

Quantitative microdialysis using stable-isotope labeled neurotransmitters improves efficiency and accuracy of neurochemical measurements

by

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## **Dedication**

To my wife Hillary, who provided endless support  
and encouragement through this entire process.

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## Abstract

The brain controls many functions including memory, learning, and addiction. Being able to measure neurochemical changes associated with these processes can elucidate and provide insight into the neural mechanisms that are responsible for each function. The main technique used to sample from the central nervous system is microdialysis. Active tissue processes can affect the recovery of microdialysis probes which can be problematic when quantifying absolute neurotransmitter concentrations.

In this work, we develop a novel approach to calibrating microdialysis probes *in vivo* based on infusing stable-isotope labeled (SIL) neurotransmitters and measuring loss of SIL standard. This allows transport across the membrane and probe recovery to be calculated enabling quantitative measurements. Additionally, the use infusion of SIL compounds allows flux and metabolism of these neurotransmitters by different pathways to be monitored by measuring downstream metabolites.

Quantitative microdialysis is performed on dopamine (DA), glutamate (Glu), and the primary glutamate precursor, glutamine (Gln). By infusing  $^{13}\text{C}_6$ -DA and  $^{13}\text{C}_5$ -Glu through the probe, the extraction fraction and the apparent concentration ( $C_{\text{app}}$ ) were shown to equal that of no-net-flux (NNF), an established but slower quantitative microdialysis technique. This SIL microdialysis method is also demonstrated to be applicable under transient conditions unlike NNF.

This new microdialysis technique was used to measure the effect of acute leptin on DA in the nucleus accumbens of rats.  $C_{\text{app}}^{\text{DA}}$  in the nucleus accumbens decreased by  $30 \pm 5\%$  after an

injection of leptin but did not decrease after a vehicle injection. The cause of this decrease was further investigated by administering raclopride, a D<sub>2</sub> antagonist, to rats that received vehicle or leptin. These experiments provide evidence that leptin increases uptake.

SIL microdialysis was also used to measure neuronal Glu release by monitoring the formation of <sup>13</sup>C<sub>5</sub>-Glu from <sup>13</sup>C<sub>5</sub>-Gln. Glu is preferentially formed from Gln in the neuron so the <sup>13</sup>C<sub>5</sub>-Glu produced should be neuronally derived. Administration of tetrodotoxin and metabotropic glutamate receptors decreased the <sup>13</sup>C<sub>5</sub>-Glu by 60 ± 7% and 56 ± 10%, respectively. An increase in <sup>13</sup>C<sub>5</sub>-Glu was detected when a stressor (tail pinch) was applied to the animal. These tests indicate that the <sup>13</sup>C<sub>5</sub>-Glu is neuronally derived.

## Chapter I

### Introduction

Measuring neurochemicals in the central nervous system can give insight into the mechanisms of cognition, mental disorders, addictions, and many other mental processes. Studies based on such measurements can be important in developing cures, preventative measures, and therapeutics for various diseases and addictions. A common approach in early studies on CNS neurochemistry was to remove the brain and measure tissue content of chemicals or prepare slices for monitoring chemical release *ex vivo*.<sup>1-3</sup> While useful, these studies are limited. The interactions of brain circuits cannot be accounted for and behavioral correlation with neurochemistry is not possible. Techniques have since been developed to measure chemicals from the brains of conscious, intact animals. These analytical methods provide additional information to brain slice studies that were previously performed. Achieving such measurements requires high spatial and temporal resolution, sensitivity, and selectivity.

