Fast capillary electrophoresis for the study of dopamine in the central nervous system

by

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LIST OF ABBREVIATIONS

3MT	3-methoxytyramine
aCSF	artificial cerebral spinal fluid
AMPH	amphetamine
AMY	amygdale
ARC	arcuate nucleus
BSA	bovine serum albumin
CE	capillary electrophoresis
CE-LIF	capillary electrophoresis with laser induced fluorescence detection
cLC	capillary liquid chromatography
COC	cocaine
COMT	catechol-*O*-methyltransferase
CNS	central nervous system
D2	dopamine autoreceptor
DA	dopamine
DAT	dopamine transporter
DOPAC	3,4-dihydroxyphenylacetic acid
EIA	enzyme immunoassay

ERK1/2	extracellular signal-related kinases-1 and -2
ESI	electrospray ionization
fMRI	functional magnetic resonance imaging
FSCV	fast-scan cyclic voltammetry
GABA	γ-aminobutyric acid
HPβCD	hydroxypropyl-β-cyclodextrin
HPLC	high performance liquid chromatography
HVA	homovanillic acid
[^{3}H]WIN35428	[^{3}H]WIN 35,428 (2β-carbomethoxy-3β-(4-fluorophenyl)-tropane)
JNK	c-Jun NH_2-terminal kinase
LC-MS	liquid chromatography with mass spectrometry detection
L-DOPA	L-3,4-dihydroxyphenylalanine
LepRb	long form of the leptin receptor
LFPP	low-flow push-pull
LHA	lateral hypothalamus
LHSS	lateral hypothalamic self stimulation
LIF	laser-induced fluorescence
LOD	limit of detection
MALDI	matrix-assisted laser desorption ionization
MAO	monoamine oxidase
METH	methamphetamine
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy

MRM	multiple reaction monitoring
MS	mass spectrometry
NAc	nucleus accumbens
NDA	naphthalene-2,3-carboxaldehyde
NNF	no net flux
PBS	phosphate-buffered saline
PET	positron emission tomography
PFC	pre-frontal cortex
PI3K	phosphatidylinositol-3-kinase
PKC	protein kinase C
RIA	radioimmunoassay
rNNF	rapid NNF
RSD	relative standard deviation
SDS	sodium dodecylsulfate
SN	substantia nigra
SNc	substantia nigra pars compacta
SPECT	single photon emission computed tomography
TH	tyrosine hydroxylase
UPLC	ultra-high performance liquid chromatography
UV-VIS	ultraviolet-visible absorption
VTA	ventral tegmental area

GLOSSARY

afferent- bearing a signal from the periphery systems of the body to the central nervous system

amygdala- a set of nuclei implicated in memory and emotional processing

anorexigenic- inhibiting feeding

arcuate nucleus- a brain region implicated in regulating energy balance; receives inputs from periphery endocrine signals

central nervous system- brain and spinal cord

denervation- the act of severing the axon of a neuron

dopaminergic tone- the baseline state of the dopaminergic system which reflects a balance of all synthesis, release, uptake, and degradation processes

hippocampus- a brain structure associated with long term memory and spatial processing

hyperphagia- excessive feeding

locus- specific location in the brain

mesocorticalimbic dopaminergic system- dopaminergic neurons originating in the ventral tegmental area and projecting to cortical and limbic nuclei

neurotransmitter- a compound released from a neuron in a Ca^{2+}-dependent manner which carries information to other cells

nigrostriatal dopaminergic system- dopaminergic neurons originating in the substantia nigra and projecting to the striatum; normal nigrostriatal dopamine function is essential for motor control and habitually learned behaviors

nucleus accumbens- target of dopamine neurons originating in the ventral tegmental area; dopaminergic activity in the nucleus accumbens has been associated with the reward value of a stimulus; the nucleus accumbens integrates inputs from main sensory, motor, and emotional systems

orexigenic- promoting feeding

Parkinson's disease- a neurodegenerative disorder characterized by loss of nigrostriatal dopamine neurons leading to tremors, rigidity, and cognitive impairments

phasic dopamine release- exocytosis of dopamine in response to external or internal stimuli

pre-frontal cortex- portion of the frontal lobes of the brain that has been associated with impulse control and planning of complex cognitive functions

schizophrenia- a neurological disorder characterized by positive symptoms (hallucinations, delusions, and thought disorder) and negative symptoms (blunted emotions, anhedonia, speech deficits, lack of motivation, and lack of social behavior)

sensitization- an increase in the response to a stimulus, particularly drugs of abuse, following pre-exposure to the stimulus; sensitization to an abused drug is used as a laboratory animal model for the acquisition of drug dependence

stereotypy- repetitive or ritualistic behavior caused by some mental diseases and exposure to certain drugs of abuse

substantia nigra- brain region from which the nigrostriatal dopaminergic system projects

systemic- administering a drug treatment so that it circulates through the bloodstream and encounters all tissues

ventral tegmental area- brain region from which the mesocorticalimbic dopamine system projects

ABSTRACT

The study of neurochemical events in the central nervous system (CNS) with temporal resolution ranging from seconds to minutes facilitates the evaluation of intercellular communication on times scales that are relevant to behavior. This dissertation demonstrates the use of capillary electrophoresis (CE) with ≤ 100 s temporal resolution to explore how the anorexigenic, adipose-derived hormone leptin modulates the dopaminergic reward system and to develop a faster method for *in vivo* calibration of a microdialysis probe.

Previous results have shown that leptin promotes dopamine (DA) synthesis in genetic knock-out models of obesity. We show that 4 hr of systemic leptin treatment in normal animals enhances amphetamine-stimulated DA efflux and activates tyrosine hydroxylase and the DA transporter in the nucleus accumbens. Leptin did not alter the basal DA concentration. These data suggested that leptin causes a balanced activation of the mesolimbic DA system. This hypothesis was supported by the observation that leptin enhances cocaine-stimulated DA. That leptin enhances DA exocytosis and uptake while leaving basal extracellular DA unaffected may indicate that leptin preferentially promotes wired phasic DA transmission over volume transmission. Leptin may affect satiety by reducing the length of phasic DA exocytosis and therefore facilitating less reward of feeding.

Previously, quantitative microdialysis of neurotransmitters has required some combination of very long experiments, between-animals experimental design, or dilution of dialysate. Rapid no net flux (rNNF) was developed to complete an *in vivo* calibration of DA in 24-36 min in one animal with no dilution. NNF calibrations using only 6 min of data for each concentration of DA accurately reproduced calibrations generated from longer analysis times and permitted sequential, replicate, within-animal calibrations. Nomifensine caused a decrease in slope of the calibration which was comparable to the published effects of dopamine uptake inhibition during NNF. rNNF permits fast, quantitative measurement of DA and could be paired with other experimental treatments. These experiments show the utility of CE-LIF for neuroscience applications and method development.

CHAPTER 1

INTRODUCTION

Over the history of natural philosophy, humans have sought to understand the relationships between the conscious mind, its control of the body, and the physiology of the brain. This pursuit has led us from the belief that the mind is wholly divorced from the physiology of the brain to the understanding that consciousness and bodily control arise from the activity of the brain. Rather than suppose, as did phrenologists, that each type of thought or emotion is governed by strictly defined regions in the brain, we now know that emotions and observable behaviors depend on dispersed neural networks. Although certain loci have been correlated with specific types of thought, such as attention and impulse control with the pre-frontal cortex (PFC)[1, 2] and reward with the nucleus accumbens (NAc),[3, 4] these regions must perform these roles in concert with other centers of the brain. Disruption of a portion of a network can lead to physical or cognitive disability, for example, loss of substantia nigra (SN) dopamine (DA) neurons causes Parkinson's disease, while disruptions in serotonergic neuronal signaling systems underlie the development of depression. Therefore, modern neuroscience seeks to understand the operation of brain from intracellular to network levels, to improve our understanding of normal and diseased states, and to point to treatments. The brain is spatially heterogeneous and is composed of a complex milieu of cells, enzymes,

neurotransmitters, and afferent signaling molecules, whose concentrations can range from picomolar to micromolar and vary on timescales as short as milliseconds. Therefore, analytical chemistry has much to contribute to neuroscience through improvements in spatial and temporal resolution, selective detection of analytes within a matrix, and *in vivo* quantitation of compounds in the brain.

This dissertation demonstrates the use of capillary electrophoresis with laser induced fluorescence detection (CE-LIF), along with *in vitro* pharmacological assays and liquid chromatography with mass spectrometry detection (LC-MS), to characterize a portion of the reward pathways involved in the control of feeding behavior. Additionally, this dissertation uses CE-LIF with one minute temporal resolution to improve *in vivo* quantitation during microdialysis of DA, a neurotransmitter associated with reward systems. This introduction first addresses neurotransmission and relevant background information on the regulation of feeding behavior by the central nervous system (CNS). It then discusses the challenges of *in vivo* analysis and quantitation.

Neuroanatomy and Neurotransmission

Neurons, the primary information units of the CNS, encode and transmit information via their specialized structures and neurochemical content. Neurons have three primary structural components that are key to their ability to transfer information: the dendrites, the soma, and the axon (Figure 1.1A). The dendrites gather excitatory or inhibitory impulses from the inputs of other neurons, causing localized hyper- or de-polarization of the cell membrane. The sum of these impulses can diffuse to the soma which contains the nucleus and other organelles for basic cellular functions. Normally,

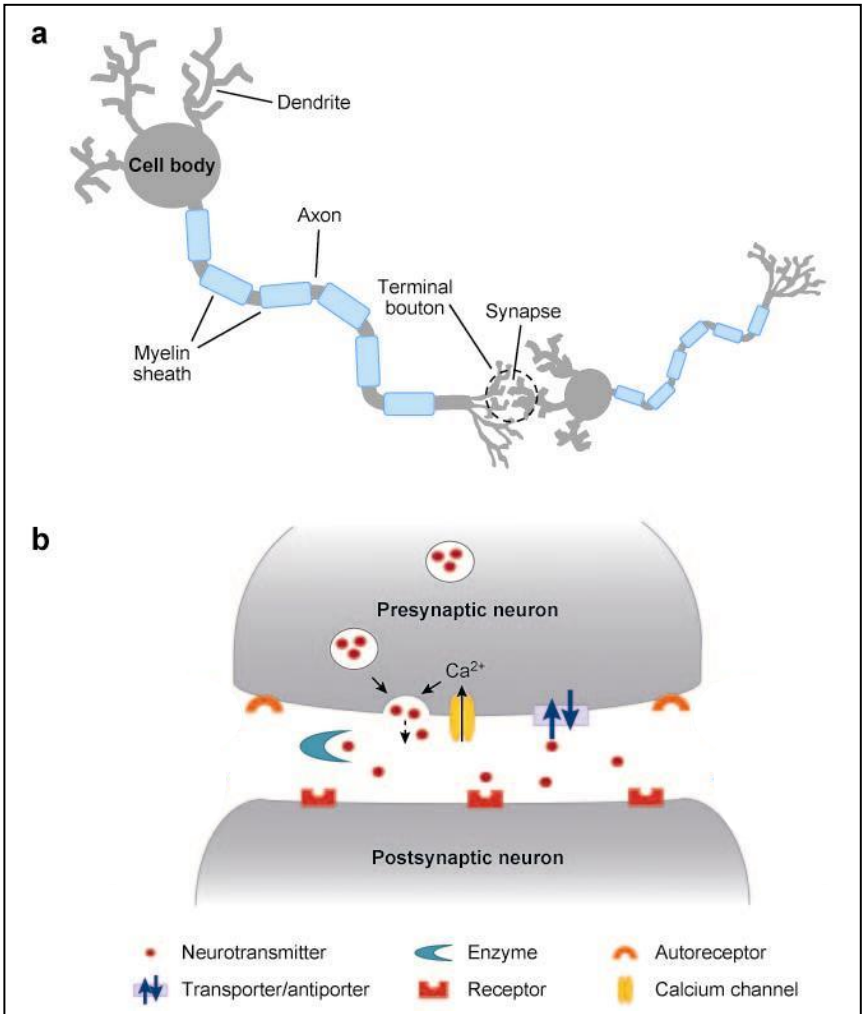

Figure 1.1: Neuroanatomy (A) Neurons consist of three primary parts: the dendrites, which collect impulses from other neurons; the soma, which contains the nucleus and organelles for metabolism; the axon, which carries an action potential to synapses with other neurons. (B) A synapse is the site of chemical communication between two neurons. An action potential causes release of neurotransmitter into the synapse. Neurotransmitter can interact with receptors on the post-synaptic cell to affect a change in that cell or with autoreceptors on the pre-synaptic cell to provide negative feedback for release. The signal from neurotransmitter is attenuated by enzymatic degradation or uptake into the pre-synaptic cell by a transporter. Neurotransmitter can also diffuse out of the synaptic cleft entirely. Figure adapted from Schultz, K.N.; Kennedy, R.T. *Annual Review of Analytical Chemistry* **2008**, *1*, 627-661.

the membrane of the neuron is maintained at a resting potential of -70 mV by the sodium-potassium pump, which pumps three Na^+ out of the cell for every two K^+ it pumps in. If the sum of the inputs collected by the dendrites causes the membrane potential at a specialized portion of the soma, the axon hillock, to reach a threshold potential (usually around -55 mV), an action potential is initiated. Voltage-gated Na^+ channels open, causing Na^+ to flow into the neuron and raising the membrane potential to a maximum of +40 mV. As the membrane potential rises, it causes voltage-gated K^+ channels to open,

which allow K^+ to flow out of the cell and reduce the membrane potential. The reduced potential closes the voltage-gated Na^+ channels, bringing the membrane back to its resting potential. This combination of Na^+ influx followed by K^+ efflux propagates down the axon to the terminal. At the terminal, depolarization of the membrane causes voltage-gated Ca^{2+} channels to open. Ca^{2+} is at a greater concentration outside the cell, so it flows into the terminal. The influx of Ca^{2+} causes vesicles loaded with neurotransmitter to migrate to the membrane for exocytosis. These vesicles fuse with the membrane, releasing neurotransmitter into the synapse.

A synapse is a site of chemical communication between two neurons (Figure 1.1B). Classically, synapses are formed on the dendrites of the post-synaptic cell; although, synapses may also form on the soma or axon. The synaptic cleft, the distance between the two neurons, is 20-40 nm.[5] Once released, neurotransmitters can diffuse across the cleft to interact with receptors on the post-synaptic cell. These receptors can be ionotropic, whereon the binding of a ligand causes an ion channel in the receptor to open or close, or metabotropic, whereon the binding of the neurotransmitter initiates a signaling cascade within the post-synaptic cell leading to changes in receptor configuration or protein expression. Neurotransmitters can also interact with autoreceptors, receptors on the pre-synaptic cell, which provide negative feedback for exocytosis. Neurotransmitters can be removed from the cleft by enzymatic degradation, by a transporter which moves the neurotransmitter back into the pre-synaptic cell, or by diffusion out of the cleft into the extracellular space. Molecules which diffuse out of the cleft can act on other neurons or glial cells in the area by means of volume transmission.

Neurotransmitters are a diverse set of molecules, from soluble gases such as nitric oxide, to small molecules such as amino acids, to peptides such as enkephalins. They are classified as excitatory, inhibitory, or neuromodulating. Glutamate and aspartate are the major excitatory neurotransmitters; γ-aminobutyric acid and glycine are the major inhibitory neurotransmitters. Most other neurotransmitters, including DA, are considered neuromodulatory because they can cause excitation or inhibition depending on the circuit in question.

Dopaminergic Pathways and the Role of DA

Dopaminergic signaling is of interest in many fields of neuroscience due to its role as mediator of survival-required behaviors and the presence of dopaminergic projections in a variety of brain regions. Traditionally, neuroscientists have divided dopaminergic neurons in the CNS into three major pathways: the tuberoinfundibular system, the mesocorticolimbic system and the nigrostriatal system. Although the distinct pathways are an over-simplification—neuronal

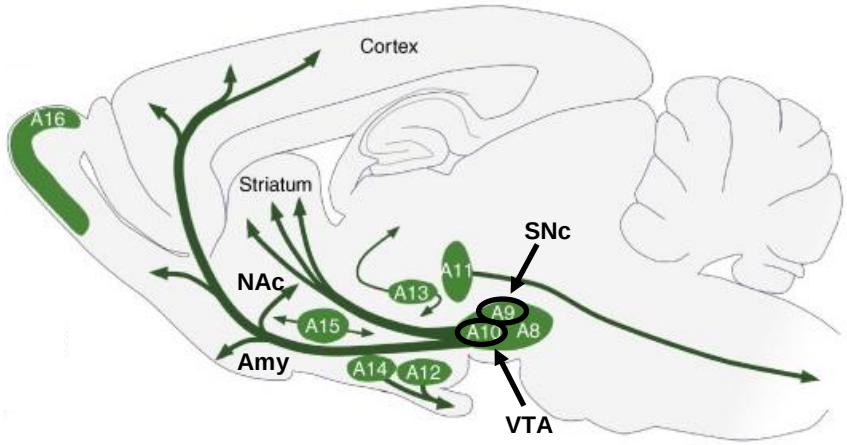

Figure 1.2: The mesocorticolimbic and nigrostriatal pathways are the two major dopaminergic projections in the CNS. The mesocorticolimbic dopaminergic neurons originate in the VTA (region A10) and project to cortical nuclei, such as the NAc and Amy, and the cortex. The nigrostriatal pathway originates in the SNc (region A9) and projects to the striatum. Figure adapted from Björklund, A.; Dunnett, S.B. *Trends in Neurosciences* **2007**, *30*, 194-202.

projections originating in regions traditionally associated with one or the other system project to a variety of brain structures[6]—for the purposes of this dissertation, the idea of distinct pathways provides a convenient starting point to discuss the roles of DA in the CNS. Mesocorticolimbic dopaminergic neurons originate in the ventral tegmental area (VTA) and project to the cortical or limbic nuclei, including the medial PFC, NAc, amygdala (Amy), and hippocampus (Figure 1.2). The nigrostriatal neurons project from the SN, especially the SN pars compacta (SNc), to the striatum (Figure 1.2). Both the NAc and striatum receive substantial inputs from other parts of the brain and are sites of integration for the sensory and cognitive states of an organism.[7] Dopaminergic tone and phasic release in these regions play key roles in modulating downstream activity and controlling behavioral outcomes.[7]

The mesocorticolimbic system mediates motivation, attention to salient stimuli, and working memory.[8, 9] Dysregulation of the mesocorticolimbic system is commonly associated with wanting of drugs and relapse to drug taking[10] and the positive and negative symptoms of schizophrenia.[11] Ambulatory sensitization to cocaine (COC, a DA transporter (DAT) blocker which inhibits DA uptake) and amphetamine (AMPH, a DAT substrate which induces reverse transport of DA through DAT) is mediated through the VTA to NAc projections.[12] The nigrostriatal system is crucial for normal locomotion and activity. Loss of nigrostriatal dopaminergic neurons causes Parkinson's disease and mice lacking DA in the nigrostriatal pathway do not eat, even when no effort is required to obtain the food.[8, 13] The stereotypy which develops with repeated exposure to COC or AMPH is mediated by the dopaminergic projections in the striatum.[12]

Both of the dopaminergic pathways are crucial for learning of new behaviors and behavioral initiation. Learning is facilitated because DA acts as a mediator of reward. In the NAc, DA has been postulated to encode the incentive salience of the reward or prediction error of a reward.[10, 14] Incentive salience is the value of a reward within the context of the organism's biological state;[10] for instance, drinking water is more rewarding when a subject has been deprived of water than when it has had free access to water. Prediction error indicates whether a particular reward was more or less valuable than expected.[14] The availability of reward (encoded by phasic DA release) facilitates operant learning (NAc) and habitual learning (striatum).[15] An increase in phasic DA release is correlated to the learning of voluntary behaviors (NAc) or rote behaviors, which once learned, require no conscious involvement (striatum).[15, 16] The mesocorticolimbic system is most associated with the modulation of motivation states while the nigrostriatal system is most known for mediating habitual behaviors.

Regulation of Feeding Behavior in the CNS

The prevalence of obesity in the American population has more than doubled since 1980 so that two thirds of American adults are categorized as overweight or obese based on their body-mass index.[17] Obese and overweight phenotypes increase the risk for the development of diabetes, heart disease, and hypertension among other conditions.[18] Therefore, research has focused on understanding the mechanisms by which body weight is normally regulated and how dysregulation leads to obesity. An individual's weight is determined by his homeostatic mechanism, which balances energy expended with energy input through food to maintain a set point of energy reserves (adipose tissue).[13]

However, this set point drifts higher when the organism has access to very palatable and calorically dense foods leading to weight gain, unless there is a concomitant increase in energy expenditure.[13] Therefore, the high prevalence of calorically dense food and more sedentary lifestyles have promoted the incidence of weight gain and obesity. While lifestyle modifications, such as diet and exercise, can reduce weight, such weight loss is rarely sustained over the long term. The need for more effective treatments for overweight and obese phenotypes necessitates improved understanding of the mechanisms by which food intake and energy homeostasis are regulated.

Under normal conditions, the CNS receives information about the body's energy reserves through a number of afferent signals released by peripheral organs. These signals include leptin, secreted by adipose (fat) cells, ghrelin, secreted by the gut, and insulin, secreted by the pancreas. Leptin and insulin are anorexigenic, meaning that they inhibit feeding, and ghrelin is orexigenic, meaning that it promotes feeding. While the remainder of this discussion will focus on leptin, all three peptides contribute to the regulation of feeding behavior in the CNS.[19]

Leptin Regulation of Feeding Behavior in the CNS

Leptin has stimulated great interest since the time of its discovery in the mid-1990s due to its ability to reverse the obese phenotype of the *ob/ob* mouse.[20] *ob/ob* mice are a naturally arising genetic knock-out strain with a mutation of the *obese* gene that leads to production of non-functional leptin.[21] The leptin deficiency in *ob/ob* mice causes hyperphagia, obesity, and health complications commonly associated with obesity. However, treatment with leptin significantly decreases the food intake and weight of

ob/ob mice and restores a physiologically normal phenotype.[20] Initially, it was thought that human obesity could be easily treated through the administration of leptin; however, relatively few humans are obese due to the production of non-functional leptin.[22] In most humans, the amount of leptin circulating in the bloodstream is proportional to the total fat mass, so obese individuals have more circulating leptin than normal weight individuals.[23] However, despite the high leptin levels, obese individuals are leptin-resistant, and lack appropriate leptin-mediated signaling and physiological control.[24]

Leptin contributes to the regulation of feeding and body weight through the control of energy balance and the modulation of food as reward. Leptin acts in the CNS via the long form of the leptin receptor (LepRb), which is expressed in a variety of brain regions including hypothalamic, midbrain, and brain stem nuclei.[25-29] The arcuate nucleus (ARC) in the medial basal hypothalamus contains sub-populations of neurons, some of which express LepRb and which act in opposition to each other to maintain energy homeostasis.[30, 31] The role of the ARC in maintaining energy homeostasis has been extensively researched,[32-35] but a majority of LepRb-expressing neurons, and leptin-mediated regulatory action, are found in other brain regions.[30] Both the mesocorticolimbic and nigrostriatal DA pathways receive inputs from LepRb-expressing neurons (Figure 1.3).[27, 28, 36, 37] LepRb-expressing neurons in the lateral hypothalamus (LHA) project to the VTA and modulate dopaminergic neurons projecting to the NAc.[36] LepRb are also expressed on dopaminergic and non-dopaminergic neurons in the VTA, which project primarily to the extended Amy.[28, 37] Most of the LepRb neurons of the VTA and SN are co-localized with tyrosine hydroxylase (TH; the enzyme which mediates the rate-limiting step in DA synthesis).[38] The expression of LepRb in

dopaminergic neurons (VTA and SN) and in neurons that project to the VTA (LHA) hint at leptin's role in modulating the dopaminergic reward system.

