factors for addiction include a family history of drug problems, male sex, impulsive traits, having another mental health disorder, peer pressure (especially in youth), and a lack of familial support.[189,190] Protective factors include positive social support and role modeling, parental controls, traits like high self-esteem, and even a few genetic traits.[189] Taken together, these factors contribute to an overall susceptibility or resilience to addiction.

Understanding the genetic factors that contribute to addiction may give us new targets for therapy. To that end, family histories and twin studies support substantial heritability of addictions (from twin studies, about 50%, of addiction vulnerability that is heritable).[191-195] Unfortunately, finding precise genetic targets through genome-wide association studies has

proved challenging. To underscore this point, linkage-based analyses find no single locus contributes a substantial fraction of the vulnerability to any addictive substance.[191] However, some targeted case control studies have defined promising gene loci. As one example, the *Slc6a3* gene for the DAT contains what is known as a variable number tandem repeat (VNTR) sequence. In some individuals, this consists of a 9-repeat allele. This polymorphism has been associated with a reduced likelihood to smoke.[196] The effect was even greater if an individual possessed the *DRD2-A2* genotype of the D2 DA receptor. Furthermore, individuals carrying the 9-repeat allele were more likely to quit smoking and had reduced novelty-seeking traits.[197] If we can pharmacologically mimic the biology of genes like these which appear to confer some resistance to addictive behavior we may be able to affect behavior in addicts.

Crosstalk between food and drug reward

Drugs of abuse evolved to utilize a system already in place to respond to natural, adaptive rewards. It should therefore come as little surprise that crosstalk between food and drug reward systems may be observed in neuronal responses and adaptations, circuits, and even hormonal regulators. It is our belief that, by understanding the regulation of behavior related to natural rewards such as the consumption of palatable foods, we may be able to discover novel therapeutic targets to approach addiction. I begin by describing the evidence for food-drug reward crosstalk (shared circuitries and shared pathologies). I finish by discussing some emerging evidence that therapies which control feeding and energy metabolism may be useful in treating drug addictions.

Shared circuitries: Feeding is regulated at multiple levels within the CNS (**Figure 8**). The mesolimbic system is thought to control hedonic or non-homeostatic feeding. Hedonic feeding generally consists of consuming palatable, calorie-dense foods beyond satiation. Several lines

of evidence suggest that the mesolimbic DA system regulates hedonic feeding in ways reminiscent of the response to drugs of abuse and may act to suppress homeostatic circuitry (i.e. the hypothalamus and brainstem). As a first point in support of this notion, both palatable food and drugs of abuse elicit rapid firing by DA neurons and an immediate phasic elevation in DA within the NAc.[139,198,199] Moreover, increases in DA are observed in anticipation of both palatable foods and previously used drugs of abuse,[200,201] supporting the idea that accumbal DA is a common neurotransmitter mediator of reward and its anticipation for both stimuli. From Palmiter's experiments deleting TH from midbrain DA neurons in mice, we also know that DA is essential for feeding.[202] The exact role of the post-synaptic DA receptors is less clear. However, it is clear that DA receptors do regulate feeding to some degree. It has been shown that chronic D2 receptor blockade promotes obesity while D1 receptor blockade has no effect on feeding or body weight.[203] On the other hand, D2 receptor overexpression increases seeking for a palatable food reward.[122]

Notably, in contrast to the literature on drugs of abuse, nigrostriatal and striatopallidal circuits – centered around the DS – have been somewhat more strongly implicated in regulating feeding behavior than the ventral striatum. In normal-weight humans, palatable food releases DA in the DS in proportion to ratings of meal pleasantness.[204] In DA-deficient mice, restoration of DA signaling selectively to the DS, but not the NAc, rescues food intake as well as motivated feeding in an operant task.[114,205-207] However, the NAc is likely still important for hedonic feeding as ablation of DA neurons projecting to the NAc does attenuate rats' motivation to work for food rewards.[208]

Shared pathologies in obesity and drug addiction: Like drug addiction, obesity is a serious public health issue which affects primarily developed nations. Obesity in the U.S. has ballooned to epidemic proportions, as rates of obesity have increased dramatically over the past 30 years. Today, approximately 1/3 of the adult U.S. population is considered obese with a body mass

index (BMI) of greater than 30.[209] The causes of obesity are multifactorial, to be sure; however, it is believed that the recent rise of obesity can be attributed in large part to the availability of low-cost calorie-dense foods.[210] These foods are highly palatable and, as described above, strongly activate the brain's reward systems in a similar fashion to addictive drugs.[211] Importantly, obese individuals disproportionately consume high energy foods and exhibit enhanced motivation to work for rewarding food stimuli.[212]

A great deal of literature has shown that obese individuals, like drug addicts, exhibit lower D2 receptor availability within the striatum, which is thought to contribute to compensatory overeating.[213] Indeed, rats which were given extended access to a cafeteria diet gained substantial weight and expressed less D2 receptor in the DS.[214] These authors further showed that the D2 receptor may play a causal role in this process as viral deletion of the D2 receptor from the DS made these rats resistant to palatable feeding inhibition by a punishment-paired conditioned stimulus. In further support of the notion of DA deficiency in obesity, human imaging studies have revealed elevated cue-induced DA elevations but decreased DA responses to actual food consumption in obese compared to lean individuals.[215,216] Palatable food stimulation induces even greater increases in striatal DA release in individuals diagnosed with binge eating disorder, a psychiatric disorder that resembles "food addiction".[217]

Interestingly, extended access to palatable food and drugs of abuse, including cocaine and heroin, increase LH ICSS reward thresholds to a similar extent compared to chow-fed, drug naïve rats.[218] This strongly supports shared pathological events within the reward system in response to both food and drugs that result in the escalation of ICSS.

Therapies to control feeding may be useful in drug addictions[1]: The evidence described so far suggests that reward for food and drugs of abuse may possess overlapping substrates within

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[1] The work presented in this section is adapted in part from Reddy, I.A., Stanwood, G.D. & Galli, A. Moving beyond energy homeostasis: new roles for glucagon-like peptide-1 in food and drug reward. *Neurochemistry International* **73**, 49-55 (2014).

the CNS. But are food and drug reward regulated in the same way? If we attempt to target feeding behavior, will we also affect circuits regulating addiction? Here, I provide evidence that suggests that brain substrates of addiction are susceptible to many of the same components regulating feeding behavior. I take this one step further in suggesting that we can use emerging therapies to control appetite, body weight, and energy metabolism to treat elements of addictive behavior.

The brain regulates homeostatic feeding behavior *via* the hypothalamus, hedonic feeding behavior through the brain's reward system, top-down control of feeding through the PFC, and modulatory control *via* regions involved in learning, memory, and emotion (e.g. the hippocampus and amygdala) (Figure 8). The brain also receives feedback from peripheral hormones which signal the body's nutritional state and energy supplies. These hormones are released from sites within the gut (stomach, pancreas, intestines, etc.) into the bloodstream where they circulate and bind to receptors in the periphery as well as the CNS (**Figure 9**). Some of these hormones function in the short-term regulation of feeding (i.e. during a meal) and include cholecystokinin (CCK), peptide YY (PYY), glucagon-like peptide 1 (GLP-1), and ghrelin, among others. Insulin and leptin regulate long-term feeding patterns and body weight.

Feeding hormones and their receptors have been major targets for the development of drugs to combat obesity. Agonists at the GLP-1 receptor (GLP-1R), for example, have already been FDA approved for obesity as they reduce body weight, appetite, and feeding.[219,220] Additionally, bariatric surgeries, or weight loss surgeries, have been shown to substantially reduce body weight in patients and alter hormonal systems involved in feeding and metabolism.[221] Importantly, evidence suggests that changes in hormonal signaling accompanying bariatric procedures may be responsible for some of their beneficial effects on body weight.[221]

Notably, the receptor targets for many of these hormones are not restricted to the homeostatic circuitry. Indeed, receptors for these hormones have been found in a number of subcortical regions including the VTA, NAc, LH, and LS.[222-225] Hormone receptors have also been shown to be expressed on DA neurons.[225,226] Their stimulation or inhibition can directly or indirectly regulate firing patterns of VTA DA neurons.[226-233] Insulin has also been shown to affect trafficking and function of the DAT.[227,234] Such anatomical and functional findings support behavioral studies showing that the acute administration of anorexigenic hormones (or their synthetic analogues) reduce the rewarding properties of palatable food as measured by CPP and operant tasks.[235,236] Additionally, antagonists of the receptor for ghrelin (an orexigenic hormone) similarly reduce hedonic feeding behavior.[237] Of note, several of these hormones have been injected directly into the VTA or NAc to generate a similar increase or attenuation in feeding.[238,239] Thus it appears that hormones regulating feeding behavior and energy metabolism likely do so through homeostatic circuits as well as hedonic regulatory mechanisms.

Hormone-induced reductions in rewarded feeding behavior prompted the hypothesis that such hormones might also modulate reward for drugs of abuse. This hypothesis has now been tested for a number of hormones. As will be discussed in great detail in the next section, GLP-1 has been shown to reduce drug reward behaviors relevant to a number of different drugs of abuse.[239] Ghrelin receptor antagonists appear to function in a similar fashion.[237] Interestingly, studies have shown that endogenous ghrelin is necessary for alcohol reward.[240] Insulin, as well, regulates the psychotropic effects of amphetamine and has been proposed to be required for normal reward for food.[52,241-244]

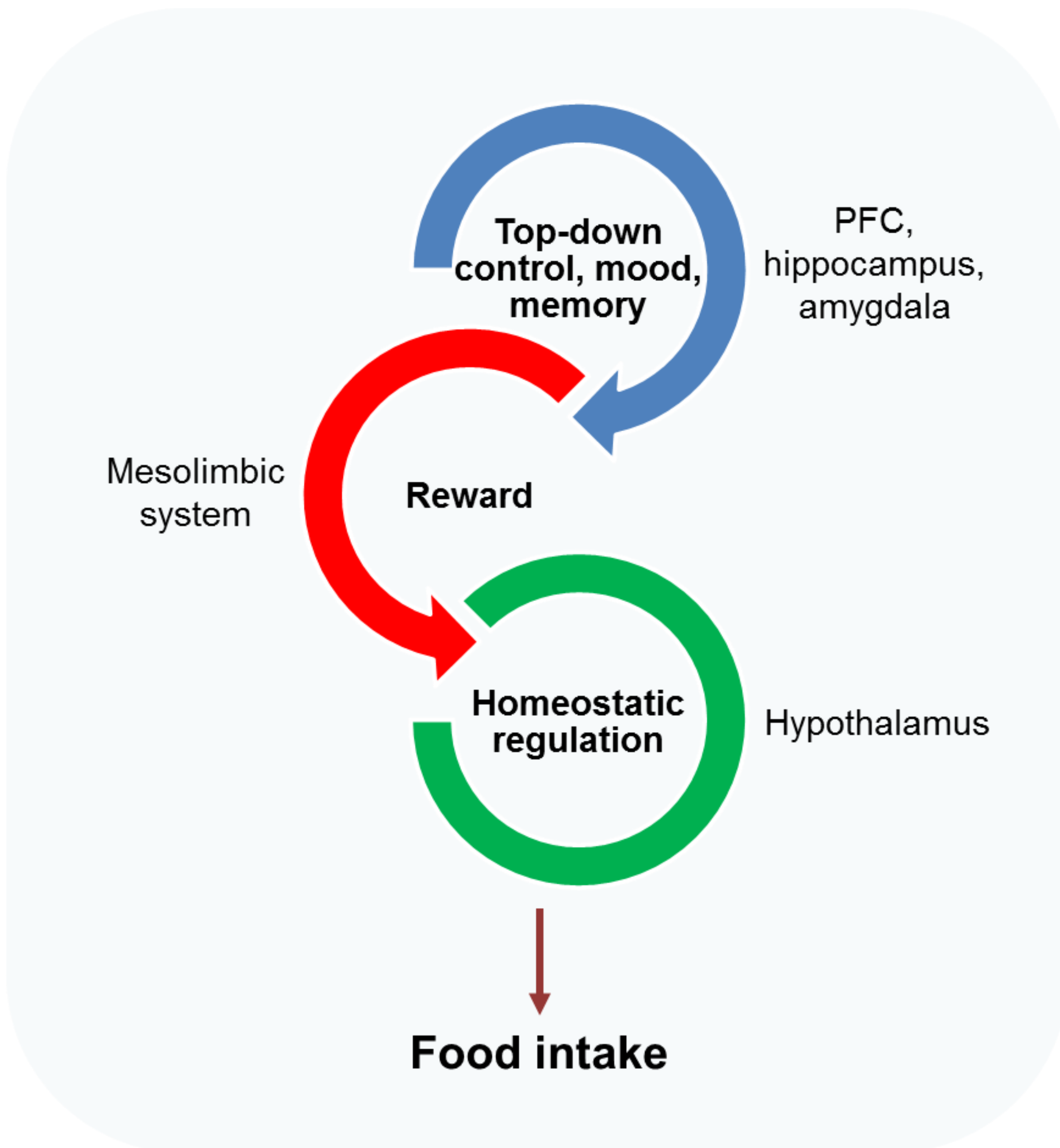

Figure 8. *Levels of central regulation of food intake*. Food intake is controlled by complex neuronal and hormonal systems. Homeostatic regulation of hunger is primarily regulated by the hypothalamus. Reward circuitry including the mesolimbic DA system is the major regulatory pathway for hedonic feeding and may have the ability to override the homeostatic regulation of feeding. Top-down regulation of feeding from the PFC allows us to make informed food choices. Additionally, memory, learning, and mood (regulated primarily by the hippocampus and amygdala) affect our feeding patterns. Circulating signals of energy availability regulate food intake *via* the hypothalamus, but also *via* some of these extra-hypothalamic regions.

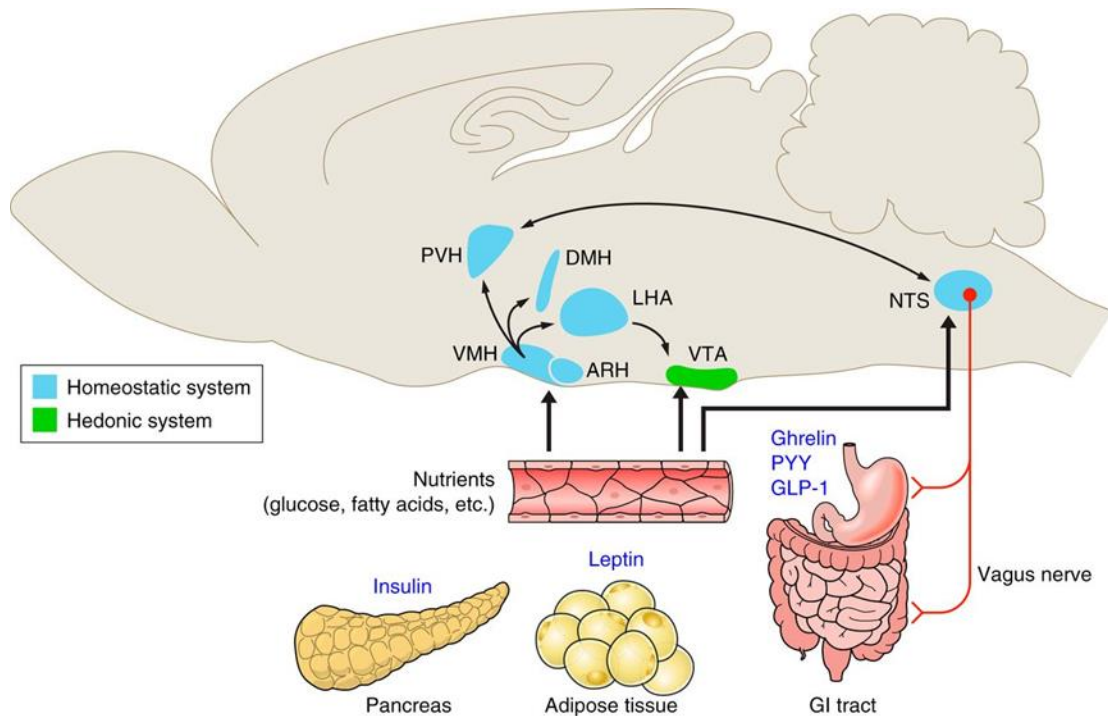

Figure 9. *Major routes for the regulation of feeding and energy balance*. This schematic illustrates routes by which peripheral signals of feeding status and energy balance communicate with homeostatic and hedonic circuits within the brain. Certain areas of the brainstem (NTS), hypothalamus (VMH, ARH), as well as the VTA can receive direct inputs from circulating signaling factors, including hormones (insulin, leptin, ghrelin, PYY, GLP-1, etc.) as well as nutrients themselves (glucose, fatty acids, etc.). These factors are capable of crossing the blood brain barrier. Additionally, the brain can receive nervous information about feeding status and stomach stretch from the vagus nerve which connects the gut to the brainstem NTS. The NTS is a major integrator of peripheral feeding signals and can communicate with the hypothalamus, as well as extrahypothalamic regions. ARH, arcuate nucleus of the hypothalamus; DMH, dorsomedial nucleus of the hypothalamus; LHA, lateral hypothalamic area; PVH, paraventricular nucleus of the hypothalamus; VMH, ventromedial nucleus of the hypothalamus. Adapted from [245].

Bariatric surgeries have also been shown to effectively reduce body weight and appetite in obese individuals. Patients who undergo the popular roux-en-Y gastric bypass (RYGB) lose significant weight, exhibit decreased appetite, and show greater interest in consuming low fat than high fat foods.[246,247] Studies using rodent models suggest that bariatric surgeries may reduce appetite for palatable foods through altered food reward. Following RYGB, rats exhibit increased hedonic reactions to a sucrose solution along with reduced consumption of a high-fat diet.[248] This same group also showed that RYGB rescues motivated feeding behavior in an operant task.[249] Human functional magnetic resonance imaging (fMRI) studies before and after RYGB have also revealed reductions in mesolimbic pathway activation (including the VTA, ventral striatum, and dorsomedial PFC) following combined audio and visual energy-dense food cues.[247] PET studies have also revealed alterations in D2 DA receptor availability and extracellular DA following RYGB.[250,251] Indeed, Ochner and colleagues have suggested that 20–45% of post-RYGB weight loss may be unexplained by change in stomach size and increases in metabolism.[221] Furthermore, weight loss after RYGB may be enhanced by changes in food intake behaviors mediated by enteric hormones like GLP-1. This is physiologically plausible since RYGB increases both basal and postprandial GLP-1 levels[252] and GLP-1 secretion is enhanced by faster delivery of food contents to the gut following the procedure.[221,253-255] Notably, RYGB "good responders" (~40% weight loss) secreted significantly more GLP-1 in response to a postoperative test meal than "poor responders" (<20% weight loss).[254,256]

Interestingly, associations have also been made between bariatric surgery and alcohol use disorder (AUD). The literature on this subject is mixed in regards to whether bariatric surgery promotes or prevents the development of AUD.[257-262] There have been two large clinical studies of AUD outcomes following RYGB. In one of these studies, 6,165 patients who had undergone RYGB surgery were assessed for alcohol intake approximately 6 months after surgery. On a 4 point scale, patients who had previously consumed alcohol "occasionally" or

“frequently” had significantly decreased their alcohol intake score as a group.[262] No changes were noted in patients who had not used alcohol before surgery. This same publication also studied the effect of RYGB on alcohol consumption in rats selectively bred to consume ethanol. Before surgery, sham rats consumed comparable amounts of alcohol to RYGB rats. After surgery, the RYGB rats consumed significantly less alcohol than sham-operated rats. In the second large clinical study, past year AUD symptoms were assessed using the standard Alcohol Use Disorders Identification Test (AUDIT) in patients who had received one of several common bariatric surgery procedures (RYGB, laparoscopic adjustable gastric banding, sleeve gastrectomy, biliopancreatic diversion with duodenal switch, or banded gastric bypass).[261] The prevalence of AUD symptoms did not significantly differ from 1 year before to 1 year after surgery but was significantly increased in the second postoperative year. Notably, this effect was associated with a number of preexisting or preoperative risk factors for the development of AUDs, including male sex, younger age, smoking, regular alcohol consumption, prior AUD, recreational drug use, and lower interpersonal support. Unfortunately, without a matched control group, it is difficult to determine whether these individuals would have been more likely to develop an AUD over time independent of their surgical status.

Alterations in ethanol consumption following RYGB may have be related to changes in circulating hormone levels. Indeed, one study revealed that GLP-1 levels increased selectively in RYGB rats following oral ethanol gavage.[262] Furthermore, intraperitoneal injection of a GLP-1R agonist reduced ethanol consumption in sham rats but not in RYGB rats, presumably because endogenous GLP-1 levels are already elevated in RYGB rats and intake is already low. However, treatment with the GLP-1R-specific antagonist, exendin-(9-39) amide (Ex-9), did not modulate ethanol consumption in either RYGB or sham rats. The lack of response to a GLP-1R antagonist calls into question the hypothesis that elevated endogenous GLP-1 is responsible for the decrease in alcohol consumption observed following RYGB. Furthermore, the effect of

RYGB on alcohol consumption in rodent models appears to be inconsistent, as two other studies found the opposite effect.[259,260] Still, these findings suggest potential avenues for future study.

The mechanisms underlying alterations in feeding through targeting different hormonal systems are still being investigated. Likely, these hormones act on multiple peripheral and central systems to regulate feeding, metabolism, and energy expenditure. However, a common theme emerges from studies of these hormonal systems, which is that many of them regulate brain reward circuitries. This concept is explored through the original work presented within this dissertation.

Glucagon-like peptide 1: Here, I present a detailed background on GLP-1's role in food and drug reward. As previously mentioned, GLP-1 is not the only hormone that bridges these two domains. However, it will serve as a detailed example and provide the necessary background to interpret the original work described within this dissertation.

Numerous studies in animals and humans have established that GLP-1, a 36 amino acid incretin hormone and neuropeptide, acts through both peripheral and central mechanisms to regulate energy homeostasis and feeding behavior.[263,264] Importantly, central GLP-1 signaling has been linked not only to the regulation of energy homeostasis, but also to non-homeostatic reinforcing and motivational processes associated with food reward.[235,265] Most notably, it was recently demonstrated that targeted activation of mesolimbic GLP-1Rs decreases preference for palatable foods as well as the motivation to work for such foods.[235,265,266] While the exact mechanisms underlying the regulation of food reward are still under investigation, these findings have inspired the hypothesis that central GLP-1R signaling plays an additional role in the hedonic response to drugs of abuse. Several recent behavioral studies in animals have provided strong support for this hypothesis. Here I examine the evidence that GLP-1R agonists

modulate the rewarding and reinforcing properties of palatable foods and drugs of abuse through the regulation of established brain reward circuitries. Furthermore, I discuss potential mechanisms of action, with an emphasis on the involvement of the neurotransmitter DA. Understanding the effects of GLP-1 on reward circuitries in the brain will allow us to better assess its utility as a pharmacotherapeutic for both obesity and substance use disorders.

GLP-1 is a peptide hormone produced by L-cells of the intestine in response to nutrient absorption within the gastrointestinal tract. While GLP-1's peripheral effects are many, it is most well-known for its glucoregulatory properties.[267] As an incretin, GLP-1 promotes glucose-dependent insulin release by binding to receptors on pancreatic beta cells. The ability to potentiate insulin secretion has made long-lasting synthetic GLP-1R agonists powerful drugs for the treatment of type 2 diabetes and more recently, obesity.[220] FDA-approved drugs in this class include exendin-4 (Ex-4) and liraglutide, which are resistant to degradation by the enzyme dipeptidyl peptidase-4 (DPP-4). Of interest, a percentage of obese diabetic patients taking these drugs lose weight, an effect that occurs primarily through reduced appetite and decreased food intake.[267,268]

GLP-1 is also a neuropeptide produced by brainstem neurons in the nucleus of the tractus solitarius (NTS) which project widely to subcortical areas containing the GLP-1R.[269,270] The NTS, in turn, receives vagal afferents which provide information regarding taste and mechanical stretch of the gastrointestinal system.[271]

Early studies established that GLP-1's anorectic effect can be induced by direct central administration. GLP-1 reduces appetite and induces weight loss when administered to the cerebral ventricles of rats.[264,272] Central infusion of GLP-1 also activates c-fos immunoreactivity in various nuclei of the brainstem and hypothalamus,[273] and injection of GLP-1 directly into the paraventricular nucleus of the hypothalamus is sufficient to suppress feeding without inducing aversion.[274]

This early research focused on the effects of GLP-1 on the hypothalamus and brainstem, regions highly involved in homeostatic metabolic control.[267,275] However, the homeostatic control of feeding does not fully account for GLP-1-mediated alterations in feeding behavior and food preference. For example, peripheral injection of GLP-1 long-lasting analogues in rodents reduces preference for sweets,[276] while both peripheral and central infusions of GLP-1 analogues reduce motivation to work for food reward.[235] These findings suggest that GLP-1 reduces food intake at least in part through the regulation of food reward in non-homeostatic circuits. In fact, various reports indicate that GLP-1Rs are expressed in components of the mesolimbic reward circuitry.[277] Recently, research exploring the central modulation of food intake by GLP-1 has shifted away from homeostatic circuits and toward areas associated with reward, motivation, and stimulus salience. This shift in focus is timely, as obesity in the U.S. has reached epidemic proportions and homeostatic control is clearly insufficient to prevent excessive food intake. In environments in which high fat and sugary foods are readily available, brain reward systems may override homeostatic systems.[233,243]

The GLP-1R appears to be expressed at low levels in the VTA and the NAc based upon autoradiographic binding studies and *in situ* hybridization; neural tracing also suggests that GLP-1-expressing neurons in the NTS project to these areas.[265,266,269,270,277,278] Furthermore, exogenous intraperitoneal injection with the synthetic GLP-1 analogue, Ex-4, induces Fos activation in the NAc.[277] The GLP-1R is also highly expressed in the LS, a region which may also play a role in modulating mesolimbic reward circuits.

Direct injection of Ex-4 into the VTA or NAc core has been shown to reduce palatable food consumption. In one study, injection of subthreshold doses of Ex-4 into the VTA or NAc core, but not the NAc shell, of food-deprived rats resulted in a significant suppression of sucrose intake at multiple time points compared to vehicle-injected animals.[265] Ex-4 also shifted their preference for high fat food to regular chow when injected into the VTA, NAc core, and NAc

shell, resulting in a reduced 24 h weight gain. Depending on the time of exposure and location of injection, the GLP-1R-specific antagonist, Ex-9,[279] increased or had no effect on high fat diet intake. This suggests that endogenous GLP-1 signaling maintains a degree of control over food intake and preference.