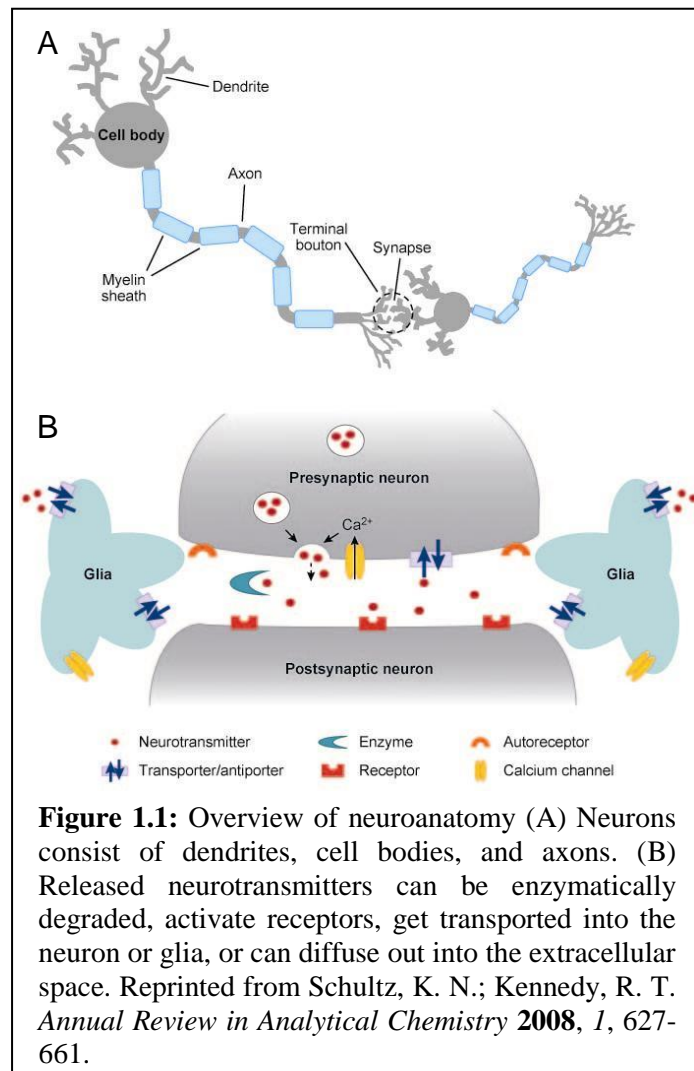
#### *Neurotransmission overview*

Neurons, which consist of dendrites, a cell body, and an axon, are the primary information units of the central nervous system. Communication between neurons is performed mainly through chemicals called neurotransmitters. Neurotransmitters can be small molecules, such as glutamate (Glu) and dopamine (DA), or larger neuropeptides, such as enkephalins. Axons from one neuron and juxtaposed cells (neuronal, muscular, or glandular) form synapses that enable chemical or electrical communication. The space between neurons in a synapse is the

synaptic cleft (~100 nm wide). Once released into the synaptic cleft, neurotransmitters can bind and activate receptors in the synapse on either pre- or post-synaptic neurons. Neurotransmitter molecules may also diffuse away from the synaptic cleft contributing to “overflow” or spill over where they may activate receptors more distal to the synapse. To terminate the signal,

neurotransmitters may be taken into the intracellular space via transporter proteins or degraded by enzymes. (Fig. 1.1)

In classical neurotransmission, the neuron is held at a resting potential of -70 mV with a sodium-potassium pump. Dendrites gather excitatory or inhibitory signals on the membrane from neurotransmitters activating receptors on the dendrites. If the sum of the inputs collected by the dendrites causes the membrane to reach a threshold potential of around -55 mV, an action potential is initiated. The



action potential propagates down the axon to the axon terminal. When the action potential reaches the axon terminal, voltage-gated  $\text{Ca}^{2+}$  channels open. The influx of  $\text{Ca}^{2+}$  causes vesicles loaded with neurotransmitters to fuse with the membrane and release neurotransmitters into the synapse.