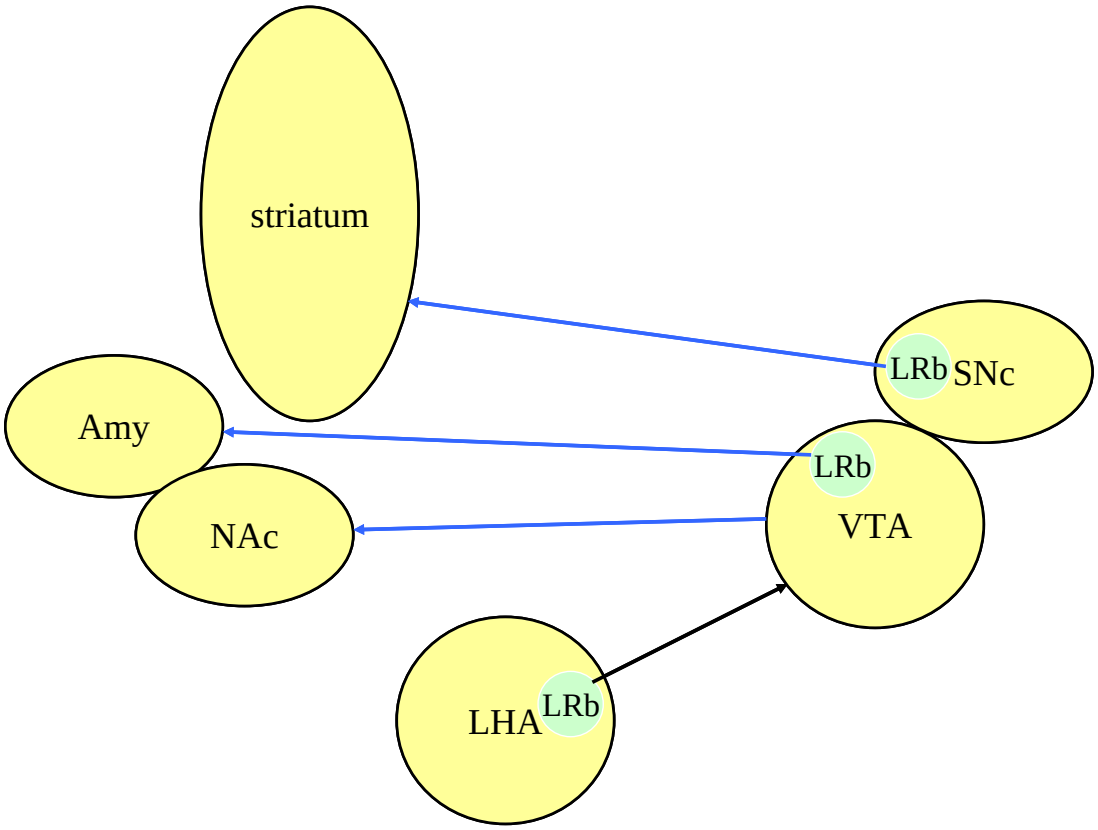

Figure 1.3: Diagram of leptin receptors which modulate dopaminergic neurons. Leptin receptor expressing neurons are represented by green circles. The black arrow indicates projections with an unknown neurotransmitter, and the blue arrows indicate dopaminergic projections. Leptin receptor expressing neurons in the LHA project to the VTA and modulate the projection to the NAc. Leptin receptors co-localize with TH in the VTA and SNc and these neurons project to the Amy and striatum respectively. This figure represents data from references Fulton 2006,[27] Hommel 2006,[28] Leinninger 2009,[36] and Leshan 2010.[37]

The modulation of both the mesocorticolimbic and nigrostriatal pathways by leptin has been demonstrated through behavioral and neurochemical measurements. Leptin directly affects neuronal activities. Systemic leptin infusion depolarizes DA neurons in the VTA.[28] *ob/ob* mice exhibit a deficiency in the expression of TH in the VTA and NAc.[27, 36] This deficiency is reversed by systemic or intra-LHA chronic leptin treatment.[27, 36] Additionally, intra-LHA treatment restores the amount of DA in the NAc of *ob/ob* mice from a deficient to normal level.[36] Mouse models which lack both leptin

and DA fail to exhibit the hyperphagia characteristic of *ob/ob* mice, indicating that this behavior is dependent on some DA being present in the CNS.[39] Consistent with reduced DA in the NAc, *ob/ob* mice fail to exhibit behavioral sensitization to AMPH, but this is reversed by chronic leptin treatment.[27]

DA signaling is required for animals to engage in feeding behavior. Mice that lack DA, referred to as DD mice, lack the motivation to feed and have reduced locomotor activity.[8] DD mice will eat if food is placed in their mouths but will not engage in food-seeking behaviors; in fact, DD mice die of starvation if not forcibly fed.[8, 13] Treatment with L-3,4-dihydroxyphenylalanine (the precursor to DA in catecholamine synthesis) restores normal feeding behavior for a period of 10 hours.[8] Viral restoration of DA synthesis to the nigrostriatal dopaminergic pathway of DD mice allows normal feeding behavior, except for re-feeding following fasting.[8, 13] DD mice with DA function restored in the striatum show a willingness to work for food equal to wild type animals;[40] however, one author later acknowledges the possibility that the virus could have restored DA function to both the striatum and NAc.[8] DD mice which received viral restoration of DA synthesis to the NAc alone do not resume normal feeding; although, without DA function in the NAc, food consumption after fasting is reduced.[8, 13] Thus, these data collectively indicate that DA signaling is essential for normal feeding behavior.

Leptin and food restriction (which depletes circulating leptin as well as causing other physiological changes) can alter the function of reward circuits. Lateral hypothalamic self-stimulation (LHSS) is an experimental framework for testing the relative value of rewards. Animals perform a specific response to receive a stimulus impulse from an electrode implanted in the LHA and an experimental treatment can

either increase or decrease their willingness to work for the stimulation. If an animal completes more responses per stimulus impulse, the experimental treatment must have created a reward deficit and increased the value of the stimulus impulse as a reward. Food restriction increases the value of LHSS at certain stimulation sites,[41] indicating that the food restriction creates a reward deficit. Leptin decreases the value of LHSS at the same sites that are sensitive to food restriction,[41] demonstrating ability of leptin to modulate the reward system.

Modulation of reward systems by leptin suggests an interesting overlap between feeding behavior and drugs of abuse, whose effect on the extracellular concentration of DA in the NAc has been extensively studied.[42] In addition to the previously discussed experiments showing that leptin is needed for sensitization to AMPH,[27] food restriction can accelerate self-administration of drugs of abuse by laboratory animals by amplifying the value of those drugs as rewards.[43] These interactions are probably due to the effects of leptin and other metabolic hormones acting on the same mesocorticolimbic pathways as drugs of abuse. In humans, commonality of these pathways causes obese individuals to be 25% less likely to develop a drug abuse disorder.[44] Overall, several lines of evidence show that leptin acts at a number of sites in the CNS, including dopaminergic pathways, causing the modulation of the value of food and other rewards. However, much remains to be learned about the precise cellular mechanisms by which leptin regulates the value of rewards.

Much of the previous research on the role of leptin has been completed with *in vitro* experiments or behavioral experiments with no neurochemical data. Each of these experimental designs has contributed to our understanding of the interaction between

LepRb and dopaminergic neurons. *In vitro* experiments demonstrated the distribution of LepRb neurons in the CNS and the dopaminergic circuits activated by leptin.[27, 28, 36, 37] Behavioral experiments pointed to the commonality of leptin- and drug-activated dopaminergic neurons[27] and the effect of brain regions involved in the mesocorticolimbic DA system on feeding behavior.[28] However, these experiments do not demonstrate how leptin might alter dopaminergic signaling or sub-cellular processes in dopaminergic neurons. Additionally, *in vitro* experiments can only provide data from a single time point in each animal. *In vivo*, freely-moving microdialysis experiments have the capacity to probe inter- and intracellular dopaminergic processes while permitting normal inputs from peripheral systems and maintaining control of behavior by the CNS. Therefore, *in vivo* experiments could demonstrate that leptin contributes to the regulation of the value of food as a reward by modulating the mesocorticolimbic DA system and probe which aspects of dopaminergic signal are regulated by leptin. *In vivo* experiments also permit longitudinal observation to determine whether certain effects of leptin develop overtime. In this dissertation, the capacity of leptin to activate the mesocorticolimbic DA system has been probed using both *in vivo* microdialysis experiments and *in vitro* assays. The benefits and restrictions of each approach are discussed in the next section.

Methods for studying the CNS

A variety of *in vitro* and *in vivo* techniques have been used to study the function and activity of the CNS. These techniques span from the greatest simplification of neural activity to studies of intact and behaving animals. *In vitro* techniques, which include tissue extracts, synaptosomes, cultured cells, and brain slices, isolate the neurons and

circuits of interest. *In vivo* techniques, which include imaging, microsensors, and sampling, allow an investigator to probe a region or circuit of interest in the context of periphery inputs and with correlation to behavior. Both *in vitro* and *in vivo* studies were used in the experiments described in this dissertation.

Tissue extracts and synaptosomes allow the study of sub-cellular aspects of the CNS. To prepare both tissue extracts and synaptosomes, a brain region of interest is quickly dissected from a euthanized animal. Tissue extracts analyze the quantity of a specific analyte, such as DA, present at the time of death. Following homogenization, the analyte is subjected to an appropriate analysis technique. The choice of analysis technique varies depending on the analyte and available equipment. For example, in chapter 4 of this work, the total amount of DA in a tissue extract was measured by high performance LC (HPLC) with electrochemical detection (EC).

Synaptosomes are nerve terminals which have been sheared off from their axons during homogenization and centrifugation. The cellular membranes seal back together at the break-point to form a vesicle containing all the cellular machinery necessary for a functioning terminal. Often, sealed vesicles of post-synaptic cells remain attached to the surface of the terminal. Therefore, synaptosomes are an ideal preparation for studying exocytotic mechanisms and receptor expression in synapses without the complicating influence of naturally arising action potentials. Synaptosomes were used for several studies in this dissertation. For reviews on the use of synaptosomes, see Nicholls[45] or Whittaker.[46]

Cell cultures allow for the study of the activity of a whole cell. Neurons are isolated from the sacrificed animal and maintained in physiological media. Experiments

with cell cultures can demonstrate how activation of a specific receptor affects intra-cellular signaling pathways or action potential frequency.

Brain slices scale up the complexity of cell cultures, permitting the study of a complete circuit. After euthanasia, the brain is cut into 0.4 mm thick slices. The slices are perfused with a medium which maintains an appropriate ionic environment and temperature, provides oxygen and glucose to the tissue, and removes waste products. Brain slices can provide excellent information on circuit function without inputs from other systems, but like all *in vitro* techniques, data obtained from brain slices must be considered in the context of how metabolism is altered in tissue removed from the body. In the case of brain slices, the healthy tissue is sandwiched between two damaged layers and activity of the cells is decreased by denervation and deficiencies in oxygen delivery and waste removal.[47]

Imaging techniques include positron emission tomography (PET), magnetic resonance imaging (MRI), and their variants. PET is used to study receptor distribution, neurotransmitter release, and the pharmacokinetics of drugs. Subjects receive an infusion of tracer agents which have been labeled with positron emitters such as ^{11}C and ^{18}F.[48] Following a decay event, the positron annihilates with an electron causing the emission of two gamma ray photons which travel in opposite directions. The detection of these photons indicates the position of the tracer agent in the CNS with a spatial resolution of 1.5-5 mm and a temporal resolution of about 30 s.[49] PET is often used to study catecholamine activity or glucose distribution in human subjects but radiation exposure limits the number of scans that a single subject can experience.[50] A variant of PET is single photon emission computed tomography in which radiation is observed in three

dimensions and is used to construct a three dimensional representation of tracer distribution.

MRI is used to study the structure of the brain. The stationary subject is placed within a magnetic field which excites the dipoles of atoms in the brain. The energy released when the dipoles relax to their original conformation is then measured, most commonly by monitoring hydrogen atoms. Using clinically available 1.5 T systems, spatial resolution of 1 mm^3 can be achieved in 5-15 minutes.[49] Variations in water content allow the tissue types of the brain to be distinguished.[49] MRI can be used to study variations in brain structure over time or between individuals. Functional MRI (fMRI) is a variant of MRI which measures changes in blood flow by observing changing ratios of oxygenated and deoxygenated blood. fMRI correlates increased blood flow with increased tissue activity.[49] It can achieve spatial resolutions of 2-3 mm in 1-4 s.[49] Magnetic resonance spectroscopy (MRS) is another variant of MRI which obtains structural and chemical information in a brain region of interest. MRS distinguishes metabolites and other compounds present in the CNS by observing the spectrum of energy that is absorbed and released. The chemical environment surrounding a dipole alters the frequency absorbed in a way that is unique to that compound. The dipoles of hydrogen and phosphorus are most commonly used for MRS.[51] It can achieve a spatial resolution of 1-2 mL in 5-8 minutes.[49]

PET, MRI, and their variants are commonly used for human studies because they do not require surgery. Although imaging requires a subject to remain stationary, humans can be instructed to visualize a scenario or emotion to probe how a brain region responds to stimuli. If imaging techniques are used in animal studies, animal must be

chemically or physically immobilized to obtain clear images. Imaging techniques are not well suited to rodent studies due to insufficient spatial resolution for the smaller brains of rodents and the inability to correlate an animal's behavior with its brain activity.

Microsensors include microelectrodes and enzyme-coated biosensors. Microelectrodes detect electroactive compounds through a variety of electrochemical methods, including amperometry and voltammetry. Voltammetry can be especially useful for the simultaneous detection of monoamines. An electroactive analyte produces a unique set of oxidation and reduction peaks at given potentials, and a number of techniques can be used to resolve the peaks of several analytes using either surface modifications or statistical methods. Although some neurotransmitters, such as catecholamines and acetylcholine, are electroactive, most are not electroactive. Some neurotransmitters, such as glutamate, can be detected electrochemically using enzyme-coated biosensors. Enzyme-coated biosensors are prepared by immobilizing an enzyme on the surface of a microelectrode. The enzyme reacts with the analyte to produce an electroactive product, such as hydrogen peroxide, which is then detected by the electrode. Microelectrodes and microsensors offer good spatial resolution (1-30 μm in diameter); the temporal resolution of an electrode could be between 100 ms and 1 minute depending on the method chosen.[52, 53] With any *in vivo* electrochemical method, resolution of analyte signal from that of other electroactive compounds is essential. The most common electroactive interferent is ascorbic acid, whose concentration in the extracellular fluid (~200 μM) is much greater than the concentration of neurotransmitter analytes. Detection of interfering species often is suppressed with a surface coating that reacts with or excludes the unwanted molecules.

Microsensors have been shown to detect neuronally-derived reserves of certain neurotransmitters (rather than neurotransmitters stored in glial cells)[54, 55] whereas the ability of sampling methods to detect neuronally derived neurotransmitters is controversial.[56-59] However, microsensors cannot detect basal extracellular concentrations of neurotransmitters due to an inability to be calibrated *in vivo*, as can be done with sampling techniques.[60, 61] Electrochemical detection techniques may experience background drift due to *in vivo* fouling of the electrode or require long periods for sample to accumulate on the sensor, both of which limit the length and frequency of their monitoring periods. Additionally, relatively few biosensor or microelectrode methods offer the ability to detect more than one analyte at a time.

Sampling techniques include microdialysis, direct sampling, and low-flow push-pull (LFPP) perfusion (Figure 1.4). Of these microdialysis is the most commonly used and will be discussed in detail in the next section. All sampling techniques extract a sample of the compounds that exist in the extracellular space which is analyzed by any number of methods. In direct sampling (Figure 1.4.A), a piece of open-tubular fused silica capillary is implanted into the brain region of interest and a vacuum (100-550 mmHg) is applied to withdraw the extracellular fluid at 1-50 nL/min.[62] The capillary serves as a storage vessel for the sample, which can be pumped out for analysis.[62] LFPP (Figure 1.4.B) is an adaptation of a technique that preceded microdialysis. Originally, a push-pull sampling probe consisted of an inlet and outlet tube through which perfusion solution flowed at 1-150 μL/min.[63, 64] However, this caused severe tissue damage. Lower flow rates ranging from 10-50 nL/min do not generate voids or lesions in the brain during LFPP.[64] Both direct sampling and LFPP sample from the very tip of the probe

which allows sampling from very small brain regions. However, both methods also suffer from a high probability of clogging, and the low flow rates present significant challenges for sample handling.

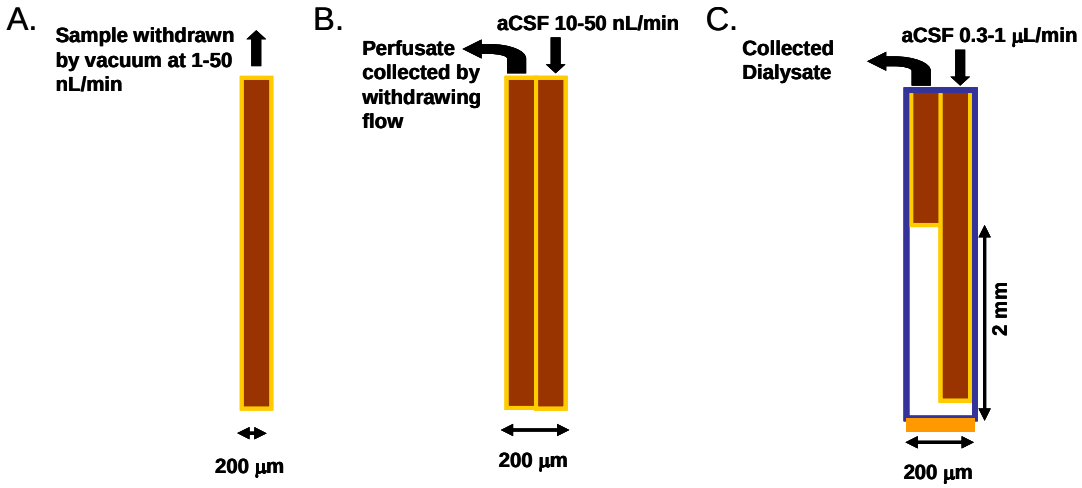

Figure 1.4: Methods for sampling from the brain. (A) Direct sampling. A capillary is inserted in to the region of interest and vacuum is applied to withdraw a sample of the extracellular fluid. (B) Side-by-side design for push-pull probe. Infusion flow is balanced by withdrawal flow from vacuum or peristalsis. (C) Side-by-side design for microdialysis. A semi-permeable membrane isolates the fluid perfusing the probe from the surrounding tissue.

Microdialysis

Microdialysis is a sampling technique in which the perfusion solution is isolated from the tissue of interest by a semi-permeable membrane (Figure 1.4.C). Small molecules can diffuse down their concentration gradients, across the membrane, and into the probe, but proteins and tissue are excluded. The membrane prevents clogging, the formation of lesions by the application of vacuum, and the infusion of bacterial contaminants. The membrane reduces the relative recovery of analytes (defined as the percentage of the analyte concentration outside the probe which is detected in the dialysate). However, the molecular-weight cut-off qualities of a membrane can provide some clean-up and preservation of the sample (as compared to direct sampling or push-pull) by excluding large molecular weight compounds, including degradation enzymes.

Microdialysis is widely used for the *in vivo* study of pharmacokinetics, disease states, and signaling pathways, with over 8,000 published neuroscience articles using the technique and nearly 17,000 articles overall.

Microdialysis was originally performed by threading a length of membrane through the skull.[65] Probes soon evolved so that the inlet and outlet entered the skull at the same point, simplifying surgical procedures.[66, 67] Currently, probes may have either concentric or side-by-side geometries. Concentric probes have more symmetrical fluid dynamics and are commercially available. However, side-by-side probes are more easily constructed in-house. Construction of probes by the experimenter offers greater flexibility in coupling the probe to an analytical method and generates probes with minimal dead volume. Therefore, side-by-side probes, constructed in-house, were used for this work.

Like all sampling methods, microdialysis can be coupled to a variety of analytical techniques. Enzyme assays, enzyme immunoassays (EIA), and radio immunoassays (RIA) are commonly used for single-analyte detection in dialysate. Enzyme assays offer simple, rapid detection and can be amenable to method development.[68] EIA and RIA are commonly used for the detection of peptide neurotransmitters because they provide the high sensitivity needed to detect analytes at 1-100 nM in the brain and in the picomolar range in dialysate.[69] However, single-analyte detection measures only one of many neurotransmitters in a dialysate sample.

Analysis by a separatory method offers the potential to detect multiple neurotransmitters at one time and to improve understanding of the interplay between neurotransmitters. HPLC is the most commonly used analytical technique due to the

wide availability of reliable commercial instruments and methods. It can be paired with detection by EC, ultraviolet-visible absorption (UV-VIS), fluorescence, or MS methods depending on the analyte of interest. The volume requirements of standard commercial columns and injection loops necessitate the collection of 10-20 μL fractions.

To reduce fraction volume and improve temporal resolution, capillary LC (cLC) and CE have been used to separate the components of the dialysate. EC detection can be coupled to either technique. However, when EC detection is used with CE, it is necessary to isolate the electric fields used for separation and detection with home-built instrumentation.[70, 71] Although UV-VIS detection can be coupled to cLC or CE, it provides poor sensitivity and limits of detection (LODs) because UV-VIS detection is pathlength dependent and the narrow diameters of capillary columns (5-100 μm in diameter) limit the number of photons available for absorption. Laser-induced fluorescence (LIF) is often used for detection on capillary columns because it is pathlength independent, giving good sensitivity and lower LODs than UV-VIS. Since most neurotransmitters are not natively fluorescent, fluorescent or fluorogenic tags are attached to the analytes, potentially degrading the quality of the separation. If pre-column derivatization is performed, the tag can mask structural differences between analytes; therefore, their differential migrations are more similar and analytes are more difficult to resolve. Post-column derivatization introduces band broadening from mixing and diffusion. Mass spectrometry (MS), with either electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI), can be used for detection following either CE or cLC. CE-MS has not gained widespread use for analysis of neurotransmitters due to the challenges of isolating the electric fields for the separation

and ionization steps and of desalting prior to ionization.[72, 73] cLC-MS has been used for the analysis of both small molecule and peptide neurotransmitters.[69] MS detection can provide excellent LODs, sufficient for the detection of most neurotransmitters, and can provide extra separatory power if coupled to a separation with incomplete resolution.

The analytical method coupled to microdialysis often dictates the temporal resolution of observation. For instance, the injection loops, column volume, and sensitivity of conventional HPLC systems frequently necessitate injection volumes of 10-20 μL and 10-20 minute fractions at a common flow rate of 1 μL/min. Such a sampling frequency is appropriate for the observation of alterations in activity occurring over the course of several hours, such as changes caused by alterations in protein expression or stimulation by some drugs. To observe a fast change, a higher sampling frequency is needed. Exocytosis occurs on the millisecond time scale and can only be observed by fast-scan cyclic voltammetry (FSCV). However, behavioral activation can be correlated to changes in neurotransmitter concentration occurring over time frames of seconds to

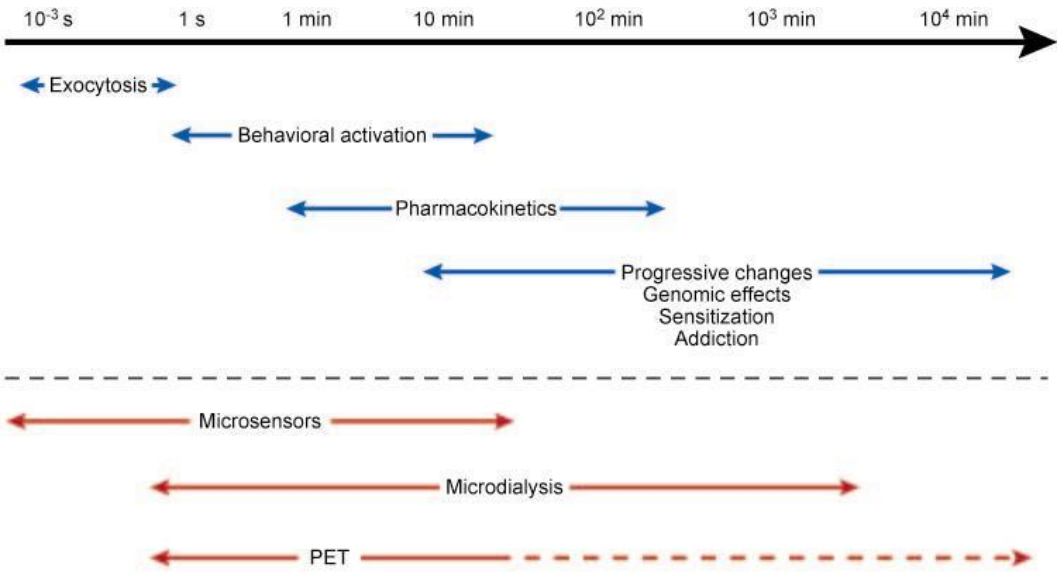

Figure 1.5: Comparison of time scale of events in the CNS with the temporal resolution achievable by in vivo monitoring methods. Figure from Schultz, K.N.; Kennedy, R.T. *Annual Review of Analytical Chemistry* **2008**, *1*, 627-661.

hours (Figure 1.5).[74] Fractions have been collected at a 20 s frequency in vials for analysis using a commercial CE instrument[75] or at a 2 s frequency as plugs segmented by an immiscible oil phase for analysis by CE-LIF.[76]

Fraction collection at high frequencies can generate an overwhelming number of samples leading to extended analysis time and presents challenges for reproducible fraction collection, prevention of evaporation, and preservation of samples. For these reasons, some researchers have chosen to perform on-line analysis, sampling from the constant flow of dialysate at regular intervals.[77, 78] On-line analysis offers regular feedback on the progress of an on-going experiment; however, sampling frequency is limited by the speed of the analysis. The choice of on-line versus off-line analysis depends on the speed of the biological change being observed and the parameters of the analytical system. Both on-line and off-line methods have been used in this work.