In another study, it was found that GLP-1 injected into the NAc core reduced 2h regular chow intake and induced c-fos expression relative to saline, but no effect was observed in the NAc shell. Again, Ex-9 had the opposite effect on chow intake.[266] Finally, Ex-4 injected into the VTA, but not the NAc shell, reduced 24h chow intake, and the activation of GLP-1Rs in the VTA maintained a significant reduction in food intake even 24h after injection.[235] The combined results of these important studies indicate that (1) the VTA and NAc core are important targets for GLP-1-mediated reduction in sucrose and high fat food preference, (2) the NAc shell is likely not an important site of action for GLP-1 in regard to food palatability, although it may still play a role in motivated behaviors,[235] (3) endogenous GLP-1 signaling in mesolimbic reward areas may be important for controlling perceived food palatability, and (4) the effects of GLP-1R signaling on reward may be relatively long-lived (>24h).

Motivation to obtain food is an important aspect of feeding behavior and is typically assessed through an operant learning paradigm called progressive ratio operant conditioning. In this task, the animal must press a lever progressively more times to receive consecutive rewards. This test has been used to assess motivational incentive following targeted injection of Ex-4 into the VTA or NAc.[235] After VTA injection, Ex-4 reduced the number of sucrose rewards obtained in a dose responsive manner, but injection into the NAc only resulted in a reduction at the highest dose. The effect of Ex-4 on motivated feeding behavior was specific to the GLP-1R, as pretreatment of animals with Ex-9 abolished the suppressive effect of intraventricular Ex-4 on operant conditioning for sucrose reward. Interestingly, this study also considered the operant responsiveness of rats that were “high reward responders” (on vehicle condition, earned 6 or

more sucrose rewards in satiated state) versus “low reward responders” (earned 5 or fewer rewards). They found that the “high reward responders” earned fewer sucrose rewards when given Ex-4 in the VTA, while the “low reward responders” were unaffected. This suggests that some animals may be predisposed to the rewarding effects of food and that Ex-4 only attenuates motivated behavior in susceptible individuals. Furthermore, these data revealed that, at least at the doses used, Ex-4 was unable to reduce the number of rewards earned to the level of vehicle-treated animals in the “low responders” group. This finding is striking as it illuminates the intrinsic challenges to overcoming reward-seeking behavior in vulnerable individuals.[280,281]

These collective findings point to a role for central signaling through the GLP-1R not only in reducing food intake and palatability, but also the motivation to work for food. It should be noted that no aversive behaviors were identified in the aforementioned studies,[235,265,266] despite reports that aversion may partially account for a reduction in food intake.[282] It may be that aversion to GLP-1 is mediated by specific brain regions implicated in aversive responses (e.g. the central nucleus of the amygdala).[235,283]

Excitingly, it was recently discovered that pretreatment with Ex-4 attenuates amphetamine- and cocaine-induced locomotion, suggesting that GLP-1R signaling reduces extracellular DA in response to psychostimulants.[284,285] Moreover, pretreatment with Ex-4 attenuates the rewarding effects of amphetamine and cocaine in a CPP test,[284,286] although preference for cocaine is not entirely abolished, suggesting that GLP-1R-independent components of cocaine reward exist.[286] Ex-4 may possess therapeutic potential in regards to psychostimulant abuse as it has been shown to reduce cocaine self-administration in mice by reducing the dose-response curve.[285] This suggests that the effect of GLP-1R stimulation cannot be overcome by administering higher doses of cocaine.

Interestingly, the ability of GLP-1R signaling to modulate the rewarding properties of drugs extends beyond psychostimulants. Similar effects have been observed for alcohol[287,288] and nicotine.[289] Alcohol use disorders, like psychostimulant abuse, are characterized by compulsive use and seeking. Alcohol promotes the firing of DA neurons in the VTA by impairing GABAergic inhibitory tone. Egecioglu and colleagues found that low doses of intraperitoneal Ex-4 attenuate alcohol-induced locomotor stimulation in rodents and reduce accumbal DA *in vivo*.[287] They also found that Ex-4 reduced alcohol intake in a two-bottle choice model and impaired alcohol CPP under both acute and chronic treatment conditions. Finally, Ex-4 diminished alcohol seeking in a progressive ratio operant conditioning paradigm, suggesting a role for Ex-4 in drug motivation. Interestingly, the rats did not exhibit any rebound increases in ethanol consumption 48h after Ex-4 administration, implying that withdrawal symptoms may be attenuated by Ex-4. A second group also found significant reductions in ethanol CPP and intake following peripheral Ex-4 administration.[288] Of interest, this study noted that the increased ethanol intake of high consumers (top 30%) over low consumers was completely abolished by peripheral GLP-1 administration in a 1h intermittent access drinking paradigm. Furthermore, this study showed that targeted microinjection of GLP-1 and Ex-4 into the VTA could significantly reduce ethanol intake. This is so far the only direct evidence that the VTA is an important site of GLP-1R signaling action in drug reward.

Evidence suggests that DAergic systems are affected by GLP-1R stimulation. Ex-4 reduces amphetamine-, nicotine-, alcohol-, and cocaine-stimulated striatal DA by microdialysis following peripheral Ex-4 injection.[284,285,287,289] In all cases, drugs were administered systemically; thus this result does not indicate whether this occurs as a direct result of striatal GLP-1R stimulation or at the circuit level. Additionally, the contribution of peripheral mechanisms cannot be ruled out. Additionally, it is also important to keep in mind that GLP-1

stimulates insulin release, which is known to regulate drug reward,[52,234] and could potentially explain the reward attenuating effects of peripherally-administered GLP-1 and its analogues.

Alterations in DA homeostasis within the mesolimbic reward system can be mediated by postsynaptic and presynaptic mechanisms in the VTA or NAc. Since GLP-1Rs are expressed at low levels within the VTA, GLP-1R signaling could alter firing rates of DA neurons, which would modulate DA release in the NAc and potentially other projection sites, including the amygdala and the PFC. Firing rates of DA cells are an important component of reward and stimulus saliency and can be influenced by hormonal signaling.[229,233] Currently, we do not know if GLP-1Rs are located on DA or GABA neurons in the VTA, but alterations in the firing properties of either neuronal type could influence DAergic output. Such a mechanism would be similar to that observed for the hormone leptin. Leptin significantly reduces DA neuronal firing rate within the VTA in vivo and in slice preparations,[226] while central leptin infusion attenuates extracellular DA by microdialysis.[231] An alternative (but not mutually exclusive) mechanism is that GLP-1 alters DA signaling and homeostasis directly within the NAc, or in regions that modulate mesolimbic output, such as the LS. Again, the existence of GLP-1Rs in the NAc and LS make this a plausible hypothesis, although we do not know whether GLP-1Rs are expressed pre- or postsynaptically. Presynaptic GLP-1R signaling within the NAc or LS might influence DA homeostasis by acting through the DAT. The DAT is normally responsible for clearing DA from the intrasynaptic space but its activity can be impaired by the actions of abused psychostimulants. It is important to note that other peripherally-generated hormones are capable of modulating DAT function. Insulin, for example, has been shown to regulate the effects of amphetamine by altering DAT surface expression and amphetamine-induced DA efflux in a PI3K-dependent manner.[52,244,290] Furthermore, insulin attenuates amphetamine-induced behaviors.[291] This lends credibility to the hypothesis that similar mechanisms of hedonic regulation are employed by GLP-1R agonists.

In summary, GLP-1 and its analogues attenuate the rewarding properties of food, alcohol, nicotine, and psychostimulants in rodents. The literature reviewed herein suggests that GLP-1 regulates feeding behavior and drug abuse, in part, through DA circuits. Given that GLP-1R agonists are already approved for the treatment of diabetes and obesity, barriers to their development to treat addiction are low.

Unifying hypothesis

The overarching hypothesis behind this work is that drugs of abuse, including cocaine, will be regulated by hormonal systems which evolved to regulate feeding and metabolism. Thus, hormones which reduce the intake of palatable foods will also impair cocaine reward. Moreover, manipulations which chronically alter hormonal systems and reduce feeding, such as bariatric surgery, will similarly reduce drug reward and provide a proof-of-concept that such chronic interventions might provide long-term regulation of reward-related DA homeostasis and reward behaviors.

Specific aims

The aims of this dissertation are as follows:

1. To determine brain substrates of GLP-1R-mediated alterations in DA homeostasis and cocaine behaviors (chapter II).
2. To determine alterations in striatal DA homeostasis following RYGB surgery (chapter III).
3. To determine whether biliary diversion surgery alters cocaine-mediated behaviors and define novel hormonal targets for the treatment of addiction (chapter IV).

CHAPTER II

GLUCAGON-LIKE PEPTIDE 1 RECEPTOR ACTIVATION REGULATES COCAINE ACTIONS AND DOPAMINE HOMEOSTASIS IN THE LATERAL SEPTUM BY DECREASING ARACHIDONIC ACID LEVELS[2]

Preface

Agonism of the GLP-1R has been effective at treating aspects of addictive behavior for a number of abused substances, including cocaine. However, the molecular mechanisms and brain circuits underlying the therapeutic effects of GLP-1R signaling on cocaine actions remain elusive. Recent evidence has revealed that endogenous signaling at the GLP-1R within the forebrain LS acts to reduce cocaine-induced locomotion and cocaine CPP, both considered DA-associated behaviors. DA terminals project from the VTA to the LS and express the DAT. Cocaine acts by altering DA bioavailability by targeting the DAT. Therefore, GLP-1R signaling might exert effects on DAT to account for its regulation of cocaine-induced behaviors. We show that the GLP-1R is highly expressed within the LS. GLP-1, in LS slices, significantly enhances DAT surface expression and DAT function. Ex-4, a long-lasting synthetic analogue of GLP-1 abolished cocaine-induced elevation of DA. Interestingly, acute administration of Ex-4 reduces septal expression of the retrograde messenger 2-AG as well as a product of its presynaptic degradation, AA. Notably, AA reduces septal DAT function pointing to AA as a novel regulator of central DA homeostasis. We further show that AA oxidation product γ-ketoaldehyde (γ-KA) forms adducts with the DAT and reduces DAT plasma membrane expression and function. These results support a mechanism in which post-synaptic septal GLP-1R activation regulates 2-AG levels to alter presynaptic DA homeostasis and cocaine actions through AA.

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[2] The work presented in this section is adapted from Reddy, I.A., Pino, J.A., et al. Glucagon-like peptide 1 receptor activation regulates cocaine actions and dopamine homeostasis in the lateral septum by decreasing arachidonic acid levels. *Translational Psychiatry* (In Press).

Introduction

Cocaine addiction is a highly prevalent disorder characterized by continued drug use in spite of negative consequences. Despite the harmful effects of cocaine, the development of pharmacotherapies to treat cocaine addiction has been slow. This is because modulators of addictive behavior are limited and we lack a comprehensive understanding of their mechanisms. Starting with the discovery that GLP-1 regulates amphetamine-induced locomotion,[292] GLP-1R agonism has been shown to reduce the rewarding properties of cocaine and other drugs of abuse in rodents.[239,285,293]

GLP-1 is both a hormone produced by L cells of the intestine and a neuropeptide produced by the NTS.[265,267] Notably, GLP-1 synthetic analogues are already approved to treat type 2 diabetes and obesity in humans.[294,295] These synthetic derivatives have a greatly extended half-life over endogenous GLP-1.[267,294] As such, they remain in circulation long enough to cross the blood-brain barrier.[296] The importance of the brain bioavailability of GLP-1 analogues is underscored by the prominent expression of the GLP-1R within the brain.[269] Ex-4 is one such synthetic GLP-1 analogue that has been used extensively to demonstrate that GLP-1R activation reduces the preference and actions of cocaine, amphetamine, alcohol, and nicotine.[235,239,284,286-289,292,297] Importantly, the *GLP-1R* 168Ser allele has been associated with increased measures of alcohol self-administration in humans.[297] These data suggest that GLP-1R signaling might regulate common mechanisms within the brain's reward system.

The neurotransmitter DA underlies the rewarding properties of drugs of abuse, as well as natural rewards.[199] It is released at DA terminals during drug intake and in anticipation of the consumption of drugs.[110,199] We therefore hypothesized that the effects of GLP-1R stimulation on cocaine reward would occur at DA nodes exhibiting GLP-1R signaling. One such node is found within the forebrain LS. The LS possesses a high concentration of GLP-1Rs and contains DA projections.[269] Additionally, the LS has historically been linked to reward; it was identified by

Olds and Milner (1954) as a potent site of electrical self-stimulation in rats.[81] Since then, several studies have found that the LS is fundamental to the rewarding properties of drugs of abuse.[130,134,135,293] Most notably, the Aston-Jones group has found that the LS is an important relay center between the hippocampus and the VTA for information about cocaine-context associations and that the LS is necessary for the formation of cocaine CPP. [134,135]

Recently, Harasta *et al.* (2015) found that endogenous signaling through the GLP-1R in the LS acts to dampen cocaine-induced locomotion and cocaine CPP, both considered DA-associated behaviors.[293] In the current study, we sought to determine the mechanism by which GLP-1R stimulation modulates septal DA homeostasis and the actions of cocaine. Cocaine blocks DA reuptake *via* the DAT, resulting in high levels of synaptic DA. Here we demonstrate that GLP-1R stimulation inhibits the ability of cocaine to increase extracellular DA levels. This phenomenon is associated with increased DAT surface expression and function mediated by a reduction in endocannabinoid and AA levels in the LS. These findings reveal a novel mechanism of GLP-1R action in brain and provide a more comprehensive understanding of how emerging GLP-1R-targeted therapies affect signaling in the brain.

Methods

Animals. Male NMRI mice (Taconic, Denmark) were used for microdialysis and c-fos experiments.[285] Sprague-Dawley rats, weighing 320-345g, were also used for the study (Taconic, Lille Skensved, Denmark). All other *in vivo* experiments used adult male C57BL/6 mice from Jackson Laboratories or bred internally at Vanderbilt University. All animals were kept at room temperature in a 12h light/dark cycle with free access to food and water. All experiments were performed during the light cycle. All experiments were in accordance with directives of “Principles of Laboratory Animal Care” (NIH publication No.85-23) and approved by

either the Danish Experimental Animal Inspectorate and the council of the European Communities or the Vanderbilt Institutional Animal Care and Use Committee.

Drugs and Materials. For in vivo studies Ex-4 was purchased either from Tocris Bioscience, UK or from Prospec, Israel. DA used as standards for HPLC was obtained from Sigma (St. Louis, MO, USA). Acetonitrile was chromatography grade (Merck, Darmstadt, Germany). All other chemicals used for microdialysis/HPLC (Merck or Fluka Chemie AG, Buchs, Switzerland) were of analytical grade or better and were used as supplied. GLP-1(7-36)-amide was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA) and Ex-9 was purchased from American Peptide Company (Sunnyvale, CA, USA) and was dissolved in artificial cerebrospinal fluid (aCSF) on the day of the experiment. Radiolabeled DA, $[^3H]$-DA (3.4-[7-^{3}H]dihydroxyphenylethylamine), was supplied by PerkinElmer Life Sciences (Waltham, MA, USA), and cold DA was obtained from Calbiochem (La Jolla, CA, USA). For in vitro experiments, AA, goat anti-γ-ketoaldehyde adduct (neuroketals antibody, C-17, sc-130089), and peroxidase (HRP)-goat anti-rat IgG (sc-2006) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rat anti-DAT (MAB369) antibody was obtained from Chemicon (Temecula, CA, USA). Bicinchoninic acid, sulfo-NHS-LC-LC-biotin, NeutrAvidin®-Agarose resin, HRP-rabbit anti-goat IgG (31402) and HRP-goat anti-rabbit IgG (31460) were obtained from Pierce (Rockford, IL, USA). Protein A and protein G Sepharose beads were obtained from GE Healthcare (Little Chalfont, UK). Lipofectamine® 2000 and 2.7-dihydrodichlorofluorescein diacetate (H_2DCFDA) were obtained from Invitrogen (Carlsbad, CA, USA). Salicylamine was obtained from Matrix Scientific (Columbia, SC, USA). Anti-dsRed was obtained from Clontech Laboratories (632496, Mountain View, CA, USA). Synthetic γ-ketoaldehydes were generously provided by Dr. Sean Davies (Vanderbilt University, USA).[1] AA used in ex vivo experiments, and all other chemicals used in this study were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO, USA).

Generation of BAC transgenic mice: BAC transgenic mice were created by the Vanderbilt Transgenic and Embryonic Stem Cell Core as previously described[2] using a BAC clone containing the *Glp1r* gene (34.6 kb), which was flanked by 6 exons of the Dnahc8 gene (27 kb) and the first two exons of the Umod1 gene (18 kb) at the 5' and 3' ends, respectively; this clone was transferred into SW105 cells. An FRT-flanked antibiotic (Kan/Neo) cassette, containing a DNA fragment of an mApple fluorescent reporter was electroporated into the normal ATG start codon of the *Glp1r* gene within the BAC clone. Antibiotic-resistant colonies with the correct insertion were selected, and the FRT-flanked cassettes were removed via bacterial FLP recombination. The final BAC vector construction was confirmed by sequencing of all recombination junctions and by pulsed-field and standard fingerprint gels to correspond with predicted restriction digests. Validated vectors were injected into B6D2 embryos via pronuclear DNA microinjection. Embryos were injected into pseudopregnant B6D2 F1 hybrid females (B6D2F1/J; Jackson Laboratories, Bar Harbor, ME). Genotypes were determined via PCR and fidelity of transgene expression in the CNS was determined by combined immunohistochemistry and ISH.

Combined immunohistochemistry and ISH *validation of GLP-1R BAC transgenic mouse.* To eliminate the possibility of aberrant mApple expression in our GLP-1R BAC transgenic mouse, we tested for co-localization of the mApple protein with GLP-1R mRNA. To accomplish this, we obtained brain sections from these mice from cortex through midbrain. Mice were deeply anesthetized with Nembutal sodium (120mg/kg; Akorn, Inc., Lake Forest, IL, USA) and transcardially perfused with RNAse-free PBS followed by RNAse-free paraformaldehyde (PFA). Brains were extracted, post-fixed for approximately 2h in 4% paraformaldehyde (PFA), cryoprotected overnight in 30% sucrose, and sectioned by microtome (60 μm). Sections were preserved in sucrose at -20°C until use. Selected sections were then processed through an ISH assay (RNAscope kit) followed by IHC. All probes, protocols, and RNAscope kit were generated

by and obtained from Advanced Cell Diagnostics (Hayward, CA, USA). Following the RNAscope assay, sections underwent IHC. Sections were blocked for 15 minutes in 0.1M PB containing 2% goat serum and 0.1% triton-X then incubated in 1% sodium borohydride in 0.1M PB for 30sec. Sections were washed and placed in primary antibody. Cells expressing mApple were identified with rabbit anti-dsRed (1:1000) and HRP-conjugated goat anti-rabbit (1:200, Perkin Elmer, NEF812001EA), and visualized by immunofluorescence with fluorescein-Cy3 amplification to produce green fluorescence (Perkin Elmer, NEL704A001K). Sections were imaged with a confocal microscope (Zeiss LSM 510 Meta).

Immunohistochemistry for DAT and mApple. Brains from GLP-1R BAC transgenic mice were extracted, post-fixed overnight in PFA and sectioned by vibratome (75μm). Sections were immunostained and imaged as described for IHC above. Cells expressing mApple were identified with rabbit anti-dsRed (1:1000) and HRP-conjugated goat anti-rabbit secondary (1:200, Perkin Elmer, NEF812001EA). DAT was identified by staining with anti-DAT (MAB369, 1:1000) and goat anti-rat secondary (1:5000, Santa Cruz Biotechnology).

High speed chronoamperometry. Slices containing LS (300μm) were prepared and chronoamperometry performed as previously described.[298] High KCl aCSF (with 30mM KCl, 97.5mM NaCl) was applied to the bath for 2 minutes to induce DA release. The oxidative signal measured in the slices is attributable to DA as the reduction/oxidation charge ratio is in the range 0.6-1.0.

c-fos expression. The procedure for c-fos IHC is as previously described.[299] Briefly, coronal brain sections (15μm) were cut through septum using a cryostat. Synthetic oligonucleotide DNA probes (DNATechnology, Aarhus, Denmark) were used for visualization of c-fos mRNA by autoradiography. The probe sequence was: 5′-CGG-GCA-GTG-GCA-CGT-CTG-GAT-GCC-GGC-TGC-CTT-GCC-TTC-TCT-GAC-TGC-3′.

In vivo microdialysis in mice. Microdialysis was performed as described previously[285] with few modifications. The dialysis probe was positioned in LS, AP: 0.5mm, ML: 0.3mm relative to the bregma and DV: -2.2mm relative to skull surface.[300] The microdialysis probe was perfused at a rate of 0.9μl/minute with aCSF. The first two 30 minute samples were discarded to obtain stable basal values. Hereafter, 30 minute fractions were collected to establish baseline DA levels. Subsequently, mice were injected with Ex-4 or vehicle, and three 30min fractions were collected. Mice were then subject to local perfusion with cocaine (50μM) for 30 minute and five 30 minute fractions were collected. All fractions were assayed immediately after collection using HPLC with electrochemical detection.[301]

In vivo microdialysis in rats. Rats were placed in a stereotaxic instrument under servoflurane anesthesia (2%) in a mixture of 20% CO2 and 80% oxygen. The anesthesia was maintained during surgery and experiment. A small hole was drilled bilaterally to allow probes to be placed into right and left LS. Probes (CMA/12, 2mm from CMA/Microdialysis AB, Stockholm, Sweden) were gently placed at the following coordinates in mm: AP:+1.0 ML:+ or- 0.4 and DV:-6.2 (relative to bregma). Rats were habituated 2h before the microdialysis sampling. Sampling (flow rate=0.9µl/minute) was at 20 minute intervals for 60 minutes (three fractions) prior to the intervention. After the seventh fraction was collected (10 total fractions), the rats were sacrificed by rapid decapitation. The brain was extracted and sliced to verify the probe location. Only results from rats with probes verified in the LS are reported here. Ex-4 (local or i.p.) or cocaine (50µM local) were administered. Local Ex-4 was administered via a plastic-one cannula glue to the microdialysis probe, and cocaine was perfused via the 2mm microdialysis probe (0.9µl/minute for 20 minutes). All compounds were dissolved in Ringer.

Ex vivo slice protein biotinylation assay. The procedure for biotinylation of live brain slices has been validated and was performed at 28°C as described previously.[302]

Ex vivo slice [^{3}H]DA transport assay. Slices were obtained as described above and allowed to recover (≥1h at 28°C) with continuous oxygenation. Following recovery, slices were treated with drug (i.e. GLP-1) for 10 minutes followed by application of 50nM [^{3}H]DA for 10 minutes with drug still present at 28°C. When Ex-9 was used, slices were pretreated with Ex-9 for 10 minutes prior to addition of drug/vehicle. Total protein was taken, and the samples were processed for protein concentration using a Bio-Rad protein assay and spectrometry at 595nm. Equivalent volumes of sample were added to 3mL of scintillation fluid (Ecoscint H, National Diagnostics, Atlanta, GA, USA) and radioactive counts were measured by scintillation counter. [^{3}H]DA was divided by protein and normalized to average control uptake from paired vehicle-treated slices from the same animal. The uptake protocol and DAT specificity were validated in slices treated with DAT blocker GBR-12909 (Sigma-Aldrich), which blocked uptake in LS by 58.2% ($t(5)=11.97$; $p<0.0001$ by Student's *t* test; n=6).

2-AG and AA measurements. Mice were injected with Ex-4 or vehicle (i.p.) 30 minutes prior to sacrifice. Mice were rapidly decapitated and brains were obtained and blocked to obtain DS and LS punches. Punches were quickly placed into Eppendorf tubes on dry ice and stored at -80°C until use. Samples were homogenized in methanol and centrifuged; water was added to the supernatant for a final ratio of 70:30 Methanol:Water. LC/MS was performed as previously described.[303] Following LC/MS, excess methanol was suctioned off the pellets. Pellets were resuspended and processed for protein concentration using a Bio-Rad protein assay and spectrometry at 595nm.

Cell culture. Rat pheochromocytoma cells (PC12) were obtained from the American Type Culture Collection (Manassas, VA), and were cultured in DMEM supplemented with 6% fetal bovine serum (heat inactivated), 6% horse serum (heat inactivated), 1mM glutamine, 100U/ml penicillin and 100µg/mL streptomycin at 37°C in a humidified 5% CO_2 incubator.[304] PC12 cells overexpressing human DAT fused to GFP (hDAT cells) were stably transfected with the plasmid

peGFP-C2-hDAT using Lipofectamine® 2000 and were cultured under the same conditions. Krebs-Henseleit (KH) buffer was used (144mM NaCl, 4.2mM KCl, 1.6mM $MgCl_2$, 1.6mM KH_2PO_4, 10mM HEPES, pH7.4) and Fe^{+3}(197μM)/ascorbate(19.7mM) solutions were prepared in KH buffer. In some cases, salicylamine, a γ-KA scavenger, was added to the culture media and preincubated with the cells for 1h at 37ºC prior to further treatments. Cell viability was measured using trypan blue staining.

Reactive oxygen/nitrogen species (ROS/RNS) measurements. In order to obtain the necessary conditions to induce γ-KAs formation from lipid peroxidation of AA, oxidative stress was promoted in hDAT cells. ROS/RNS formation in hDAT cells was measured using the fluorescence of H_2DCFDA according to previous reports from our laboratory.[3]

PC12 Transport assays. DAT-mediated uptake of DA in PC12 cells overexpressing a GFP-tagged DAT (hDAT) has been described previously.[305]

Cell surface biotinylation in hDAT cells. Cell surface biotinylation was performed as described by Egaña et al. (2009) with some modifications.[305]

Immunoprecipitations. Immunoprecipitations were performed using 1mg total protein. To immunoprecipitate DAT-GFP, cell homogenates were incubated overnight at 4°C with a monoclonal anti-GFP antibody (G-6539, 1:100), followed by the addition of 50μl of a mixture of protein A and protein G Sepharose beads (2h at 4°C in rotatory shaker). Immunoprecipitated proteins were recovered by centrifugation at 14.000xg for 2 minutes (4°C), washed with ice-cold buffer D, and resuspended in protein sample buffer containing βME. Proteins were separated by SDS-PAGE and transferred to PVDF membranes before incubation with an antibody against γ-KA-lysil adduct (neuroketals antibody, C-17, 1:400) and anti-DAT (MAB369, 1:1000).