The communication between neurons is mediated by gap junctions and, more commonly, activation of receptors by neurotransmitters. Many different types of receptors are located on distinct areas of both neurons and astrocytes. In this thesis, DA and Glu receptors will be discussed. Five different DA receptors have been identified and designated D<sub>1-5</sub>. D<sub>1</sub> and D<sub>5</sub> receptors share similar structures in their trans-membrane domains and are classified as D<sub>1</sub>-like family. These receptors activate adenylyl cyclase. Activating D<sub>1</sub> receptors has been shown to be involved in reward seeking behavior. For example, administering a D<sub>1</sub> agonist systemically decreased the reinstatement of drug seeking behavior following a cocaine prime.<sup>4</sup> D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub> receptors belong to the D<sub>2</sub>-like family which, when activated, inhibit the formation of cAMP by inhibiting the enzyme adenylyl cyclase.<sup>5</sup> D<sub>2</sub> receptors are located on the presynaptic and post-synaptic neuron. The presynaptic D<sub>2</sub> receptor acts in a modulator function, affecting synthesis, storage, and release of DA through feedback mechanisms.<sup>6</sup> The post-synaptic D<sub>2</sub> receptor transmits inhibitory or excitatory signal to the neuron. Because D<sub>2</sub> receptors can act as modulators of DA, blocking D<sub>2</sub> receptors can stop the negative feedback of presynaptic neurons caused by extracellular DA.<sup>7-10</sup> This mechanism is exemplified in multiple studies including one where raclopride (1.2 mg/kg, i.p.), a D<sub>2</sub> antagonist, was administered to a rat while DA was measured in the nucleus accumbens (NAc). This dose of raclopride increased extracellular DA by 250%.<sup>7</sup>

Two classes of Glu receptors have been identified, ionic Glu receptors, which mediates fast synaptic transmission, and metabotropic Glu receptors (mGluR), which couple to G-proteins and are responsible for modulating slow synaptic transmission.<sup>8,9</sup> The different types of ionotropic glutamate receptors are 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA), kainite, and N-methyl-D-aspartate (NMDA) receptors.<sup>8</sup> When Glu binds to these

receptors, an ion channel opens where  $\text{Na}^+$  and  $\text{K}^+$  can cross the membrane which can induce an excitatory postsynaptic current. 3 groups of mGluRs have been discovered: group I, group II, and group III. In rat hippocampus slices, group II and group III mGluRs were shown to be localized mainly to the presynaptic neuron, while group I was located in the postsynaptic neuron.<sup>10</sup> Group II mGluRs appear to act as autoreceptors, decreasing Glu release in synaptosomes and electrically stimulated Glu release in the striatum of rats when activated.<sup>11, 12</sup>

#### *Importance of measuring Glu in the central nervous system*

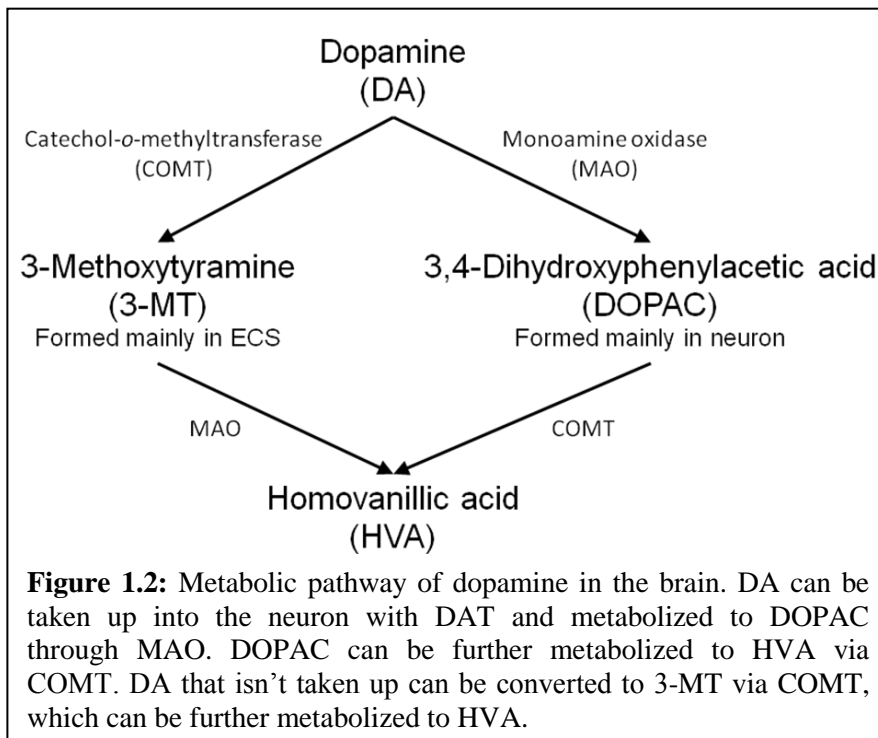
Glu is the main excitatory neurotransmitter found in the brain. Several disorders can arise when Glu is not regulated correctly. Thus, measuring extracellular Glu can provide information on the cause of these disorders and how to better treat them. For example, an excess of Glu in the medial prefrontal cortex has been linked with schizophrenia<sup>13-15</sup> and an excess of Glu in the hippocampus has been associated with epilepsy.<sup>13, 16</sup> Administering a group II mGluR agonist (lowers extracellular Glu concentrations in brain) reduced seizures in epileptic models of mice<sup>17</sup> and decreased schizophrenic symptoms in humans.<sup>18</sup>