As mentioned earlier, microdialysis can measure basal concentrations of neurotransmitters. Several methods of quantitation have been used to account for the effects of the probe and the tissue on recovery, though these methods are controversial.[56-59] Two methods, extrapolation to zero flow rate and no net flux (NNF), perform an *in vivo* calibration which accounts for the matrix effects of the probe and tissue. Alternatively, the slow flow rate method uses flow rates around 50 nL/min to achieve relative recoveries approaching 100%. With near-100% recovery, the dialysate concentration equals the extracellular concentration. All three of these methods require very lengthy experiments to measure the extracellular concentration and cannot be applied to stimulated conditions. An external calibration with correction for the *in vitro* recovery of the probe is sometimes used for quantitation of non-steady-state systems.

However, correction for the *in vitro* recovery can only account for the effect of the probe but not the tissue. Therefore, use of an external calibration cannot accurately report the extracellular concentration of an analyte. Transient NNF,[79] slow flow monitored by CE,[80, 81] and slow flow with make-up flow[82, 83] modify *in vivo* quantitation methods so that they can be applied to changing systems. All of these methods will be discussed further in Chapter 4.

Quantitation with microdialysis is controversial because the acute damage of probe implantation yields data which contradict information generated by microelectrodes. NNF quantitation indicates that the extracellular concentration of DA in DA-rich areas of the brain is in the low nanomolar range;[84] however, infusion of a glutamate antagonist caused a voltammetric microelectrode to measure a decrease in extracellular DA of 485 nM.[85] The difference between the microdialysis and microelectrode data has been attributed to a trauma layer around a microdialysis probe characterized by impaired DA release and uptake.[84, 86] This trauma layer yields a reduced slope for *in vivo* NNF following an acute implant as compared to *in vitro* NNF;[84] however, this difference between *in vivo* and *in vitro* NNF was not observed if the probe was implanted the day prior to experimentation.[87] Therefore, impaired DA release and uptake may be characteristic of tissue following an acute implant but not after an overnight implant which allows the tissue to recover to some degree prior to experimentation.

Specific Aims of Dissertation

This work demonstrates the routine application of CE with high temporal

resolution (<90s) for the microdialysis study of DA in the central nervous system, specifically, that leptin activates the mesocorticolimbic DA system to regulate food as reward. Other methods, such as microdialysis coupled to microbore LC-MS and *in vitro* pharmacological assays, have also been used to characterize how leptin acutely modulates the mesolimbic DA system. Additionally, this dissertation presents a method for rapid *in vivo* quantitation of DA during microdialysis which expands the range of conditions under which *in vivo* quantitation can be performed.

Characterization of the acute modulation of DA reward by leptin

Previous work on the effects of leptin has primarily been performed with chronic treatments of *ob/ob* mice and other genetic knock-out models. It is unclear to what extent the effects of leptin on the mesolimbic DA system reflect the normal actions of leptin or the chronic leptin deficiency of the model. This work explored the acute effects of leptin in normal animals and the common neuronal pathways acted on by leptin and drugs of abuse. Acute, systemic leptin treatment of Sprague-Dawley rats caused a significant amplification of the magnitude of AMPH-stimulated DA efflux. *In vitro* studies indicated that this amplification could be attributed to leptin-mediated increases in the activity of TH and DAT. However, leptin did not alter the basal concentration of DA measured *in vivo* or DA metabolism measured *in vitro*. These data suggested that leptin caused a balanced activation of the DA system which did not alter basal concentrations but primes the system for enhanced response when stimulated.

Subsequent studies further probed the relative effects of leptin on DA release and uptake. Leptin did not alter DA metabolism measured *in vivo.* Leptin enhanced COC-stimulated DA, suggesting leptin increases DA exocytosis. Such an increase in

exocytosis would need to be balanced by an increase in DA uptake in order for the basal concentration of DA to remain stable. Microinjection of DA into the NAc provided further evidence that leptin acutely modulates DAT activity in vivo, though further study is required to determine the exact parameters of DAT function altered in the microinjection experiment. These data supported the hypothesis that leptin causes a balanced activation of the mesolimbic DA system which contributes to setting the overall dopaminergic tone of an animal.

Rapid NNF for on-line calibration of microdialysis

The preceding leptin studies highlighted the potential importance of a method that could rapidly and quantitatively measure extracellular DA while providing information on the activity of DAT. Therefore, this study demonstrated that rapid switching of the concentration of DA in the microdialysis perfusion solution permits a NNF calibration to be completed in 24-36 minutes. The rapid NNF (rNNF) calibration accurately measured the extracellular concentration of DA and the activity of DAT as compared to a NNF calibration generated from a longer measurement period. The accuracy of this measurement was improved when the perfusion flow rate was 0.3 μL/min as compared to 1 μL/min, perhaps indicating that lower flow rates cause less perturbation of normal tissue function. The within-animal reproducibility of the slope of rNNF calibrations was excellent, with 3-4 % relative standard deviation (RSD) at both 0.3 and 1 μL/min. The RSD of the extracellular concentration was greater and more varied but typically represented a range of several nanomolar, perhaps reflecting natural fluctuations in the extracellular concentration of DA. In the presence of nomifensine, which blocks DA uptake by DAT, calibration by rNNF recorded the expected increase in DA concentration

and decrease in slope. rNNF could be performed before and after an experimental treatment which would not be possible with traditional NNF techniques.

As a whole, this dissertation expands the application of high speed analytical techniques for the analysis of dialysate and neurochemical systems. The acute leptin studies contribute to the greater body of research on the ability of leptin to modulate DA reward. rNNF demonstrates a method for faster in vivo quantitation which increases the conditions under which quantitative analysis of DA can be performed.

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CHAPTER 2

LEPTIN PROMOTES DOPAMINE TRANSPORTER AND TYROSINE HYDROXYLASE ACTIVITY IN THE NUCLEUS ACCUMBENS OF SPRAGUE-DAWLEY RATS

Introduction

Multiple observations link energy balance to the function of brain reward pathways: not only are obese humans 25% less likely to develop substance abuse disorders,[1] but also food restriction increases the incentive value of drugs of abuse.[2, 3] Thus, the regulation of feeding and drug abuse likely act on shared brain pathways. Leptin plays a key role in regulating feeding behavior and other aspects of energy balance.[4-7] In addition to potentiating satiety signals, leptin modulates the incentive salience of rewards.[8, 9] While much is known about the mechanisms by which leptin influences satiety, only recently have investigators begun to explore the mechanism by which leptin regulates the incentive salience of food and drug rewards, and our understanding of this aspect of the action of leptin remains incomplete. The mesolimbic dopamine (DA) system controls the incentive salience of food[10] (as well as other natural rewards and drugs of abuse).

Populations of LepRb-expressing neurons in the lateral hypothalamus (LHA)[11] and the ventral tegmental area (VTA)[12, 13] contribute to regulation of the mesolimbic DA

system by leptin, and leptin may modulate the mesolimbic DA system in several ways. Leptin treatment of midbrain tissue slices slightly hyperpolarizes VTA DA neurons.[13] Also, leptin-deficient *ob/ob* mice exhibit decreased VTA and NAc tyrosine hydroxylase (TH; which catalyzes the rate-limiting step in DA synthesis) expression and DA content.[11, 12] Consistent with decreased mesolimbic DA content in *ob/ob* mice, amphetamine (AMPH, which acts via the DA transporter, DAT, to promote DA efflux) fails to promote normal locomotor activity and sensitization in these animals, and chronic leptin treatment restores the AMPH responsiveness of *ob/ob* mice.[12]

While these data collectively demonstrate that leptin regulates dopaminergic reward systems, and suggest that chronic leptin deficiency attenuates both DA production and responsiveness to AMPH, it is possible that the decreased mesolimbic TH and DA content in *ob/ob* animals represents a secondary compensatory response to chronic leptin deficiency. Furthermore, while TH and DA content represent important variables in the function of the mesolimbic DA system, the potential leptin-mediated modulation of other determinants of mesolimbic DA function has not been carefully examined, or as in the case of the modulation of DAT, not explored at all.

To address these important outstanding issues in leptin action, our present study aimed to determine the acute regulation of the mesolimbic DA system by leptin in normal animals. We used *in vivo* microdialysis in freely moving animals to monitor the basal and AMPH-stimulated extracellular DA concentrations in the NAc and assayed VTA and NAc tissue for TH and DAT expression and activity 4 h after leptin treatment of Sprague-Dawley rats.

Methods

Materials

Unless otherwise noted, all materials were purchased from Sigma-Aldrich and were used without further purification.

Animals and surgery

All experiments were performed on male Sprague-Dawley (Harlan) rats 60-100 days old. Animals were maintained on a 12:12 light:dark cycle with lights on at 08:00 hr. Following surgery to implant a guide cannula, animals were housed individually. All animal procedures were approved by the University Committee for the Use and Care of Animals at the University of Michigan.

For the implantation of a guide cannula (Plastics One, Inc.), rats were anesthetized with a combination of ketamine (65 mg/kg, Fort Dodge Animal Health) and medetomidine (0.5 mg/kg, Pfizer Animal Health) i.p. Using aseptic technique, a guide cannula was implanted anterior +1.6 mm, lateral ± 1.3 mm, ventral -5.0 mm from bregma and secured in place with dental cement (A-M Systems, Inc.). The guide cannula was sealed by means of a solid metal stylet (Plastics One, Inc.). Animals were allowed to recover for 5-10 days before experimentation. Several times during the recovery period, the animals were brought to the experimental room for acclimation to the experimental environment.

Microdialysis

The extracellular DA concentration in the NAc was measured *in vivo* by microdialysis coupled online to capillary electrophoresis with laser-induced fluorescence (CE-LIF). Microdialysis probes were prepared as described previously.[14] Before

implantation, the probes were sterilized in ethanol (Fisher Scientific) for 10 min. Between 17:00 and 20:00 hr on the day before experimentation, the animal was weighed, was briefly anesthetized with isoflurane (Baxter Healthcare Corporation), and a microdialysis probe with a 2 mm long sampling region was implanted, extending 3.4 mm past the tip of the guide cannula. The animal was placed in a Ratturn (Bioanalytical Systems, Inc.) to prevent the probe from being disturbed by normal movements of the animal. Between 08:10 and 08:50 hr on the day of experimentation, the animal received 1 mg/kg leptin or phosphate buffered saline (PBS, 20 mM phosphate pH 5.2, 0.13 M NaCl) vehicle i.p. This supraphysiological dose was selected to match the systemic doses previously given to rats by us and others.[15-17] Four h later, the animal received 1 mg/kg D-AMPH s.c. One h before and four h after the AMPH stimulation, the extracellular DA was monitored as previously described.[18] Briefly, the dialysate was mixed online with naphthalene-2,3-dicarboxaldehyde (Invitrogen) and KCN (Fisher Scientific) to derivatize the amine functionality of DA. Injections were made from the derivatized sample stream onto a separation capillary by means of a flow-gated interface. CE separations, lasting 100 to 120 s, were performed with a buffer of 30 mM phosphate, 6.5 mM sodium dodecylsulfate (SDS), and 2 mM hydroxylpropyl-β-cyclodextrin and detected on-column by laser induced fluorescence. The separation and detection were controlled using software written in Labview 5.0 (National Instruments). Following the observation period, the probe was removed, and the animal was euthanized with Fatal Plus (Vortech Pharmaceuticals, Inc.). The brain was removed and preserved in 4% paraformaldehyde for histology. An external calibration was performed through the probe and the *in vitro* recovery was measured.

Histology

One week prior to histological study, the preserved brains were transferred to 30% sucrose in 4% paraformaldehyde. Each probe tract was visualized by cryostatic sectioning. Data from those probe tracts which fell outside of the NAc were excluded from final analysis.

Tissue collection

The expression and activity of TH and DAT were measured in VTA and NAc tissue samples. Prior to the collection of tissue from the VTA and NAc, animals experienced a mock-implant of a microdialysis probe and were housed overnight in their home cages in the experimental room. On the day of tissue harvesting, between 08:10 and 08:50, the animals received 1 mg/kg leptin or PBS vehicle i.p. Four h later, the animals were decapitated. The brain was rapidly removed and cooled for a few minutes in ice cold artificial cerebral spinal fluid (aCSF, 145 mM NaCl, 2.7 mM KCl, 1.0 mM $MgSO_4$, 1.2 mM $CaCl_2$, 2.0 mM phosphate, 200 μM ascorbic acid, pH 7.4). The VTA and NAc were dissected by means of a brain matrix. Tissue samples for qPCR were stored at -80ºC until analysis. Tissue samples for other assays were treated as described below on the day of collection.

TH and DAT mRNA qPCR

RNA was prepared from microdissected VTA and NAc using Trizol (Invitrogen) and 1μg samples were converted to cDNA using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen). Sample cDNAs were analyzed in triplicate via quantitative RT-PCR for *Gapdh* and either *Th* or *Dat* (Applied Biosystems) using an

Applied Biosystems 7500. Relative mRNA expression values are calculated by the $2^{-\Delta\Delta Ct}$ method, with normalization of each sample ΔCt value to the average vehicle-treated ΔCt.

Preparation of synaptosomes

The western blots for TH and DAT, the TH activity assay, the [^{3}H]WIN 35,428 (2β-carbomethoxy-3β-(4-fluorophenyl)-tropane) ([^{3}H]WIN35428) binding assay, the DAT biotinylation assay, and the [^{3}H]DA uptake assay were all performed in synaptosomes. Tissue samples were homogenized on ice as follows: VTA samples in 100 μL and NAc samples in 300 μL 0.32 M sucrose for western blots; VTA samples in 150 μL and NAc samples in 400 μL 0.32 M sucrose containing a cocktail of protease inhibitors (Complete Mini, Roche Diagnostics) for the TH activity assay; and in 1.3 mL 0.32 M sucrose containing a cocktail of protease inhibitors for the [^{3}H]WIN35428 binding, DAT biotinylation, and [^{3}H]DA uptake assays. Homogenized samples were centrifuged at 800g for 10 min. The supernatant was centrifuged at 12,000g for 15 min. The supernatant was discarded. The pellet was resuspended as described.

TH and DAT western blot

TH and DAT protein expression were measured by quantified western blot. Synaptosomes were lysed for 1 h in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40 (Triton X-100), 1% sodium doxycholate, 1% SDS), 100 μL for VTA samples and 400 μL for NAc samples. The lysed synaptosomes were centrifuged at 12,000g for a half h. The protein concentration in the supernatant was measured with a D_C protein assay kit (Bio-Rad) using bovine serum albumin (BSA, Proliant Biologicals) for calibration. Ten μg of protein for the TH western blots and 20 μg of protein for the DAT western blots together with SDS sample buffer (2% SDS, 10% glycerol, 0.1%

bromophenol blue, 100 mM dithiothreitol, 50 mM Tris-Cl pH 6.8) were loaded onto a 10% polyacrylamide gel and the proteins of interest were resolved. Following protein transfer and blocking with 2.5% BSA, the membranes were cut between the 43 and 56 kDa markers. The lower molecular weight portion of each membrane was treated with actin HRP antibody (Santa Crux Biotech Corp.), diluted 1:2,000 for the TH blot and 1:5,000 for the DAT blot in 2.5% BSA, for 1 h. The higher molecular weight portion was treated overnight with monoclonal TH antibody (Zymed Laboratory), diluted 1:800 in 2.5% BSA, or MAB 16 monoclonal DAT antibody (generous gift of Dr. Roxane Vaughan), diluted 1:2,000 in 2.5% BSA, followed by goat anti-mouse HRP antibody (Santa Cruz Biotech Corp.), diluted 1:15,000 in 2.5% BSA for the TH blot and 1:20,000 in 2.5% BSA, for the DAT blot for 1 h. The membranes were imaged following treatment with the Pierce ECL Western Blotting Substrate (Thermo Scientific) for 5 min. Quantification of the bands was performed with Scion Image software (Scion Corporation).

[^{3}H]DA uptake assay

The activity of DAT was measured by the uptake of [^{3}H]DA into NAc synaptosomes. Synaptosomes were resuspended in 2 mL KRB. [^{3}H]DA (specific activity 23.5 Ci/mmol, PerkinElmer Life and Analytical Sciences) uptake activity was determined in triplicate in 200 µL for 2 min at 37ºC at final [^{3}H]DA concentrations of 30 nM, 100 nM, 300 nM, and 1 µM. Non-specific activity was determined using 100 µM cocaine (Sigma) and 1 µM unlabeled DA. The reaction was stopped by the addition of 5 mL ice cold KRB and rapid filtration through GF/C filters (Fisher Scientific). The filters were washed with two 5 mL portions of KRB, and the radioactivity on the filters was measured

using a Beckman LS5801 scintillation counter (Beckman Coulter). DA binding affinity for DAT (K_m) and DA uptake velocity (V_{max}) values were determined by nonlinear regression analysis using Prism 5 (Graphpad Software, Inc.).

[^{3}H]WIN 35,428 binding assay

Binding of the cocaine analogue [^{3}H]WIN35428, specific activity 85 Ci/mmol, PerkinElmer Life and Analytical Sciences) to assess DAT surface expression was measured by a method modified from Reith and colleagues.[19, 20] Synaptosomes were resuspended in 1.2 mL of the assay buffer (30 mM sodium phosphate pH 7.4 and 0.32 M sucrose). [^{3}H]WIN 35,428 binding was measured in triplicate in 200 µL with 103 nM WIN 35,428 for 1 h at 4ºC. Nonspecific binding was determined in the presence of 1 mM unlabeled [^{3}H]WIN 35,428. The reaction was stopped by the addition of 5 mL ice cold 30 mM phosphate and the rapid filtration through GF/C filters. The filters were washed with two 5 mL portions of 30 mM phosphate and the radioactivity on the filters was measured using a liquid scintillation counter.

DAT biotinylation assay

DAT surface expression was confirmed by surface biotinylation. Synaptosomes prepared from NAc tissue were suspended in 500 µL PBS/Ca/Mg containing 1.5 mg/mL sulfo-NHS-SS-biotin (Fisher Scientific) for 2 h at 4°C. Excess biotin was quenched by incubating and washing synaptosomes with 100 mM glycine in PBS. Synaptosomes were lysed overnight in RIPA buffer containing protease inhibitors. Surface proteins (500 µg) were pulled down by streptavidin beads (Thermo Scientific) overnight, and eluted by SDS loading buffer containing 100 mM DTT. The surface protein fraction and 10 µg protein from the lysed synaptosomes (the total DAT protein) were loaded onto a

10% polyacrylamide gel, and the proteins of interest were resolved. After blocking with 2.5% BSA, the membranes were treated overnight with MAB 16 monoclonal DAT antibody diluted 1:500 for the surface DAT and 1:1000 for the total DAT, followed by goat anti-mouse HRP antibody, diluted 1:15,000 for the surface DAT and 1:20,000 for the total DAT, for 1 h. The membranes were imaged following treatment of the surface DAT with Pico Enhanced Chemiluminescence (Millipore) and the total DAT with the Pierce ECL Western Blotting Substrate for 5 min. Quantification of the bands was performed with Scion Image software (Scion Corporation).

TH activity assay

TH activity was measured using a method modified from Levine *et al.*[21] L-[3,5-^{3}H]tyrosine (Perkin-Elmer) was purified on a 6 cm long x 0.6 cm diameter column of Dowex 50WX4-200 beads**.** After application of the L-[3,5-^{3}H]tyrosine to the column, it was washed with 10 mL H_2O and 20 mL 0.5 M HCl. The L-[3,5-^{3}H]tyrosine was eluted with 20 mL 1 M HCl. The purified solution was stored at -20ºC.

Synaptosomes were lysed in 30 mM sodium acetate pH 6.0 and 0.1% Triton X-100 containing a cocktail of protease inhibitors (10 volumes in µL per mg of tissue wet weight). Ten µg protein were used for each VTA assay; 50 µg protein for each NAc assay. The blank was determined in the absence of enzyme. After purification, the activity of the L-[3,5-^{3}H]tyrosine solution was 5,000 cpm/µL. The reaction mixture was added to 200,000 cpm of L-[3,5-^{3}H]tyrosine dried under N_2 at 50ºC. In addition to L-[3,5-^{3}H]tyrosine, the assay mixture contained 0.1 mM unlabeled tyrosine, 1 mM ascorbic acid, 0.1 mM $Fe(NH_4)_2(SO_4)_2$, 750 units catalase, 1 mM tetrahydrobiopterin, 40 µM potassium phosphate pH 6.6. TH was assayed in triplicate in a total volume of 100 µL at

37ºC for 15 min. The reaction was stopped by the addition of 100 µL 1 M trichloroacetic acid.

The radioactive species other than [^{3}H]H_2O were removed from the assay solution by means of ion exchange chromatography. Ion exchange columns were prepared in 14.4 cm x 0.6 cm glass pipettes (Fisher Scientific) and consisted of 0.7 mL 50% slurry Dowex 50WX4-200**,** 80 µL 50% slurry activated charcoal, and 6 drops 50% slurry Dowex 1X2-400. One assay product mixture was purified per column. After application of the reaction product mixture to the column, the column was washed with two 0.7 mL portions of H_2O. Radioactivity was measured by a liquid scintillation counter.

Total DA in tissue assay

Following rapid dissection, tissue samples were homogenized in 1.3 mL 0.32 M sucrose with a cocktail of protease inhibitors. The homogenate was mixed 1:1 with 2 M perchloric acid. The sample solution was centrifuged at 10,000g for 30 min. For analysis of DA by HPLC-EC, the supernatant was diluted 1:100 in perchloric acid containing 14.5 nM 2-aminophenol (internal standard).