Analyses. Data are presented as means ± standard error of the mean (SEM). Data were examined with Student's *t* test for comparisons of only two data sets and one-way or two-way

ANOVA for comparisons of multiple groups, followed by Tukey's- or Bonferroni-corrected comparisons. In *ex vivo* [^{3}H]DA uptake experiments, samples with low protein content (<6µg/mL) were excluded from analysis (n=1). Optical density of bands was determined using Image J software. Statistical analysis was performed with GraphPad Prism software, version 5.02 (GraphPad Software, San Diego, CA, USA). A p-value <0.05 defined statistical significance.

Results

GLP-1R expressing neurons are juxtaposed to DA terminals in the LS

The GLP-1R is highly expressed within the LS,[269] a brain region in which DAT-expressing DA terminals reside.[306] Here we first describe their proximity and anatomical distribution in this region (**Figure 10**). To this end, we generated a transgenic mouse expressing a bacterial artificial chromosome (BAC) for the protein mApple under the control of the promoter for the GLP-1R. mApple signal was amplified by immunostaining (Figure 10A, C, D, and F) and DAT was labeled in parallel (Figure 10B, C, E, and F). Within the rostral LS (Figure 10A-C), DA terminals are found medially in the intermediate LS subregion (LSi), directly adjacent to GLP-1R-expressing cells found mostly in the dorsal LSi. We observed visible overlap between these cell bodies/projections and DA terminals. Within the caudal LS (Figure 10D-F), there is similar evidence for this anatomical overlap. The DA terminals are labeled as two strips extending dorsomedially to ventrolaterally in the LSi. The GLP-1R-expressing cells are also found in the caudal LSi, but the cell bodies additionally extend further into the more medial laterodorsal tegmental area (Ld). Importantly, we show that DA terminals within this region release DA: superfusing LS slices with an artificial cerebrospinal (ACSF) solution containing high [K^+] causes DA release as recorded by high-speed chronoamperometry (Figure 10G; approximate electrode

placement is denoted by * in Figure 10F). The use of mApple for identifying GLP-1R-expressing neurons was validated by *in situ* hybridization (ISH; **Figure 11**).

Systemic administration of a GLP-1R agonist reduces cocaine-induced DA elevation and cfos expression in the LS

We next tested whether a therapeutically relevant administration (i.e. systemic administration) of Ex-4 was capable of altering neuronal activity in the LS. We first evaluated the effect of Ex-4 on the activation of septal neurons by measuring c-fos expression. Ex-4 administration (i.p.) 30 minutes prior to sacrifice decreased basal c-fos gene expression by ISH relative to vehicle-treated mice (Figure 10H and I). This result indicates that systemic administration of Ex-4 alters neuronal activity in the LS.

The psychogenic and addictive properties of cocaine are mediated, at least in part, through the blockade of DAT-mediated DA reuptake and an increase in extracellular DA.[66,307] Therefore, signaling pathways able to modulate or diminish this DA increase offer enormous therapeutic potential. We determined the effect of systemic Ex-4 on local cocaine-induced changes in DA homeostasis *in vivo* within the LS, as measured by microdialysis in freely moving mice. Perfusion of cocaine through the microdialysis probe increased septal DA ~5-fold (Figure 10J; $F(9,49)=2.73$, $p=0.014$ by ANOVA). Systemic pretreatment with Ex-4 (i.p.) 90 minutes prior impaired cocaine's ability to significantly increase extracellular DA. These results were paralleled in rats (**Figure 12**A), demonstrating across species that GLP-1R activation diminishes cocaine's ability to increase extracellular DA in the LS.

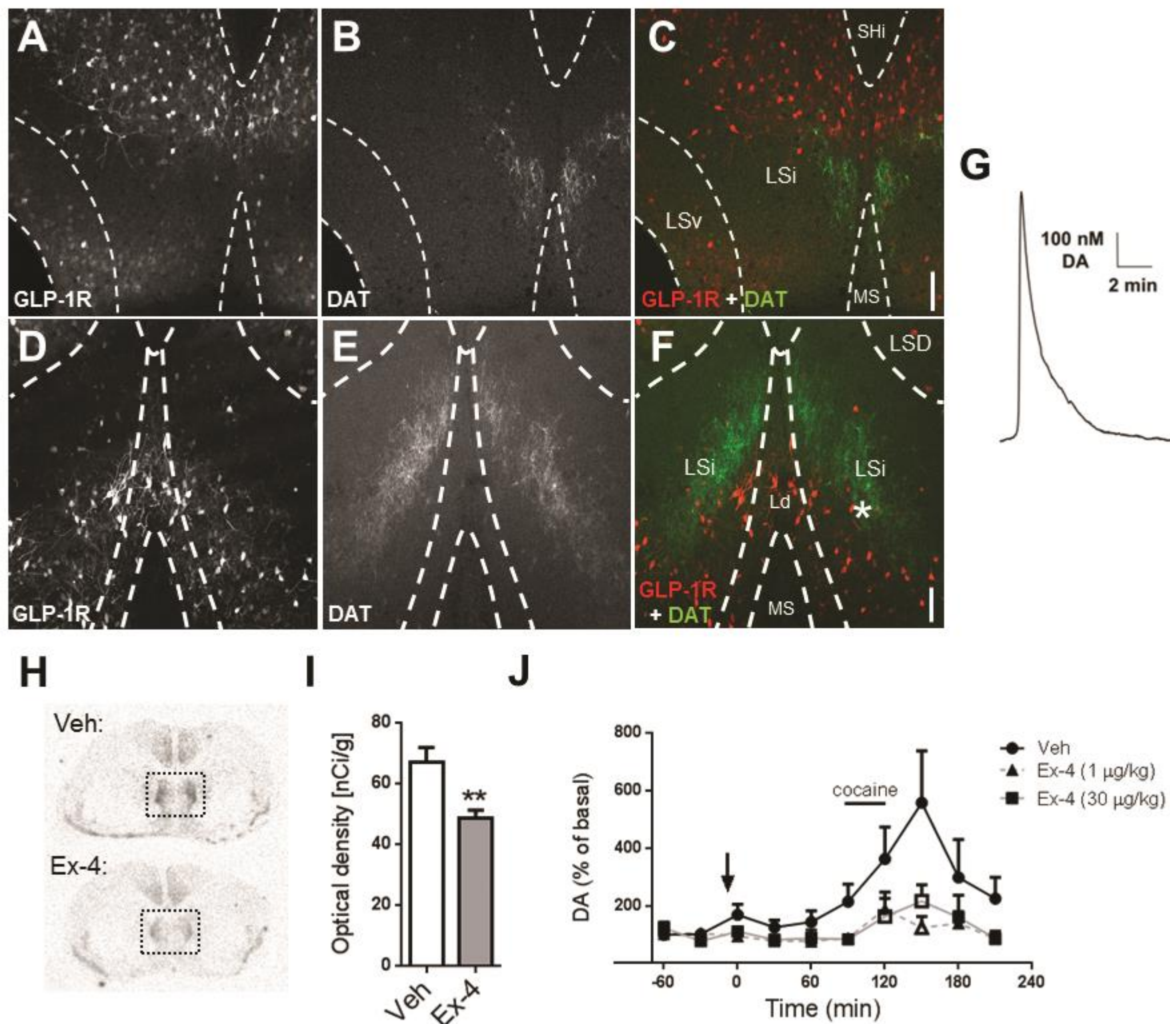

Figure 10. *The GLP-1R is highly expressed in LS where GLP-1R agonists block cocaine-induced DA in vivo.* (**A-C**) Cells expressing the GLP-1R (white in A, red in C) and terminals expressing DAT (white in B, green in C) in rostral LS (GLP-1R in A, DAT in B, merge in C). Confocal images in z stack through 13 planes taken with a 10X objective. (**D-F**) Cells expressing the GLP-1R (white in D, red in F) and terminals expressing DAT (white in E, green in F) in caudal LS (GLP-1R in D, DAT in E, merge in F). Confocal images in z stack through 14 planes taken with a 10X objective. (**G**) DA release induced by 30mM KCl (representative of n=3) in section from the caudal LS. Approximate site of recording is demarcated by * in F. Ld=laterodorsal tegmental nucleus; LSd=dorsal LS; LSi=intermediate LS; LSv=ventral LS; MS=medial septum; SHi=septohippocampal nucleus. Dotted lines delineate region boundaries.[300] Scale bars=100µm. (**H**) Representative cfos expression in the LS 30 minutes following vehicle (top) or Ex-4 (30µg/kg, i.p., bottom) injection. Dotted box includes the LS. (**I**) Quantification of optical density analysis of cfos autoradiographs in LS (t(14)=3.26; **p<0.01 by Student's *t* test; n=8). (**J**) Cocaine (50µM) greatly increased septal DA levels in mice pretreated with saline (i.p., solid circles). This cocaine-induced DA increase is significantly diminished in mice pretreated with Ex-4 (1µg/kg i.p., solid triangles; 30µg/kg i.p., solid squares). Extracellular concentrations of DA are expressed as the percentage of basal levels in two fractions collected before the intervention; significant ANOVA. Open symbols indicates significance by *post hoc* test where p<0.001 at 150 minutes and p<0.05 at 120 minutes; n=5-6 per group.

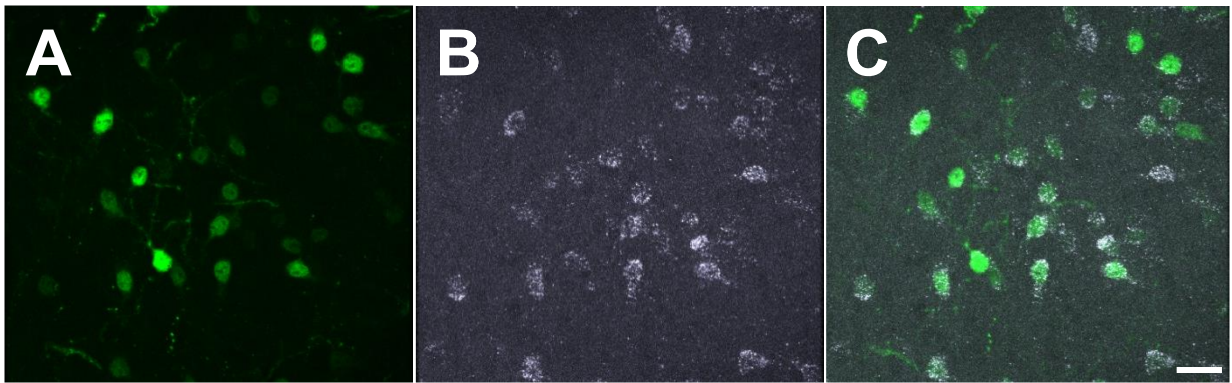

Figure 11. *GLP-1R mRNA and mApple reporter are highly co-expressed in the LS of GLP-1R BAC reporter mice*. Mice expressing mApple protein under the GLP-1R promoter (A, green) express GLP-1R mRNA (B, white) mApple in the same cell bodies (C, merge). Confocal images of a z stack through 23 planes of view taken with a 40X objective. The scale bar in C represents 25 µm.

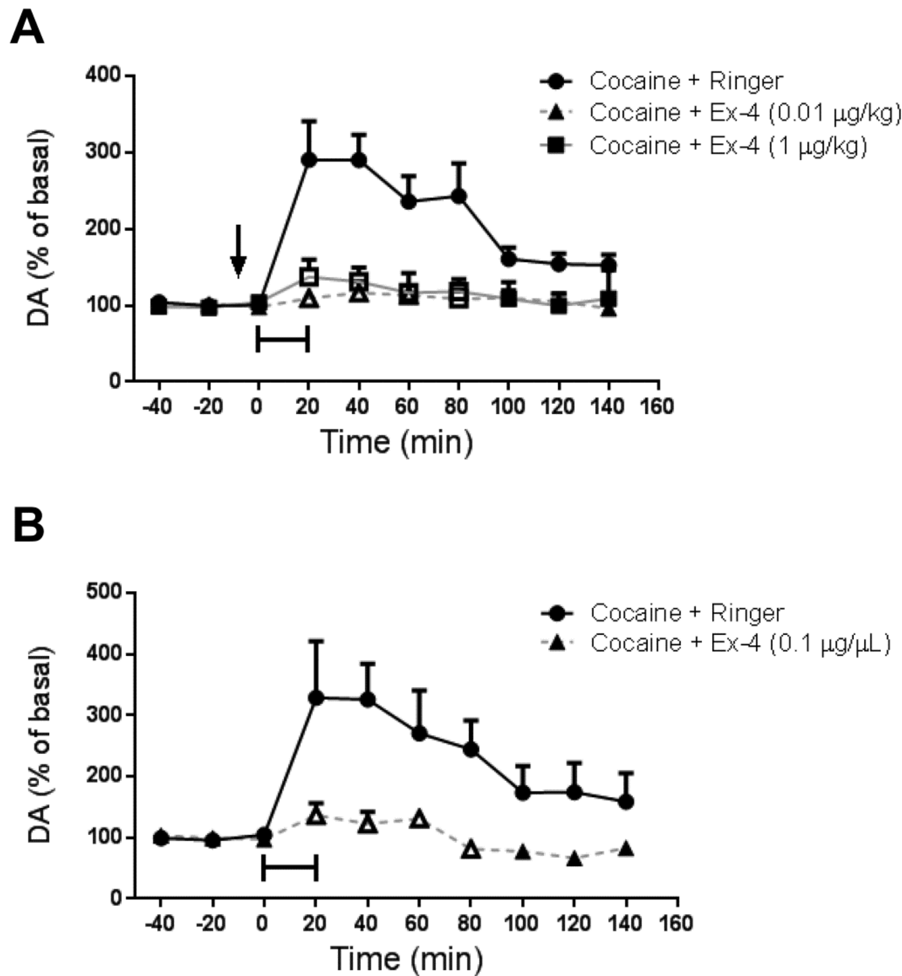

Figure 12. *Extracellular concentrations of DA measured by microdialysis in the LS in anesthetized rats following systemic or local septal application of vehicle or Ex-4 concurrent with local cocaine perfusion.* (**A**) Effect of Ex4 0.01µg/kg i.p (solid triangles), Ex4 1µg/kg i.p. (solid squares), and cocaine alone (solid circles, 50µM, 0.9µl/minute in 20 minute). Extracellular concentrations of DA are expressed as the percentage of basal levels in three fractions collected before the intervention. Significant two-way ANOVA. Open symbols indicate significance by *post hoc* test ($p<0.001$ for all open symbols compared to cocaine + ringer, n=4). (**B**) Effect of Ex4 0.01µg/µL (solid triangles), cocaine 50µM alone (solid circles). Extracellular concentrations of DA are expressed as the percentage of basal levels in three fractions collected before the intervention. Significant two-way ANOVA. Open symbols indicate significance by *post hoc* test ($p<0.001$ at time 20 and 40, $p<0.05$ at time 60, and $p<0.01$ at time 80, n=4-5).

Local GLP-1R signaling blocks cocaine-induced elevation in DA, promotes septal DAT membrane expression, and increases DA uptake

We next sought to determine whether local GLP-1R activation could account for observed systemic effects of Ex-4 on the LS. Ex-4 or vehicle was perfused through the microdialysis probe concurrently with cocaine (Figure 12B). Local administration of Ex-4 impaired the ability of cocaine to increase extracellular DA levels. This finding further highlights the role of septal GLP-1Rs in modulating cocaine's ability to enhance DA function.

Multiple mechanisms could explain how local GLP-1R signaling impairs cocaine-induced increases in extracellular DA in the LS. However, DAT-mediated DA reuptake is the primary mechanism regulating extracellular DA levels,[65] a process dynamically regulated by DAT plasma membrane expression.[241,244,308-310] Notably, DAT expression is inversely proportional to the ability of cocaine to cause its behavioural effects.[68,69] Therefore, we hypothesized that GLP-1R signaling regulates DA homeostasis in the LS, as well as cocaine's ability to increase extracellular DA, by controlling DAT surface expression. In support of this mechanism, GLP-1 application increased surface levels of DAT as measured by LS slice biotinylation[311] (**Figure 13**A and B). In order to determine whether the elevation of DAT surface expression translated to functional changes in DAT activity, we measured uptake of [^{3}H]DA in septal slices treated with GLP-1. GLP-1 significantly increased DA uptake (Figure 13C). This increase was specific to GLP-1R signaling, as demonstrated by its blockade by the selective GLP-1R antagonist, exendin-(9-39)-amide (Ex-9; Figure 13D).[279] Ex-9 alone had no significant effect (91% ± 13%; $t(3)=0.6749$; $p=0.55$ by Student's *t* test; n=4). These studies were performed in slices in which only the local circuits are intact; therefore, we can assume that our observations were the result of local GLP-1R stimulation and not the result of feed-forward circuit-level changes. Together, these data demonstrate that GLP-1R signaling increases both DAT expression at the plasma membrane and DA uptake, suggesting that GLP-1R signaling might regulate cocaine actions in the LS through DAT-dependent mechanisms.

Ex-4 reduces levels of the retrograde messenger, 2-AG, and lowers levels of AA in the LS

Our findings thus far suggest that septal GLP-1R agonism affects DAT expression and function locally. However, the GLP-1R is predominantly expressed postsynaptically (Fig. 10 and 11), while the DAT is located on presynaptic DA terminals (Figure 10). This raised the possibility that GLP-1R signaling regulates DAT function through a retrograde signal. 2-AG is a well-established endogenous cannabinoid retrograde messenger whose signaling has been shown to modify reward and feeding.[229,312-314] Of note, cannabinoid CB1R antagonists, which decrease 2-AG signaling, have been shown to decrease cocaine reward.[315] 2-AG is synthesized post-synaptically, but degraded into free AA in presynaptic axon terminals. Indeed, 2-AG hydrolysis via MAGL is the primary mechanism for free AA generation in the CNS.[316] Importantly, AA impairs DAT-mediated DA uptake.[55,317,318] Thus we sought to determine whether GLP-1R activation modulates 2-AG-AA signaling. To this end, mice were injected with Ex-4 (i.p.) 30 minutes prior to sacrifice, and the LS was punched and analyzed by mass spectrometry for 2-AG and free AA levels.[316] We found that Ex-4 significantly reduced both 2-AG and AA levels in the LS (**Figure 14**A and B), pointing towards reduced endocannabinoid metabolism in the LS. This phenomenon was not observed in the DS, where DAT is expressed in high quantities but GLP-1R-expressing cells are only sparsely distributed (**Figure 15**). These data demonstrate region-specific regulation by GLP-1R signaling of 2-AG levels and its metabolism.

AA reduces DA uptake and DAT surface expression

Among its functions as a signaling molecule, AA has been previously shown to impair DAT-mediated DA uptake.[55,317,318] Consistent with findings from heterologous cell lines and striatal synaptosomes[55,317,318] we first demonstrated that AA application to LS slices significantly reduced DA uptake (Figure 14C).

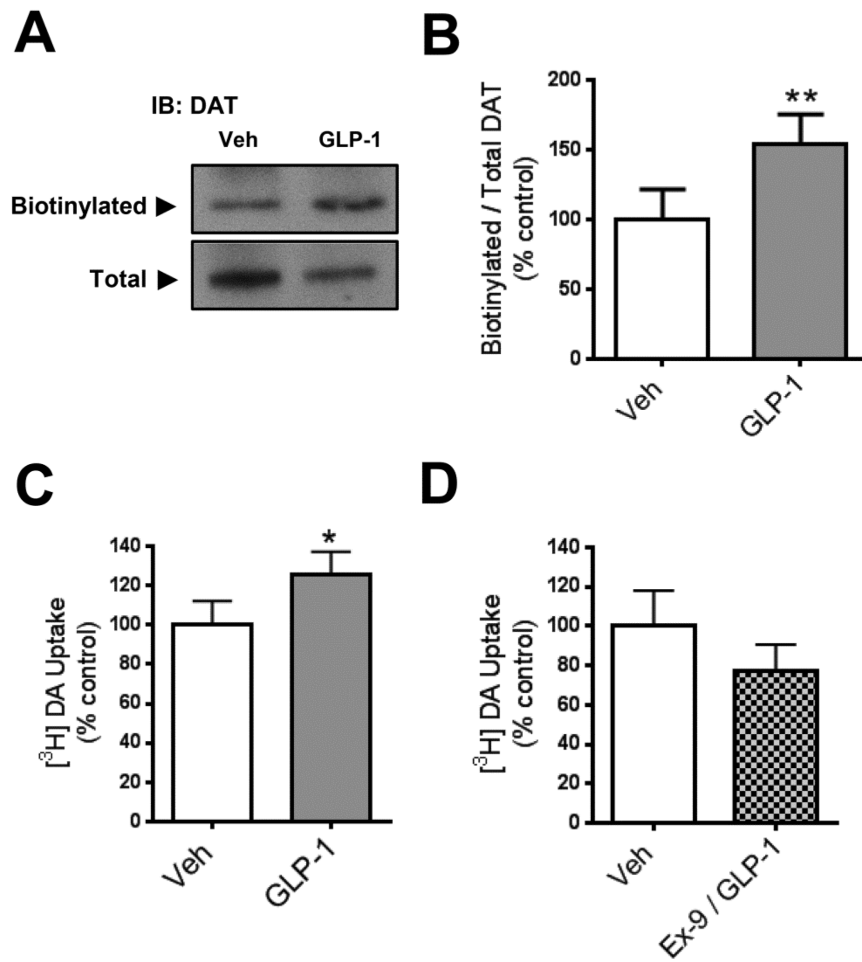

Figure 13. *Local activation of the GLP-1R promotes septal DAT membrane expression and [^{3}H]DA uptake.* (**A**) Representative immunoblot of surface and total DAT following slice treatment with vehicle (aCSF) or 1nM GLP-1. (**B**) Ratio of DAT surface expression in GLP-1-treated *ex vivo* septal slices as percentage of average vehicle values ($t(3)=10.12$; $^{**}p<0.01$ by Student's *t* test; $n=4$). Slices were treated with 1nM GLP-1 for 20 minutes. (**C**) [^{3}H]DA uptake in the LS from *ex vivo* slice preparation following 20 minute treatment with vehicle (aCSF) or 10nM GLP-1. Uptake was significantly elevated ($t(4)=2.35$; $^{*}p<0.05$ by Student's *t* test; $n=5$). (**D**) Pretreatment with 100nM Ex-9 for 10 minute blocks the increase in [^{3}H]DA uptake following treatment with 10nM GLP-1 ($t(3)=1.047$; $p=0.19$ by Student's *t* test; $n=4$).

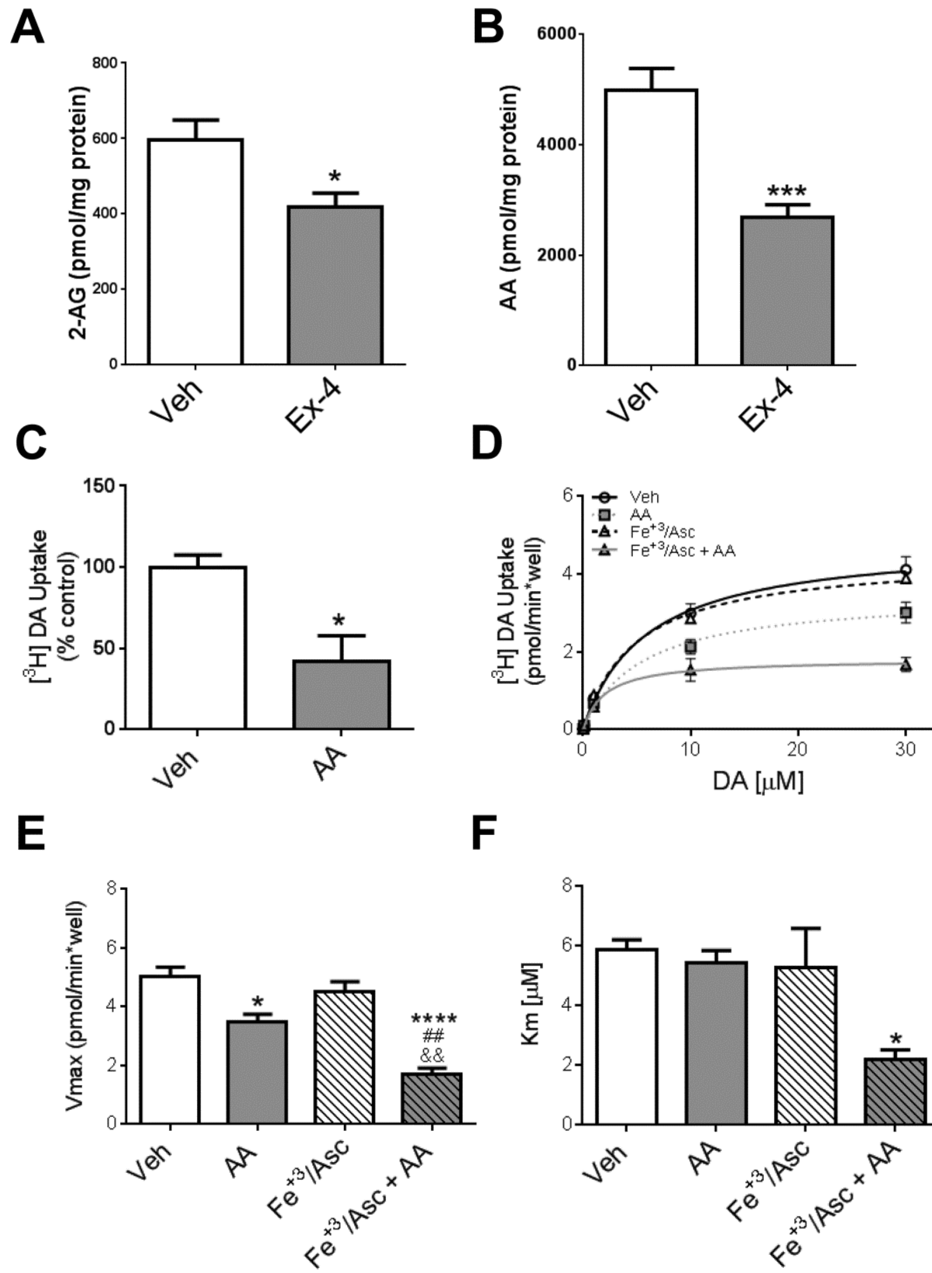
A
B
C
D
E
F
2-AG (pmol/mg protein)
AA (pmol/mg protein)
Veh
Ex-4
[3H] DA Uptake (% control)
[3H] DA Uptake (pmol/min*well)
AA
Fe+3/Asc
Fe+3/Asc + AA
DA [μM]
Vmax (pmol/min*well)
Km [μM]
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Figure 14. *Treatment of mice with Ex-4 reduces septal AA levels and AA is a strong modulator of DAT function in LS.* (**A**) Ex-4 (2.4µg/kg, i.p.) administered *in vivo* 30 minutes prior to sacrifice resulted in lower levels of septal 2-AG ($t(11)=2.62$; *$p<0.05$ by Student's *t* test; n=6-7). (**B**) Ex-4 (2.4µg/kg, i.p.) administered 30 minutes prior to sacrifice reduces levels of septal AA ($t(10)=5.018$; ***$p<0.001$ by Student's *t* test; n=6). (**C**) 80µM AA applied for 20 minutes to slices containing the LS reduces [^{3}H]DA uptake to 42% of vehicle- (DMSO) treated slices ($t(3)=2.59$; *$p<0.05$ by Student's *t* test; n=4). (**D**) [^{3}H]DA uptake kinetics in hDAT cells treated either with vehicle (open circles) or Fe^{+3}/Asc (open triangles), or with 40µM AA for 1h at 37°C in absence (gray squares) or presence of Fe^{+3}/Asc (gray triangles). The kinetic parameters (**E**) Vmax and (**F**) Km were obtained from the Michaelis-Menten fit to the influx of [^{3}H]DA. Each value represents mean ±S.E.M. of ≥three independent experiments. *$p<0.05$, ****$p<0.001$, $^{\#\#}p<0.01$, and $^{\&\&}$ $p<0.01$ indicate significant differences between Veh and AA, Veh and Fe^{+3}/Asc+AA, AA and Fe^{+3}/Asc+AA, and Fe^{+3}/Asc and Fe^{+3}/Asc+AA, respectively. Significant ANOVA followed by *post hoc* tests.