Glu has also been shown to be an integral part in drug seeking behavior. In a previous study, rats were allowed to press a lever to self administer cocaine daily for 5 days. The reward seeking behavior was then extinguished by substituting saline for cocaine. Rats then had a DA receptor antagonist (fluphenazine) or an AMPA receptor antagonist (decreases extracellular Glu) microinjected into the NAc prior to a systemic injection of cocaine. Rats that received the DA receptor antagonist produced the same lever presses as before, while rats that received AMPA receptor antagonist had a reduction in lever presses.<sup>19</sup> This study showed that Glu, and not DA, was responsible for the reinstatement behavior.

These studies relied on measuring Glu dependent behavioral changes and Glu concentrations in tissue slices. Measuring Glu in an intact, conscious animal can lead to better therapeutics for diseases like schizophrenia, epilepsy, and addiction. Analytical techniques capable of making these measurements are necessary to gain further understanding of the role of Glu in the brain.

Like Glu, DA is associated with addiction and schizophrenia, along with a number of other functions. DA is considered to be the main “reward” neurotransmitter in the brain. Understanding how it is regulated can aid in interpreting extracellular DA measurements and can provide insight into incentive salience, reward seeking behavior, and therapies for diseases such as Parkinson’s disease.

*Role of DA in the central nervous system*



DA is regulated differently than Glu. In addition to point-to-point communication between dopaminergic neurons, DA synapses can be more “open” compared to Glu synapses in that they rely on diffusing to neighboring synapses for neural communication.<sup>20</sup>

Once released from a neuron, DA can reach different metabolic fates. It can get taken up by the

neuron via the DA active transporter (DAT) where it can be repackaged in vesicles for release later, or be metabolized into 3,4-dihydroxyphenylacetic acid (DOPAC) via the monoamine oxidase (MAO) enzyme (Fig. 1.2). DOPAC can be further metabolized to homovanillic acid via catechol-*O*-methyltransferase (COMT). DA that is not taken up into the neuron can be metabolized to 3-methoxytyramine (3-MT) by COMT in the extracellular space. 3-MT can be metabolized to HVA via MAO.

Dopaminergic neurons are found in, but not limited to, the midbrain, hypothalamus, and olfactory bulb.<sup>21</sup> Multiple diseases arise when DA is not regulated correctly, including a lack of DA in the striatum causes Parkinson's disease. Symptoms can be alleviated with L-DOPA (precursor to DA) treatments.<sup>22</sup> One cause of schizophrenia is believed to be over activation of DA receptors. Antipsychotics, which block D<sub>2</sub> receptors, are the most common treatment for schizophrenia.

DA is also associated with addiction and reward seeking behavior. In a review by Robinson and Berridge, the authors hypothesize that DA in brain regions associated with drug "wanting" are responsible for addiction. Sensitization of the mesotelencephalic DA system, associated with "wanting," caused by repeated drug use increases the incentive salience of stimuli associated with the drug.<sup>23</sup> As a consequence of sensitization, normal "wanting" can become compulsive drug craving.