DA turnover assay

Following rapid dissection, VTA tissue samples were homogenized in 2 M perchloric acid; NAc tissue samples were treated as described for the total DA assay. DA and the DA metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 3-methoxytyramine (3MT), were detected by HPLC-EC as described for the total DA assay. The content of each metabolite was normalized by the content of DA in each tissue sample for a metric of DA turnover.[22-24]

Results

Leptin enhances AMPH-evoked NAc DA efflux in normal animals

To investigate whether acute leptin treatment could modify DA signaling in normal animals, we initially examined the potential regulation of AMPH-stimulated DA efflux by leptin in the NAc of Sprague-Dawley rats. *In vivo* microdialysis in the NAc of freely-moving rats was coupled on-line to CE-LIF to measure the extracellular concentration of DA. Extracellular DA concentration was monitored beginning 3 h after leptin treatment and 1 h before the administration of AMPH (1 mg/kg, s.c.) (Figure 1A). We chose this duration of treatment in order to permit sufficient time for leptin to access its targets and acutely modulate neurophysiology, but to avoid potential long-term compensatory or secondary alterations in the

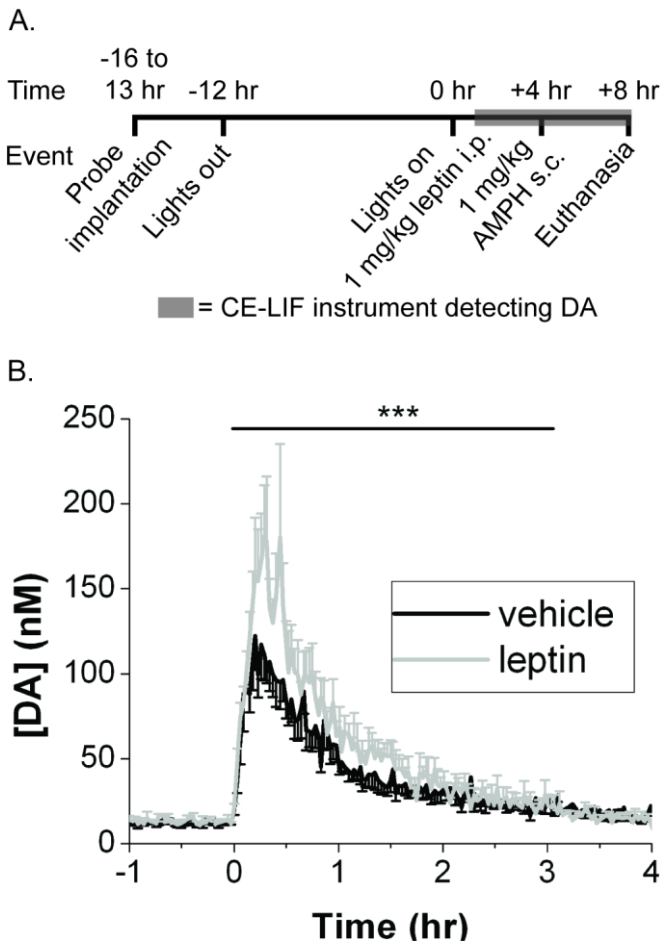

Figure 2.1: Procedure and results for microdialysis experiment. (A) Protocol for probe implantation and experimental treatments. (B) Trace of average AMPH evoked-DA efflux following treatment with leptin or vehicle 4 hours prior. Vehicle n = 6. Leptin n = 4. The [DA] is the dialysate concentration corrected for the *in vitro* recovery of the probe. The error bars represent SEM of [DA]. The black bar indicates the time period from time = 0-3.25 hrs which was subjected to 2 way ANOVA with repeated measures. *** indicates p value for interaction term (treatment X time) < 0.001.

mesolimbic DA system. While leptin treatment did not alter the basal extracellular DA prior to AMPH administration, leptin pretreatment increased the magnitude of AMPH induced DA efflux (Figure 1B). Accurate probe placement was confirmed by sectioning (See Appendix). Thus, systemic leptin acutely potentiates AMPH-induced DA efflux in the NAc. These data reveal that leptin rapidly modulates the mesolimbic DA system of an intact rat.

Leptin acutely increases TH activity without altering TH expression or total DA in normal animals

Previous studies with *ob/ob* animals suggest that leptin deficiency blunts AMPH-mediated behavioral changes at least in part by decreasing NAc DA content, and that increased DA stores contribute to the reversal of this defect during chronic leptin

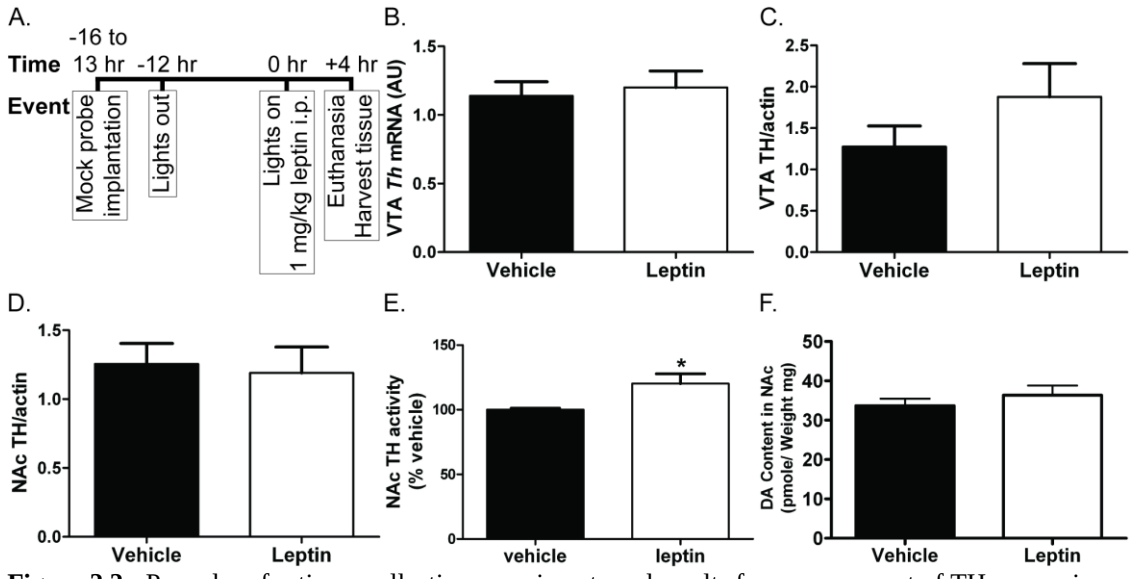

Figure 2.2: Procedure for tissue collection experiments and results for measurement of TH expression, TH activity, and total DA in tissue. All error bars represent SEM and treatment groups were compared by t test. (A) Protocol for animal treatment prior to tissue harvesting. The protocol was designed to mimic that used for the microdialysis experiments. (B) Expression of *Th* mRNA in the VTA as measured by qPCR. Vehicle n = 26. Leptin n = 24. (C) and (D) Expression of TH protein in the VTA(C) and NAc(D) as determined by quantified western blot. Actin band for used for standardization of the TH signal. Vehicle n = 6. Leptin n = 6. (E) Activity of TH in NAc collected from leptin or vehicle treated animals as measured by turnover of [^{3}H]tyrosine. Vehicle n = 6. Leptin n = 5. * indicates p value < 0.05. (F) Total DA in the NAc measured by LC-EC. Vehicle n = 8. Leptin n = 9.

treatment.[12] These alterations in DA stores mirror changes in TH expression, suggesting that chronic leptin treatment modulates mesolimbic DA content by controlling TH levels. We tested the ability of an acute leptin treatment to alter TH expression or DA content, by examining parameters of DA production in the mesolimbic DA system (Figure 2). We performed these experiments essentially as described for the AMPH-stimulated DA release in Figure 1, except that the animals received no AMPH treatment, but were sacrificed for tissue collection 4 h after the administration of leptin or vehicle (Figure 2A). We initially examined *Th* mRNA expression in the VTA, and TH protein content in the VTA and NAc (Figure 2B-D). These data demonstrated that 4 h of leptin treatment was insufficient to alter TH content by any of these measures, suggesting an alternate mechanism for the enhancement of AMPH-stimulated DA release by leptin in the normal rat. However, NAc TH activity was significantly increased by leptin treatment (Figure 2E). Despite the increase in TH activity, NAc DA content was not altered by leptin treatment (Figure 2F) although a non-significant trend toward increased DA turnover was observed in the NAc (Figure 3A). No trend regarding DA turnover was observed in the VTA (Figure 3B). These results show that a single systemic leptin injection can increase

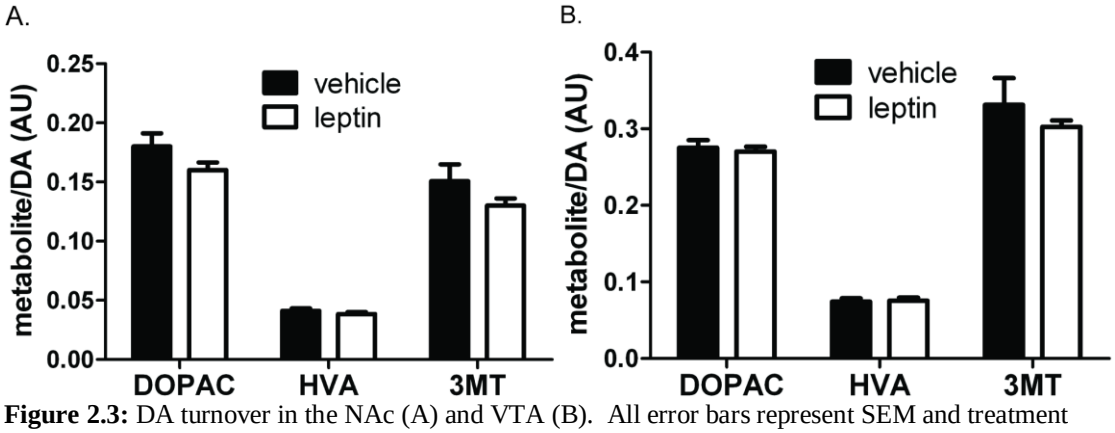

Figure 2.3: DA turnover in the NAc (A) and VTA (B). All error bars represent SEM and treatment groups were compared by t test. For all data, n = 9 except HVA in the NAc of leptin treated rats where n = 8.

the activity of TH after 4 h but is not sufficient to promote increased expression of the enzyme or DA content. The lack of effect on DA content may be due to insufficient time to accumulate enough DA to affect the large pool of DA in the NAc or a lack of storage of local changes in DA.

Leptin increases DAT activity but not expression

Since NAc DA content is not increased following 4 h leptin treatment in Sprague-Dawley rats, some other mechanism must underlie the increase in AMPH-stimulated DA efflux observed following leptin treatment under similar conditions. As AMPH promotes the efflux of cytosolic DA stores via DAT,[25] we examined NAc DAT activity by measuring the uptake of [^{3}H]DA into synaptosomes following pre-treatment with leptin (Figure 4A and B). This analysis revealed a 2-fold increase in synaptosomal NAc DA uptake in leptin-treated animals compared to controls, and that the increased DAT activity reflected an increased V_{max} for transport. However, this increased DAT activity was not the consequence of increased cell-surface DAT (Figure 4C-D). Nor were *Dat* mRNA or DAT protein expression increased by leptin treatment (Figure 4E-G). Thus, after 4 h of a single systemic leptin injection, DAT activity is increased independent of changes in total DAT content or cell surface expression. These results suggest that leptin can activate NAc DAT and this increased DAT activity may underlie the enhanced response to AMPH in acutely leptin-treated rats.

Discussion

Leptin modulates behavior related to reward pathways[6, 9, 26] suggesting a partial basis for leptin effects on feeding and interaction of leptin and feeding with drugs of

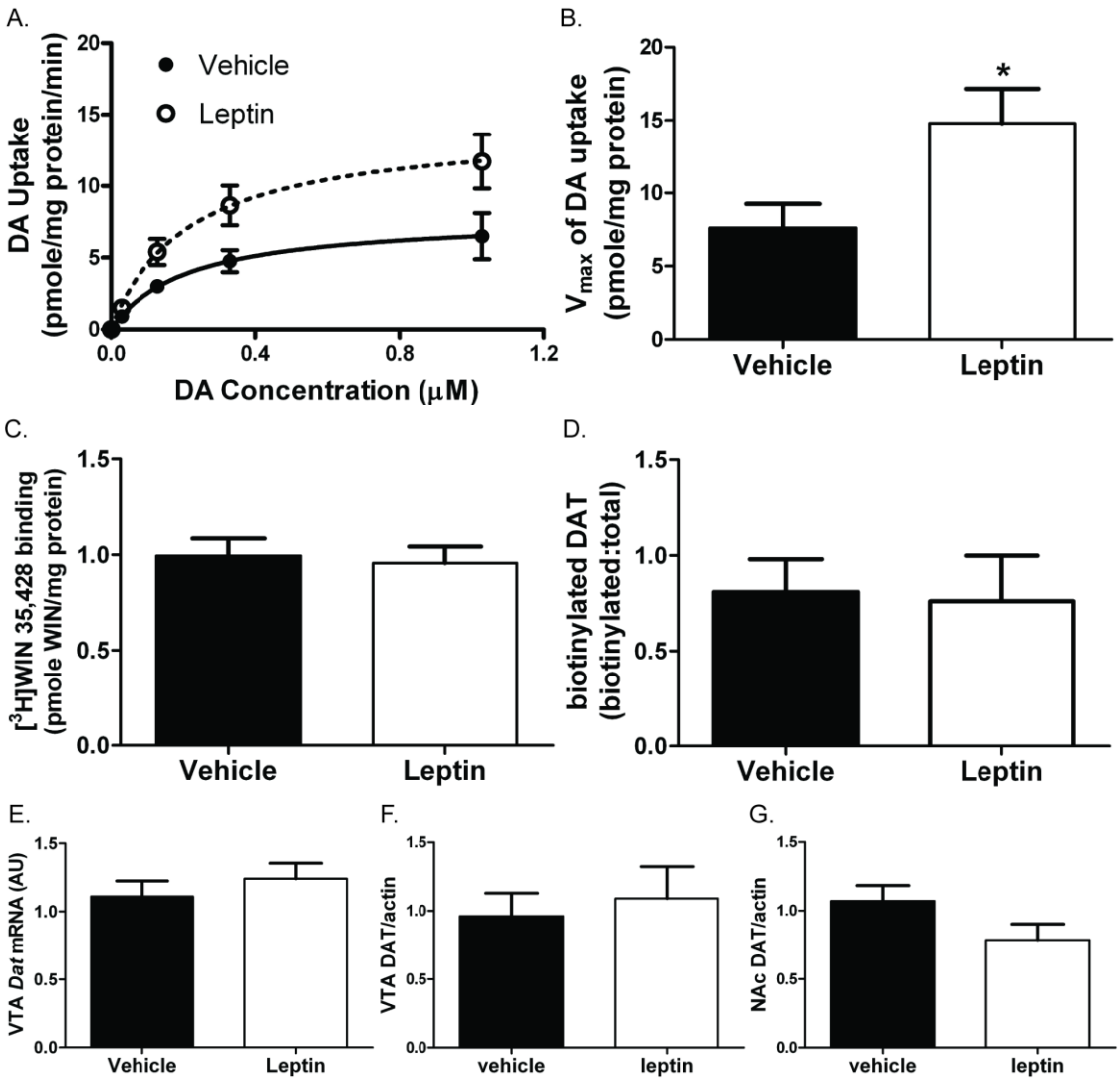

Figure 2.4: Results for measurement of DAT activity and expression. All error bars represent SEM and treatment groups were compared by t test. (A) DA uptake in the NAc as a function of [DA]. Vehicle n = 7. Leptin n = 8. DA uptake data were fit to the Michaelis-Menton equation. (B) The V_{max} for DA uptake in tissue from the leptin treated animals was significantly greater than in vehicle treated animals. * indicates p value < 0.05. (C) DAT surface expression in the NAc as measured by [^{3}H]WIN35428 binding. Vehicle n = 6. Leptin n = 6. (D) DAT surface expression as measured by biotinylation. Vehicle n = 5. Leptin n = 5. (E) Expression of *Dat* mRNA in the VTA as measured by qPCR. Vehicle n = 14. Leptin n = 14. (F) and (G) Expression of DAT protein in the VTA(F) and NAc(G) as determined by quantified western blot. Actin band for used for standardization of the DAT signal. Vehicle n = 6. Leptin n = 6.

abuse. Studies of the neurochemical changes that underlie the effects of leptin in *ob/ob* mice have pointed to the mesolimbic DA pathway as a site of leptin action, specifically regulation of TH expression, DA content,[11, 12] and somatodendritic vesicular packing.[27] As lifelong leptin deficiency may result in adaptations that mask direct regulation of the

DA system, understanding the neurochemical effects of leptin requires examination of acute leptin effects in normal animals. In this vein, leptin has been shown to decrease firing of VTA dopaminergic neurons due to hyperpolarization in anesthetized rats,[13] to decrease extracellular DA concentration as measured by microdialysis in food-deprived rats,[28] and augment the reward-potentiating effect of AMPH on lateral hypothalamic self-stimulation.[29] Here we find that a single i.p. leptin injection acutely increases the activity of DAT and TH, without altering their expression or changing overall DA content or turnover in normal (fed) rats.

Previous work in leptin-deficient models, which have reduced *Th* mRNA and protein expression,[11, 12] has shown that chronic leptin treatment increases TH content in mesolimbic DA neurons. Our present work in normal animals shows that acute exposure to leptin regulates TH activity rather than its expression, as well as regulating DAT activity, revealing heretofore unknown mechanisms by which leptin modulates the mesolimbic DA system. While the acute effects of leptin on the mesolimbic DA system occur independently of alteration in TH and DA content, they are consistent with the notion that leptin tends to promote pathways leading to DA synthesis, as previously shown in *ob/ob* animals. It is not yet clear whether the differences between the effects of acute versus chronic leptin action represent the consequences of long-term leptin action, *per se*, or rather differences due to compensation for the lifelong loss of leptin in *ob/ob* animals.

While the brief leptin treatment that we employed in this study does not alter basal concentration of extracellular DA in normal (fed) rats, AMPH treatment amplifies the effects of increased TH and DAT activity and promotes a significant increase in DA

efflux in leptin-treated rats. Thus, increased activity of TH and DAT are acute downstream effects of leptin in normal rats that can lead to changes in dynamic responses to perturbations such as AMPH challenge.

The stable basal DA concentration observed following leptin treatment in our microdialysis experiments, together with increased TH activity, increased DAT activity, and suppressed dopaminergic firing rate,[13] suggest that these cellular level effects of acute leptin treatment, which would individually be predicted to cause opposing changes to the extracellular DA concentration, yield a balanced response that maintains consistent synaptic DA concentrations under baseline conditions. While leptin does not alter extracellular DA concentration under basal conditions, the dynamics of DA signaling during perturbations of the system may be altered by the change in activity of key regulatory proteins. The effect of AMPH exemplifies how these effects of leptin can give rise to a change in DA signaling.

While our present results and the previous data of others reveal no effect of leptin deficiency or treatment on baseline extracellular DA concentrations or D2-mediated IPSCs,[27] leptin reportedly suppresses basal and feeding-related extracellular DA levels in food-restricted rats.[28] In view of the decreases in V_{max} of DAT associated with food restriction,[30] it seems likely that the effect of leptin on DA concentrations after food restriction could be mediated, at least in part, by the increased DAT activity evoked by leptin. It will be of interest to determine if leptin also alters DA concentration dynamics associated with food seeking[31] and use of abused drugs.

In agreement with our observations on AMPH-evoked DA efflux, leptin increases behavioral sensitivity to AMPH.[12, 29, 32] This observation has previously been considered

paradoxical to the finding that food restriction increases sensitivity to drugs of abuse.[3] However, our results, showing increased activity of TH and V_{max} of DAT by leptin combined with the reported hyperpolarizing effect of leptin on dopaminergic neurons[13] provide an explanation for this effect of leptin on AMPH-evoked DA efflux. That is, the DA efflux elicited by the AMPH-induced reversal of DAT[25] is dependent on TH activity.[33, 34] Moreover, the velocity of DA uptake through DAT is enhanced at hyperpolarizing potentials.[35] Therefore, the leptin evoked changes would be expected to yield a system more responsive to AMPH.

The activation of DAT by leptin is reminiscent of the effect of insulin, another neuromodulating hormone involved in regulating energy balance and metabolism. Like leptin in this study, insulin has been shown to promote DAT activity without significantly affecting surface expression (although a trend toward increased expression was observed).[36] Furthermore, hypoinsulinemic animals exhibit reduced DA uptake but not reduced surface binding.[30] Previous studies have shown that both hypoleptinemic[12] and hypoinsulinemic[37] animals have reduced locomotor activity during their initial AMPH stimulation. The similarity of these data suggest that leptin and insulin act on the mesolimbic DA system via partially overlapping mechanisms.

Conclusions

This work shows that leptin acutely modulates the mesolimbic DA system in normal animals through enhanced DAT and TH activity. These effects interact in a way that does not alter the basal extracellular DA concentration but can alter DA extracellular

concentration dynamics with perturbations, such as AMPH treatment. These data suggest new mechanisms for regulatory actions of leptin on the mesolimbic DA system.

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CHAPTER 3

LEPTIN CAUSES BALANCED ACTIVATION OF THE MESOLIMBIC DA SYSTEM

Introduction

As discussed in Chapter 2, leptin has been shown to chronically regulate the mesolimbic dopamine (DA) system in genetic knock-out models of obesity. The data presented in Chapter 2 showed that leptin acutely modulates the mesolimbic DA system in normal animals by activating both tyrosine hydroxylase (TH), the enzyme that mediates the rate-limiting step of DA synthesis, and the DA transporter (DAT), which is responsible for attenuating the signal of DA in the synaptic cleft. Together, these increases in activity enhance amphetamine (AMPH)-evoked DA efflux. However, acute leptin treatment did not alter the basal extracellular DA concentration in the nucleus accumbens (NAc). These results led to the hypothesis that the activation of TH and DAT by leptin caused a balanced activation of the DA system with no change in basal concentration.

The data showing that leptin activates TH and DAT were obtained using *in vitro* synaptosomal and tissue extract assays. These assays permitted the examination of the effects of leptin on the individual components which control DA neurotransmission. Each assay was performed under a set of optimized non-physiological conditions. *In vitro* assays are valuable for the insight they provide into sub-cellular systems, but their

controlled conditions may not necessarily reflect phenotypes actually expressed by an intact animal. With these assays, we demonstrated the capacity of leptin to increase the activity of TH and DAT, but *in vivo*, it is possible that other processes constrain or minimize these effects. Therefore, it is desirable to follow up on the *in vitro* assays to determine if the balanced activation of the DA system, which we posited to be the effect of leptin, could be observed *in vivo* by microdialysis.

Increased activity of TH could support elevated DA exocytosis. DA released by exocytosis is primarily cleared from the synaptic cleft through the action of DAT;[1] although, some DA is converted to the metabolites 3-methoxytyramine (3MT), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) by enzymatic degradation. If the rate of DA exocytosis changed, it might be reflected in altered rates of DA metabolism, especially of 3MT, which is only produced extracellularly.[2, 3] The concentration of 3MT has sometimes been used a metric for extracellular DA; although, the concentration of 3MT correlates with the concentration of DA better for decreases in DA rather than increases.[4] Therefore, if leptin increased DA exocytosis, the concentration of 3MT may also increase.

A change in DA exocytosis caused by leptin could also be observed by an altered response to the administration of cocaine (COC), which inhibits transport of DA by DAT. The basal concentration of DA reflects a balance between the rates of DA exocytosis and uptake. If leptin does cause a balanced activation of DA exocytosis and uptake, inhibiting uptake would make exocytosis the dominant factor controlling the extracellular concentration of DA. Therefore, if the balanced activation hypothesis is correct, leptin-treated animals should have an enhanced DA stimulation in response to COC.

More active DAT would facilitate faster clearance of exogenously applied DA. DA can be microinjected adjacent to a microdialysis probe so that the clearance of the exogenous DA by an exponential decay is observed. The amplitude of the observed microinjection has been correlated to the amount of DAT expressed on the surface of the cell membrane[5] and the rate of clearance has been correlated to the rate of DA uptake by the DAT.[6] If leptin increases the rate of DA uptake *in vivo*, the rate of clearance for microinjected DA should be greater in leptin-treated animals.

Therefore, we used off-line analysis by liquid chromatography-mass spectrometry (LC-MS) to monitor the levels of DA and its metabolites for 4 hr after leptin treatment and following administration of COC. The clearance of exogenously applied DA was observed using capillary electrophoresis (CE) with sheath flow laser induced fluorescence (LIF) detection and 9.5 s temporal resolution.

Methods

Materials

Unless otherwise noted, all materials were purchased from Sigma-Aldrich and were used without further purification.

Animals and surgery

All experiments were performed on male Sprague-Dawley (Harlan) rats 60-85 days old. Animals were maintained on a 12:12 light:dark cycle with lights on at 08:00 hr. Following surgery to implant a guide cannula, animals were housed individually. All animal procedures were approved by the University Committee for the Use and Care of Animals at the University of Michigan.

For the implantation of a guide cannula (Plastics One, Inc.), rats were anesthetized with a combination of ketamine (65 mg/kg i.p., Fort Dodge Animal Health) and medetomidine (0.5 mg/kg i.p., Pfizer Animal Health). Using aseptic technique, the guide cannula was implanted anterior +1.6 mm, lateral ± 1.3 mm, ventral -5.0 mm from bregma and secured in place with dental cement (A-M Systems, Inc.). The guide cannula was sealed by means of a solid metal stylet (Plastics One, Inc.). Animals were allowed to recover for 5-10 days before experimentation. During the recovery period, the animals were brought to the experimental room for acclimation to the experimental environment.