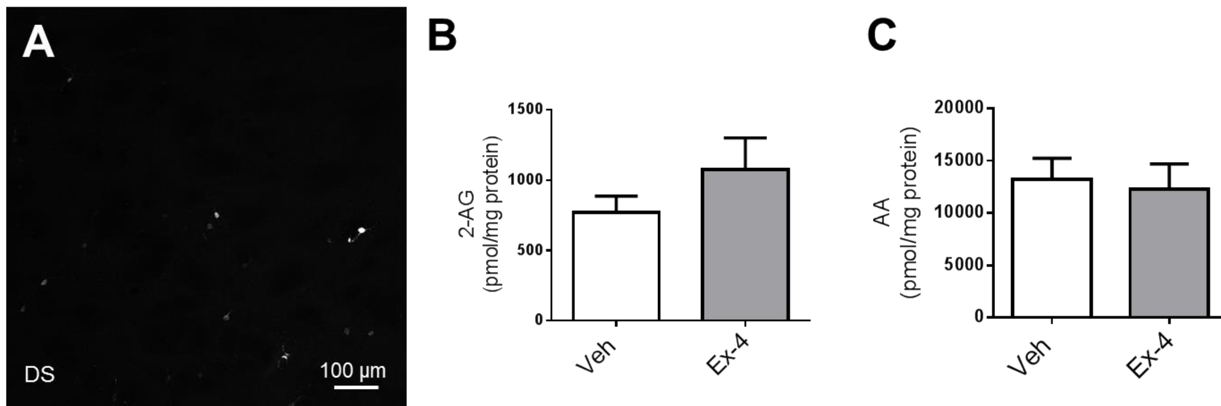


Figure 15. *Ex-4 does not alter levels of 2-AG or AA in the DS.* (**A**) Cells expressing the GLP-1R (white) are found at low levels in the DS, while the DAT is highly expressed (not shown). Confocal images of z stack through 13 planes taken with a 10X objective. (**B**) Ex-4 (2.4μg/kg, i.p.) administered 30 minutes prior to sacrifice does not alter 2-AG levels in the DS (t(12)=0.29; p=0.78 by Student's *t* test; n=5-9). (**C**) Ex-4 (2.4μg/kg, i.p.) administered 30 minutes prior to sacrifice does not alter DS AA content (t(12)=1.33; p=0.21 by Student's *t* test; n=5-9).

While we have established that AA inhibits DAT function in the LS, the mechanism(s) responsible for this effect are unknown. To define these molecular mechanisms we adopted PC12 cells stably transfected with the human DAT (hDAT cells). PC12 cells contain vesicles capable of neurotransmitter release.[319] These vesicles hold catecholamines (mostly DA) and as such they were suitable for our molecular studies. Incubation of hDAT cells with AA alone decreased DAT maximal velocity (Vmax) to 69% of control condition (Figure 14D and E). Notably, psychostimulants are known to induce oxidative stress,[320-322] while GLP-1R signaling, which reduces AA levels, is known to reduce it.[323] Thus we further explored mechanistically the interplay between oxidative stress and AA levels in terms of DAT function. Here, we treated hDAT cells with AA along with an oxidizing solution containing Fe^{+3} (197μM) plus ascorbate (19.7mM, Fe^{+3}/Asc) (Figure 14D-F). Importantly, Fe^{+3}/Asc induces the lipid peroxidation of AA, a process which produces reactive species capable of modifying protein structure and function.[324-327] Notably, DAT is highly susceptible to oxidant injury.[328-330] We thus posited that AA might exhibit greater inhibitory effects under oxidative conditions. Indeed, a greater decrease in Vmax to 34% of control was obtained when AA was applied in the presence of Fe^{+3}/Asc (Figure 14D and E; See also **Figure 16** for validation of oxidizing conditions and cell viability). However, when hDAT cells were incubated with Fe^{+3}/Asc alone, there were no significant changes in DAT activity (Figure 14D and E). These results show that in the presence of AA there is a significant reduction in DAT function under both oxidizing and non-oxidizing conditions. Notably, a greater loss of function was observed under oxidizing conditions which also affects DAT's apparent affinity for DA (Figure 14F).

DAT plasma membrane trafficking is a key regulator of DA transport capacity.[35,331] Therefore, the effect of AA on DAT function could be explained by changes in cell surface expression. Our results indicate that in hDAT cells, AA, under both oxidative and non-oxidative conditions, decreases significantly and to the same extent plasma membrane DAT (**Figure 17**A

and B) as determined by biotinylation. Therefore, the decrease in DAT function induced by AA alone parallels the decreased transport capacity induced by AA (Figure 14). These data also point to additional mechanisms promoted in terms of DAT function by oxidative stress.

AA oxidative product, γ-KA, forms a complex with DAT and regulates its function

Oxidation of AA (mediated by free radicals and other reactive oxygen/nitrogen species) yields a series of prostaglandin H_2 isomers that rearrange to their corresponding γ-KAs, also named isoketals. These γ-KAs react rapidly with lysine residues forming stable adducts, which can modulate the activity of proteins.[324-327] At the plasma membrane DAT localizes to microdomains including lipid rafts. Lipid rafts are regions highly enriched in phospholipids containing AA, and DAT localization to lipid rafts has been associated with regulation of DAT function and trafficking.[34,332,333] Therefore we determined whether post-translational modifications of DAT by γ-KAs are associated with changes in DAT function.

To determine if DAT is a target of AA oxidative products (e.g. γ-KAs) and whether this interaction is relevant for its function, we conducted experiments using synthetic isoketals (IsoK). hDAT cells were incubated with IsoK or vehicle for 1h at 37°C and cell extracts were used for immunoprecipitation experiments. We observed a significant increase (78%) in the amount of isoketal adducts that co-immunoprecipitated with DAT compared to the control condition (**Figure 18**A and B). Importantly, incubation with IsoK resulted in a 59% decrease in function compared to vehicle control (Figure 18C and D), with no change in Km (Figure 18E). Furthermore, we evaluated the effects of salicylamine, a selective scavenger of γ-KA, on DA uptake (Figure 18F). In the presence of salicylamine, there were no significant changes in Vmax (Figure 18G) or Km (Figure 18H) when cells were treated with AA under non-oxidative or oxidative conditions with respect to the control condition. This is in contrast to our earlier results in the absence of salicylamine (Figure 14D and E), suggesting that the ability to form γ-KA-DAT

adducts is necessary for AA to impair DAT function. Importantly, our findings demonstrate that DAT is a target of AA oxidative products, and that this interaction has profound functional implications.

Discussion

Since the discovery that the LS is a potent site of electrical self-stimulation in both rodents and humans,[81,334] it has historically received relatively little attention as a reward center. Recent studies, however, suggest that it integrates output from traditional reward areas including the VTA and lateral hypothalamus. [134,135] In this context, the LS has been described as a relay node from the hippocampus (area CA3) to the VTA, and has been suggested to play a pivotal role in encoding context-reward associations for cocaine.[135] Notably, functional inactivation of the rostral LS abolished cocaine CPP. [134] These reports and others [130,335,336] characterize the LS as a brain region critically involved in coordinating the activity of multiple reward centers and reinforce its importance in cocaine reward.

GLP-1Rs are highly expressed in the LS and their signaling has been shown to attenuate the locomotor response to the psychostimulant d-amphetamine,[292] to reduce cocaine CPP,[284,286] and to block self-administration for cocaine in rodents.[285] Therefore it is conceivable that their signaling within the LS may modulate cocaine CPP and reward. To support this hypothesis, a recent study shows that GLP-1R expression in the LS controls cocaine CPP and locomotion.[293] However, the mechanism by which GLP-1R signaling fine tunes DA homeostasis and cocaine reward in the LS remains elusive.

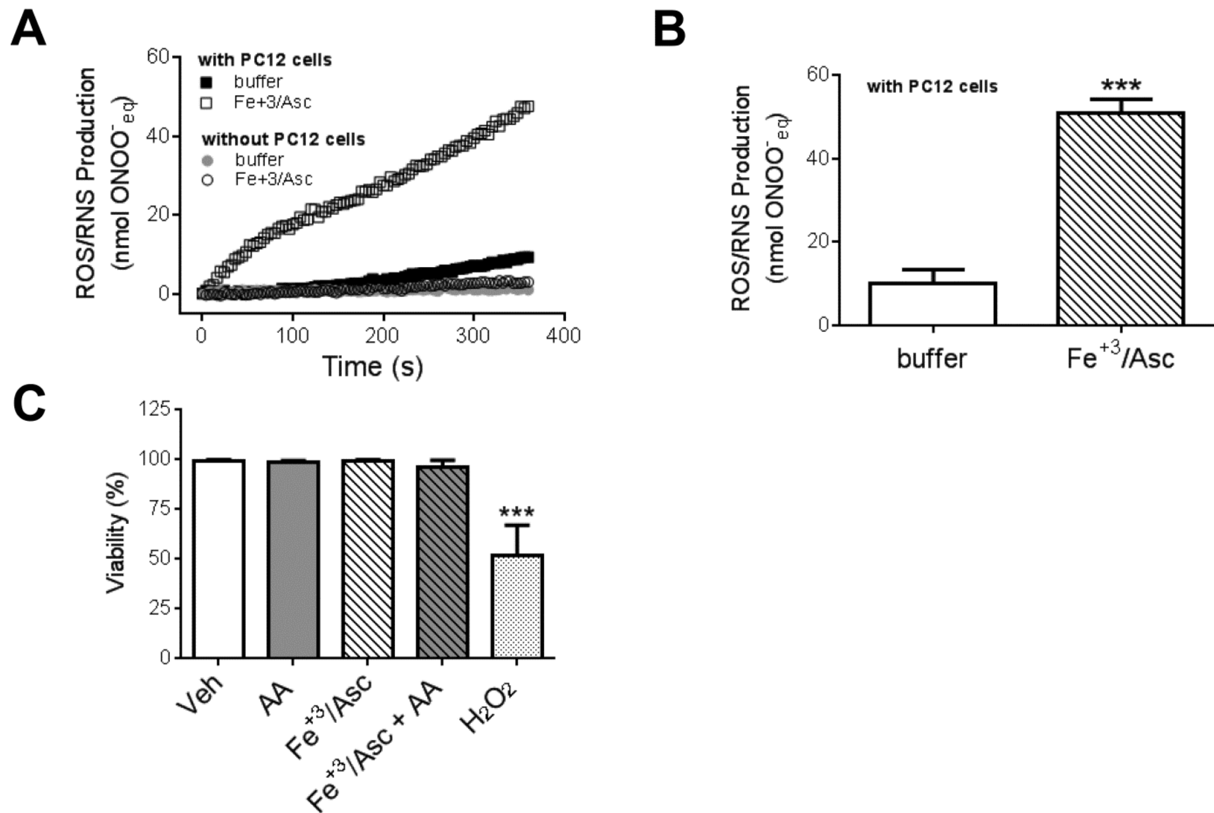


Figure 16. *Fe^{+3}/Ascorbate solution induces reactive oxygen species/reactive nitrogen species formation in PC12 cells without changes in cell viability.* (**A**) Time course of reactive oxygen species/reactive nitrogen species (ROS/RNS) production in PC12 cells in absence (buffer, black squares) or presence of Fe^{+3}/Asc (open squares) at 37°C, evaluated with the fluorescent probe H_2DCFDA (2.5μM). Control experiments without cells in absence (buffer: gray circles) or presence of Fe^{+3}/Asc (open circles) were also performed. (**B**) Data are expressed as ROS/RNS production (in nmol of $ONOO^-$) in the first 6 minutes of incubation with the Fe^{+3}/Asc solution. Each value represents the mean ±S.E.M. of at least three different experiments. *** indicates significant differences between KH buffer and Fe^{+3}/Asc conditions ($t(5)=8.49$; ***$p=0.0004$ by Student's *t* test; n=5-9). (**C**) Cell viability of PC12 cells treated with AA (40μM) for 1h at 37 °C in absence or presence of Fe^{+3}/Asc. As positive control of cellular death cells were incubated with 2.2mM H_2O_2 (1h at 37°C). Each value represents the mean ±S.E.M. of at least three independent experiments.*** indicates significant difference (***$p < 0.0001$) with respect to control condition (Veh: 0.1% ethanol in KH buffer). ANOVA followed by *post hoc* test.

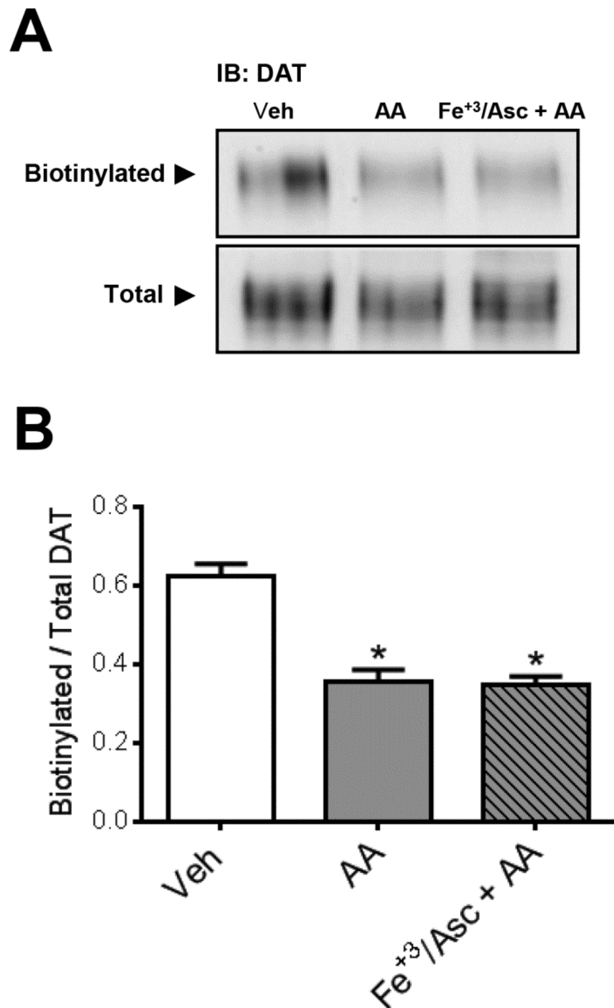


Figure 17. *AA decreases DAT cell surface expression in hDAT cells.* (**A**) Cell surface biotinylation and immunoblots for DAT using anti-DAT in extracts from hDAT cells treated with AA (40µM) for 1h at 37°C in absence or presence of Fe^{+3}/Asc. (**B**) Results expressed as the ratio of optical density between biotinylated (surface) and total DAT signals for each experimental condition. Each value represents mean ±S.E.M. of ≥three independent experiments. *$p<0.05$ indicates significant differences with respect to vehicle condition (0.1% ethanol in KH buffer). Significant ANOVA followed by *post hoc* tests.

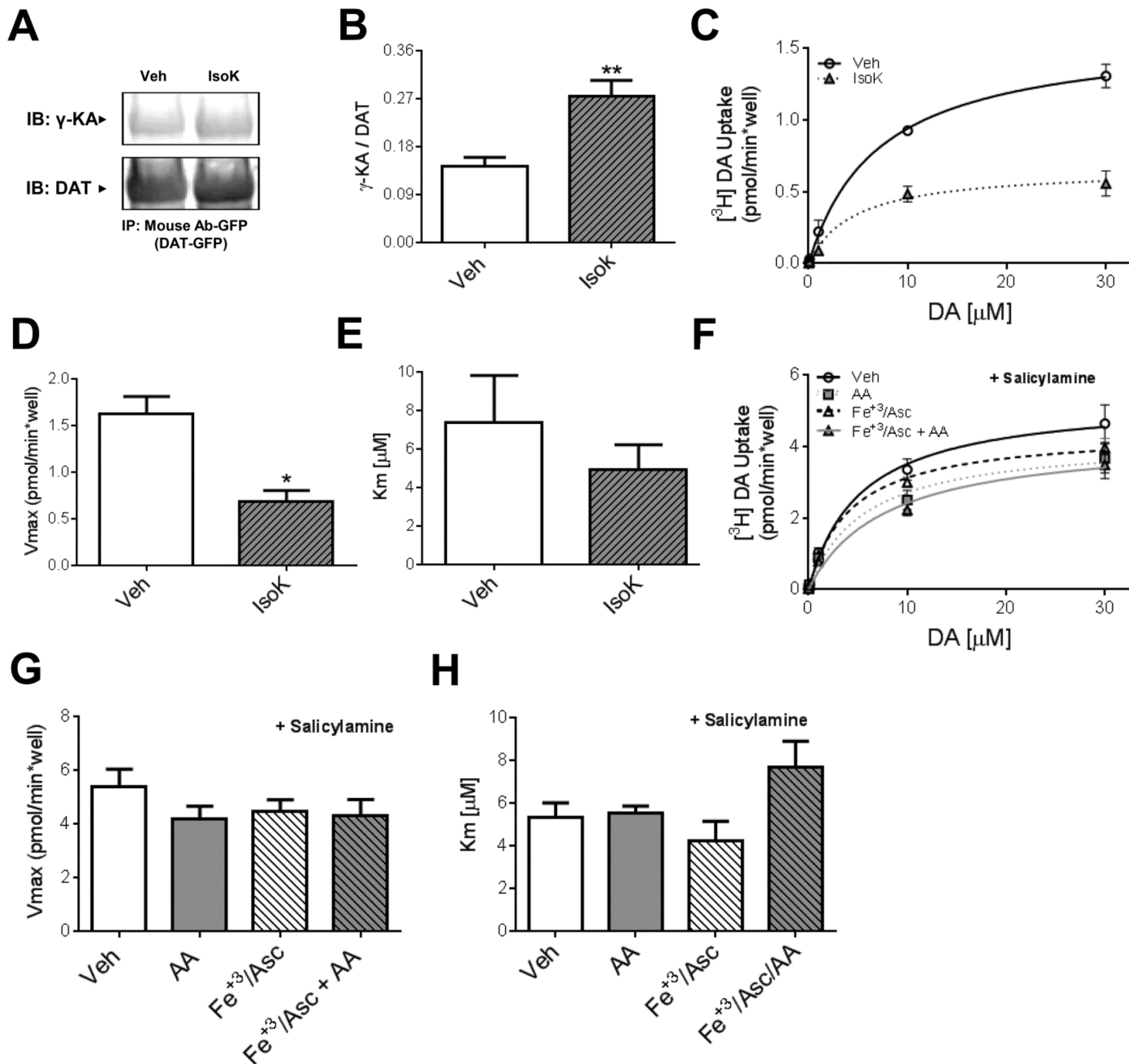


Figure 18. *Synthetic isoketals form adducts with hDAT and decrease its function.* (**A**) Immunoblots with anti-γ-KA protein adduct and anti-DAT antibodies using GFP-DAT immunoprecipitates from hDAT cells treated with synthetic isoketals (1μM) for 1h at 37°C. (**B**) Quantitation of immunoprecipitates as a ratio of γ-KA:DAT. Each value represents mean ±S.E.M. of ≥four independent experiments ($t(6)=3.94$; $^{**}p<0.01$ by Student's *t* test; n=4). (**C**) Uptake of [^{3}H]DA in hDAT cells treated with vehicle (open circles) or with 1μM synthetic isoketals (gray triangles) for 1h at 37°C. The kinetic parameters (**D**) Vmax and (**E**) Km were obtained from the Michaelis-Menten fit to [^{3}H]DA uptake. Each value represents mean ±S.E.M. of three independent experiments (Vmax: $t(4)=4.21$; $^{*}p<0.05$ by Student's *t* test; n=3; Km: $t(4)=0.88$; $^{*}p=0.43$ by Student's *t* test). (**F**) Influx of [^{3}H]DA in hDAT cells pretreated with salicylamine (0.5mM) and then treated with vehicle (open circles, 0.1% ethanol in KH buffer), Fe^{+3}/Asc (open triangles) or with 40μM AA for 1h at 37 °C in absence (gray squares) or presence of Fe^{+3}/Asc (gray triangles). The kinetic parameters (**G**) Vmax and (**H**) Km were obtained from the Michaelis-Menten fit to the influx of [^{3}H]DA. Each value represents mean ±S.E.M. of ≥three independent experiments. No significant differences were observed between groups. Statistical analysis was performed with ANOVA.

Cocaine, by targeting the DAT, increases extracellular DA levels which leads to cocaine's behavioral actions. DAT expression level is an important factor in determining the effects of cocaine, likely through effects on basal DA tone. For example, extended access to cocaine, which results in escalating cocaine intake, causes lower expression of striatal DAT and reduced DA clearance in rats.[337-341] The involvement of DAT expression in cocaine's behavioral actions is further supported by the finding that hyperlocomotor responsiveness to cocaine is significantly enhanced in two different mouse models expressing low levels of DAT,[68,69] likely resulting from reduced basal DAT-mediated DA uptake [69]. Additionally, experiments from heterologous cell lines show that cocaine is more potent in inhibiting DA uptake in cells expressing lower amounts of DAT.[342] Notably, a high percentage of striatal DAT (at least 47%) needs to be occupied by cocaine in addicts before they report subjective effects of the drug,[343] implying that modulating DAT surface expression alters the reinforcing effects of cocaine in humans. However, it is clear to us that other mechanisms in addition to DAT occupancy participate in the behavioral actions of cocaine. These include altered firing activity of DAergic neurons and/or other mechanisms that modify DA release. These kinds of mechanisms have been defined for other drugs of abuse, such as morphine, and may be contributing in parallel to altered cocaine reward.[344]

In this study, we hypothesized that GLP-1R signaling controls, at least in part, cocaine actions by regulating DAT expression/function in the LS. We find that GLP-1R agonism enhances membrane expression of DAT. This increase translates into an increase in transport capacity, and is paralleled by reduced ability of cocaine to increase extracellular DA levels. Our data suggest that increasing the amount of DAT expressed at the cell surface may reduce the extent to which cocaine is effective at increasing extracellular DA within the LS. These data offer an additional mechanism for GLP-1R signaling regulation of the reinforcing and rewarding

properties of cocaine without precluding the possibility that GLP-1R signaling may also affect DA release and firing properties of VTA neurons.

Here we find that stimulating the GLP-1R through administration of Ex-4 in a therapeutically relevant manner (systemic administration) reduces septal 2-AG levels. One possibility is that the decrease in 2-AG is due to GLP-1R-mediated stimulation of PI3K,[345] or inhibition of PTEN[346] which will decrease the levels of plasma membrane PIP_2, one of the precursors for 2-AG synthesis.[347] We show that in the LS GLP-1R is located postsynaptically. Indeed, 2-AG diffuses across synapses. Therefore, we believe that this loss of 2-AG is causal for the observed decrease in LS AA content as AA is primarily produced in the brain through the presynaptic conversion from 2-AG.[316] This reduction is important since AA has been shown to inhibit DA uptake in *ex vivo* and *in vitro* preparations,[55,317,318] and that psychostimulants such as amphetamine cause release of AA by activation of PLA_2.[348] Consistent with AA-mediated inhibition of septal DA uptake, we show that GLP-1R-mediated reduction in AA signaling is associated with an increase in DA uptake in LS slices. This increase in DAT function stems at least in part from augmented DAT surface expression, pointing to DAT trafficking as the underlying mechanism by which GLP-1R signaling and AA levels controls DA homeostasis in the LS, as depicted by our schematic model (**Figure 19**).

Of note, AA is known to facilitate a pro-inflammatory state in the brain;[349] GLP-1 analogues, on the other hand, possess both anti-inflammatory and neuroprotective effects.[323,350,351] GLP-1R stimulation has demonstrated positive effects in rodent models of Alzheimer's disease as well as Parkinson's disease (a degenerative disorder of DAergic neurons).[351-353] Furthermore, psychostimulants themselves enhance AA levels and may exacerbate oxidative stress in the brain,[320-322,348] possibly increasing levels of harmful AA metabolites. Based on our findings, GLP-1R signaling may oppose the effects of psychostimulants by affecting levels of AA as well as oxidative stress.

In this study, we provide biochemical and functional evidence supporting the notion that AA regulates DAT function by regulating DAT trafficking. We believe that the increase in DAT surface expression promoted by GLP-1R signaling represents a mechanism by which this neuropeptide controls both cocaine-induced increase in LS DA as well as cocaine actions. This AA-mediated mechanism is enhanced by peroxidation of AA leading to the formation of AA metabolites such as γ-KA. Treatment of hDAT cells with AA under conditions favoring lipid peroxidation significantly decreased DAT surface expression and transport capacity; the latter effect was also demonstrated with synthetic isoketals. These results are consistent with the known ability of isoketals to impair the function of plasma membrane proteins.[354] Interestingly, AA alone was also able to significantly reduce DAT function. However, this reduction was smaller than that obtained under conditions favoring lipid peroxidation or in the presence of isoketals. These results provide a mechanism for the results reported by Chen *et al.* (2003) and Zhang and Reith (1996), wherein the activity of DAT expressed in heterologous systems was reduced by AA.[55,318] They also mechanistically support the notion that GLP-1R signaling regulates DA homeostasis in the LS by decreasing AA levels. How exactly AA and its metabolites control DAT function and trafficking remains elusive. However, we hypothesize that covalent interactions of AA metabolites with specific lysine residues on the DAT support these actions.[324] Notably, we were able to demonstrate a physical interaction between γ-KA and DAT by immunoprecipitation.