Multiple studies have provided evidence that this theory is correct. For example, in one report rats were given 4 daily injections of amphetamine (increases DA in the mesotelencephalic system) or saline. Amphetamine was then given to both groups on day 5. The amphetamine treated rats showed greater behavioral sensitization compared to saline treated rats. When the rats were allowed to self-administer amphetamine following daily injections, the rats that received



amphetamine self-administered more than the saline treated rats.<sup>24</sup> This supports the theory because sensitization elicited greater self administration. All rats had been exposed to the amphetamine but the sensitized rats “craved” the drug more.

In addition to drug addiction, DA is also important in feeding behavior. Transgenic mice that cannot produce DA exhibited low locomotor activity and did not feed. These mice usually died within a few weeks. When given chronic injections of L-DOPA, the mice exhibited behavior close to wild-type mice.<sup>25</sup> In a separate study, increasing DA in the NAc of rats by microinjecting of amphetamine into the NAc increased the incentive salience of sucrose.<sup>26</sup> Although DA is important for feeding, it is not the only factor. Feeding is a complex behavior and understanding the interactions that DA plays with other neurotransmitters, neuropeptides, and hormones, may help provide information to why feeding can become irregular (e.g. anorexia and obesity).

#### *Leptin affects DA and reward circuitry*

One hormone that may be involved in regulating DA signaling is leptin. Leptin has generated interest due to the profound effects of the absence of leptin or leptin receptors on feeding behavior. Leptin deficient mice (*ob/ob*) exhibited obesity and low energy expenditure.<sup>27</sup> The same is true with leptin receptor deficient mice (*db/db*).<sup>28, 29</sup> When treated with leptin, *ob/ob* mice exhibited phenotypes closer to wild-type mice, but *db/db* mice did not.<sup>30, 31</sup> In humans, people born with congenital leptin deficiencies tend to be overweight. Chronic treatment with leptin increased energy output and reduced body fat in a human with this leptin deficiency.<sup>32</sup> Studying where leptin receptors are located and how these areas affect feeding can lead to a better understanding of obesity.

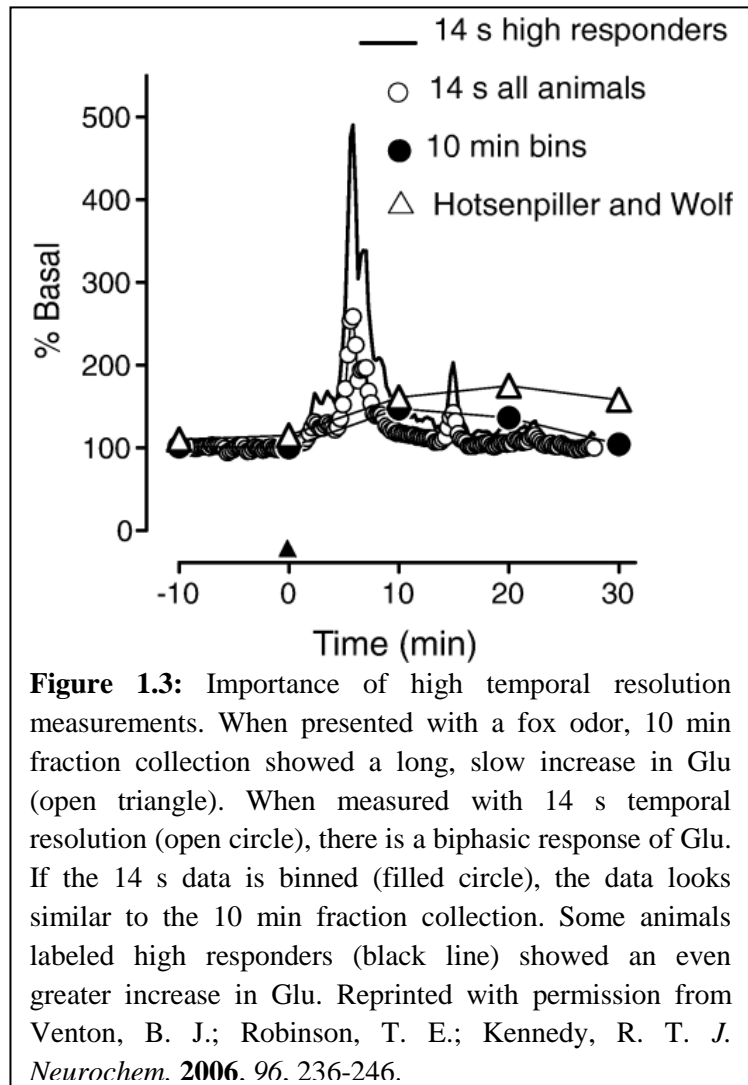
Leptin receptors have been found in multiple brain areas, including the lateral hypothalamus (LH), ventral tegmental area (VTA), and brain stem.<sup>33-35</sup> Using patch-clamp electrophysiological measurements, leptin was shown to depolarize 34% of leptin receptor containing neurons in the LH area.<sup>36</sup> Fluorescent tagging of neurons with leptin receptors in the LH revealed that these neurons innervated the VTA, but did not innervate the NAc.<sup>34, 36</sup> Although no leptin receptor containing neurons from the LH area innervate the NAc, neurons in the VTA, which are affected by leptin, have been demonstrated to innervate the NAc.<sup>37</sup> These brain areas are often associated with reward and reinforcement.<sup>23, 38</sup> Measuring how leptin affects DA in these areas may provide insight into how leptin alters feeding behavior. In this dissertation, we use novel techniques to further study how leptin interacts with the DA system as discussed in chapter 3.