Microdialysis

Microdialysis probes were prepared as described previously.[7] Before implantation, the probe was sterilized in ethanol (Fisher Scientific) for 10 min. Between 16:00 and 20:00 hr on the day before experimentation, the animal was weighed, was briefly anesthetized with isoflurane (Baxter Healthcare Corporation), and was implanted with a microdialysis probe with a 2 mm long sampling region, extending 3.4 mm past the tip of the guide

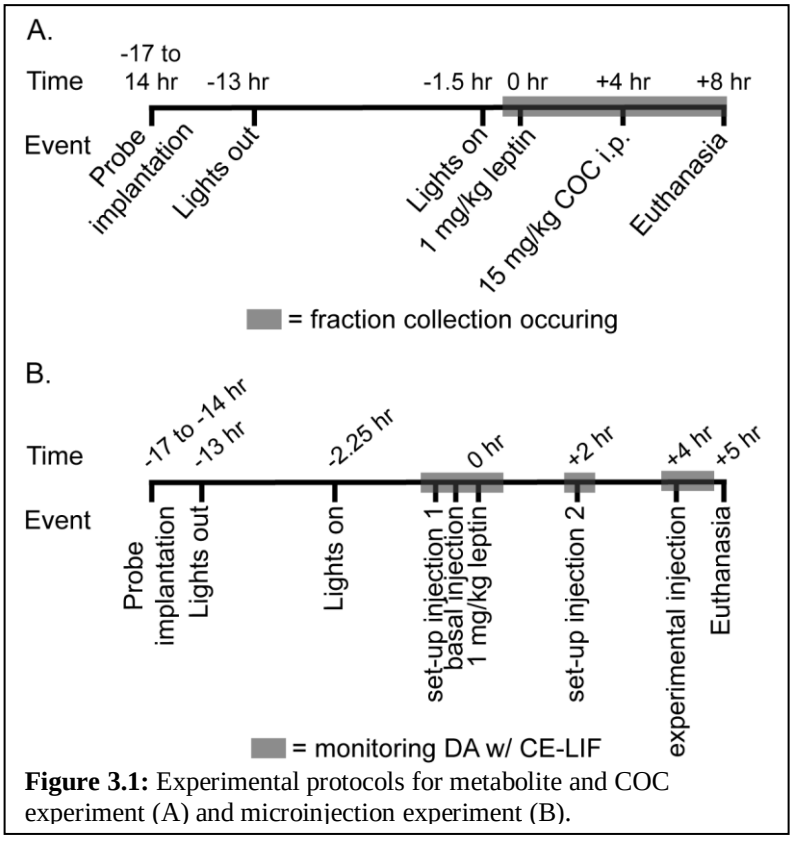

Figure 3.1: Experimental protocols for metabolite and COC experiment (A) and microinjection experiment (B).

cannula. The animal was placed in a Ratturn (Bioanalytical Systems, Inc.) to prevent the probe from being disturbed by the normal movements of the animal.

For experiments studying the effect of leptin on DA metabolism and response to COC, the probe was perfused with artificial cerebral spinal fluid (aCSF, 145 mM NaCl, 2.7 mM KCl, 1.0 mM $MgSO_4$, 1.2 mM $CaCl_2$, 2.0 mM phosphate, 200 μM ascorbic acid, pH 7.4) at 0.5 μL/min for a 30 min equilibration period beginning about 08:10 hr on the day of experimentation. Three 20 min fractions of dialysate were collected on ice. Then, the animal received 1 mg/kg leptin or phosphate-buffered saline (PBS, 20 mM phosphate pH 5.2, 0.13 M NaCl) vehicle i.p. This supraphysiological dose of leptin was selected to match the systemic doses previously given to rats by us and others.[8-10] Twenty minute fractions were collected for the next 4 hr until the animal received 15 mg/kg COC i.p. After the administration of COC, fractions were collected every 10 min for the next 4 hr. All fractions were frozen at -80°C immediately following collection until derivatization and analysis, as described below. The protocol for the leptin-metabolite-COC experiment is summarized in Figure 3.1A.

For the microinjection experiments, the microinjectors consisted of 5 cm of 20 μm i.d./90 μm o.d. fused silica capillary. Four millimeters of 100 μm i.d./200 μm o.d. fused silica capillary were glued around the outside of the microinjector, 2 mm from the tip. The microinjector was glued to the microdialysis probe so that the tip of the microinjector was about halfway along the length of the microdialysis active area. The microdialysis probe plus microinjector were implanted as described above. During the overnight equilibration prior to experimentation, the microinjector was filled with aCSF and connected with a butt-end Teflon connector to a 100 μm i.d./360 μm o.d. fused silica

capillary, which functioned as an aCSF solution reservoir. The probe was perfused at 0.5 μL/min. Between 09:15 and 09:45 hr on the day of experimentation, a different solution reservoir was filled with 200 μM DA in aCSF. This buffer reservoir was connected to a picospritzer (General Valve Corp.) and to the microinjector, replacing the overnight buffer reservoir. Eight to 10 min after replacing the buffer reservoir, an initial set-up microinjection (47 nL over 1 s) was performed to flush the aCSF from the microinjector. Twenty min after the set-up injection, the rat received the first microinjection (47 nL over 1 s) of 200 μM DA to probe basal DA uptake. Fifteen minutes after the first microinjection, the animal received 1 mg/kg leptin or vehicle i.p. About 2 hr after leptin administration, a new 200 μM DA solution was prepared and loaded into the microinjector as before to ensure that the DA in the microinjector was unoxidized. A set-up microinjection was delivered between 2 and 2:20 hr after leptin administration. Four hours after leptin administration, the animal received the second experimental microinjection. During all microinjection steps—connection, flushing, and experimental treatment—the dialysate was derivatized on-line and monitored by CE-LIF, as described below. The protocol for the microinjection experiment is summarized in Figure 3.1B.

Following the observation period, the probe was removed, and the animal was euthanized with Fatal Plus (Vortech Pharmaceuticals, Inc.). The brain was removed and preserved in 4% paraformaldehyde for histology. Following the metabolite and COC experiment, the *in vitro* recovery of the probe was measured. Following the microinjection experiment, an external calibration was performed through the probe and the *in vitro* probe recovery was measured.

LC-MS analysis

For the metabolite and COC experiments, 12 neurotransmitters and 6 monoamine metabolites were analyzed by an ultra-high performance LC (UPLC)-tandem MS method developed by Peng Song.[11] This method derivatizes the phenol and amine functionalities of these polar analytes with a benzoyl (Bz) group to improve their retention on a reverse phase column. Benzoyl chloride was chosen as a derivatization agent because [$^{13}C_6$]benzoyl chloride is commercially available and can be used as an internal standard for quantitation. Benzoylation was performed by mixing 5 μL sample with 2.5 μL 100 mM sodium tetraborate and 2.5 μL 2% benzoyl chloride in acetonitrile (v/v), vortexing briefly after each addition. The mixture reacted for 10 min at room temperature. Then, 2.5 μL internal standard solution (1 mM glycine (Gly), serine (Ser), and taurine (Tau), 100 μM aspartate (Asp), glutamate (Glu), γ-aminobutyric acid (GABA), histamine (Hist), adenosine (Ado), HVA, 5-hydroxyindoleacetic acid (5-HIAA), L-3,4-dihydroxyphenylalanine (L-DOPA), and DOPAC and 10 μM serotonin (5HT), norepinephrine (NE), epinephrine (Epi), DA, normetanephrine (NM), and 3-MT derivatized with [$^{13}C_6$]benzoyl chloride using identical procedure as ^{12}C reagent and then diluted 100-fold with dimethyl sulfoxide with 1% formic acid) was added to the derivatized sample.

The derivatized samples were analyzed using a nanoAcquity UPLC (Waters). Nine microliters of the derivatized sample were injected onto a HSS T3 column (Waters; 1 mm · 100 mm, 1.7 μm, 130 Å pore size) and separated at 27°C with a binary gradient at a flow rate of 100 μL/min. Mobile phase A was 10 mM ammonium formate, 0.15% (v/v) formic acid in water. Mobile phase B was acetonitrile. The gradient conditions were as

follows: initial, 0% B; 0.1 min, 15% B; 2 min, 20% B; 2.3 min, 25% B; 2.31 min, 50% B; 5.31 min, 50% B; 5.57 min, 65 % B; 6.57 min, 65%B. Eluting analytes were ionized by an atmospheric pressure ionization source in positive electrospray ionization mode (capillary voltage 3 kV; source temperature, 140ºC; desolvation temperature, 400ºC; cone gas flow rate, 150 L/hr; desolvation gas flow rate, 500 L/hr). Multiple reaction monitoring (MRM) was performed with a Waters/Micromass Quattro Ultima triple quadrupole mass spectrometer. The interchannel delay and intercycle delays for MRM were both 10 ms and other MRM conditions are listed in Table 3.1. Automated peak integration was performed using Masslynx v. 4.1 (Waters).

analyte	precursor m/z	product m/z	cone voltage (V)	collision energy	dwell time (ms)
Bz-Gly	180	105	35	10	75
$^{13}C_6$Bz-Gly	186	111	35	10	75
Bz-GABA	208	105	35	20	150
$^{13}C_6$Bz-GABA	214	111	35	20	150
Bz-Ser	210	105	35	20	50
$^{13}C_6$Bz-Ser	216	111	35	20	50
Bz-Hist	216	105	35	20	50
$^{13}C_6$Bz-Hist	222	111	35	20	50
Bz-Tau	230	105	35	10	50
$^{13}C_6$Bz-Tau	236	111	35	10	50
Bz-Asp	238	105	35	10	75
$^{13}C_6$Bz-Asp	244	111	35	10	75
Bz-Glu	252	105	35	20	150
$^{13}C_6$Bz-Glu	258	111	35	20	150
Bz-Ado	372	136	35	30	75
$^{13}C_6$Bz-Ado	378	136	35	30	75
Bz-5HT	385	264	60	20	150
$^{13}C_6$Bz-5HT	397	270	60	20	150
Bz-NE	482	105	60	30	150

$^{13}C_6$Bz-NE	500	111	60	30	150
Bz-DA	466	105	70	30	150
$^{13}C_6$Bz-DA	484	111	70	30	150
Bz-Epi	496	105	60	30	150
$^{13}C_6$Bz-Epi	514	111	60	30	150
Bz-L-DOPA	302	105	35	15	75
$^{13}C_6$Bz-L-DOPA	308	111	35	15	75
Bz-HVA	304	105	35	15	150
$^{13}C_6$Bz-HVA	310	111	35	15	150
Bz-5HIAA	313	146	35	15	150
$^{13}C_6$Bz-5HIAA	319	146	35	15	150
Bz-NM	374	105	60	15	150
$^{13}C_6$Bz-NM	386	111	60	15	150
Bz-3MT	376	105	35	20	150
$^{13}C_6$Bz-3MT	388	111	35	20	150
Bz-DOPAC	394	105	35	20	150
$^{13}C_6$Bz-DOPAC	406	111	35	20	150

Table 3.1: MRM conditions of 18 benzoylated neurotransmitters and metabolites and their internal standards

CE-LIF analysis

For the microinjection experiments, seven neuroactive amines were analyzed by CE-LIF as has been reported previously.[12] Briefly, the dialysate was derivatized online with *o*-phthaldehyde and β-mercaptoethanol to derivatize the amine functionality. Injections were made from the derivatized sample stream onto a separation capillary by means of a flow-gated interface. CE separations were performed on a 9 cm long 10 μm i.d./150 μm o.d. fused silica capillary with a buffer of 75.9 mM borate and 0.90 mM hydroxypropyl-β-cyclodextrin, pH 10.0, in overlapping injection mode. The total length of the separation was 13 s and the second injection was made 6.5 s after the initial injection. Including injection processes, the temporal resolution was 9.5 s. LIF detection occurred off-column in a sheath flow cuvette. The sheath flow buffer was 75.9 mM

borate. The separation and detection were controlled using software written in Labview 5.0 (National Instruments).

Histology

One week prior to histological study, the preserved brains were transferred to 30% sucrose in 4% paraformaldehyde. Each probe tract was visualized by cryostatic sectioning. Data from those probe tracts which fell outside of the NAc were excluded from final analysis.

Results

Leptin does not alter the extracellular concentration of DA metabolites under basal condtions

To determine whether acute leptin DA treatment modulates DA exocytosis, we examined whether leptin treatment altered the concentration of the DA metabolites 3MT, DOPAC, and HVA. During 4 hr post-leptin administration, leptin did not cause a difference in how the concentrations of the three metabolites varied over time (Figure 3.2A-C). The concentration of DOPAC was elevated in leptin-treated animals but this elevation existed prior to the experimental treatment; the leptin treatment did not change the concentration of DOPAC from its basal concentration. In agreement with the data presented in Chapter 2, leptin did not alter the extracellular concentration of DA (Figure 3.2D). These data indicate that leptin does not alter DA metabolism, suggesting that leptin either does not affect, or only subtly, affects exocytosis.

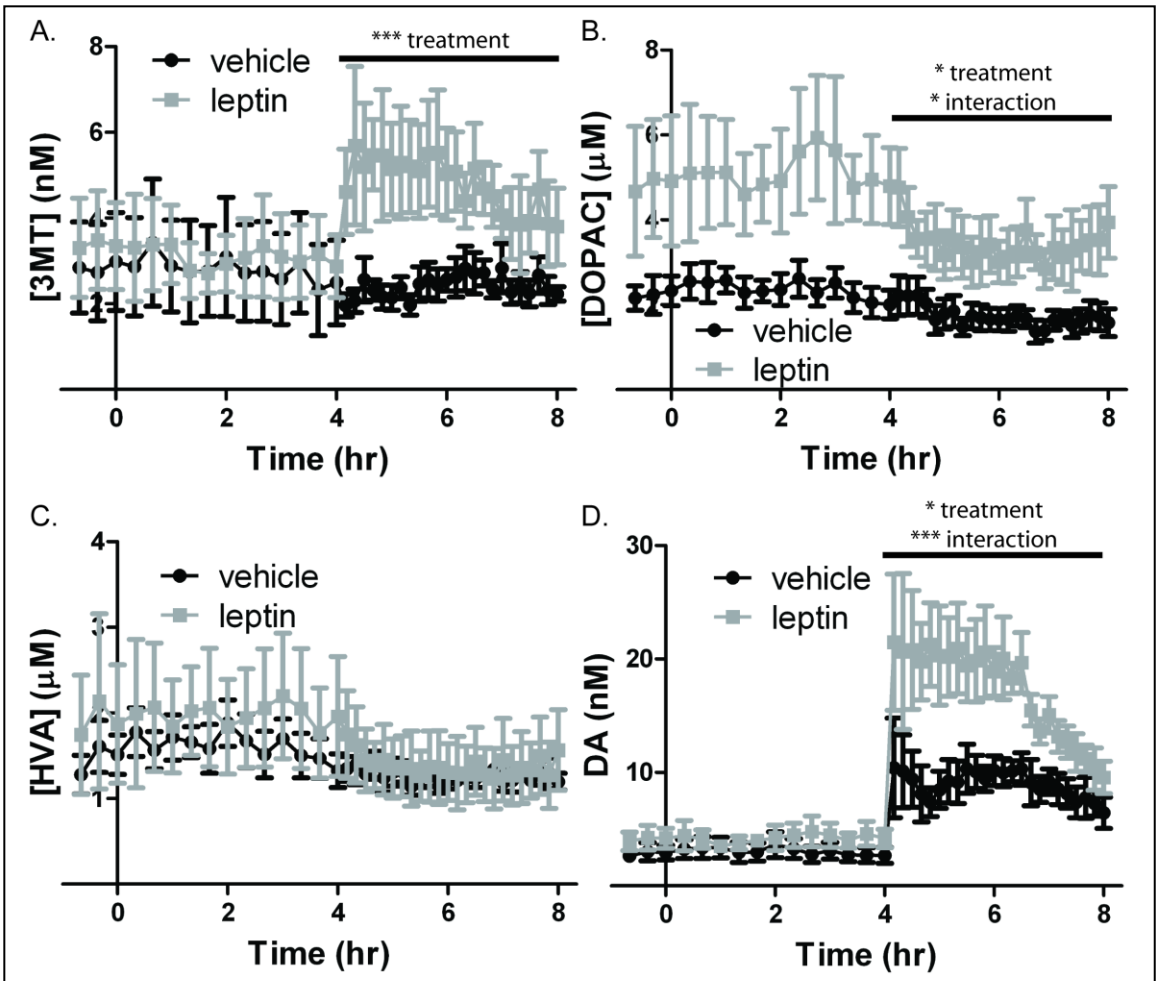

Figure 3.2: Effect of leptin and COC on DA metabolites (A-C) and on DA (D). Leptin was given at time = 0 hr; COC was given at time = 4 hr. Vehicle n = 5 for pre-COC time period, n = 4 for COC time period; leptin n = 4. For the effect of leptin on the COC stimulation, significance was tested with 2-way ANOVA performed by Prism. * $p < 0.05$. *** $p < 0.001$.

Leptin increased the stimulation of DA by COC

In addition to assessing the effect of leptin on DA metabolism, DAT inhibition was also used to assess the ability of leptin to affect exocytosis. Using the same 4 hr treatment period from the leptin-AMPH experiment (See Chapter 2), leptin significantly enhanced the DA response to COC (Figure 3.2D). Enhancement of COC-stimulated DA suggests that leptin does regulate DA exocytosis. Additionally, there was a significant increase in 3MT during the COC period only in leptin-treated animals. DOPAC decreased during the COC period in both treatment groups and the decrease was

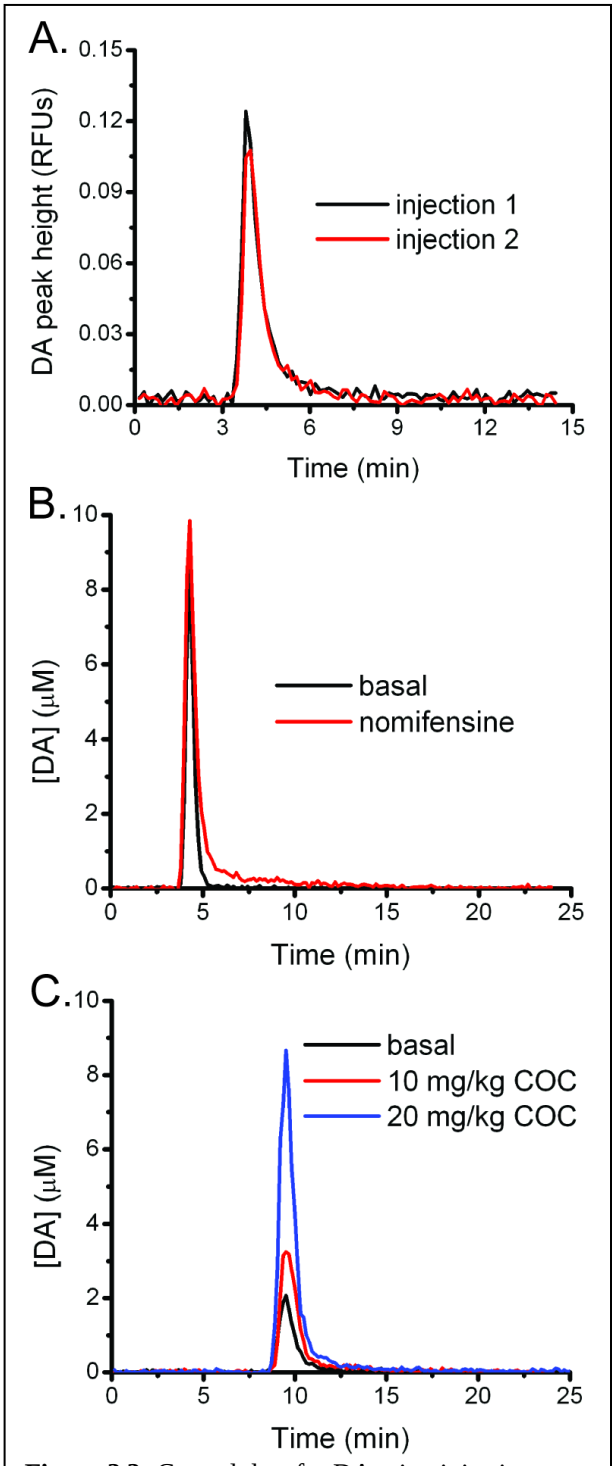

Figure 3.3: Control data for DA microinjections. (A) 200 μM DA with 2 hr between injections (B) basal: 100 μM DA; nomifensine: 100 μM DA and 1 mM nomifensine (C) 200 μM DA injected under basal conditions and after COC treatment

proportional to the original concentration of DOPAC. HVA was not significantly altered during the COC period.

Leptin prevents the normal response to DA microinjection

To determine whether leptin activates DAT *in vivo*, we observed the clearance of exogenously-applied DA. Prior to the leptin-microinjection experiment, we performed several control experiments to show that microinjecting DA did indeed probe the function of DAT. Two hundred micromolar DA, microinjected into the NAc twice, with 2 hr between the injections, showed similar reproducibility to that reported in the literature (Figure 3.3A).[5] The difference in amplitude between the two microinjections was 13% and the difference in clearance rate was 12%.

Additionally, DA was microinjected together with the DAT uptake inhibitor nomifensine or 10 min following systemic injection of COC (Figure 3.3B-C). The co-injection of DA and nomifensine caused a 14% increase in amplitude and a 35% decrease in clearance rate, consistent with DAT inhibition. Microinjection of DA in the presence of COC caused 57% (10 mg/kg COC) and 319% (20 mg/kg COC) increases in amplitude. The lower dose of COC caused a 17% decrease in clearance rate, while at the higher dose of COC, there was no change in the clearance rate. These data supported the use of DA microinjection to probe DAT function.

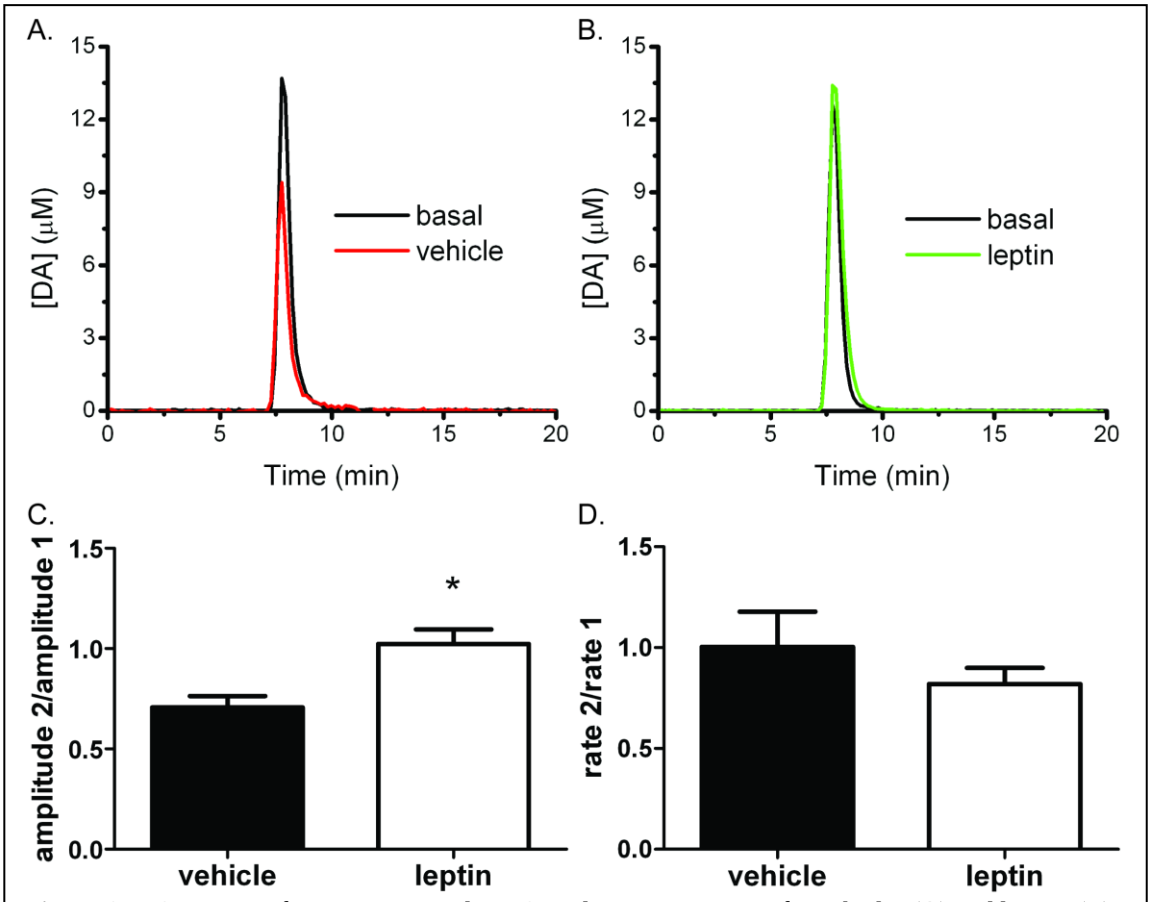

Figure 3.4: Summary of microinjection data. Sample microinjections for vehicle- (A) and leptin- (B) treated animals. The amplitude (C) and clearance rate (D) from the experimental microinjection was normalized to the basal microinjection. Vehicle n = 3; leptin n = 3. Significance was determined by t test. * $p < 0.05$ as compared to vehicle-treated group.