In summary, we have shown that GLP-1R agonism increases DAT function and membrane expression, decreases cocaine-induced increases in extracellular DA, and reduces endocannabinoid and AA signaling in the LS. This decrease is important since cocaine has been shown to increase 2-AG tone in brain.[355] This suggests that other brain centers targeted by cocaine and expressing the GLP-1R, albeit at lower levels, may exhibit a similar mechanism of reward modulation. These brain regions include the NAc and VTA which have been identified

as targets of Ex-4 actions on brain reward.[235,288,289,356,357] Nevertheless, this mechanism has still to be validated outside the LS. Our study points to septal GLP-1R as a potential novel target for the treatment of drug abuse. Targeting GLP-1R signaling represents an enormous translational opportunity since GLP-1 analogues are already available for clinical use.

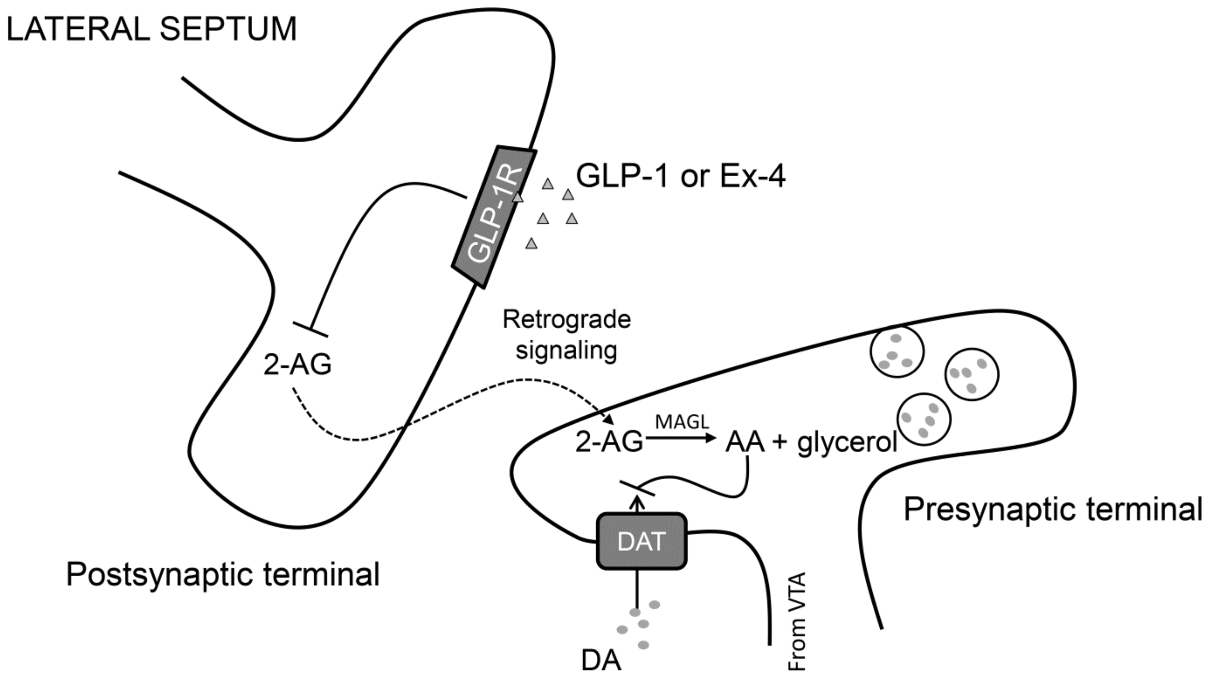


Figure 19. *Schematic depicting model for GLP-1R-mediated retrograde regulation of DA uptake.* 2-AG, 2-arachidonylglycerol; DA, dopamine; DAT, dopamine transporter; Ex-4, exendin-4; GLP-1, glucagon-like peptide 1; GLP-1R, GLP-1 receptor; MAGL, monoacylglycerol lipase; VTA, ventral tegmental area.

CHAPTER III

STRIATAL DOPAMINE HOMEOSTASIS IS ALTERED IN MICE FOLLOWING ROUX-EN-Y GASTRIC BYPASS SURGERY[3]

Preface

RYGB is an effective treatment for obesity. Importantly, weight loss following RYGB is thought to result in part from changes in brain-mediated regulation of appetite and food intake. DA within the DS plays an important role in feeding behavior; we therefore hypothesized that RYGB alters DA homeostasis in this subcortical region. In the current study, obese RYGB-operated mice consumed significantly less of a high-fat diet, weighed less by the end of the study, and exhibited lower adiposity than obese sham-operated mice. Interestingly, both RYGB and caloric restriction (pair feeding) resulted in elevated DA and reduced NE tissue levels compared with ad libitum-fed sham animals. Consequently, the ratio of NE to DA, a measure of DA turnover, was significantly reduced in both of these groups. The RYGB mice additionally exhibited a significant increase in phosphorylation of TH at position Ser31, a key regulatory site of DA synthesis. This increase was associated with augmented expression of ERK1/2, kinases targeting TH Ser31. Additionally, RYGB has been shown in animal models and humans to improve insulin sensitivity and glycemic control. Curiously, we noted a significant increase in the expression of insulin receptor-β in RYGB animals in striatum (a glucosensing brain region) compared to sham ad libitum-fed mice. These data demonstrate that RYGB surgery is associated with altered monoamine homeostasis at the level of the DS, thus providing a critical foundation for future studies exploring central mechanisms of weight loss in RYGB.

[3] The work presented in this chapter is modified from Reddy, I.A., et al. Striatal dopamine homeostasis is altered in mice following Roux-en-Y gastric bypass surgery. *ACS Chemical Neuroscience* **5**, 943-951 (2014).

Introduction

Obesity is a growing health epidemic in both youth and adult populations. Over 15% of American children and over 30% of adults are obese.[358] The associated comorbidities of obesity, including type 2 diabetes and heart disease, are a major source of mortality.[359] Notably, bariatric surgery has been an effective means of weight loss for obese individuals who are able to undergo surgery.[360] RYGB is the most widely used bariatric surgical procedure performed in the USA, which results in sustained weight loss and improved metabolic parameters.[361,362] The procedure reroutes the upper stomach to a more distal portion of the small intestine (proximal jejunum), thus bypassing the major portion (~90%) of the distal stomach, the duodenum and the proximal jejunum (**Figure 20**).[363]

A number of non-mutually exclusive mechanisms have been suggested to account for weight loss following RYGB. Ochner et al. (2011) estimated that restrictive effects of limiting stomach size and malabsorptive effects of bypassing a portion of the proximal gut account for approximately 55-80% of observed weight loss, while the additional weight loss must be explained by alternative mechanisms.[364] One of the more striking consequences of RYGB is a sustained loss of appetite associated with a reduction in food intake, likely mediated by adaptations within the central nervous system to post-operative changes in levels of circulating hormones, including insulin.[239,256,365-367] The brain plays an important role in regulating appetite and feeding, which requires the integration of information about nutritional requirements, energy stores, the availability and desirability of foods, and the motivation to work for palatable foods.[4,368,369] The latter may be particularly relevant to the etiology of the contemporary obesity epidemic in which the evolutionarily adaptive drive to consume energy-dense foods becomes maladaptive in the setting of abundance.

Functional brain imaging studies in humans have demonstrated significantly blunted MRI responses to the consumption of palatable food in obese individuals compared with healthy-

Figure 20. *RYGB in mice.* The RYGB procedure in our mice consisted of ligation of the stomach between the glandular portion and the gastric fundus. A portion of the jejunum, 4 cm from the Ligament of Treitz and 6 cm from the site of gastroenterostomy, was transected. The distal segment was anastomosed to the stomach in a side-to-side fashion. GI continuity was established by performing a side-to-side jejuno-jejunostomy. The sham procedure involved mobilization of the forestomach and proximal and distal jejunum and ileum without any intersection. Adapted from [370].

weight controls,[371] suggesting a model by which deficits in the experience of foods promotes compensatory overconsumption. Under this model, weight loss in RYGB might be explained, in part, by amelioration of central dysfunction. The aim of the current study was to begin to define the central neurochemical and molecular candidates by which RYGB may regulate feeding behavior in the setting of obesity.

In this study, we focused on the nigrostriatal DA axis. This pathway has been strongly implicated in the motivation to seek food.[140,233] Importantly, impairments in striatal DA signaling have been repeatedly associated with obesity.[213,214,372] In this context it is important to point out that circulating hormones which are altered in states of obesity and diabetes, such as insulin and GLP-1, promote changes in DA homeostasis within the striatum.[234,239] Further, it has been previously shown that appropriate insulin signaling at the level of the striatum prevents excessive high fat feeding.[234,373] These findings provide a theoretical framework in which DAergic tone is impaired under the state of altered hormonal signaling in obesity and, importantly, that RYGB would enhance striatal DA neurotransmission in the setting of obesity.

Methods

Mice: Male C57BL/6J mice arrived from Jackson Laboratory (Bar Harbor, ME) at 6 weeks of age and were initially group-housed at the Vanderbilt Mouse Metabolic Phenotyping Center (Nashville, TN, USA) with a 12h light/dark cycle (lights on at 6:00 a.m.). Mice were given one week to acclimate to the facility before being switched from a standard chow diet to a 60% kcal fat diet (Research Diets, Inc., New Brunswick, NJ, USA) for seven weeks prior to surgery to establish diet-induced obesity (**Figure 21**). Mice were kept on this diet for the remainder of the study. Animals which had undergone sham surgery were split into pair-fed and ad libitum-fed groups. On day 7 following surgery, all animals were moved from group housing to single

housing. Feeding from this point on was measured every 2-4 days (grams of food given minus grams of food remaining). Mice which were noticeably crumbling food were excluded from food intake analysis (n=1). Pair feeding was started on day 9 post-surgery; this consisted of providing animals with a measured amount of food each morning and evening, which matched food consumed per half day in the RYGB animals from the same cohort in the prior 2-4 days. Body weight throughout the study was measured on a weekly basis. All experiments, procedures, and surgeries involving mice were performed in compliance with and were approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

RYGB surgical preparations: RYGB and sham surgeries were performed under inhaled 3-5% isoflurane anesthesia as previously described ("RYGB" procedure).[370] Mice which did not achieve a body weight of at least 33g on the day before surgery were excluded from the study. Prior to surgery, mice were fasted for approximately 12h. On the morning of surgery, the mice received 0.03 mg of buprenorphine analgesic (Patterson Veterinary via Hospira Inc., Nashville, TN, USA) and 0.1 mL 0.9% saline (intraperitoneal). Animals were administered 0.017 mg ketoprofen (Cayman Chemical, Ann Arbor, MI, USA) once/day as needed for 1-2 days following surgery and weekly iron dextran injections (10 mg/kg; Durvet, Inc., Blue Springs, MO, USA) to prevent anemia.

Whole body composition: Body composition was measured in conscious mice using the mq10 NMR analyzer (Bruker Optics Inc., Billerica, MA, USA) at the Vanderbilt University Mouse Metabolic Phenotyping Center as described previously.[370] Measurements were made one week prior to surgery (+/- 1 day) and each week thereafter.

Monoamine content: Mice were sacrificed four weeks following surgery under inhaled isoflurane anesthesia. A section of brain including the striatum was blocked. A small portion of the DS was taken by punch microdissection for determination of monoamine content, while the rest of the

striatum was dissected and saved for western blotting. Tissue saved for both monoamine determination and western blotting was immediately placed in tubes on dry ice. Tissue punches were analyzed at the Vanderbilt University Neurochemistry Core by high performance liquid chromatography (HPLC) with amperometric detection as described previously.[302]

Tissue preparation and immunoblotting: Tissue punches from DS were collected and homogenized on ice in buffer containing 22 mM HEPES, 133 nM NaCl, 1% triton, 0.1% each of leupeptin, pepstatin, and aprotinin, 1% phosphatase inhibitor cocktail 3 (Sigma Aldrich, St. Louis, MO, USA), and 0.5 mM PMSF, then spun at 13,000×g for 30 minutes at 4°C. The supernatant was taken and combined with pulldown buffer containing 24 mM HEPES, 146 nM NaCl, 0,1% triton, 0.1% each of leupeptin, pepstatin, and aprotinin, 1% phosphatase inhibitor cocktail 3 (Sigma Aldrich), and 0.5 mM PMSF. The protein content was then assessed, compensated so that each sample contained the same amount of total protein, and analysis performed. Protein was eluted with 2X sample buffer for 5 minutes at 95°C, cooled, and separated by 10% SDS-PAGE. Resolved proteins were then transferred to polyvinylidene difluoride (PVDF) membrane and blocked in either 5% milk or 2.5% BSA in 0.1% Tween20 in Tris-buffered saline. Blots were then incubated in primary antibody rocking either at room temperature for 1h or overnight at 4°C. The primary antibodies used in this study included: tyrosine hydroxylase (1:1000; Cell Signaling Technology; Danvers, MA, USA), phospho-tyrosine hydroxylase serine 31 (1:500; Cell Signaling Technology), DAT (1:10,000; Dr. Roxanne Vaughan, University of North Dakota School of Medicine), ERK 1/2 (1:1000; Promega; Madison, WI, USA), phospho-ERK 1/2 (3:2000; Promega; Madison, WI, USA), and insulin receptor-β (1:300; Santa Cruz Biotechnology, Santa Cruz, CA, USA). All proteins were detected using HRP conjugated secondary antibodies (1:5000; Santa Cruz Biotechnology). After chemiluminescent visualization (Amersham ECL-Plus; Piscataway, NJ, USA) on Hyblot CL film (Denville Scientific, South Plainfield, NJ, USA), protein band densities were quantified using ImageJ software

(ImageJ, National Institutes of Health, Bethesda, MD, USA). All measures of protein were normalized to measures of β-actin from the same samples and expressed as a percentage of the average optical density of the sham control group.

Statistics: Results are presented as mean ± standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism version 6.00 for Windows (San Diego, CA, USA). Comparisons between sham, sham pair-fed, and RYGB groups were made by one-way ANOVA with Dunnett's post test against the sham group unless otherwise noted. For all measures, data points which fell ≥ an interquartile range outside the first and third quartiles for the surgical/feeding group were excluded from analysis as outliers. Correlation analyses were performed by linear regression in GraphPad Prism. Significance was defined as a p value <0.05.

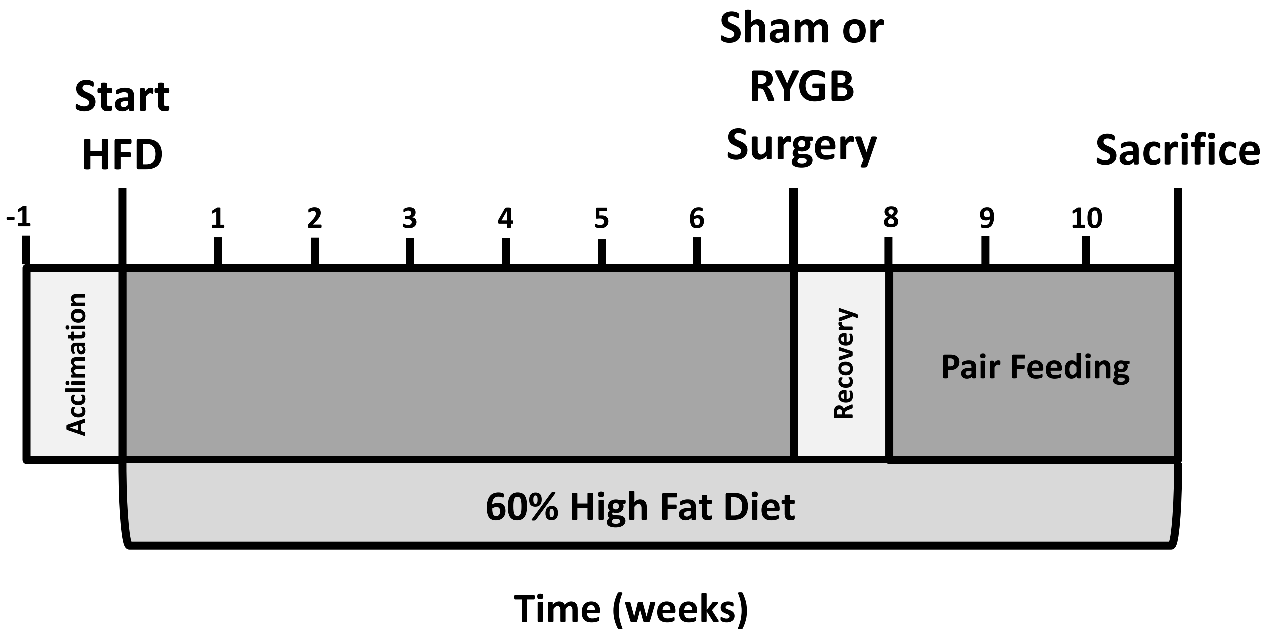


Figure 21. *RYGB study design*. Mice were acclimated to the mouse metabolic phenotyping core facility for one week. They were then placed on a 60% high-fat diet for 7 weeks. At week 7, the mice underwent surgery, either RYGB or sham. They were given one week for recovery, after which they were singly housed and the sham group was split into pair-fed animals and *ad-libitum*-fed animals. At week 11 after starting the high fat diet, all mice were sacrificed and striatal tissue was collected for analysis (n=6-8 per group).

Results

RYGB reduces body weight, food intake, and adiposity in mice:

After 7 weeks on a high fat diet, mice were randomly assigned to receive sham or RYGB surgery (Figures 20 and 21). All mice were allowed to feed on a high-fat diet ad libitum for one week following surgery, after which the sham group was randomly assigned to either continue ad libitum feeding, or to be pair-fed to the RYGB group (see methods for details). Body weights for animals in each of these three groups were monitored for the duration of the study (n=6-8 per group). Following the initial seven weeks on the high-fat diet, the average weight of the animals was 38.4 ±0.8g with no significant differences among animals (**Figure 22**A; $p>0.05$). At the end of the study (4 weeks after surgery), the RYGB group weighed significantly less than the sham animals (Figure 22A; 27.5 ±0.9g vs. 40.0 ±1.7g; ####$p<0.0001$ by two-way ANOVA and Bonferroni's multiple comparison test), with an average weight loss of 26.9 ±1.4% from peak weight immediately before surgery to four weeks post-surgery. In contrast, the sham pair-fed group only lost 11.1 ±2.4% of their peak body weight, which was significantly different from the ad libitum sham group at week 11 (*$p<0.05$). Importantly, the RYGB group also weighed significantly less than the pair-fed group (††$p<0.01$).

The RYGB animals consumed significantly less food by weight compared to the shams (Figure 22B; **$p<0.01$ in last 4 days of intake measurement by two-way ANOVA and Bonferroni's multiple comparison test). At four weeks post-surgery, the body fat in the RYGB group was 10.25 ±0.55% and 31.05 ±3.5% in the sham group (**Figure 23**A; ***$p<0.001$). In contrast, the adiposity of the pair-fed group (28.08 ±2.3%) was no different than that of ad libitum-fed shams ($p>0.05$). RYGB also resulted in greater lean mass composition by the end of the study with respect to sham ad libitum mice, our control group for the remainder of the study (Figure 23B; ****$p<0.0001$).

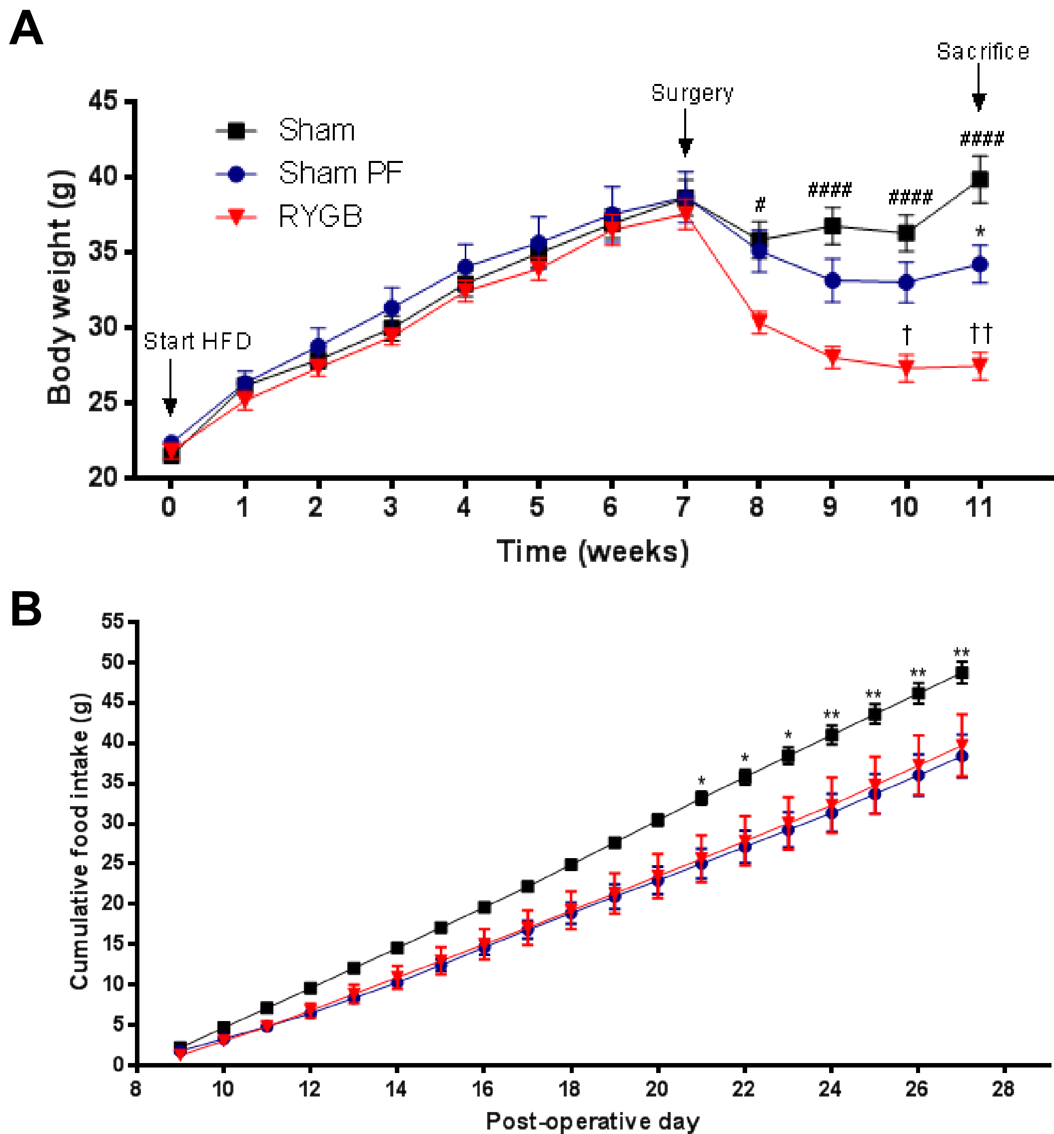


Figure 22. *RYGB decreases body weight and food intake*. (A) Body weights of sham, sham pair-fed, and RYGB mice over the course of the study. There were no significant differences in body weight prior to surgery. # represents significant differences between sham and RYGB groups. * indicates significant differences between sham and sham pair-fed groups. † represents significant differences between RYGB and sham pair-fed groups (n=6-8). (B) Cumulative intake of a 60% high-fat diet was measured starting at day 9 (start of pair-feeding) through day 27 post-surgery. * indicates a significant difference between the sham-operated *ad-libitum*-fed group and the RYGB-operated group (n=5-8).

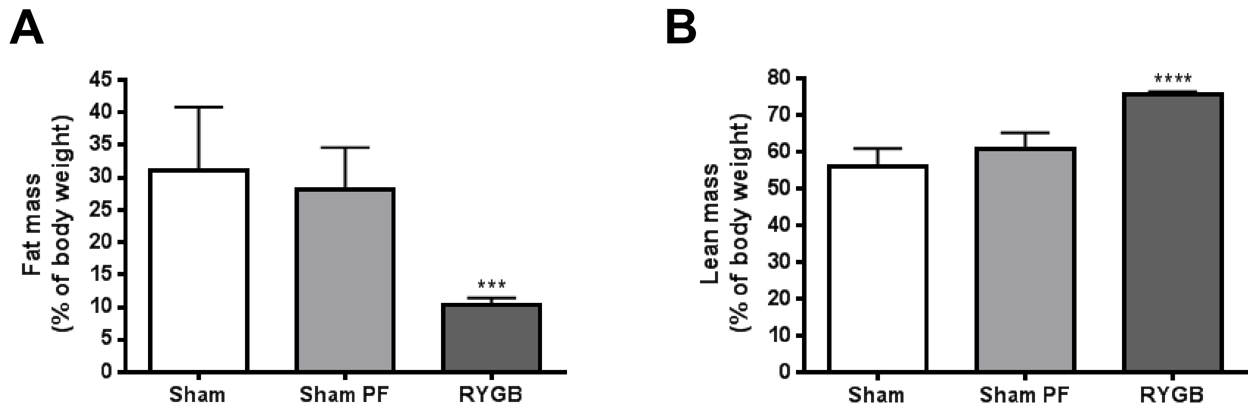


Figure 23. *RYGB decreases fat mass and increases lean mass*. (A) Fat mass and (B) lean mass in each of the surgical/feeding groups quantified as percent of total body weight at week 11 (n=5-8). Together, fat mass and lean mass compose the entirety of body weight with the exception of free fluid mass. Significant differences were noted between the sham and RYGB groups for both fat (***p<0.001) and lean mass (****p<0.0001). Data are represented as mean ± SEM.

Mice that have undergone RYGB or caloric restriction exhibit higher levels of DA and reduced levels of NE in the striatum:

Four weeks post-surgery, all groups were sacrificed and DS tissue was collected. Levels of monoamines and their metabolites were determined by HPLC. DA levels were significantly elevated in both the RYGB and sham pair-fed groups with respect to shams fed ad libitum (**Figure 24**, top; $^{**}p<0.01$ and $^{*}p<0.05$ for DA in RYGB and sham PF animals, respectively; mean HPLC values were 120.9 ±9.7 ng/mg protein, 148.9 ±4.1, and 168.8 ±8.9 for sham, sham PF, and RYGB groups, respectively; n=5-8 per group). On the other hand, NE levels were significantly reduced in the RYGB and sham PF mice (Figure 24, middle; $^{*}p<0.05$ in RYGB and sham PF mice; mean HPLC values were 3.1 ±0.1 ng/mg protein, 2.0 ±0.2, and 2.0 ±0.4, respectively; n=5-7 per group), while there were no significant differences in the levels of the non-catecholaminergic monoamine, serotonin (5-HT; Figure 24, bottom; $p>0.05$).