The microinjection procedure caused the amplitude of the second experimental microinjection to be reduced by 30% as compared to the basal microinjection (Figure 3.4A). This drop in amplitude was unexpected, but significantly blocked by leptin (Figure 3.4B-C). The decay rates for the two microinjections were unchanged in the vehicle-treated group and the leptin-treated group was not significantly different (Figure 3.4D). This result was unexpected. These data demonstrate not only the dynamic regulation of DAT surface expression and activity in response to exogenous DA but also the *in vivo* regulation of DAT by leptin.

Discussion

The data presented in Chapter 2 provided the foundation for the theory that leptin causes a balanced activation of the mesolimbic DA system in normal animals by activating both TH and DAT. Since the data on TH and DAT were obtained from *in vitro* studies, we wanted to return to intact animals to consider whether leptin could achieve this balanced activation in the presence of the many other inputs of a whole brain.

While the data in the previous chapter show that leptin activates TH, this activation does not necessarily mean that leptin affects exocytosis because newly synthesized DA could be part of non-vesicular DA reserves. If the activation of TH primarily increased cytosolic DA, it could cause an increase in AMPH-evoked DA efflux without altering exocytosis. We examined the effect of leptin on exocytosis in two ways: DA metabolism and DAT inhibition. When DA is released from the terminal, it is primarily cleared from the synapse by DAT[1] but it can also be degraded by two enzymes, monamine oxidase (MAO) and catechol-*O*-methyltransferase (COMT). MAO converts

DA to DOPAC which can then be transformed by COMT to HVA. COMT converts DA to 3MT and subsequent action by MAO produces HVA. Since COMT is only present on post-synaptic cell membranes[2, 3] while MAO is expressed inside dopaminergic neurons as well as in the extracellular space,[4] the level of 3MT has sometimes been used as a metric for DA release.[4] However, leptin did not alter the extracellular concentration of 3MT or the other DA metabolites measured prior to the administration of COC. If leptin caused an increase in DA basal exocytosis, and 3MT could be used as a metric for DA exocytosis, the concentration of 3MT would be expected to increase. Brown *et al.* reported better correlation between DA and 3MT following treatments that caused decreases in the concentration of DA, such as the exocytosis inhibitor tetrodotoxin, rather than treatments which caused increases in DA, such as the DAT inhibitor bupropion.[4] They concluded that 3MT may be a better marker for decreases in DA release rather than increases. Therefore, a leptin-mediated increase in DA exocytosis might not be reflected in a change in 3MT. Additionally, the concentration of 3MT detected in the dialysate was near the limit of detection (LOD) for the LC-MS method and the peak areas were noisy leading to high inter-fraction variation in the concentration of 3MT. If there were a change in 3MT concentration, it could have been masked by the noise of the 3MT peak.

Since monitoring DA metabolism does not seem to be the most sensitive means to probe DA exocytosis, we used COC to perturb the DA system following leptin treatment. The extracellular concentration of DA reflects a balance between exocytosis and uptake. An effect of leptin on exocytosis could be observed by blocking uptake so that exocytosis was the primary determinant of the extracellular concentration. Therefore, 4 hr after the leptin treatment, the animals received COC to inhibit DAT. Leptin caused a significant

enhancement in the overall magnitude of the COC stimulation, suggesting that leptin increases DA exocytosis. Additionally, the concentration of 3MT increased during the COC stimulation only in leptin treated animals. There was a significant difference between the leptin- and vehicle-treated groups in the change in the concentration of DOPAC during the COC stimulation but the change of each group was proportional to the original concentration of DOPAC. The difference between the treatment groups probably reflects the pre-existing difference in DOPAC concentration rather than an effect of leptin. These data support the hypothesis that leptin causes a balanced activation of the mesolimbic DA system because the increase in DA release was only apparent in the presence of DAT inhibition. Alternatively, leptin may prime exocytotic and uptake mechanisms for amplified response to COC and AMPH. The increase in 3MT during the COC stimulation in the leptin- but not vehicle-treated animals may also suggest either that leptin activates COMT or that the greater magnitude of COC-stimulated DA leads to sufficient DA reaching glial-bound COMT to produce more 3MT.

To provide additional support for the theory of balanced activation, we intended to observe DAT activity *in vivo*. Data presented in Chapter 2 showed that the V_{max} for DA uptake was significantly elevated following leptin treatment but DAT surface expression was unchanged. Therefore, we concluded that the activity of DAT was enhanced by leptin pre-treatment. Microinjection of DA was chosen to probe DAT *in vivo* because it allowed for the brief application of a high concentration of DA that was capable of saturating the transporter, the condition under which differences in V_{max} are most apparent.

Prior to the leptin-microinjection experiment, control experiments were performed to ensure that the microinjections would be replicable and would probe DAT function. The co-injection of nomifensine and DA probably provides a better model for the leptin experiment for several reasons. Without pretreatment of the tissue by nomifensine, the extracellular concentration of DA should be the same at the time of each microinjection. Since the CE-LIF method could not detect basal concentration of DA, elevation of the basal DA concentration by DAT inhibition would also increase the amplitude of the observed DA microinjection. Additionally, the concentration of nomifensine (1mM) was an order of magnitude greater than the concentration of DA so all DAT exposed to the microinjected DA also experienced a larger concentration of nomifensine to ensure that nomifensine would successfully compete for DAT. The increase in amplitudes of the microinjections following the COC treatments can be partially attributed to elevation of the extracellular concentration of DA by COC prior to the microinjection. This elevation was below the LOD of this analysis method, but was expected as an established effect of COC. The stability of the decay rate following the higher dose of COC may be due to the ability to detect more of the microinjected DA as a result of elevated extracellular DA prior to microinjection. Both the nomifensine and the lower dose of COC (which should cause a smaller change in extracellular DA concentrations) experiments demonstrated that the clearance rate of microinjected DA decreased following DAT inhibition. Therefore, DA microinjection could be used to probe DAT function.

Given the control data showing reproducible microinjection of DA with a 2 hr interval between injections, it was expected that the *in vivo* activity of DAT could be

easily assessed by observing the clearance rate of the microinjection. Our data agreed with that of Gulley, Doolen, and Zahniser[5] who reported reproducible microinjection of 70 nL 200 μM DA into the striatum and NAc at 5 min intervals over a 15 min period. The relative standard deviation (RSD) of the observed amplitude was 12.7% for the striatum and 17.8% for the NAc; the RSDs of the time to 80% of clearance were 8.5 and 14.1%, respectively. Therefore, we expected that the two experimental microinjections in the vehicle-treated group would substantially equivalent. The 30% difference in amplitudes when the two microinjections were 4 hr apart suggests that the time course of the experiment is crucial when measuring DAT function.

There is considerable evidence that DAT surface expression and function is rapidly modified upon exposure to substrate and that the length of exposure is a controlling factor in the type of the response observed. Exposure of cultured cells and synaptosomes to DA or AMPH causes the surface expression of DAT to rise within seconds of exposure, to peak a couple of minutes after the beginning of exposure, and to drop below baseline levels 20-30 minutes later.[13, 14] If synaptosomes are prepared from rats pretreated with methamphetamine (METH), they show a significant reduction in DA uptake and DAT surface expression with 1 hr of pretreatment but the DA uptake is partially restored 24 hours after the METH treatment.[15] Switching from microinjections of DA at 5 min intervals to microinjections of DA at 2 min intervals causes a reversible increase in the amplitude and clearance time for each microinjection.[5] Additionally, the increase in the amplitude of the *in vivo* DA microinjection correlates to *in vitro* studies to conclude that the amplitude of a DA microinjection is controlled by the amount of DAT expressed on the cell surface.[5] In light of this inverse correlation between the amplitude

of a microinjection and the surface expression of DAT, the decrease in amplitude that we observed for the second microinjection in vehicle-treated animals suggests that the protocol used for this experiment causes an increase in DAT surface expression

DA uptake is a function of the number of DAT expressed on the cell surface and the activity of DAT at transporting DA across the membrane. Rapid changes in DAT function, such as those described above, are primarily mediated through alterations in DAT trafficking, the process by which DAT is continually internalized and recycled to the surface.[1] Exposure to substrate induces rapid changes in the relative rates of internalization and recycling leading to the rapid changes in the amount of DAT expressed on the cell surface. The above examples clearly demonstrate that the time scale and protocol for exposure have a strong impact on DA function. We are unaware of other work demonstrating longitudinal observation following *in vivo*, rapid, and local application of substrate, such as affected by our experimental protocol, but it is not unreasonable that the 30% reduction in amplitude observed in the treatment group reflects an increase in DA uptake mediated by an increase in DAT surface expression. Synaptosomal studies could be used to confirm that the microinjection protocol modifies DA uptake capacity.

Leptin blocked the normal response to microinjection, possibly by preventing trafficking of DAT and reduction of DAT activity. Unlike vehicle-treated animals, leptin-treated animals did not exhibit a change in the observed amplitudes of the microinjections, suggesting that leptin blocks the increase in surface DAT caused by this microinjection protocol. Leptin may also modulate DAT activity to prevent the normal response to the microinjection protocol. Both the vehicle- and leptin-treated rats did not

exhibit a significant change in clearance rate between the two experimental microinjections. The increase in DAT surface expression in the vehicle-treated animals might be predicted to cause an increase in DA uptake leading to observation of an increase in the clearance rate. The clearance rate is proportional to DA uptake, which itself is a function of the activity and availability of DAT. For the clearance rate to stay constant in the presence of increased DAT expression, the activity of DAT must be decreased by the microinjection procedure in the vehicle-treated animals (Figure 3.5). By contrast, the leptin-treated animals exhibit neither a change in amplitude nor clearance rate, indicating that changes to rate of DA uptake, DAT surface expression, or DAT activity are blocked by leptin administration. Although the activity of DAT is not

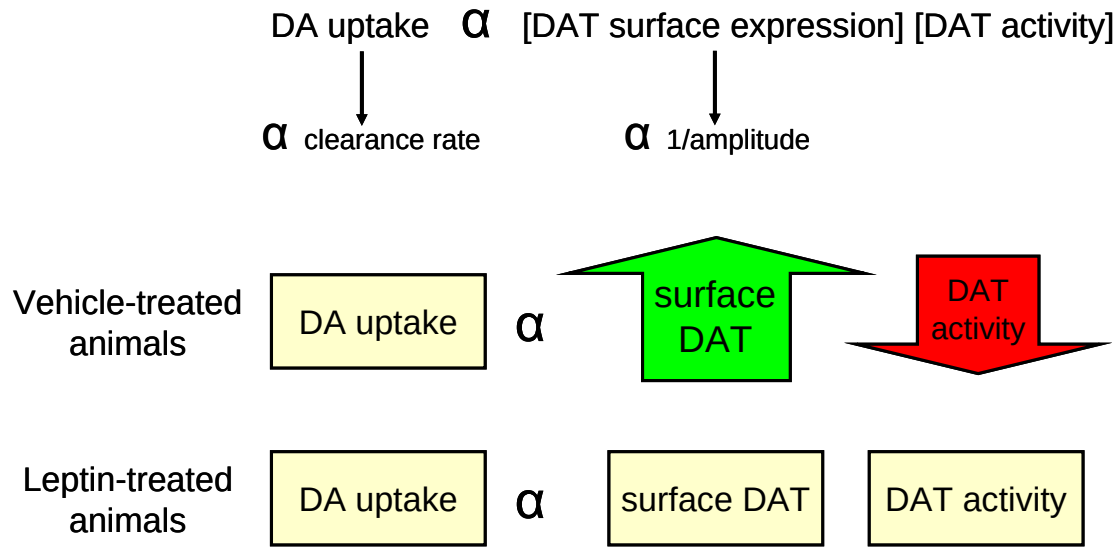

Figure 3.5: Schematic of a possible interpretation of microinjection data. DA uptake refers to the accumulation of DA intracellularly following transport by DAT across the membrane. DAT surface expression is the subset of total DAT expression that is incorporated into the cell membrane and available to transport DA. DAT activity refers to the rate at which DAT completes a DA transport cycle; greater activity would cause more DA to be transported in a set period of time. DA uptake (dependent on the clearance rate) is a function of DAT surface expression (inversely dependent on the amplitude) and the activity of DAT. In vehicle-treated animals, the amplitude of the second microinjection decreased, indicating an increase in DAT surface expression, while the clearance rate and DA uptake were unchanged; therefore, DAT activity decreased. In leptin-treated animals, neither amplitude nor the clearance rate changed; therefore, DAT uptake, surface DAT expression, and DAT activity were constant. Under this interpretation, DAT activity was greater in leptin-treated animals than vehicle-treated animals. Careful characterization of how the clearance rate and amplitude reflect DAT function would be required to confirm this interpretation.

significantly different between the two experimental microinjections in leptin-treated animals, it is elevated relative to the vehicle-treated animals, suggesting that leptin promotes elevated DAT activity *in vivo* in addition to in synaptosomal preparations. Careful characterization of how specific aspects of a microinjection decay curve relate to DAT function would be needed to state with certainty that leptin blocks DAT trafficking or loss of DAT activity precipitated by the microinjection protocol. Without further experiments, the current microinjection data demonstrate that acute leptin treatment does regulate DAT function *in vivo*.

The changes in DAT function caused by microinjection and blocked by leptin were probably caused by overlapping activation of intracellular signaling pathways by the experimental protocol and leptin. DAT function is regulated by a number of intracellular signaling pathways including protein kinase C (PKC), the extracellular signal-related kinases-1 and -2 (ERK1/2), and phosphatidylinositol-3-kinase (PI3K).[1] Binding of agonists to dopamine autoreceptors (D2) has been shown to activate ERK1/2, c-Jun NH_2-terminal kinase (JNK), and PI3K.[16, 17] The D2 agonist quinpirole increases DAT surface expression and V_{max} by activation of ERK1/2;[16] therefore, the increase in DAT surface expression caused by the microinjection protocol might be due in part to D2 activation. Leptin activates multiple intracellular signaling pathways including ERK1/2 and PI3K, both of which mediate some of the anorexigenic effects of leptin.[18] In contrast, activation of JNK, which can be mediated by binding of DA to D2,[17] can partially reverse the anorexic action of leptin.[19] *In vitro* and *in vivo* pharmacological studies could be used to determine which pathways mediate the microinjection-induced

increase in DAT surface expression and by which extracellular DA pathways leptin acts to block the effects of DA microinjection.

While both the current work and the data presented in Chapter 2 show leptin acting to provide balanced activation of the mesolimbic DA system, the *in vivo* modulation of DA by leptin under normal conditions is likely more subtle. These data show leptin regulating the mesolimbic DA system under extreme treatments of AMPH and COC exposure and acute DA microinjection. In most individuals, the dopaminergic system never experiences the challenging conditions presented by these experiments nor do normal individuals experience acute fluctuations in circulating leptin, such as precipitated by a leptin injection. However, these *in vivo* protocols along with *in vitro* experiments have been necessary to probe the cellular circuitry which underlies the normal effects of leptin. Since circulating leptin levels reflect the fat mass of an organism,[20] leptin's ability to activate TH and DAT contribute to setting the dopaminergic tone of an organism and controlling phasic DA exocytosis. A balanced, though subtle, leptin-mediated increase in DA exocytosis and uptake could contribute to more precise control of the mesolimbic DA system. Reward precipitating events would cause greater release of DA

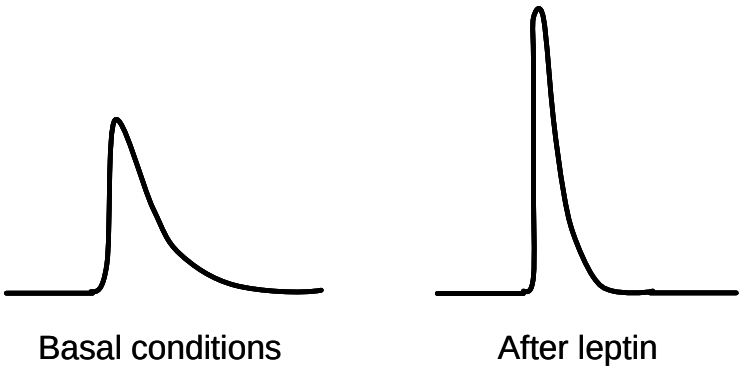

Figure 3.6: Hypothesized intra-synaptic time course of individual DA transients. Leptin increases exocytosis, causing an increased concentration of DA within the synaptic cleft per exocytotic event. Leptin-mediated increases in DA uptake rapidly clear the released DA and reduce the length of each transient, preventing the increased concentration of DA from leaving the synaptic cleft.

into the synaptic cleft providing more stimulation of post-synaptic neurons, but the elevated activity of DAT would efficiently clear DA from the synaptic cleft to prevent excessive stimulation. If we were able to observe phasic DA within individual synapses, release events would have greater amplitude but be shorter in duration (Figure 3.6). Balanced activation of exocytosis and uptake by leptin might modify dopaminergic neurotransmission so that wired transmission is promoted (as indicated by greater DA exocytosis) but volume transmission unaltered (because increased DA uptake prevents the released DA from leaving the synaptic cleft). The reward-modulating satiety signal of leptin may be affected because it limits volume transmission of phasic DA. Since volume transmission is a large component of dopaminergic transmission,[21] limiting volume transmission would also limit the degree to which an animal completes potentially rewardable behaviors, like feeding. Additionally, the mesolimbic DA system receives inputs from a number of endocrine and neural factors which report on the metabolic, cognitive, and emotional state of the body and also can modulate the DA system. Under normal conditions, leptin is one of many factors which provide inputs to the dopaminergic system and which together establish the reward parameters under which the organism functions.

Conclusions

This work supports the hypothesis that leptin causes balanced activation of the mesolimbic DA system. The enhanced COC-stimulated DA suggests that leptin increases DA exocytosis which must be balanced by increased DA uptake to maintain a stable basal concentration. Since leptin did not alter basal DA metabolism or basal DA,

the posited leptin-mediated increase in exocytosis is likely subtle under basal conditions, perhaps reflecting preferential promotion of wired transmission. Comparison of microinjections in vehicle- and leptin-treated animals shows that leptin blocks the normal response to the microinjection protocol. Under normal conditions, leptin is one of many factors that modulate the mesolimbic DA system and act together to set the overall dopaminergic tone of an animal.

Acknowledgements

Dialysate fractions were analyzed by UPLC-MS by Peng Song.

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CHAPTER 4

RAPID NO NET FLUX FOR ON-LINE CALIBRATION OF MICRODIALYSIS

Introduction

Quantitation of the extracellular concentration of analytes remains a challenge in microdialysis experiments due to the difficulty in modeling the physical characteristics of the brain tissue into which the probe is implanted. Often, absolute quantification is unnecessary and data are reported as a percentage of the basal value. However, absolute concentration is needed to compare data from microdialysis experiments to published receptor binding constants[1] or to measurements of neurotransmitters by other techniques. Relying on knowledge of the *in vitro* recovery of the probe and an external calibration to estimate the extracellular concentration of a neurotransmitter is inadequate because uptake and degradation processes and the tortuosity of the tissue can yield an *in vivo* recovery significantly different from the *in vitro* recovery.[2]

Three main methods for *in vivo* quantitcation have been used. In the extrapolation to zero flow method, the probe is perfused at several flow rates. The concentration recovered at each flow rate is used to extrapolate to the equilibrium state in existence under conditions of zero flow to yield the apparent extracellular concentration.[3, 4] The slow flow method relies on relative recoveries that approach 100% at probe perfusion

rates around 50 nL/min so that the dialysate concentration equals the extracellular concentration.[3] In the no net flux method (NNF), the probe is perfused with multiple concentrations of the analyte, above and below the expected concentration.[5, 6] The change in concentration of analyte from entering to exiting the probe is plotted versus the concentration perfused into the probe. The *x*-intercept of this plot, the point at which there is no net flux between the probe and the tissue, yields the apparent extracellular concentration. For the analysis of dopamine (DA), the slope of the linear regression is dependent on the rate of uptake by the DA transporter (DAT).[7, 8] Using traditional high performance liquid chromatography (HPLC) analysis of the dialysate, the quantitative measurement of a neurotransmitter by one of these methods is a very long process (perhaps taking the better part of a day)[3] and is limited to the observation of steady-state systems.

Many experiments could benefit from quantitative measurements of extracellular neurotransmitter concentrations performed more rapidly than is possible with the preceding techniques. Such experiments include the monitoring of extracellular concentrations before and after a drug treatment or at various time points during long term changes. Transient NNF has been used for quantification during stimulation with cocaine and amphetamine.[9] Transient NNF requires that several groups of animals be administered the stimulating drug, and microdialysis be performed while each group is perfused continuously with a different concentration of the analyte. These data are combined to produce NNF calibrations at each point in the stimulation across the groups. Drawbacks to this method include requiring a between-animals design that uses several times more animals than standard NNF and assuming equal response from all animals at

all drug concentrations, which may not always be appropriate. The challenge of small sample volumes generated by the slow flow method has been addressed by the use of an analytical method with high mass sensistivity or make-up flow following microdialysis. Capillary electrophoresis (CE) has much greater mass sensitivity as compared to HPLC, which is normally used for analysis of dialysate. Therefore, CE can handle low nanoliter injections, making it compatible with flow rates less than 100 nL/min. Microdialysis has been coupled on-line to CE for quantitative *in vivo* monitoring of aspartate and lactate[10] and amino acids[11] with temporal resolutions of 45 s to 3 min. The use of make-up flow with the slow flow method, also called MetaQuant, can be used to achieve 100% recovery of analytes with more manageable fraction collection times and fraction volumes;[12, 13] however, the dilution of dialysate by make-up flow could be incompatible with limits of detection for established analytical methods. Also, unlike NNF, the slow flow method does not provide added information that can be obtained from the slope of a NNF curve. For example, changes in slope for DA NNF calibration curves have been related to changes in uptake rates.[7] The data pertaining to the effects of leptin on DAT presented in Chapters 2 and 3 demonstrate a situation when a rapid *in vivo* metric for measuring DA uptake with minimal perturbation of tissue would be very useful.

To address these issues, we have explored the possibility of analyzing DA by NNF in dialysate using rapid CE for analysis. We find that upon perfusion of a new DA concentration through the probe, concentrations stabilize in less than 2 minutes, allowing complete NNF curves to be obtained in 24-36 min. This rapid NNF (rNNF) method has application under transient conditions where more information is required than can be

obtained by low flow methods. The rNNF technique is applied to the measurement of DA before and after treatment with the DA uptake inhibitor nomifensine.

Methods

Materials

Unless otherwise noted, all materials were purchased from Sigma-Aldrich and were used without further purification.

Animals and surgery

All experiments were performed on anesthetized male Sprague-Dawley (Harlan, Indianapolis, IN, USA) rats. Animals were maintained on a 12:12 light:dark cycle with lights on at 08:00 h. All animal procedures were approved by the University Committee for the Use and Care of Animals at the University of Michigan.

Rats were anesthetized with a combination of ketamine (Vetpo Distributors, 65 mg/kg i.p.) and Dexdomitor (Vetpo Distributors, 0.5 mg/kg i.p.). A guide cannula (Plactics 1, Inc.) was used to promote accurate placement of the probe in the nucleus accumbens (NAc). The cannulae were implanted anterior +1.6 mm, lateral ±1.3 mm, ventral 5.0 mm from bregma and secured in place with dental cement (A-M Systems, Inc.). Following each experiment, rats were euthanized with Fatal Plus (Vetpo Distributors). The brains were removed and preserved in 4% paraformaldehyde for histology.

Microdialysis

Microdialysis probes were constructed as described previously with 2 mm long active area.[7] The probes were attached inside push-pull caps (Plastics 1, Inc.) which

mated with the guide cannulae. The implanted probe extended 3.4 mm past the end of the cannula. Probes were implanted in the NAc 2-4 h before the beginning of experimentation and perfused with artificial cerebral spinal fluid (aCSF; 145 mM NaCl, 2.7 mM KCl, 1.0 mM $MgSO_4$, 1.2 mM $CaCl_2$, 200 μM ascorbic acid, 2.0 mM phosphate, pH 7.4). Characterization of rNNF was performed with perfusion flow rates of 0.3 and 1 μL/min. The slower flow rate was used to obtain a greater relative recovery, and the higher flow rate was used to compare results to other published data that used a more typical microdialysis flow rate.

All syringes used to perfuse the probe were driven by a four-channel syringe pump (Chemyx) to minimize variations in flow rate between syringes. Between the pump and the probe, the perfusion solution flowed through a six port valve (Valco), which was set up with two inlets, two wastes, and a single outlet (Figure 4.1). At any time, the perfusion of the probe could be switched from the solution entering one inlet to the other. The solution entering each inlet could be rapidly changed by means of butt-end Teflon-tubing connectors attached to the capillaries in the inlets. For experiments in which six concentrations of DA were perfused through the probe, syringes not connected to the valve at that time could be switched for other syringes containing other concentrations of DA. The flow rates generated by each syringe were tested prior to each experiment to ensure that all syringes delivered the same flow rate. The pressure drop

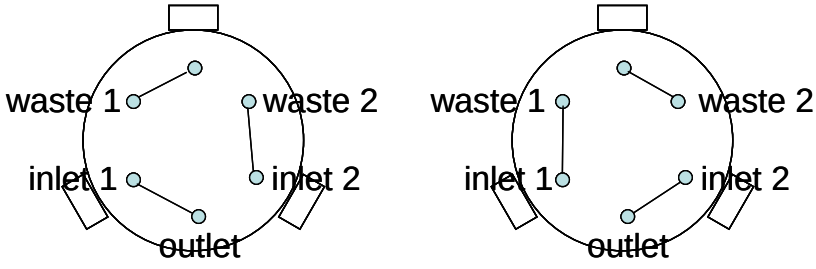

Figure 4.1: Fluidic connections for 6-port valve. Valve position for perfusion from inlet 1 is shown on left and from inlet 2 is shown on right.

across each waste line was balanced with the pressure drop from the valve outlet to the end of the derivatization capillary to prevent switching-related pressure pulses from changing the amount of DA detected. It is essential that any changes in observed DA be due to the concentration perfused or the biological response and not from flow rate variations caused by the syringes or the valve.

Capillary electrophoresis-laser induced fluorescence

Dialysate was derivatized online with naphthalene-2,3-dicarboxaldehyde (NDA, Invitrogen) and potassium cyanide (Fisher Scientific) and detected by CE with laser induced fluorescence (LIF) detection largely as described previously.[14] Briefly, the same flow rate (0.12 μL/min) was used for the derivatization agents whether the dialysate flow rate was 0.3 or 1 μL/min; however, when the dialysate was pumped at the higher flow rate, the concentrations of NDA and cyanide were increased proportionally. Derivatized sample was injected onto the separation capillary by means of a flow-gated interface. CE separations, lasting 90-110 s, were performed in overlapping injection mode with a buffer of 30 mM phosphate, 6.5 mM SDS, and 2 mM HPβCD and detected on-column by LIF. The second injection was made 60 s after the first injection and the first separation was completed prior to detection of the second injection. Hereafter, an "injection period" will refer to one of these 60 s inter-injection periods. The separation and detection were controlled using software written in Labview 5.0.

Results

Stability of observed DA peak following switch in concentration of DA

To determine the time needed for equilibration following a switch to a new

concentration of DA, a probe was implanted in the NAc, and the perfusion solution was switched from aCSF to 25 nM DA. The height of the DA peak was observed for at least 40 min following the switch. Following the switch from aCSF to 25 nM DA during perfusion at either 0.3 or 1 μL/min, the peak height of DA in the dialysate rose in less than 2 minutes to a new, stable height (Figure 4.2). The relative standard deviation (RSD) for the DA peak height during perfusion with 25 nM DA was 5.5 % at 0.3 μL/min and 5.0% at 1 μL/min.

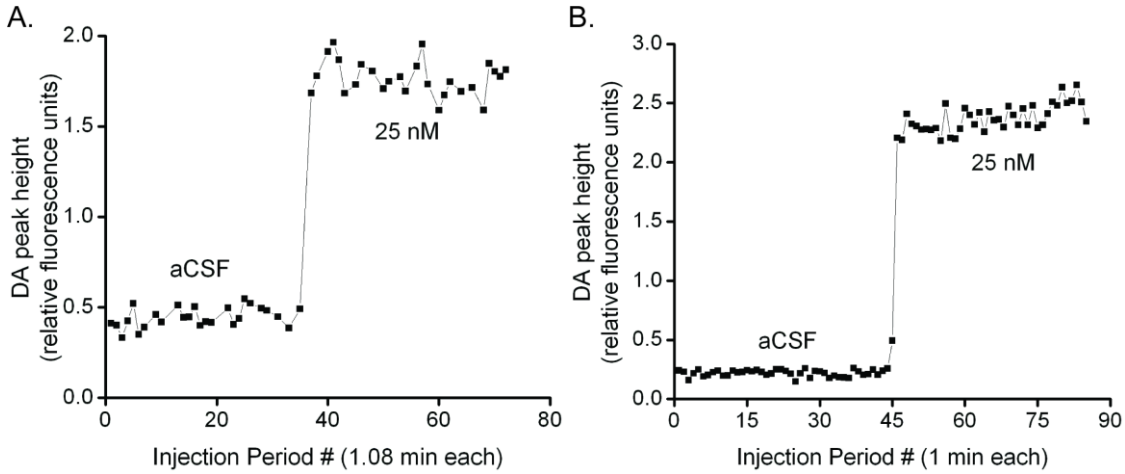

Figure 4.2: Following a switch from perfusion with aCSF to 25 nM DA, the peak height of DA quickly stabilized. The RSD of the height during perfusion with 25 nM is 5.5% at 0.3 μL/min (A) and 5.0% at 1 μL/min (B).

Response time for switching between concentrations of DA

It was necessary to determine the number of injection periods needed to respond to a switch between the concentrations of DA perfused through the probe and the contribution that the valve, the probe, and the tissue made to that response time. The effect of the valve was judged by switching between concentrations of DA during direct injection in absence of a probe. The effect of the probe was measured by performing an *in vitro* NNF experiment where the probe was placed in ~0.8 L of rapidly stirred aCSF at 37°C. The effect of the tissue was observed by implanting the probe in the NAc. The

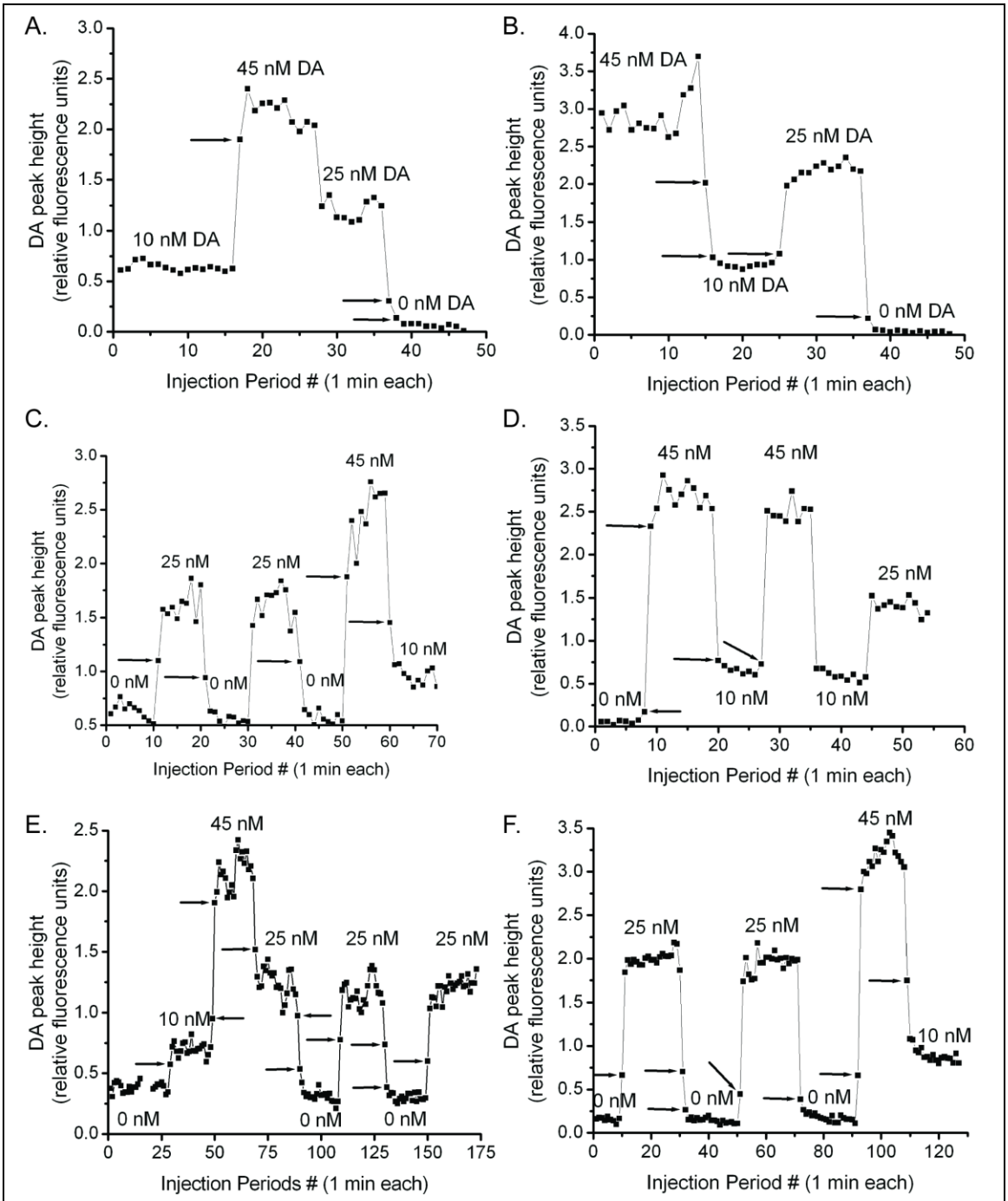

Figure 4.3: Characterization of the number of injection periods needed to switch to a new concentration of DA at at 0.3 µL/min (A, C, E) and 1 µL/min (B, D, F). (A and B) The valve was used to switch between DA standards which were pumped directly to the CE column. (C and D) A microdialysis probe was placed in a well stirred solution of aCSF and an *in vitro* NNF calibration was performed. (E and F) A microdialysis probe was placed in the NAc and an *in vivo* NNF calibration was performed.

period of perfusion at each concentration of DA was 10 min for direct injection and *in vitro* NNF and 20 min for *in vivo* NNF. At both flow rates, zero to two injection periods

were needed to transition between concentrations of DA during direct injection of dopamine standards (Figure 4.3A-B). The response time was not increased by the addition of the probe during *in vitro* NNF (Figure 4.3C-D) or tissue during *in viv*o NNF (Figure 4.3E-F).

Minimum number of injection periods for each concentration of DA

The minimum number of injection periods for each concentration of DA during rNNF was determined by adding the number of transition injection periods to the four injection periods to be averaged for quantitation. Since the maximum number of transition injection periods was 2, the minimum number of injection periods needed for quantitation of DA at each perfusion concentration was 6. Therefore, the probe was perfused at each concentration for 6 min.

Table 4.1A

Rat ID	% difference in NNF conc.	% difference slope
1	1.14	3.0
2	0.83	12.4
3	7.69	8.7
average	3.22	7.7
standard deviation	3.87	6.6

Table 4.1B

Rat ID	% difference in NNF conc.	% difference in slope
4	32.59	26.6
5	11.01	7.8
6	19.44	3.1
average	21.02	12.5
standard deviation	10.87	12.5

Table 4.1: Comparison of the apparent extracellular concentration and slope measured by NNF using 20 minutes per concentration of DA and only the first 6 minutes of each concentration when the flow rate was 0.3 μL/min (A) or 1 μL/min (B). The difference between the full data and the pseudo-6 minute data is expressed as a percentage of the values generated by the full data.

To confirm that using only 6 min for each concentration of DA could give an accurate measurement of the extracellular concentration, a NNF calibration using 20 min of perfusion for each concentration was compared with a NNF calibration using only the first 6 injection periods, as if 6 min of perfusion had been used (Figure 4.4). When the flow rate was 0.3 μL/min (Table 4.1A), the difference between the extracellular concentration of DA yielded by the

calibration from the full data and the calibration using only the first 6 min was less than 8% for all experiments and 3.2% on average. The average difference in the slope for the two calibrations was 7.7%. When the flow rate was 1 μL/min (Table 4.1B), the average difference between the extracellular concentration for the full data and the short period was 21.0% and the average difference for the slope was 12.5%.

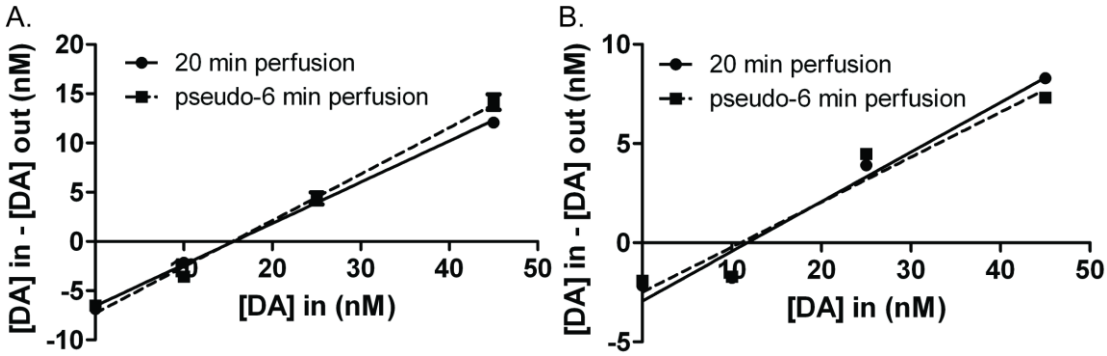

Figure 4.4: A NNF calibration using only the first 6 injection periods of a 20 minute perfusion accurately determined the extracellular concentration as compared to the complete data set when the flow rate was 0.3 μL/min (A) or 1 μL/min (B).

Reproducibility of in vivo *rNNF*

The reproducibility of rNNF was measured *in vivo* by performing three sequential rNNF calibrations under basal conditions. Each calibration consisted of perfusion with six concentrations of DA from 0-200 nM for 6 minutes each. The average RSD for the slope of the three rNNF calibration was 3.2% at 0.3 μL/min and 3.0% at 0.3 μL/min (Figure 4.5 and Table 4.2). The relative variation of the apparent extracellular concentration of DA was greater, 31% and 41% on average (Table 4.2); however, this typically represented absolute variation of a couple of nanomolar. For example, in one rat, the apparent extracellular concentration of DA ranged from 5.7 to 8.8 nM, yielding an RSD of 23.0%.

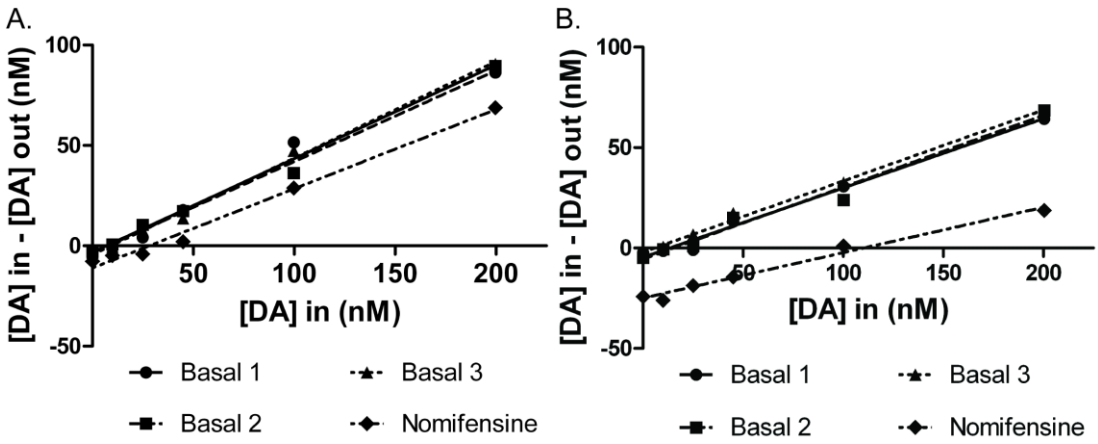

Figure 4.5: Reproducibility of rNNF and the effect of nomifensine. Nomifensine has a smaller impact on the DA concentration and slope at 0.3 μL/min (A) than 1 μL/min (B). At 0.3 μL/min, the concentration of DA increased by 377% and the slope decreased by 16.5%. At 1 μL/min, the concentration of DA increased by 982% and the slope decreased by 34.8%.

Table 4.2A

Rat ID	[DA] RSD	slope RSD
7	14.9	3.7
8	70.7	3.2
9	15.6	3.0
10	23.0	2.9
average	31.0	3.2
standard deviation	26.7	0.4

Table 4.2B

Rat ID	[DA] RSD	slope RSD
11	43.0	1.8
12	0.6	4.5
13	119.7	6.3
14	17.5	3.9
average	45.2	4.1
standard deviation	52.6	2.3

Table 4.2: Relative standard deviation of apparent extracellular concentration and slope for sequential replicate rNNF measurements when the flow rate was 0.3 μL/min (A) or 1 μL/min (B). The high RSD for the apparent DA concentration represents variation of a couple of nM and may reflect natural fluctuations in extracellular DA concentrations over time.

Effect of nomifensine on rNNF

After measurement of the NNF concentration and slope of the NNF calibration under basal conditions, the perfusion of the probe was switched to 5 μM nomifensine (Research Biochemicals, Inc.), a DAT inhibitor, for 30 min. Then, a rNNF calibration was performed in presence of 5 μM nomifensine using the same concentrations of DA as for the basal measurement. The perfusion of 5 μM nomifensine increased the concentration of DA on average to 26.6 nM at 0.3 μL/min and 86.6 nM at 1 μL/min, representing 377% and 982% increases respectively (Table 4.3 and Figure 4.5). Nomifensine caused the slope to decrease by an average of 16.5% at 0.3 μL/min and 34.8% at 1 μL/min (Table 4.3 and Figure 4.5).

Table 4.3A

Rat ID	[DA] basal (nM)	[DA] w/ nomifensine (nM)	% Δ slope
15	1.65	29.8	-13.3
9	8.05	28.2	-15.4
10	7.04	21.7	-20.7
average	5.6	26.6	-16.5
SD	3.4	4.3	3.8

Table 4.3B

Rat ID	[DA] basal (nM)	[DA] w/ nomifensine (nM)	% Δ slope
11	11.5	110.3	-35.8
13	1.7	18.9	-32.3
14	10.9	131.3	-36.2
average	8.0	86.8	-34.8
SD	5.5	59.8	2.1

Table 4.3: The effect of 5 μM nomifensine on the concentration of DA and the slope of the rNNF calibration when the flow rate was 0.3 μL/min (A) or 1 μL/min (B). On average, nomifensine caused the concentration of DA to increase 377% at 0.3 μL/min and 982% at 1 μL/min. The change in slope is expressed as a percentage of the slope under basal conditions.

Discussion

Following a switch to perfusion with a new concentration during traditional NNF, most experimenters allow equilibration periods of 20-90 minutes. This work shows that the extracellular DA concentration stabilizes quickly following a switch. Therefore, a long equilibration time is not needed following a change in the microdialysis perfusion solution.

The number of injection periods needed to transition between perfusion with two different concentrations of DA was 0-2 runs regardless of whether the switching occurred during direct injection, *in vitro* NNF or *in vivo* NNF. Therefore, the valve used for switching was the primary contributor to the response time. Shorter transition times could be obtained if a valve with a smaller dead volume were used and would offer an opportunity for completing a rNNF calibration in a shorter period of time.

The NNF calibrations performed at the lower flow rate showed better agreement between the full data and the first 6 min. The larger relative differences between full data and the pseudo-rNNF data at the high flow rate could be related to the decreased linearity of the calibrations but also could indicate that higher flow rates cause more perturbation

of *in vivo* cellular processes. If indeed lower flow rates have less of an impact on tissue activity, then lower flow rates should be preferred as giving more accurate observations of un-probed tissue.

Using traditional or transient NNF, measurement of with-in animal variability is precluded by the long analysis times; however, rNNF allowed three sequential calibrations to be completed in 108 min, only 36 min each. Although it was impossible to prevent either the extracellular concentration of DA or the rate of DA uptake by DAT from changing by natural processes over the course of the three measurements, these sequential rNNF measurements provide the best possible approximation of the within animal variability of technique. The three sequential rNNF measurements show that there is minimal variation in the slope of the calibrations. In light of the work of Smith and Justice,[7] which showed that only inhibiting uptake, and not synthesis, release, or metabolism, altered the NNF slope, the low variation in the slope of the replicate rNNF calibrations suggests that the rate of DA uptake under basal conditions was relatively constant. Although the relative variation of the DA concentration for the three rNNF calibrations was greater than usually accepted for analytical work, the RSDs typically represented absolute variations of a couple of nanomolar. This small variation could represent natural fluctuations in DA concentration.

At the higher flow rate, DAT inhibition had a greater impact on both the concentration of DA and the slope of the calibration. The higher flow rate should cause greater flux, leading to more diffusion of nomifensine out of the probe, resulting in a greater concentration change at the higher flow rate. The percent decrease in slope at

1 μL/min is comparable to the decreases in slope reported by Smith and Justice during the perfusion of the DAT inhibitors cocaine and GBR-12909.[7]

Twenty-four to thirty-six minutes does not represent a lower limit on the time needed to complete a NNF calibration. The use of a valve with a smaller dead volume could decrease the number of transitional injection periods to 1 or 0 and decrease the time for a calibration with four standards to 16 minutes if the same CE system were used for analysis. If a different analysis method with a faster temporal resolution, such as that reported by Bowser and Kennedy[15] or Wang *et a*l.,[16] were characterized for transitions between perfusion concentrations, a rNNF measurement might be completed in minutes. Therefore, rNNF might reasonably be performed at multiple time points over the course of an experiment.

Conclusions

A NNF calibration can be performed in 24-36 minutes with each concentration perfused for 6 min each. An extended equilibration period following a change in the concentration of DA is not necessary, and with the equipment used in this work, the valve used for switching perfusion solutions was the primary contributor to the response time. Under basal conditions, rNNF calibrations show good reproducibility, especially with regards to the slope of the calibrations. As with traditional NNF, the slope of a rapid rNNF calibration is sensitive to changes in dopamine uptake. rNNF could be used to measure quantitatively and quickly the extracellular concentration of DA before and after an experimental treatment. Such an analysis would be impossible with the long analysis times of traditional NNF.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

This work demonstrates the advantages of high temporal resolution and multi-analyte detection in the analysis of dialysate. Sampling frequencies of one-per-90 s or more facilitated the development of a faster method of *in vivo* quantitation and observation of the acute effects of leptin on the central nervous system (CNS). Multi-analyte liquid chromatography-mass spectrometry (LC-MS) analysis minimized the number of animals required for these studies. Thus, this work broadens the analytical techniques available to neuroscientists and improves our understanding of the role of dopamine (DA) in the leptin-mediated regulation of feeding behavior.