DA levels within terminals are highly regulated by a number of homeostatic mechanisms, including metabolism, synthesis, and synaptic reuptake.[374] In relation to DA metabolism, there were no significant differences between groups in terms of levels of DA metabolites (**Figure 25**). However, a small proportion of DA is also converted to NE in the striatum by the enzyme DA β-hydroxylase.[375] Thus, it was important to determine whether the conversion of DA to NE was affected by RYGB by measuring changes in the ratio of NE to DA. This ratio was significantly reduced both in the pair-fed and RYGB animals relative to sham ad libitum-fed animals (Figure 24B; $^{**}p<0.01$). These findings demonstrate that, in the setting of high-fat feeding, both food restriction and RYGB act to increase striatal DA content and that this effect is potentially mediated in small part by reduced conversion of DA to NE.

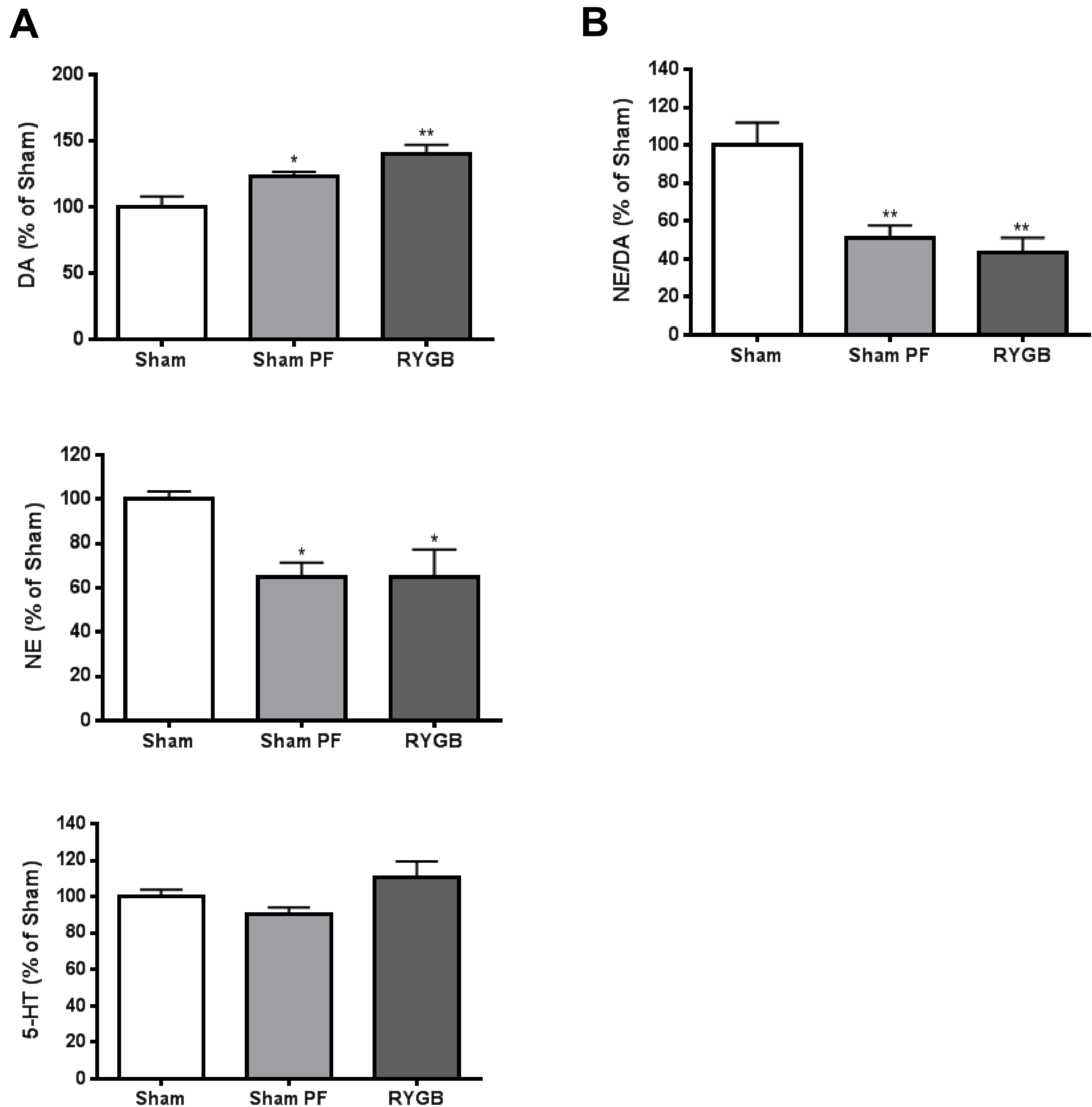


Figure 24. *DA levels are elevated in the DS of sham PF and RYGB-operated mice.* (A) Striatal DA measured by HPLC was significantly elevated in both sham pair-fed (*p<0.05) and RYGB mice (**p<0.01) compared to sham-operated animals (top; n=5-8). In contrast, NE was significantly decreased in both sham pair-fed and RYGB mice compared to sham-operated animals (middle; *p<0.05; n=5-7) while 5-HT was not significantly different between groups (bottom; n=6-7). (B) Conversion of DA to NE was significantly impaired in the pair-fed and RYGB mice, as noted by the ratio of NE to DA (**p<0.01; n=5-6). Data are represented as mean ± SEM.

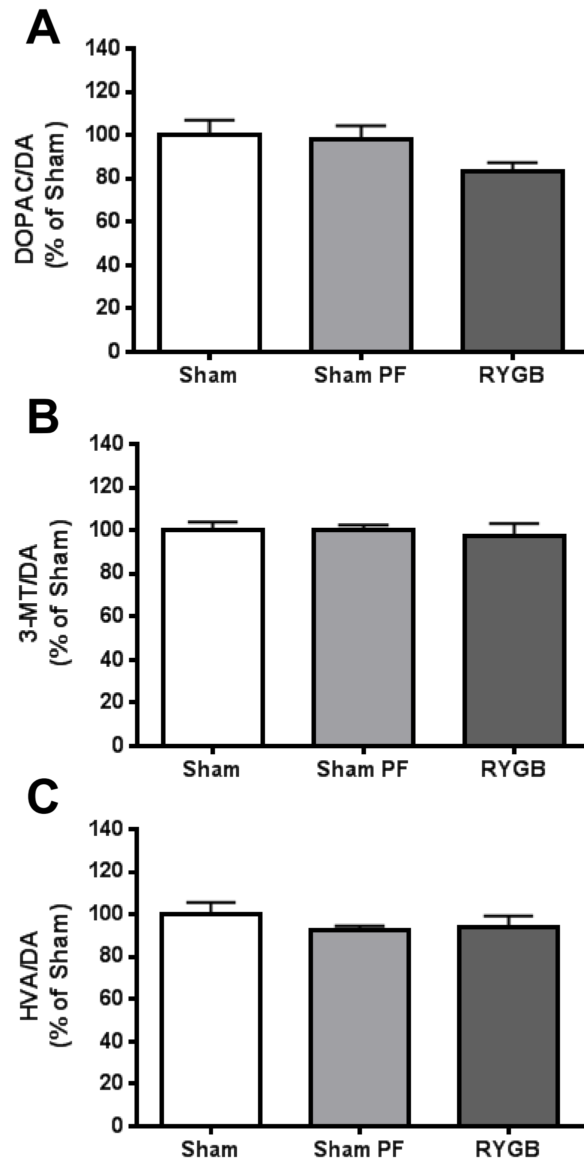


Figure 25. *Striatal content of DA metabolites is not altered by RYGB or pair-feeding.* Levels of DA metabolites (A) DOPAC (n=5-8), (B) 3-MT (n=5-8), and (C) HVA (n=6-8) normalized to total DA were the same across surgical and feeding groups ($p>0.05$). Data are represented as mean ± SEM.

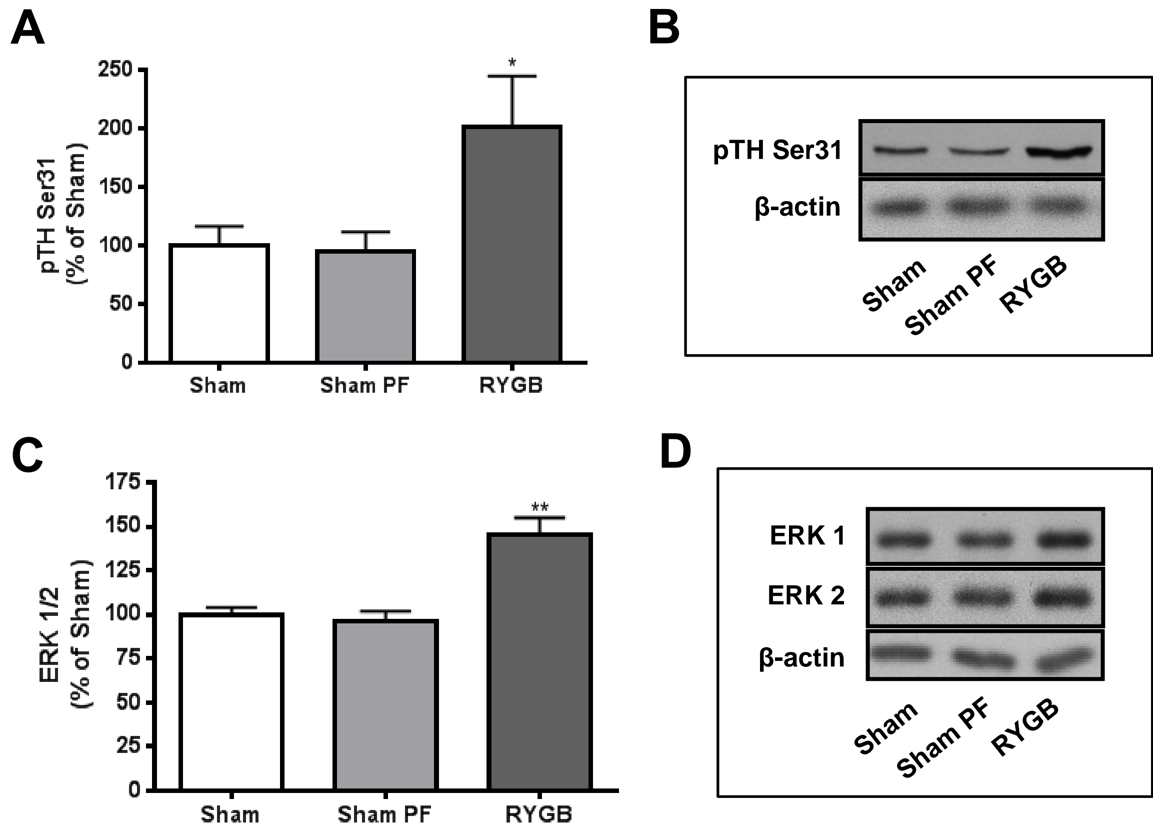


Figure 26. *TH phosphorylation at Ser31 and expression of ERK 1/2 are elevated in RYGB-operated mice.* (A) Phosphorylation of tyrosine hydroxylase at residue Ser31 (pTH-Ser31) was significantly elevated in the RYGB group compared to the sham *ad-libitum*-fed animals (*$p<0.05$; n=6-8). Data were normalized to β-actin. (B) Representative immunoblots. (C) Expression of ERK 1/2 was elevated in the RYGB mice compared to shams (**$p<0.01$; n=5-6). Data were normalized to β-actin and (D) a representative immunoblot is shown. Data are represented as mean ± SEM.

RYGB but not caloric restriction increases TH phosphorylation at residue Ser31:

In addition to DA metabolism, DA levels in neurons are also homeostatically regulated by reuptake and synthesis. Given that altered conversion of DA to NE can only explain partially the increase in DA in the RYGB animals, we thus asked whether these other components of DA homeostasis were altered by either RYGB or caloric restriction (pair-feeding). First, we determined whether expression of the DAT is altered by either intervention. The DAT acts to clear DA from the synapse by active uptake into the terminals. Total expression of DAT was not significantly different across the three different experimental groups (100.0 ±9.1%, 115.2 ±9.4%, and 140.5 ±35.4% in sham, sham PF, and RYGB, respectively; n=6). Similarly, total expression of TH, the rate-limiting enzyme in DA synthesis, was unchanged across groups (100 ±24.7%, 104.9 ±10.7%, and 124.9 ±32.8% in sham, sham PF, and RYGB, respectively; n=4-6).

The activity of TH in relation to DA synthesis is regulated, in part, by phosphorylation of the residues Ser31 and Ser40. Phosphorylation at either of these residues results in increased activity of TH and increased DA synthesis.[376] Due to the low stoichiometric presence of Ser40 in striatum,[377] we focused on measuring striatal changes in phosphorylation of TH at Ser31. Interestingly, RYGB animals showed increased expression of phospho-TH Ser31 compared with sham animals (**Figure 26**A and 4B; *p<0.05), while there was no significant difference between sham pair-fed animals and shams fed ad libitum.

The phosphorylation of TH at Ser31 is known to be regulated by ERK1/2.[376,378] Although we did not observe an increase in ERK1/2 phosphorylation (100 ±18.7%, 127.7 ±30.5%, and 98.6 ±14.1% in sham, sham PF, and RYGB, respectively; n=6-8) a significant increase in ERK1/2 expression in the RYGB animals was noted, which may account for the observed increase in phospho-TH Ser31 (Figure 26C and D; **p<0.01). Individually, both ERK 1 and ERK 2 were significantly increased in the RYGB group (100 ±12.5%, 91.3 ±9.9%, and 167.8 ±9.9%

for ERK1 in sham, sham PF, and RYGB, respectively; $p<0.001$; 100 ±10.5%, 125.7 ±11.1%, and 177.3 ±25.1% for ERK2 in sham, sham PF, and RYGB, respectively; $p<0.01$; n=6-8 per group).

RYGB promotes expression of the insulin receptor β subunit in striatum:

It is well documented that levels of circulating hormones are altered by RYGB surgery.[221] The brain receives constant information about the body's nutritional status via these circulating hormones including, but not limited to, insulin, leptin, GLP-1, orexin, and ghrelin. Receptors for each of these hormones have been found in brain regions involved in energy homeostasis and feeding behavior, including striatum.[379-381] These enteroendocrine signaling factors have been shown to regulate feeding and body weight through central mechanisms.[382] In particular, within the central nervous system, insulin signaling has previously been linked to changes in DA homeostasis and food reward.[290,308,383] Given that circulating insulin levels are proportional to adiposity,[384] that RYGB improves insulin sensitivity,[385] and that in our study the RYGB group exhibited lower levels of adiposity than the pair-fed animals, possible changes in striatal insulin signaling were determined in our experimental groups. Here, expression of the β subunit of the insulin receptor was elevated in the striatum of animals that underwent RYGB, but not in the pair-fed group (**Figure 27**A and B; *$p<0.05$).

After uncovering a number of molecular changes associated with RYGB, we were interested to know whether any of these changes correlated with reductions in body weight. Such an analysis might provide insight into the contribution of body weight and associated metabolic changes toward adaptations in the brain DAergic system. Incorporating all animals from all groups, we measured the correlation between body weight and striatal DA, DAT, pERK1/2, ERK1/2, TH, pTH-Ser31, and the insulin receptor β subunit (**Table 2**). Significant inverse correlations were found between body weight and DA, ERK1/2 expression, pTH-Ser31

expression, and expression of the insulin receptor β subunit. Given that the RYGB group lost more weight than the pair-fed group, it is difficult from this study to determine whether the changes were caused primarily by a reduction in body weight/adiposity or through a more surgery-dependent, weight-independent mechanism.

Discussion

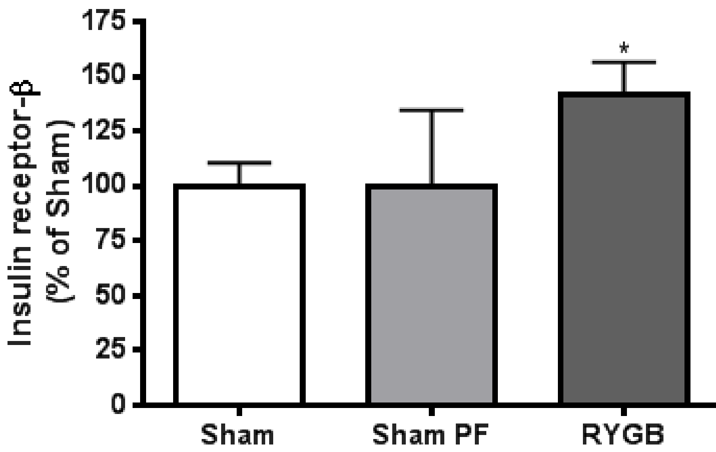
Both human and rodent studies indicate that RYGB alters the rewarding properties of palatable foods.[366,386-389] People who undergo bariatric surgery self-report less desire to consume palatable foods than before surgery[366,386-388] and reduce their intake.[390] Shin et al. (2010) performed RYGB in rats and found that, compared with sham animals, they exhibited more positive orofacial responses to a low concentration sucrose solution and a lower rate of licking of a high concentration sucrose solution.[248] Importantly, RYGB resulted in reduced consumption of a high-fat diet. This same group also noted that RYGB rescued food motivation to the level of lean controls in both an incentive runway paradigm and a progressive ratio operant paradigm.[248,249] These results promote a model by which RYGB reduces the need to consume large quantities of palatable food by restoring appropriate levels of hedonic stimulation. These behavioral correlates implicate adaptations within brain reward circuitry; yet the neural mechanisms driving changes in feeding behavior in bariatric surgery are unclear.

Dysregulated DA signaling within the striatum has been strongly associated to high-fat feeding, obesity, and reward.[4,213,214,244,391,392] PET studies point to altered DA signaling in the DS of obese individuals.[213,393] Similarly, rodent models of obesity, including diet-induced obesity models,[214,244,391,394] and obesity-prone genetic lines,[395] have all exhibited deficits in striatal DA homeostasis. Importantly, viral-mediated knockdown of the striatal D2 receptor suggest that impaired DA signaling may be a causal factor in the etiology of obesity.[214] Of note, amphetamine as well as other DA-targeting drugs possess potent anorectic properties,[396,397]

supporting our hypothesis that RYGB reduces intake of obesogenic food by enhancing DA neurotransmission. Of course, these drugs possess addictive properties; thus, defining other targets of regulation within this system through the study of RYGB has the potential to reveal novel and safer pharmacological targets for the treatment of obesity.

Although the literature makes strong reference to differences in levels of appetite-regulating gut hormones following bariatric surgery,[239,256,364,367,398] few attempts have been made to define the neurobiological adaptations that result from this altered neuro-hormonal milieu.[250,399,400] The aim of the current study was to define the neurochemical and molecular phenotype of RYGB within the striatum in a well-controlled preclinical mouse model. Furthermore, we sought to determine which of these phenotypes resulted from a simple reduction in caloric intake versus an effect of the surgical procedure. We focused our study on monoamine signaling within the DS, as DA within this region plays a critical role in the consummatory drive for food.[140,233] We observed altered catecholamine (DA and NE) levels in the DS of both RYGB and pair-fed (chronically food-restricted) animals, but not the non-catecholaminergic neurotransmitter 5-HT. This finding is consistent with the idea that DA signaling within the DS supports the rewarding properties of palatable food,[204] and that food is more rewarding under conditions of restriction.[236] A potential explanation for a small component of this effect may lie in alterations in DA conversion to NE, as observed in the current study. As this process occurs within vesicles and without changes to DA metabolism, we would expect a reduction in DA processing to NE to result in an increase in DA accumulation in vesicles with enhanced DA release in both chronically food-restricted and RYGB mice. In fact, it has previously been reported that electrically-evoked DA release in the DS and NAc is attenuated in slices taken from rats fed a cafeteria diet for 15 weeks versus rats on a regular chow diet.[395] Future studies will determine whether evoked DA release is corrected in the RYGB model.

A



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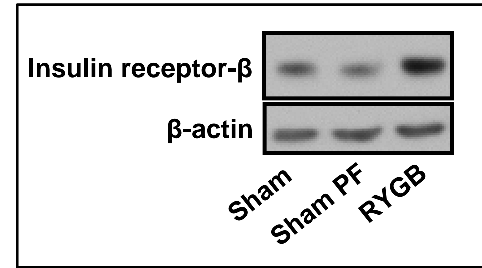

Figure 27. *Expression of the insulin receptor β subunit is elevated in RYGB-operated mice.* (A) Expression of the insulin receptor β was higher in the RYGB mice compared to shams (*$p<0.05$; n=6-7). Data were normalized to β-actin and (B) a representative immunoblot is shown. Data are represented as mean ± SEM.

Table 2. Correlation between body weight and studied molecular markers of DA homeostasis.

	R^2	df	P value
DA	0.182	20	**0.048**
DAT	0.091	19	0.183
pERK1/2	0.001	19	0.921
ERK1/2	0.454	17	**0.002**
pTH-Ser31	0.204	20	**0.035**
TH	0.003	17	0.812
IR-β	0.265	18	**0.020**

Body weight was significantly inversely correlated with DA levels, ERK1/2 expression, phosphorylation of TH at residue Ser31, and IR-β expression. R^2 = coefficient of determination; df = degrees of freedom.

To further explain this increase in striatal DA content, we next focused on determining possible changes in the function of key regulators of the DA synthetic pathway. We determined that animals which underwent RYGB had significantly greater levels of TH phosphorylation at Ser31. This phenotype was not observed in either the sham or the sham pair-fed groups, suggesting that the increased DA levels in the sham pair-fed group stems from a mechanism independent of changes in TH function. Consistent with the increase in phospho-TH, the expression of ERK1/2 was elevated in the RYGB mice. Since phosphorylation of Ser31 is targeted by ERK1/2, the increase in expression of ERK1/2 may suggest a possible mechanism by which TH activity is upregulated.[378] Finally, given the extensive literature on altered neuro-hormonal levels following RYGB, we explored markers of differential hormonal signaling in the RYGB group which could connect changes in gut anatomy with the changes DA homeostasis observed in the DS. Our group and others have previously found that insulin signaling in the DS acts to regulate DA homeostasis,[52,290] and that this signaling is dysregulated in rodents fed a high-fat diet.[244] Importantly, insulin resistance and type 2 diabetes often develop in the setting of obesity and are corrected by RYGB.[385,401] We found that expression of the insulin receptor β subunit was significantly upregulated in mice that underwent RYGB surgery, but not in either of the sham groups. The relevance of these data is enhanced considering that the striatum has been described as glucosensing brain region[402,403] and that increased brain glucose availability (i.e. hyperglycemia) and glucose oxidation disrupt both nigrostriatal neurotransmission and striatal DA turnover.[404,405]

Here, we report for the first time neurochemical changes at the level of the striatum in a preclinical model of RYGB. These changes include an elevation of DA levels with reduced conversion to NE, increased phosphorylation of TH, increased expression of the regulatory kinase ERK1/2, and increased insulin receptor-β expression. RYGB, while generally effective as a treatment for obesity, is not equally effective for all individuals.[406] Here, we have speculated that neural mechanisms may be an important factor mediating weight loss in RYGB.

Understanding these neural contributions to weight loss may allow for the development of pharmacotherapeutic interventions to improve clinical outcomes in patients undergoing bariatric surgery. Of course, pharmacological weight loss approaches, or so-called "knifeless surgery," if sufficiently effective would be beneficial as a means to avoid surgery altogether and bring relief to a larger patient population.[407,408]

CHAPTER IV

BILIARY DIVERSION SURGERY ENHANCES BILE ACID SIGNALING AND IMPAIRS COCAINE REWARD IN MICE

Preface

The gut-to-brain axis regulates diverse behavioral phenotypes; however, mechanisms supporting this communication are poorly understood. We reveal that a gut-based bariatric surgical approach chronically elevates systemic bile acids and attenuates cocaine-induced elevations in accumbal DA. Notably, this surgery reduces cocaine reward and sensitization. These findings define bile acid signaling and bile acid analogues as a pharmacological target for the treatment of cocaine abuse and reveal a mechanism of gut-to-brain communication.

Introduction

Traditionally, bile acids have been seen as simple detergents participating in the emulsification of ingested fats. However, it is increasingly apparent that bile acids also function as steroid hormones with targets in the intestine, liver, and brain.[409-411] Bile acids produced from cholesterol in the liver enter the proximal small intestine at the duodenum and are taken back up into portal circulation at the distal ileum, a segment of the small intestine densely populated by bile acid receptors and reuptake transporters. Biliary diversion - a newly developed bariatric surgical procedure in mice - is capable of chronically elevating circulating bile acids through ligation of the common bile duct and anastomosis of the gallbladder to the ileum (**Figure 28**A; GB-IL).[412] In the control surgery, the gallbladder is anastomosed to the duodenum (Figure 28A; GB-D), restoring normal bile flow independent of the sphincter of Oddi.

Biliary diversion was recently developed in mice to treat high fat diet-induced obesity.[412] GB-IL mice exhibited reduced high fat food consumption and weight loss. This reduction in the intake of rewarding, calorically-dense food could stem at least in part from altered reward for palatable food. Dysregulated mesolimbic DA circuitry has been linked to high fat, high calorie food consumption.[214,413,414] Psychostimulants such as cocaine hijack these circuits to cause addiction. We thus hypothesized that bile diversion to the ileum, which reduces high fat intake, might also reduce the rewarding properties of cocaine.

Methods

Mice: Male wild-type C57BL/6J mice used for surgeries or for OCA treatment were acquired from Jackson Laboratories (Bar Harbor, ME, USA) at 5 weeks of age. Mice were acclimated to a Vanderbilt University housing facility for one week prior to surgery. Surgery (GB-D or GB-IL) occurred at 6 weeks of age. Mice were given at least 2 weeks to recover from surgery and were handled for 3 days prior to the start of the CPP paradigm. At this point, mice either underwent behavioral testing (beginning with CPP) or were sensitized to cocaine without behavioral testing. Mice from the surgical model were sacrificed at either 4-5 or 7-8 weeks post-surgery. *Gpbar1* (TGR5) knockout heterozygous breeder mice were obtained from Dr. David Wasserman at the Vanderbilt University School of Medicine and their generation is described in Vassileva et al.[415] These mice were maintained on a C57BL/6J background. Heterozygous mice were mated to generate male and female knockout mice and wild-type mice used in behavioral experiments. These mice were handled for 3 days prior to the start of the CPP paradigm, which began at 8 weeks of age. All mice were group housed at a Vanderbilt University housing facility with *ad libitum* access to standard chow and water. The temperature- and humidity-controlled facility is maintained on a 12:12 h light:dark cycle (lights on 07:00-19:00 h) and all experiments were

performed during the light phase. All protocols were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Surgery: The control surgery (GB-D) and experimental surgery (GB-IL) were performed as previously described.[412] Body weights were measured immediately prior to surgery and following surgery up until sacrifice. Body weights for each mouse were averaged within 4 day bins and are represented as such in the group averages.

Amperometry in ex vivo *slice preparation*: Following recovery from surgery as described above, GB-D and GB-IL mice were treated with saline or cocaine along the same schedule as used for cocaine CPP using 20 mg/kg cocaine (see below; briefly, i.p. injections of saline every other day for 8 days and injections of cocaine on alternate days). After each injection, mice were placed back in their home cages. Mice were sacrificed by rapid decapitation under isoflurane anesthesia 1-2 days following their final cocaine injection. Slices were prepared as previously described[302] and electrically-evoked DA release in the NAc was measured by standard methods.[416] DA release was stimulated with a bipolar electrode placed on the surface of the slice and recorded with a carbon fiber electrode.

HPLC: Mice were sacrificed by decapitation under isoflurane anesthesia at 4-5 weeks following GB-D or GB-IL surgery. The brain was quickly dissected, blocked, and the NAc was punched bilaterally. Punches were placed into tubes on dry ice and stored at -80°C until processing. HPLC to measure monoamines from tissue was performed as previously described.[417]

CPP and locomotor sensitization: CPP was performed as previously described, with modifications.[286] Briefly, 2-chamber CPP apparati (MED-CPP2-MS; Med Associates, St. Albans, VT, USA) with distinct rod and mesh floor inserts were used. The associated software allowed for automated measurement of beam breaks on X-Y-Z axes (16 infrared beams, 50 ms intervals). Mice were weighed and then acclimated to the testing room for 20 minutes prior to

testing each day. During the first phase (pre-conditioning, day 1), mice were placed on the grid floor side of the 2 chamber apparatus. For 30 minutes, the mice had free access to both sides of the apparatus. During the second phase (conditioning, days 2-9), on alternate days mice were restricted to one side or the other of the apparatus for 30 minutes by use of a dividing door. Just prior to being placed in the chamber, each mouse was given an injection of either cocaine (20 mg/kg, i.p.) or saline (i.p.). Cocaine was paired with the side of the apparatus less preferred during pre-conditioning. Approximately half of the mice were started on cocaine, while the other half were started on saline. During this time, each mouse's locomotor activity was measured and used to determine cocaine-induced locomotor sensitization. The final phase of CPP (post-conditioning, day 10) consisted of placing the mouse on the cocaine-paired side initially with the dividing door removed; however, no drug was given on this day. Thus, mice were given full access to both compartments and their time spent on each side was measured. %CPP was calculated as the time spent on the cocaine-paired side during post-conditioning minus the time spent on the cocaine-paired side during pre-conditioning divided by the time spent on the saline-paired side during pre-conditioning. The first 20 minutes of pre-conditioning and post-conditioning were used in the calculation of %CPP. Locomotor sensitization to cocaine was determined by the ambulatory distance on days when mice received cocaine during the conditioning phase of CPP (4 exposures). All CPP was performed during the first phase of the light cycle. Activity Monitor v5.10 (MED Associates) was used to analyze CPP activity.

Open field (OF) locomotion: 4-7 days following CPP, GB-D and GB-IL mice from selected cohorts were tested for OF locomotion. Mice were initially weighed. Following 20 minutes of acclimation to the testing room, mice were placed in clean automated OF chambers (28x28 cm; MED-OFA-510; MED Associates) under constant illumination for 60 minutes and ambulatory distance was recorded. Time spent in the center of the chamber relative to the outer edges was calculated using the default center-surround analysis in the accompanying software. Mice were

then removed from the chamber, injected with saline (i.p., equivalent volume to a 20 mg/kg dose of cocaine at 1.2 mg/mL) and placed back in the chamber for 90 minutes. Finally, mice were removed again and injected with cocaine (20 mg/kg, i.p.) before being placed back in the chamber for an additional 120 minutes. Activity Monitor v5.10 (MED Associates) was used to analyze OF activity. AUC was calculated from the cocaine phase of testing.

Morris water maze – Hidden Platform (HWM): Following CPP and OF locomotion, GB-D and GB-IL mice from selected cohorts were tested on the HWM. The water maze protocol here was modified from a protocol previously described.[418] A round tub measuring 92x92 cm was filled with clean water the day before the first day of behavioral testing. On each morning of testing, mice were acclimated to the testing room for at least 10 minutes after which behavioral testing began. For the first 5 days, a platform was placed just under the water in the northeast corner of the maze such that mice could not see it. Each day for 4 trials per day, mice were placed into the pool facing the wall and were given 60 seconds to find and stand on the platform. If they found it, they were allowed to stand on it for 10 seconds before being removed by the experimenter. If they did not find the platform in the 60 seconds, they were placed on the platform by the experimenter for 20 seconds. After each trial, mice were allowed to dry in a clean cage on top of a warming pad, with at least 10 minutes in between each trial. On the final day of testing, the platform was removed. The mice were placed in the pool for a single trial and % time in the target quadrant was measured. All sessions were recorded by video and analyses of behavioral performance on the water maze were made using ANY-maze software (Stoelting Co., Wood Dale, IL, USA).

Tail Suspension Test (TST): Following CPP and OF locomotion, GB-D and GB-IL mice from selected cohorts were tested on the TST. This involved individually suspending each mouse by the tail using adhesive tape to a flat, stainless steel force sensor connected to a computerized monitoring system (v3.30, MED Associates). The force sensor measured the amount of time

each mouse spent struggling to right itself. The mouse was suspended from the sensor for a total 6 minutes. The last 4 minutes of the trial were used to calculate time immobile, which was defined as the total time during which the mouse movement did not exceed a preset threshold of seven for 200 ms. A single trial was performed for each mouse.

Rotarod: Following CPP and OF locomotion, GB-D and GB-IL mice from selected cohorts were tested on the rotarod. The rotarod consisted of a rotating, grooved rubber cylinder (approx. 3 cm in diameter) with dividers so that multiple mice could be placed on the machine at once. Mice were placed on the cylinder, which rotated for 5 minutes gradually increasing from 4 to 40 rpm. The amount of time spent on the cylinder before safely falling was recorded. Three trials were performed each day for 4 days, with 5 minutes in between trials.

Bile acid determination: Serum bile acids were measured by mass spectrometry *via* the Vanderbilt Mass Spectrometry Core facility using methods previously described.[412] Bile acids were measured from trunk blood taken immediately following decapitation at sacrifice at 4-5 weeks and 7-8 weeks post-surgery.

OCA administration: To allow for gut bioavailability of OCA without the stress of oral gavage, OCA was administered to mice by voluntary oral administration.[419] OCA (Adipogen, San Diego, CA, USA) was initially dissolved in beta cyclodextrin (20% w/v) and then dissolved within palatable drug-laced jellies. Jellies were composed of gelatin (10% w/v), sucralose (18.5% w/v), artificial strawberry flavoring (8% v/v); beta cyclodextrin (2% w/v) in water. Jellies containing OCA were made to contain 10 mg/kg based on each mouse's original weight on the first day of drug or vehicle administration. Jellies containing vehicle were identical to drug-laced jellies except that they contained beta cyclodextrin without dissolved drug. Mice were given jellies by voluntary oral administration on six consecutive days per week for 4 weeks. They were given the jellies between 3pm and 6pm by placing each mouse into an OF chamber containing the

jelly for 20 minutes. To ensure that mice consumed the jellies consistently, all mice were initially trained to eat jellies without drug for 5 days prior to drug/vehicle jelly administration. Mice in this group underwent behavioral testing for cocaine CPP as described above 2 weeks into chronic drug administration. Drug administrations were continued until sacrifice at 4 weeks following the start of drug/vehicle administration.

Whole-cell electrophysiology: Ex vivo mouse sagittal brain slices were prepared as previously described.[177] Briefly, mice were acutely anesthetized using isoflurane and sacrificed by rapid decapitation. Brains were submerged in ice-cold sucrose dissecting solution (80 mM NaCl, 2.5 mM KCl, 7 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 75 mM sucrose, and 25 mM glucose) and 250 µm thick slices were cut using the Leica 200VT Vibratome. Slices were allowed to recover for 10-15 minutes at 35°C in an N-methyl D-glucamine (NMDG) based recovery solution (2.5 mM KCl, 20 mM HEPES, 1.2 mM NaH_2PO_4, 25 mM glucose, 93 mM NMDG, 30 mM $NaHCO_3$, 5 mM sodium ascorbate, 3 mM sodium pyruvate, 10 mM $MgSO_4$, and 0.5 mM $CaCl_2$) before being transferred to a room temperature ACSF holding chamber for 1h prior to use. ASCF used for holding chamber and recordings contained 120 mM NaCl, 2.5 mM KCL, 1.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 10 mM glucose. The nucleus accumbens shell was identified by the shape of the anterior commissure, the corpus callosum, and the absence of stria in the brain slice. Accumbens shell medium spiny neurons were patched using the Scientifica Patch Star system and 4-7 MΩ glass micropipettes made using the Sutter Brown/Fleming P1000 Micropipette Puller. Patch pipettes were filled with a cesium-based internal solution (120 mM $CsMeSO_3$, 15 mM CsCl, 8 mM NaCl, 10 mM HEPES, 0.2 mM EGTA, 10 mM TEA-Cl, 4 mM ATP, 0.3 mM GTP, 0.1 mM spermine, and 5 mM QX-314 bromide). Evoked responses were elicited by electrical stimulation via the Iso-flex stimulation isolator and recorded using the Axopatch Multiclamp 700B amplifier and Axon Digidata 1550 low-noise data acquisition digitizer (Molecular Devices, Sunnyvale, CA, USA). All recordings

were performed in 50 μM picrotoxin to isolate excitatory events and cells with an access resistance greater than 20 MΩ or those in which the access resistance changed greater than 20% during recording of a single experiment were excluded from analysis. For measurements of EPSC amplitude in the presence of OCA, OCA (Adipogen) stock aliquots in DMSO were diluted 1:1000 in ACSF (final concentration: 10 μM). OCA-containing ACSF was perfused into the recording chamber for 10 minutes following baseline acquisition while holding at -70 mV. Peak EPSC amplitude was measured for the duration of the experiment and normalized to the first 10 minutes. Analysis was performed by averaging six sweeps of identical interstimulus intervals and dividing the mean amplitude of the second event by the initial.

Cecal content sampling and microbiota analysis: Cecal content samples were collected from GB-D and GB-IL mice at sacrifice 4-5 weeks after surgery and stored at -80°C. The DNA extractions, amplification, library prep, and sequencing were done by the Gut Microbiome Core, at the University of California at Davis. The 16S universal Eubacteria primers (PCR primers 515/806) were used to amplify the V4 variable region. A single-step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) were used under the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 seconds; 53°C for 40 seconds and 72°C for 1 min; after which a final elongation step at 72°C for 5 minutes was performed. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA). Microbial sequencing was analyzed by bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP) using a Roche 454 pyrosequencer and titanium reagents and 3-5K nominal sequences per sample of high quality extracted DNA. Sequences were depleted of barcodes and primers, then short sequences <200 base pairs are removed, sequences with >1 ambiguous base calls removed, and sequences with homopolymer runs exceeding 6 base pairs removed using the statistical software package Quantitative Insights Into Microbial Ecology

(QIIME). A total number of 491,009 sequences passed a quality filter with a minimum score of 25 and an average length of 460 base pairs. Operational taxonomic units were defined after removal of chimeric and singleton sequences, clustering at 3% divergence (97% similarity).[420] OTUs were then taxonomically classified using BLASTn against a curated GreenGenes database.

Statistical analysis: Data are presented as means ± standard error of the mean. Statistical analysis was performed with GraphPad Prism software, version 5.02 (GraphPad Software, San Diego, CA, USA). Data were analyzed by statistical tests noted in figure legends, which included Student's *t* test, multiple *t* test, two-way RM ANOVA, and linear regression functions through Prism as appropriate. Outliers were defined as having values outside of quartile1 – 1.5×interquartile range (IQR) and quartile3 + 1.5×IQR and were excluded. A p-value <0.05 defined statistical significance for all tests except for multiple *t* test analysis which determined significance using the Holm-Sidak method for correction of multiple comparisons, with alpha=5%.

Results

Cocaine directly alters DA neurotransmission and produces its rewarding effects by increasing available extracellular DA in specific brain regions, including the NAc.[413,421] We first studied the effect of the surgery on cocaine's ability to enhance DA availability in NAc. We performed our experiments in animals fed a chow diet. In GB-IL mice, there were no significant differences in body weight as compared to GB-D as early as day 12 post-surgery (Figure 28B). This strongly suggests that, on a chow diet, the long-term homeostatic regulation of body weight in GB-IL parallels that of the GB-D mice. Here, we measured the effect of cocaine on electrically- evoked DA release in NAc slices. Three stable baseline recordings were taken at

five minute stimulation intervals and no differences were noted between GB-D (Figure 28C, baseline) and GB-IL (Figure 28D, baseline) in terms of peak amperometric current (Figure 28E). However, the increase in electrically-evoked DA release promoted by 10 μM cocaine was significantly reduced in the GB-IL mice (Figure 28C-D, cocaine; quantitation in 28F, as area under the curve). Quantitation of the area under the curve (AUC) of the amperometric trace in the presence of cocaine expressed as a percentage of average AUC for the three baseline recordings is shown in figure 28F. Importantly, GB-IL mice do not exhibit an overt neurochemical phenotype, as total accumbal tissue levels of DA and its related monoamines, NE and serotonin (5-HT), were not significantly altered with respect to GB-D (Figure 28G).

We next determined whether GB-IL mice display reduced behavioral responses to cocaine. Mice were tested for cocaine conditioned place preference (CPP; 20 mg/kg, i.p.) in a dual compartment apparatus (**Figure 29**A). During conditioning sessions, locomotor behavior was measured. On first exposure to cocaine, cocaine-induced hyperlocomotion was indistinguishable between the two groups, suggesting that GB-D and GB-IL mice experienced similar levels of cocaine centrally. Notably, while control mice exhibited significant locomotor sensitization to cocaine over multiple exposures (*$p<0.05$), the GB-IL mice did not (Figure 29B; **Figure 30**). Prior work strongly suggests that psychomotor sensitization is associated with the development of molecular adaptations within the mesocorticolimbic system in the development of an addiction.[422] The lack of locomotor sensitization in our biliary diversion model may thus support impairments in the central encoding of cocaine reward. Importantly, while both groups formed a place preference for cocaine, the preference of GB-IL mice for the cocaine-paired side was significantly less than that observed for GB-D mice (Figure 29C). In an open field, neither spontaneous nor saline-induced locomotion in GB-IL mice significantly differed from GB-D mice; however cocaine-induced locomotion (20 mg/kg, i.p.) was significantly attenuated between 10 and 30 minutes post-injection (Figure 29D and *inset*).

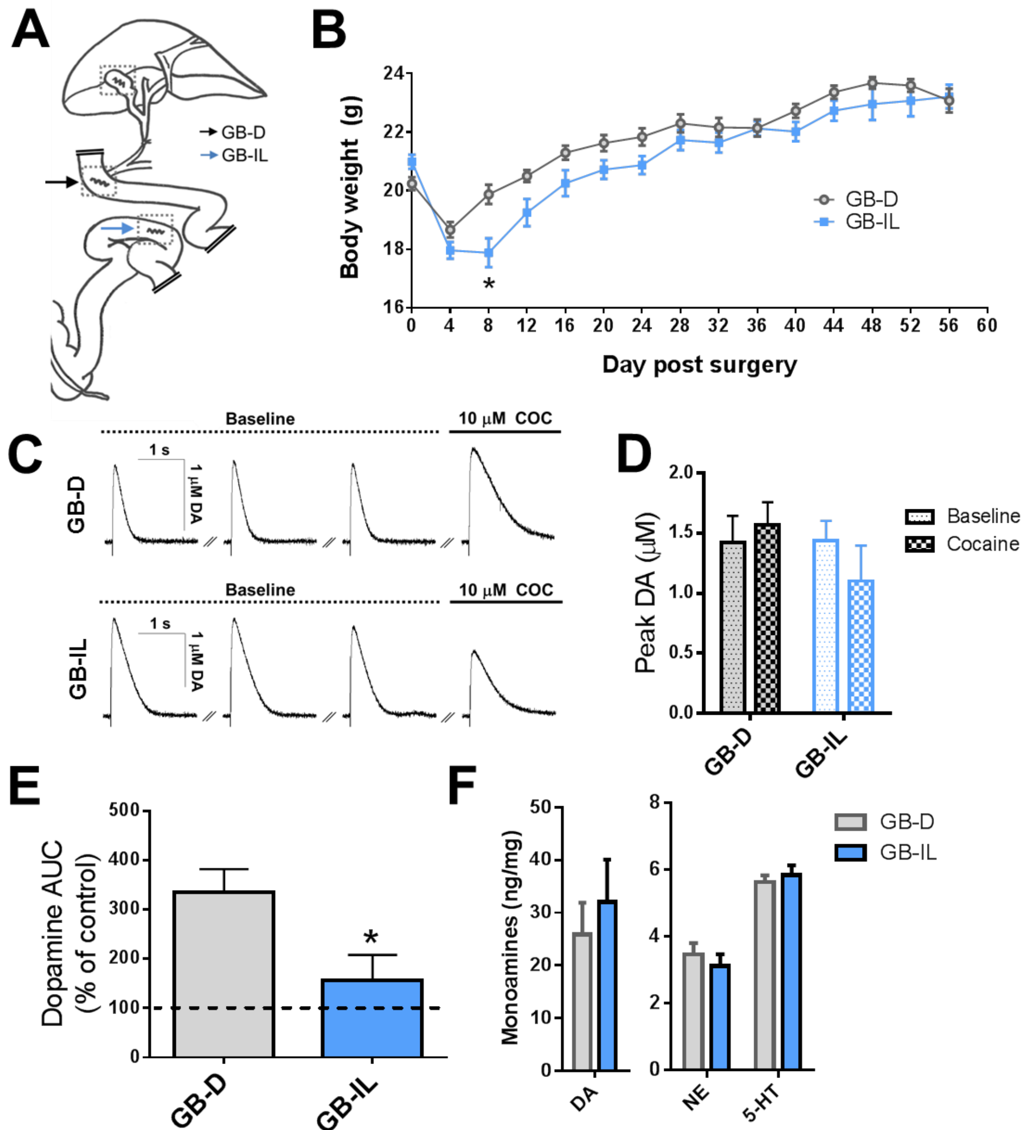

Figure 28. *Biliary diversion blocks cocaine's ability to increase DA levels in the NAc.* (**A**) Schematic representation of GB-D (black arrow) surgery and GB-IL (blue arrow) surgery. Dotted boxes outline incision sites in gallbladder and intestine. (**B**) Body weight following GB-D or GB-IL surgery (n=17-21; *p<0.001 by multiple *t* test comparison). (**C**) Amperometric recordings of DA from the NAc in GB-D (top) and GB-IL (bottom) mice. Electrically-evoked DA responses are stable at 5 minute intervals. Cocaine robustly enhanced the evoked DA response in the GB-D mice (n=4). This effect is blunted in GB-IL with respect to GB-D mice (n=5). (**D**) Quantitation of the peak amplitude of amperometric recordings under baseline (dotted bar) or cocaine (checkered bar) conditions in GB-D (black) or GB-IL (blue) animals (p>0.05 comparing baseline to cocaine for each surgical group). (**E**) AUC of the evoked DA response normalized to the average of the pre-cocaine baseline values from each slice (n=4-5; *p<0.05, Student's *t* test). (**F**) Levels of DA, NE, and 5-HT in NAc tissue punches from GB-D and GB-IL mice. No significant differences were noted (n=4-6; p>0.05 by Student's *t* test).

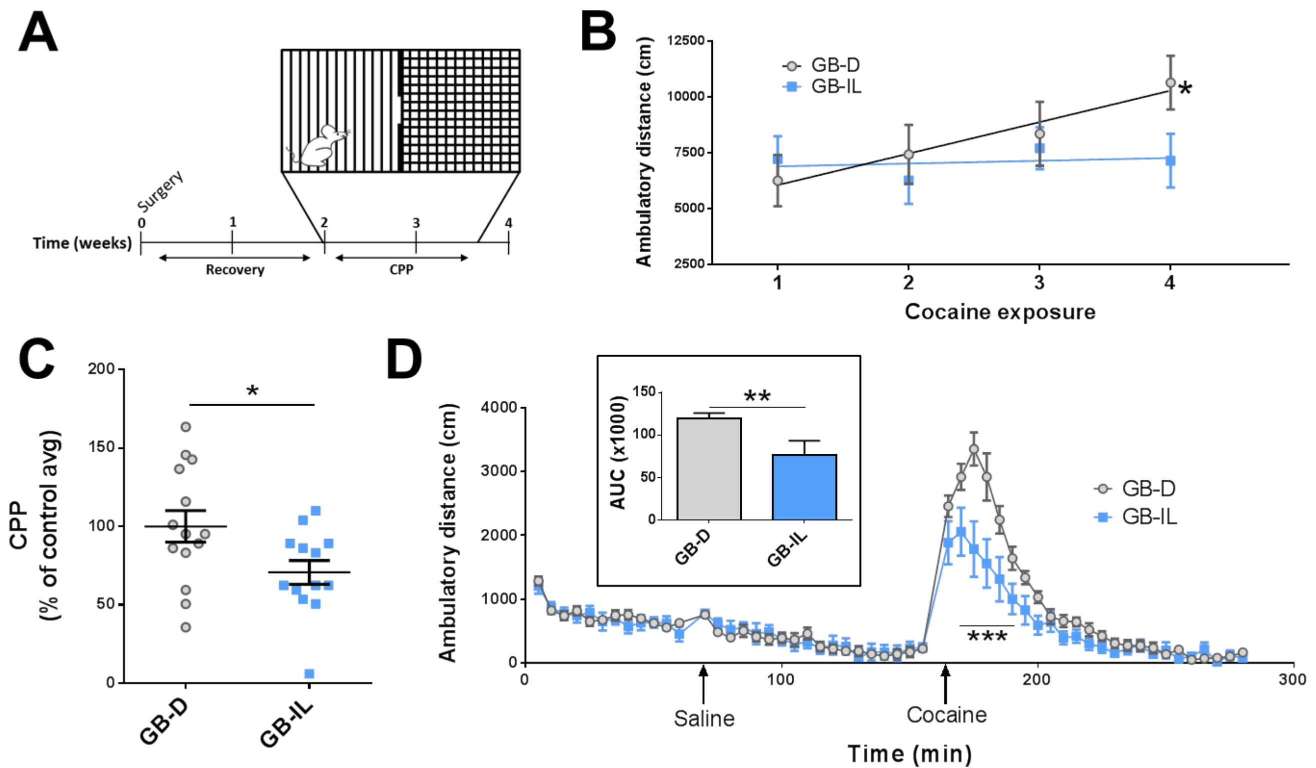

Figure 29. *Bile diversion to the ileum blocks cocaine locomotor sensitization and reduces CPP.* (**A**) Mice underwent surgery (GB-D or GB-IL), recovered for 2 weeks, and began the cocaine CPP paradigm. (**B**) Average group locomotor activity in CPP chambers during four cocaine exposures (20 mg/kg, i.p.) with linear regression of activity over the four exposures (n=12-15; *$p<0.05$ indicates significant regression from zero slope; $F(1,46)=6.280$). (**C**) Cocaine CPP expressed as %CPP normalized to GB-D average (n=13-14; *$p<0.05$ by Student's *t* test). (**D**) Open field locomotion. GB-IL mice exhibit a reduced response to cocaine with no difference in basal locomotion (n=8-10; $p<0.05$ by two way RM ANOVA for cocaine administration, $F(1, 16)=6.544$; ***$p<0.001$ between 10 and 30 minutes following cocaine injection by multiple *t* test comparison). (**inset**) Area under the curve for cocaine locomotor responses in GB-D and GB-IL mice (**$p<0.01$ by Student's *t* test).

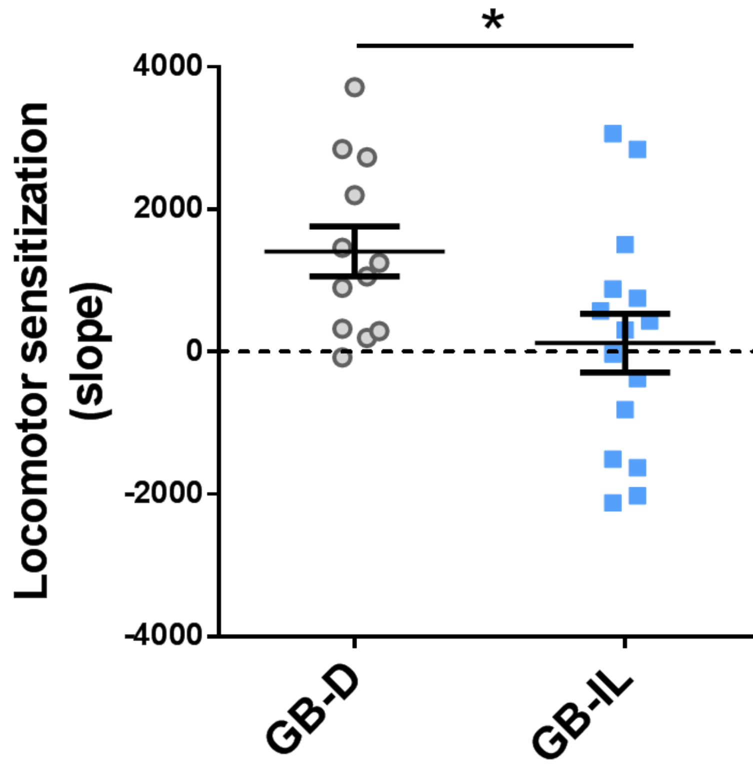

Figure 30. *Biliary diversion reduces locomotor sensitization to cocaine.* Each dot indicates the slope of the linear regression of the ambulatory distance induced by the four cocaine exposures in each individual mouse (n=12-15; *p<0.05 by Student's *t* test).

The reduction in conditioning to cocaine cannot be attributed to impaired spatial learning or memory capabilities, as we did not observe any significant impairment in performance on a hidden water maze task (**Figure 31**A-C). Moreover, no generalized impairments in motor abilities (Figure 31D) or motivated behavior/affective state in a tail suspension test (Figure 31E) were observed. However, in the open field, we did observe a small but significant increase in center time in the GB-IL group (Figure 31F), suggesting that the surgery may also affect systems regulating exploratory behavior or anxiety.