Analytical Conclusions

Microdialysis is a work-horse method for *in vivo* neuroscience studies but quantitative measurement of *in vivo* concentrations of analytes is challenging. The tortuosity of diffusion, active uptake mechanisms, and enzymatic degradation which occur in tissue are difficult to model *in vitro*; therefore, many studies only report results relative to basal values. However, quantitative measurements are necessary to compare the data obtained by microdialysis to values reported by other methods or to binding constants for receptors. The extrapolation to zero flow, low flow, and no net flux (NNF)

methods have been used to make quantitative measurements of neurotransmitters sampled by dialysate. These methods are time consuming, especially when coupled with high performance LC (HPLC), the analysis method most commonly coupled to microdialysis. The low flow method has been coupled to capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) or combined with make-up flow to reduce the time needed to collect sufficient sample for analysis. However, for the analysis of DA, only NNF can provide information on the rate of DA uptake in addition to quantitative measurement of the extracellular concentration. Therefore, this work demonstrated the development of rapid NNF (rNNF), using CE-LIF with 1 minute temporal resolution, so that quantitative measurements of DA and information on DA uptake can be coupled to other experimental treatments.

When the concentration of DA perfused through the probe was rapidly switched, the amount of DA in dialysate re-stabilized in less than 2 minutes. The transition time for switching between perfusion concentrations was 0-2 injection periods. NNF calibrations derived from data for pseudo-6 min perfusion for each standard accurately reproduced the NNF calibration obtained from 20 min perfusion; although, perfusion at 0.3 μL/min produced a more accurate rapid calibration than perfusion at 1 μL/min. The improved accuracy of 6 min NNF calibrations produced at lower flow rates suggested that lower flow rates caused less perturbation of tissue function than flow rates more commonly used for microdialysis. Sequential rNNF calibrations, with 6 min of perfusion per standard, were completed in 36 minutes each. The relative standard deviation (RSD) of slope of the replicate rNNF calibrations was 3- 4%; the relative variation in extracellular concentration was more variable but typically represented a range of a couple of

nanomolar. Treatment with the uptake inhibitor nomifensine caused the expected increase in extracellular DA and decrease in slope. rNNF calibrations are accurate and reproducible and can be completed in 24-36 minutes, permitting them to be performed before and after experimental treatments.

Biological conclusions

With the increasing prevalence of overweight and obese phenotypes in the global population, there is a critical need to understand how feeding behavior is regulated under normal conditions and how it becomes dysregulated leading to weight gain. Several periphery metabolic hormones contribute to the regulation of feeding behavior in the CNS. The adipose-derived satiety signal leptin has been shown to regulate energy balance and the value of rewards including food. Most of the research into the ability of leptin to modulate the reward systems of CNS has used genetic knock-out models in which compensatory changes due to life-long deficiencies may have confounded the results. This work explored the acute effects of leptin on the mesolimbic dopamine system of normal rats.

Previous work in normal, unfasted animals had primarily used chronic treatments, which probably evoked homeostatic regulatory mechanisms, preventing observation of the immediate effects of leptin on the dopaminergic system. This work showed that acute treatment of normal animals with leptin increases the activity of tyrosine hydroxylase (TH) and the DA transporter (DAT) but without changing their expression. Activation of TH and DAT both contributed to an enhanced magnitude of amphetamine (AMPH)-evoked DA release when AMPH was administered 4 hours after leptin. The acute leptin

treatment did not change the total amount of DA in the tissue nor DA metabolism, measured either in the extracellular space or in bulk tissue.

The basal concentration of DA in the nucleus accumbens (NAc) was not changed by leptin pre-treatment. This lack of change suggested that the activation of TH and DAT counter each other so that the extracellular DA concentration was unaltered. The hypothesis of balanced activation was supported by the leptin mediated enhancement of cocaine (COC)-stimulated DA. The enhanced COC stimulation in leptin-treated animals suggested that leptin increased DA exocytosis, an effect which was balanced by an increase in DAT activity and which was best observed under DAT inhibition. The DA microinjection protocol supplied more evidence that leptin modulates DAT in vivo although the precise nature of that regulation merits further study.

These data expanded knowledge of the cellular effects of leptin on the reward systems of normal animals. They demonstrate that leptin is capable of modulating the mesolimbic DA system in normal animals (Figure 5.1) and expand our understanding of the neural circuitry normally modulated by leptin. Without the perturbations of AMPH, COC, or DA microinjection, leptin most likely contributes toward regulation of the dopaminergic tone, promoting

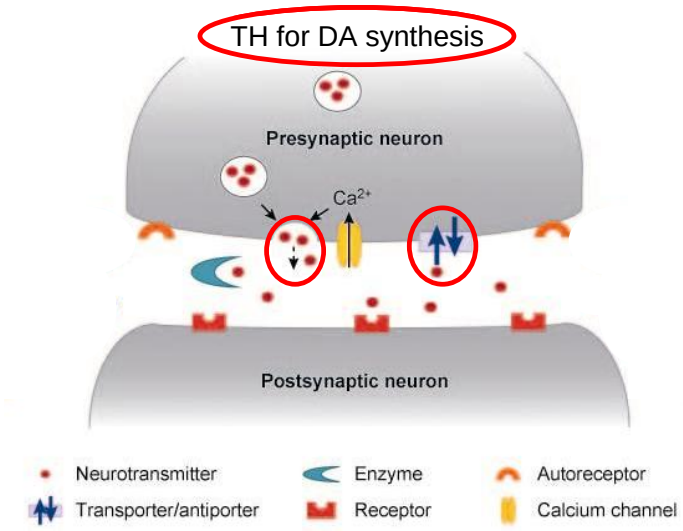

Figure 5.1: Summary of effects of leptin on the mesolimbic DA system. TH activity, DAT activity, and DA exocytosis (circled in red) are increased by acute leptin treatment. Figure adapted from Schultz, K.N.; Kennedy, R.T. *Annual Review of Analytical Chemistry* **2008**, *1*, 627-661.

wired transmission over volume transmission. Promotion of wired transmission likely limits facilitation of reward for behaviors like feeding. Under normal physiological conditions, leptin is one of many endocrine factors which contribute to the dopaminergic tone of an animal and regulate the rewarding nature of food. Therefore, the development of effective treatments for obesity depends on a comprehensive understanding of how both leptin and other endocrine factors contribute to normal energy homeostasis and how perturbations of this system lead to increased food intake and weight.

Future directions—analytical improvements

rNNF greatly decreases the time needed to complete a NNF calibration, but the method, as presented, does not represent the lower limit on the time period for completion of a calibration. The time for each rNNF calibration is currently limited by the dead volume of the valve used for rapid switching and by the temporal resolution of the analytical method.

If a valve with a smaller dead volume were chosen, the transition time for a switch might be reduced to less than one minute, the temporal resolution of the CE-LIF analytical method used here. Reduction of the transition time could decrease the time for perfusion with each standard from 6 minutes to 4 minutes and the total calibration time from 24-36 minutes to 16-24 minutes.

The time for each calibration could be decreased further by selecting an analysis method that provided greater temporal resolution. The sheath flow CE-LIF instrument, used in Chapter 4, has a temporal resolution of 14 s when operated in its normal overlapping injection mode. If it were chosen for analysis, rNNF calibrations might be

performed for glutamate, aspartate, γ-aminobutyric acid, serine, taurine, glycine, or even DA, if an inability to observe basal concentrations of DA were not a concern. High temporal resolution could also be achieved by segmenting the dialysate into plugs separated by an immiscible oil carrier phase.[1] Segmented dialysate has already been analyzed by CE[2-4] and enzyme assays.[5] Segmented flow offers the ability to maintain excellent temporal resolution and permits analysis by the most appropriate method for detection of the analyte of interest. With either a faster analysis method or use of segmented flow, rNNF calibrations might be completed in a few minutes.

As currently described, rNNF can be laborious due to the difficulty in keeping all perfusion solutions flowing at precisely the same flow rate. Small changes in the quality of the seal produced by the plunger of the syringe cause changes in the pressure applied by the plunger and consequently produce variations in the flow rates of each standard solution. This relatively small variation in flow rate produces pressure pulses upon switching that cause instrumentally, and not biologically, derived changes in the DA detected in dialysate. These artificial DA fluctuations impair the linearity of the NNF calibration. In this work, the problem was addressed by measuring the flow rate produced by each syringe following each experiment. This approach was time consuming and could not rule out alteration in the quality of the plunger seal during the course of an experiment. Flow rates could be more constant if all perfusion solutions were driven by the same forces. This might be accomplished by peristalsis on a microfluidic chip such as that described by Cellar *et al.*[6] Although small variations in flow rate may still occur if the perfusion solutions were driven by peristalsis, all standard

solutions would experience these changes equally, minimizing artificial changes in signal.

Although rNNF is substantially faster than traditional NNF, it still requires that a calibration be performed outside of a wider experimental design. It also cannot account for changes in the extraction fraction of an analyte over the course of an experiment as the slow flow method and transient NNF are able to do. Perfusion of the probe with a ^{13}C-labeled analyte at a set concentration coupled with analysis by the UPLC-MS method, used in Chapter 3, could allow continuous quantitation at any flow rate and with corresponding information about the rate of DA uptake when DA is the analyte. When any analyte is perfused through the probe, a fraction of it will diffuse out of the probe even as analyte from *in vivo* sources diffuses into the probe. If the exogenous analyte were ^{13}C-labeled, it would be possible to distinguish the exogenous and endogenous analyte to determine the *in vivo* recovery of the analyte. Changes in recovery of ^{13}C-labeled analyte would reflect changes in the recovery of the unlabeled analyte and alterations in the endogenous processes that control recovery. Therefore, increased recovery of ^{13}C-labeled DA would indicate a decrease in DA uptake by DAT. To prove that perfusion with a ^{13}C-labeled analyte can provide continual quantitation of dialysate, a NNF calibration will need to be performed in the presence of a small concentration of the ^{13}C-labeled analyte to demonstrate that the extraction fraction of NNF is equivalent to the recovery of the ^{13}C-labeled analyte. A small concentration ^{13}C-labeled analyte in the perfusion solution would be preferred for continuous quantitation so that exogenous analyte causes the least perturbation of normal tissue function. It will also be necessary

that the recovery of ^{13}C-labeled analyte responds as expected to pharmacological manipulation.

Development of a continuous quantitation method by perfusion of ^{13}C-labeled analyte might prove especially useful to the neuroscience community. Some of the analytical techniques described in this dissertation can be challenging to those without training in separations and are labor intensive as compared to HPLC analysis. On the contrary, there is a growing availability of LC-MS core facilities at many institutions, which offer fee-for-service analysis of whatever samples are provide by the client. Therefore, adoption of quantitation by perfusion of ^{13}C-labeled analyte would not require any special skills for the average neuroscientist and would increase the convenience of *in vivo* quantitation of dialysate.

Future directions—modulation of DA by leptin

While this work has shown acute effects of leptin on TH and DAT, other aspects of the reward-regulating effects of leptin remain to be explored. The data from the COC experiments indirectly suggest that acute leptin treatment elevates DA release in the NAc. Direct measurement of individual dopaminergic exocytotic events is not possible with microdialysis but can be observed using fast scan cyclic voltammetry (FSCV). FSCV could be used to confirm that leptin elevates DA exocytosis.

In addition to the attenuative effects of DAT, the D2 autoreceptor also acts to reduce the rewarding signal of DA. Activation of the D2 autoreceptor causes inhibition of DA exocytosis. Recent work has shown that chronic administration of leptin to normal mice reduces D2 expression.[7] Acute leptin treatment may modulate the effects of

the D2 autoreceptor prior to decreases in expression, analogous to the changes in activity observed for TH and DAT. The ability of leptin to act acutely on D2 function could be explored by observing whether the effects of D2 agonists and antagonists are altered by leptin pre-treatment.

The NAc is not the only dopaminergic target region that receives signals from leptin receptor (LepRb)-expressing neurons. There are LepRb-expressing neurons in the VTA that project to the extended amygdala (Amy) and in the substantia nigra pars compacta (SNc) which presumably project to the striatum.[8] The acute effects of leptin on dopaminergic projections to the Amy and striatum should be probed to determine whether the effects observed in the NAc are repeated in other brain regions.

All leptin-DA experiments described in this dissertation used an acute treatment procedure to avoid activating homeostatic mechanisms which could obscure the effects of leptin in normal animals. However, the existence of such mechanisms has not been conclusively demonstrated. For instance, chronic leptin treatment decreased D2 expression in the striatum and NAc.[7] It would be interesting to explore the time course of the modulation of DA by leptin to determine whether the effects of leptin are subject to homeostatic control and when this control becomes apparent.

Conclusion

Microdialysis is widely used in studies of the CNS. This dissertation demonstrates a faster method for quantitative measurement of DA sampled by microdialysis. One-minute temporal resolution and rapid switching of perfusion solutions allowed rNNF calibrations to be completed in 24-36 minutes. Further research

into quantitative microdialysis methods could decrease the time needed for rNNF calibrations or develop a method for continuous quantitation using perfusion with ^{13}C-labeled analytes.

Microdialysis, coupled to high temporal resolution or multi-analyte detection techniques, was used to explore the acute modulation of the dopaminergic projections to the NAc by the anorexigenic hormone leptin. Leptin acutely modulates the mesolimbic DA system by activating TH and DAT and promoting exocytosis. This activation profile explains why the responses to AMPH and COC are enhanced following treatment with leptin. Further study of leptin's effects on dopamine could demonstrate the effects of leptin on other dopaminergic cellular processes or on other dopaminergic projections. As a whole, this work demonstrates the usefulness of cutting-edge analytical methods for improvements to neurochemical monitoring techniques and for observing the interaction of periphery signals and the CNS.

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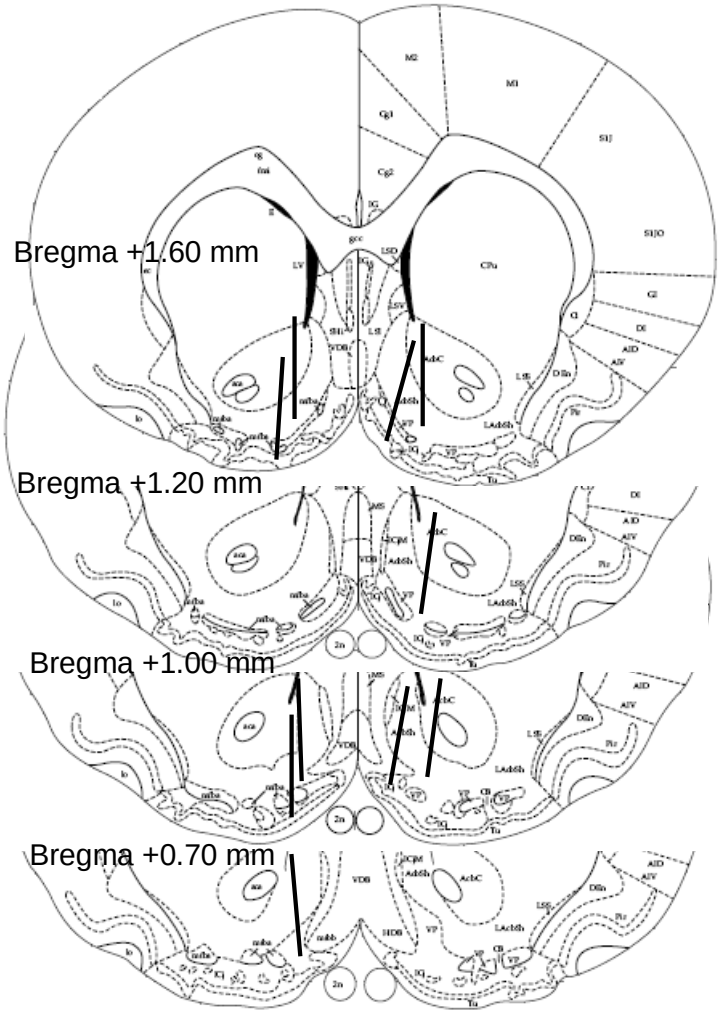
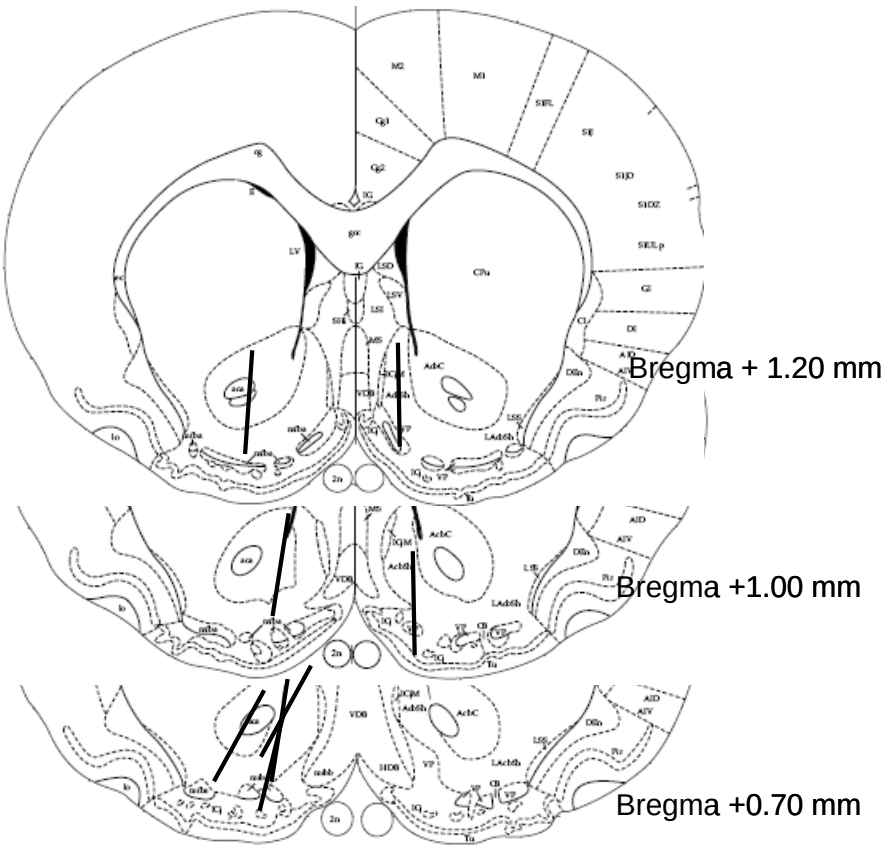
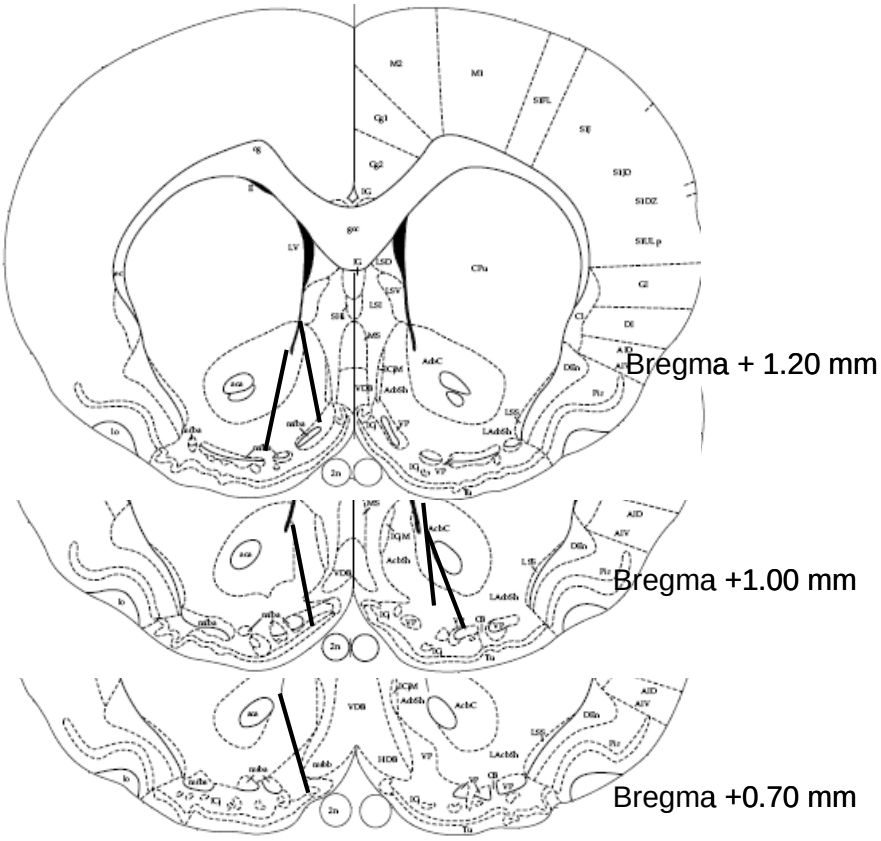
APPENDIX



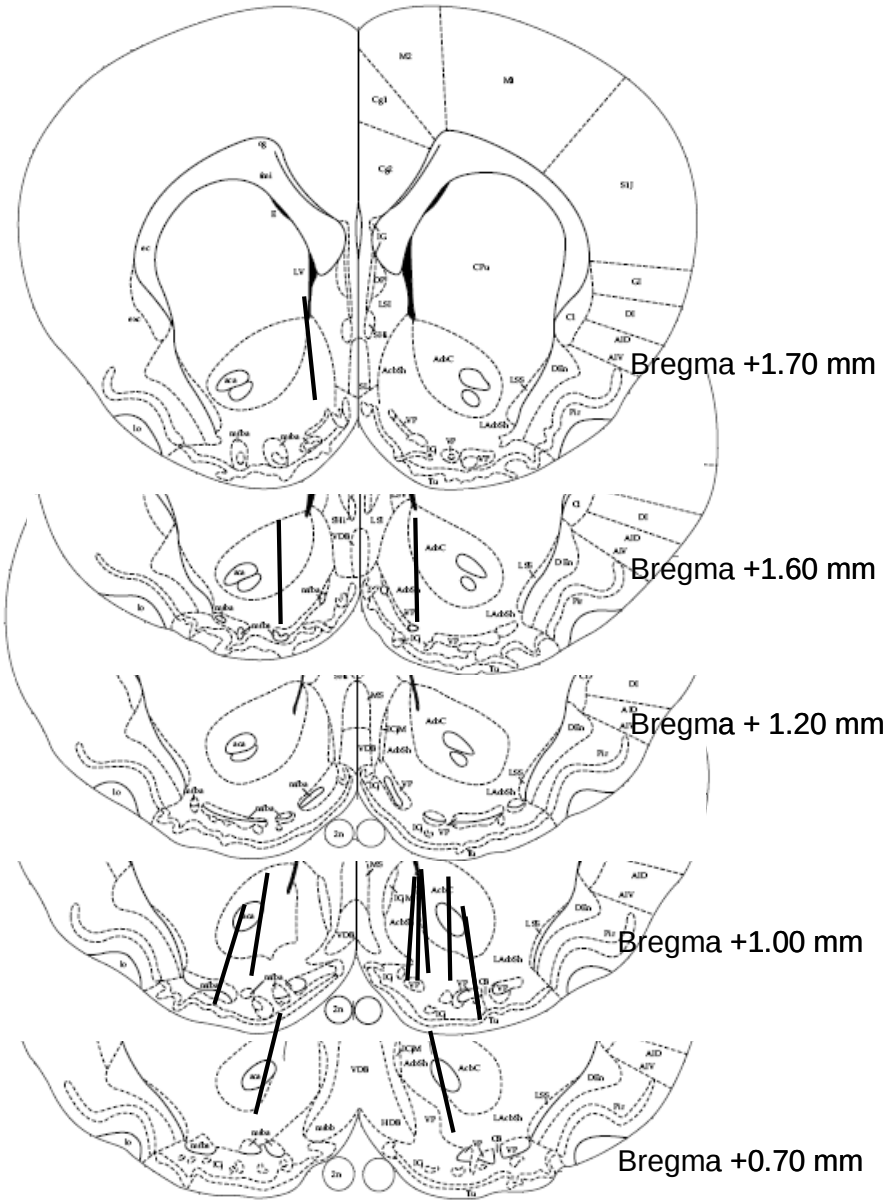
Appendix Image 1: Probe placements for leptin-amphetamine experiment.



Appendix Image 2: Probe placements for experiments showing the effects of leptin on DA metabolism and COC



Appendix Image 3: Probe placements for microinjection experiments.



Appendix Image 4: Placement of probes for NNF experiment.