GB-IL mice exhibit greatly increased levels of total and conjugated circulating bile acids relative to GB-D, while levels of primary, secondary, and unconjugated bile acids remain unchanged (**Figure 32**A). This finding, in conjunction with our behavioral data, points to a potent and previously unexplored role for conjugated bile acids as regulators of cocaine reward. However, whether bile acids signal directly within the brain is unknown. Prior work suggests that AMPAR signaling in the NAc participates in molecular mechanisms underlying cocaine-seeking behavior.[173,423] Here we show that the synthetic bile acid obeticholic acid (OCA) induces a robust depression of AMPAR-mediated EPSCs in the NAc (Figure 32B). This result indicates that bile acid signaling acutely regulates excitatory neurotransmission in the NAc, an important component of the development of reward-context associations in the CPP test.[91,173] Using an *in vivo* pharmacologic model, we further demonstrate that chronic OCA administration is sufficient to reduce cocaine CPP (Figure 32). For two weeks prior to the initiation of cocaine CPP, mice were treated with OCA (10 mg/kg, p.o.) or vehicle (Figure 32C). The treatment continued until sacrifice 4 weeks following drug initiation. Mice treated with OCA, compared with vehicle treatment, exhibited decreased cocaine CPP (Figure 32D).

In addition to their canonical role as fat emulsifiers, bile acids signal in a hormonal fashion mainly through two bile acid receptors, the farnesoid x receptor (FXR) and g protein-coupled bile acid receptor 1 (TGR5);[424] only TGR5 is expressed in the brain.[410,425] To test

whether TGR5 receptor signaling contributes to reduced susceptibility to the rewarding properties of cocaine, we measured cocaine CPP in TGR5 knockout mice. We found that deletion of the TGR5 receptor results in significantly increased preference for the cocaine-paired chamber relative to wild-type littermates (Figure 32E). This enhancement in cocaine reward identifies a role for the TGR5 receptor in reward processes and supports basal signaling through TGR5 as a contributor to resilience to cocaine reward.

Discussion

Our findings support a pivotal role for bile acid signaling in neuronal function as well as in reward behaviors. This was first revealed by a novel surgery where bile acids were diverted to the ileum to increase reabsorption and augment levels of circulating bile acids. We demonstrate that this surgery was able to modify reward acquisition for cocaine. GB-IL blocks both sensitization and the rewarding properties of cocaine, which relies on increases in extracellular DA levels. Notably, the surgery alters cocaine's ability to increase DA levels in the NAc. These results thus reveal that a surgery designed for weight loss also regulates psychostimulant reward. We also show that bile acid signaling may be critical to this process. This notion is supported by the finding that chronic OCA treatment impairs cocaine reward. Conversely, TGR5 knockout mice exhibit enhanced cocaine preference compared to their wild-type counterparts. Together, these results point to a role for bile acid signaling in susceptibility or resilience to cocaine reward. Of note, we did not find any differences in the gut microbiome composition in our surgical model (**Figure 33**), suggesting that potential alterations to the gut microflora are unlikely to mediate altered gut-to-brain communication in this setting.

Interestingly, both DAergic and glutamatergic systems in the NAc appear to be affected by alterations in bile acid signaling as we were able to alter cocaine-induced increases in

extracellular DA in our surgical model and acutely induce a long-lasting depression of EPSCs pharmacologically. Notably, both of these actions contribute to the rewarding and reinforcing effects of cocaine.[426] Thus, further studies exploring whether pharmacologic, or even surgical, enhancement of bile acid signaling could intervene in established addictions are warranted. Importantly, the bile acid receptor agonist used in the current study (OCA) has already completed a phase III clinical trial to treat hepatic steatosis.[427] This drug showed clinical efficacy in this setting with an excellent safety profile, thereby reducing barriers to its development for addiction.

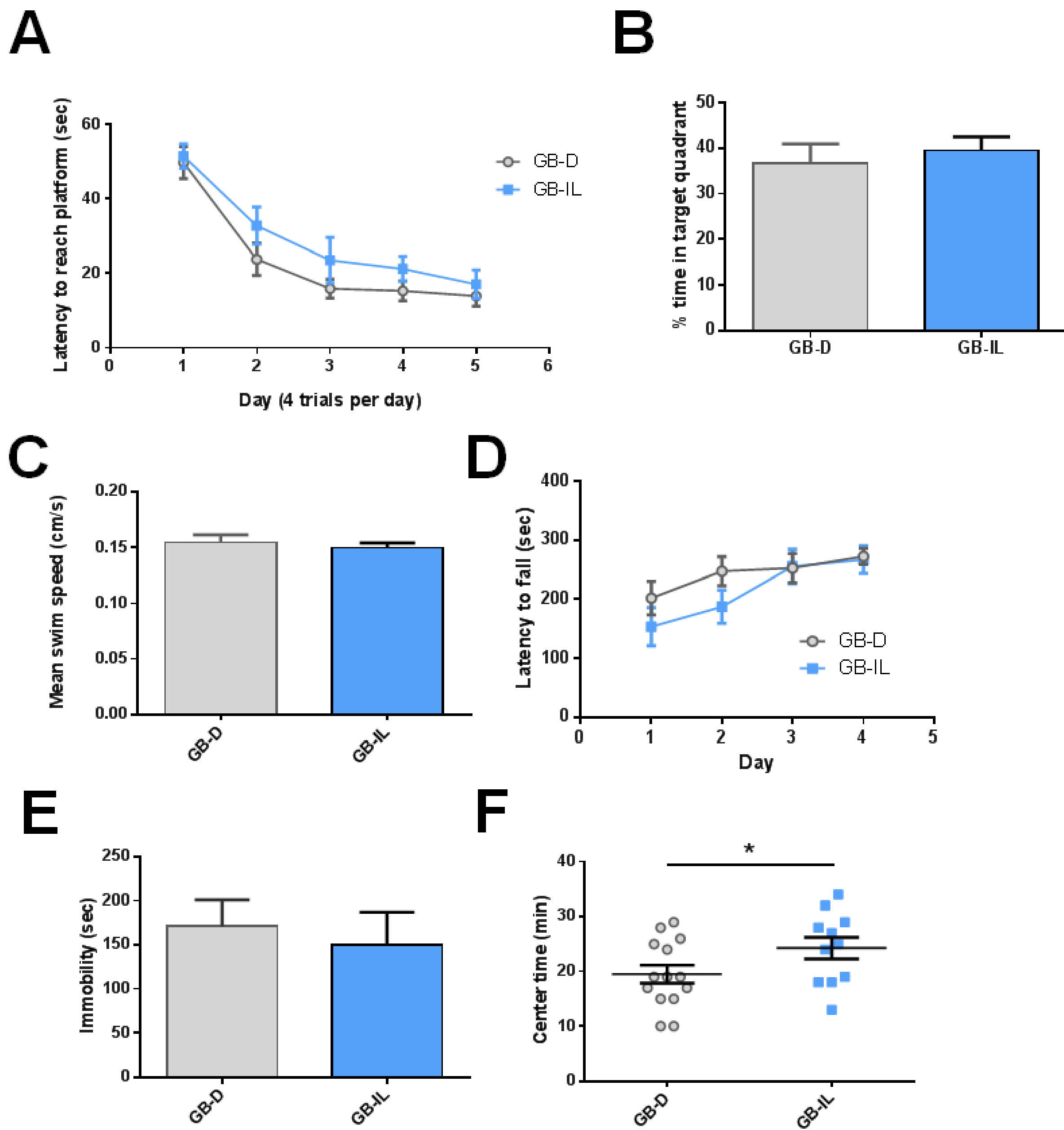

Figure 31. *Biliary diversion does not alter learning, memory, motor function, or affective behavior.* (**A**) There were no significant differences between GB-D and GB-IL mice in a Morris Water Maze acquisition task (n=7-8; p>0.05 by two-way RM ANOVA). (**B**) There were no significant differences between groups in a Morris Water Maze recall task (p>0.05 by Student's *t* test). (**C**) Mean swimming speed in GB-D and GB-IL mice (p>0.05 by Student's *t* test). (**D**) There were no significant differences in latency to fall from a rotarod (n=5-8; p>0.05 by two-way RM ANOVA). (**E**) Time immobile on a tail suspension task was similar between groups (n=7-8; p>0.05 by Student's *t* test). (**F**) Open field locomotion revealed a significant increase in center time in the GB-IL mice (n=11-14; *p<0.05 by Student's *t* test).

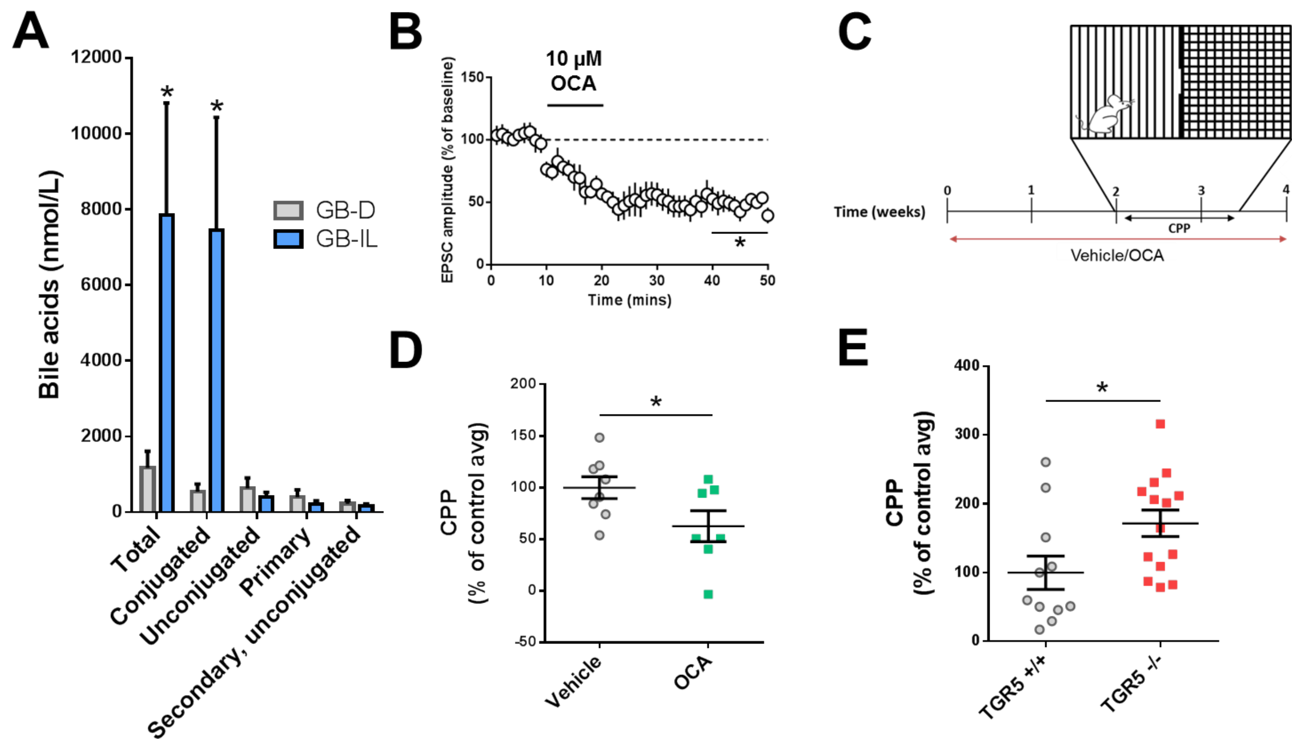

Figure 32. *Bile acid signaling regulates cocaine reward.* (**A**) GB-IL mice exhibit elevated levels of serum total and conjugated bile acids with respect to GB-D (n=7-8; *p<0.05 by Student's *t* test). (**B**) 10 µM OCA reduced EPSC amplitude in NAc medium spiny neurons (n=4; *p<0.05 by Student's *t* test). (**C**) Mice were treated orally with either vehicle or 10 mg/kg OCA six days a week for four weeks. After 2 weeks the mice were started on the cocaine CPP paradigm. (**D**) OCA-treated mice showed reduced preference for the cocaine-paired chamber (n =7-8; *p<0.05 by Student's *t* test) expressed as %CPP normalized to GB-D average. (**E**) Constitutive deletion of the TGR5 receptor (-/-) results in increased preference for the cocaine-paired chamber, expressed as %CPP normalized to TGR5 (+/+) littermate average (n=11-14; *p<0.05 by Student's *t* test).

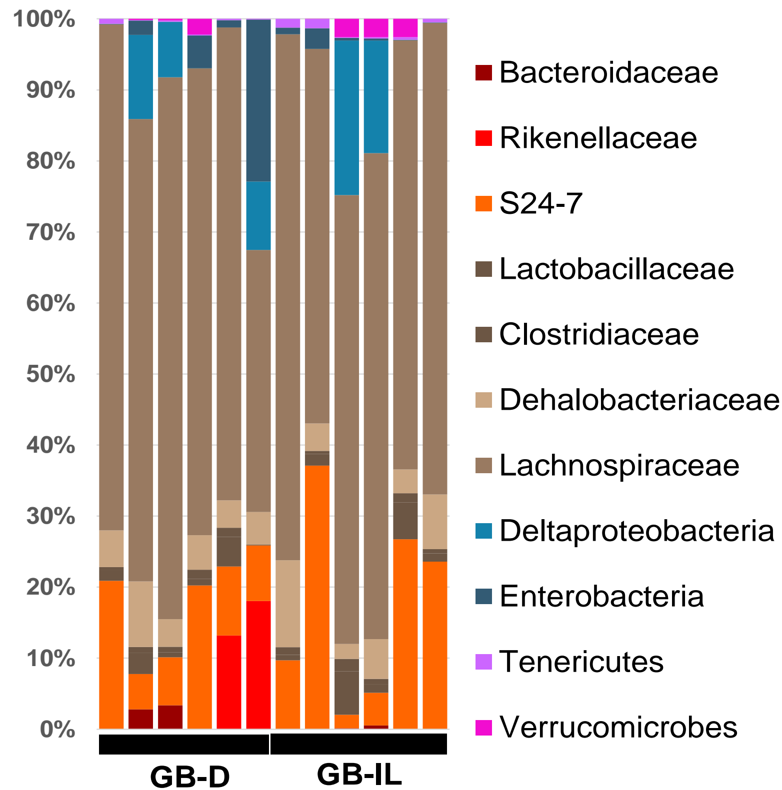

Figure 33. *GB-IL mice do not exhibit altered gut microbiota compared to GB-D controls.* Stacked column bar graph depicting the relative abundances and distributions of the most highly abundant resolved bacterial phyla across the 12 fecal samples analyzed. Cecal contents from mice subject to GB-D (n=6) or GB-IL (n=6) were subjected to 16S RNA sequencing. Bacteroidetes are colored in shades of red, proteobacteria in shades of blue, tenericutes in purple and verrucomicrobes in pink. Each column represents a single mouse. No significant differences in bacterial abundances were noted.

CHAPTER V

SYNTHESIS, GENERAL DISCUSSION, AND FUTURE DIRECTIONS

In summary, we have shown that the hormone GLP-1 regulates DA homeostasis and cocaine actions through a novel retrograde mechanism in the LS (Chapter II), that RYGB surgery alters striatal DA homeostasis (Chapter III), and that a surgery designed for weight loss increases systemic bile acids which appear to play a role in regulating reward for cocaine (Chapter IV). These findings are bound together by the concept that the gut and the hormones produced by it to regulate feeding and energy metabolism can modify DA homeostasis and behavioral manifestations of cocaine action.

While we are far from understanding the complete mechanisms by which the gut communicates with the brain to regulate reward, we believe that we have helped to expand a new frontier in neuroscience. More and more we learn that the periphery plays an important role in shaping central function. The gut and its associated signaling systems have garnered much attention recently in this regard. We now know – from our work and that of others – that alterations to the gut or gut-generated hormones can affect complex behaviors related to stress, affective state, and reward.[389,428,429] These studies reveal that the gut should not be viewed as a simple feedback system, but a system that plays an integral part in shaping our day-to-day moods and desires. Furthermore, a better understanding of the gut-to-brain axis may allow us to probe its role in the development of serious neuropsychiatric disorders and harness newly defined mechanisms of central regulation towards the generation of novel therapies.

We believe that substance use disorders, highly-prevalent disorders caused at least in part by reward system dysfunction, could benefit from this enhanced understanding of gut-to-brain regulation. Currently, the individual and public health impact of substance use disorders,

particularly for psychostimulants, is compounded by the fact that we have very few effective therapies to combat them. Behavioral therapies, although effective for some patients, have not provided a systemic solution.

Movement through the translational pipeline for pharmacotherapies to fight addiction has been slow in part, I believe, because we have traditionally sought to intervene at direct sites of drug action. This is problematic because this type of intervention interferes with normal reward processes, which may lead to anhedonia (e.g. CB1R antagonists) or prolonged withdrawal symptoms (e.g. opioid receptor antagonists).[430,431] Furthermore, directly impairing DA or opioid systems can lead to severe side effects (e.g. antipsychotics which block D2 receptors; opioid receptor antagonists),[432-434] limiting the utility of such drugs. I believe that we need to find modulators of reward systems which may not affect reward processes quite so directly or dramatically but which have the potential to reduce drug intake without serious side effects. Understanding how hormones and other gut-to-brain signaling mechanisms regulate neurotransmission, reward, and drug use may provide some of the modulatory regulation necessary to fight addiction.

In this vein, long-lasting synthetic analogues of GLP-1 have been shown to reduce the rewarding and reinforcing properties of food, psychostimulants, alcohol, and nicotine, and are now approved as weight-loss therapy in obese individuals.[220,235,284-289] Given their strong safety profile in patients, GLP-1 analogues have great potential to be developed as therapy for substance use disorders. However, a few questions should first be addressed: At what stages of addiction can GLP-1 analogues be effectively used? What are the targets of GLP-1 analogues in the brain and how are they working?

Regarding the first question, it is important to remember that the development of substance use disorders involves at least (1) susceptibility to the drug in naïve individuals, (2)

the potentially damaging physiologic effects of the drug, (3) the enjoyment of the drug, (4) the motivation to work to obtain more of the drug, (5) learning to associate the drug with environmental cues, (6) withdrawal effects from the drug, and (7) susceptibility to relapse in prior users. In designing novel therapies against substance use disorders, it is important to characterize the intervention at each of these levels. For example, it would be inappropriate to prescribe a therapy that decreases the hedonic properties of a drug but increases the motivation to consume it. The animal literature has primarily shown that GLP-1 analogues reduce acute effects of drugs (in locomotor assays),[284,287,289,292] drug intake (in choice tests and self-administration),[285,287] and drug reward (as measured by CPP)[284,286,287,289,435] through acute pre-administration of GLP-1 analogues. Unfortunately, these results don't tell us much about how GLP-1 analogues might function in the context of an individual who has been taking drugs for a long time (and has potentially developed some of the central adaptations to the drug described in Chapter I). They also fail to inform how GLP-1 analogues might work if administered chronically and out of sync with drug administrations. Indeed, central resistance is thought to develop in response to chronic insulin and leptin administration.[436-438] There is little evidence to suggest that this happens at the GLP-1R;[439] however, this possibility should be explicitly tested. Additionally, one of the most important tests of the utility of GLP-1 analogues would be in a reinstatement model of drug-seeking. Reinstatement models are a way for us to test in animals how a therapy might be able to block or attenuate drug-seeking following an abstinence period. This is thought to model the very relevant condition of relapse drug-seeking in humans. To our knowledge, no experiments have been performed to determine whether GLP-1 analogues might prevent reinstatement, but such tests will be crucial going forward. Finally, there is some evidence that signaling through the GLP-1R may play a role in the susceptibility or resilience to drug reward. GLP-1R knockout mice exhibit augmented cocaine-induced locomotor responses and CPP compared with wild-type controls.[293] This effect relies, of course, on endogenous GLP-1R-mediated signaling. It will thus be interesting to determine whether polymorphisms in the

GLP-1R or differences in endogenous GLP-1 signaling contribute to drug reward vulnerability. In fact, there are common variants within the GLP-1R,[297,440] and at least one is associated with alcohol dependence in males.[297] Taken together, it seems that signaling through the GLP-1R plays a definitive role in modifying drug reward. Nevertheless, additional work will be required to determine how to effectively implement GLP-1-based therapies in the treatment of human addictions.

In our study of GLP-1R signaling in the LS, we attempted to address the second key question regarding the targets of GLP-1 analogues in the brain and mechanism of action. One of the goals of neuroscience is not just to find therapies that work, but also to understand how they work in order to refine our use of them as new technologies and targeted approaches become available. In doing so, we first showed that administration of Ex-4 reduces septal levels of the endocannabinoid 2-AG. This finding is interesting on its own because 2-AG promotes hedonic reactions and feeding behavior, processes opposed by Ex-4. Furthermore, a recent study showed that cocaine is able to mobilize 2-AG;[153] thus Ex-4 may act to oppose the hedonic properties of cocaine in this manner. Future work will be required to determine the exact mechanisms by which increased stimulation of GLP-1Rs leads to the downregulation of 2-AG. A lipidomics analysis of precursors to 2-AG may suggest where a bottleneck may be occurring and point to potential enzymes inhibited by GLP-1R signaling. It would also be reasonable to determine whether the effect on 2-AG alone contributes to altered reward mechanisms. Behavioral and molecular studies of Ex-4 action using the drug rimonabant to block CB1Rs will determine whether any of Ex-4's effects on reward are CB1R-dependent. This may be important as reduced CB1R signaling has been associated with anhedonia and suicidal ideation. While there is no evidence of these symptoms in patients taking GLP-1R agonists for the treatment of diabetes,[441] this may be a consideration in patients suffering from addiction.

In this same study we also showed that AA levels are reduced by treatment with Ex-4, likely an effect of a processing bottleneck upstream of 2-AG. Focusing our studies in a cell model, we further show that AA reduces DAT function – consistent with prior literature – and likely does so through reduced surface expression. Given that AA generates γ-KA species capable of covalently binding the DAT and reducing its function, we suggest that the DAT is permanently altered by this interaction. In this scenario, the DAT is likely tagged for degradation, perhaps by ubiquitination, and trafficked into the lysosomal degradation pathway. Recovery of DAT expression would require trafficking from a reserve pool of DAT and increased *de novo* DAT production. This is in contrast to much of the DAT trafficking literature which has focused on the role of signaling kinases in trafficking DAT to recycling endosomes. Additional work will be necessary to test the hypothesis that γ-KA-DAT is targeted for degradation and clarify which of these two internalization pathways dominates in response to AA.

Based off of this work, we further hypothesize that the γ-KA scavenger, salicylamine, may modify reward processes related to cocaine. Interestingly, one study revealed that salicylamine prevents working memory deficits in a mouse model of Alzheimer's disease and could be safely administered *in vivo*.[442] Future studies will explore whether salicylamine alters reward in behavioral assays.

We might also consider the therapeutic possibilities that arise from the biliary diversion model. This surgical manipulation of the gut allowed us to reduce the development of cocaine reward in mice as measured by CPP. Biliary diversion (GB-IL) surgery, which chronically elevates systemic bile acid levels, reduced the formation of cocaine CPP as well as the sensitization to cocaine over time. Chronic oral treatment with a synthetic bile acid leading up to and throughout the CPP protocol also impaired the development of a preference for cocaine. On the other hand, genetic deletion of a major bile acid receptor involved in hormonal signaling was able to enhance preference for cocaine. All of these interventions altered bile acid signaling well

before the mice were ever exposed to cocaine. Therefore, these results suggest that bile acid signaling may play a role in the resilience or susceptibility to the rewarding effects of cocaine. While it is currently difficult to imagine a way to *prevent* addictive behavior clinically, we believe that a better understanding of the possible modulators of the reward system will inform future work regarding therapeutic targets. Furthermore, the things that make us susceptible to disease are of interest, particularly in the age of increasingly affordable genetic testing. It is possible that components of bile acid processing may be polymorphic or dysregulated from one individual to another and thus contribute to disease potential. This is plausible, as endogenous signaling through the TGR5 bile acid receptor appears to be an important contributor to cocaine CPP. In the future it will be also interesting to determine whether biliary diversion is able to affect drug-seeking behavior in animals with prior experience with cocaine. We believe that biliary diversion may have an effect on already established cocaine responses as the surgery has been shown to reduce high fat feeding in mice which had been fed such a diet for 12 weeks prior to surgery.

The mechanism(s) by which biliary diversion or bile acids affect reward is still, admittedly, poorly understood. First and foremost, experiments will be required to determine whether the effect of increased bile acid signaling relies on a direct central effect. To determine whether the effect of biliary diversion or bile acid signaling on cocaine CPP relies on central mechanisms, bile acids could be perfused directly into the brain or into specific brain regions. Additionally, the TGR5 bile acid receptor could be genetically deleted from the brain or specific brain regions using conditional mouse lines, viral approaches, or RNA silencing. Secondly, describing the distribution of bile acid receptors in the brain will also be important. Several studies have suggested that TGR5 receptors are expressed at low levels in the brain,[410,425] but their distribution and co-expression profiles are unknown. This information may help fuel future mechanistic studies. For instance, it would be helpful to know if the TGR5 receptor is expressed on accumbal MSNs and, if so, whether it is expressed on the D1 or D2 subtype MSNs. Such

information might inform, for example, the interaction with DAergic and glutamatergic systems that we observed in our study. Beyond TGR5, there is also the possibility that there may be alternative bile acid targets within the CNS. These include neurosteroid receptors as well as the pregnane X receptor,[443,444] which have been shown to bind bile acids or structurally related species.

Finally, a behavioral study to follow-up our findings of altered striatal DA homeostasis in RYGB is likely warranted. As was reviewed in the introductory chapter, there is evidence that RYGB alters food and alcohol intake and reward; however, its effect on psychostimulants has not been tested. It should be noted that food and alcohol are ingested, caloric substances and may therefore be absorbed and processed differently following RYGB than a drug that is not taken by oral administration. Therefore we might expect a different result for a drug like cocaine. If RYGB does show effects on cocaine reward, this might open up the possibility of surgical interventions in humans as RYGB is already commonly performed on obese individuals. Of course, this would be a very extreme treatment for addiction, but the benefits might outweigh the risks for cases of intractable addiction. Moreover, a number of different hormonal systems are affected by RYGB,[252] and there are likely many changes that we are yet unaware of. Further study of the RYGB model and its effects on reward could therefore uncover novel mechanistic or therapeutic targets related to reward and addiction.

In conclusion, through our studies we have revealed, as proof of principle, that hormonal systems and gut-based interventions can have profound effects on brain DA homeostasis and behavioral responses to cocaine. As described above, these interventions may have therapeutic potential or inspire the discovery of novel therapeutic targets. In the face of an addiction crisis and a failing pharmaceutical pipeline, such inspiration is sorely needed.

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