Understanding how neurotransmitters like DA and Glu affect feeding, addiction, disease, and cognition can provide insight into cures, preventative measures, and a more general understanding of the brain. Behavioral measurements alone yield a limited amount of information; however, being able to quantify these neurochemical changes in intact brains is hampered by large challenges and methodological requirements.

#### *Challenges in measuring neurochemicals*

One challenge with measuring neurochemicals in rodents is the physical size of the brains. Further, the brain is a heterogeneous structure with discrete regions. Neurochemical events can occur in specific areas that can be 0.5 mm<sup>3</sup> or smaller. Therefore, the spatial resolution of the sensor or probe used has to be adequate for measuring from the area of interest exclusively without interference from other brain structures.

In addition to using small probes, neurotransmitters should be measured with a high temporal resolution. Neurochemical events can occur on the ms timescale (e.g. exocytosis) up to the month timescale (e.g. addiction). In order to gain as much information as possible, making measurements at high temporal resolutions can measure changes that would otherwise be unseen with slower measurements. For example, when a fox odor was presented to rats, a long, slow increase in Glu was



detected when measured with a 10 min temporal resolution (Fig. 1.3).<sup>39</sup> With a 14 s temporal resolution, Glu had a rapid biphasic response to fox odor which was not detected in the slower method.<sup>40</sup>

Sensitivity is also an issue when measuring from the brain. Neurotransmitter concentrations range from pM to mM. The detection method must be sensitive in order to measure the analyte of interest. Finally, high selectivity is required. The brain consists of, but is not limited to, tissue, blood, proteins, enzymes, and neurotransmitters. Being able to differentiate

select neurotransmitters from other chemicals in the brain is necessary for performing accurate measurements.

A problem specific to Glu measurements is the pool of extracellular Glu in the ECS can be derived from both neurons<sup>12,41</sup> and astrocytes<sup>42-45</sup>. Glu is highly regulated in the brain due to its neurotoxicity at higher concentrations.<sup>46,47</sup> To prevent neurotoxic Glu concentrations, astrocytes rapidly clear Glu via excitatory amino-acid transporters (EAATs) and convert the Glu to glutamine (Gln).<sup>48-50</sup> A technique that can measure Glu derived specifically from the neuronal pool is required to better understand diseases and addictions caused by abnormalities in neuronal Glu regulation.

Being able to distinguish the neurotransmitter of interest from the other chemicals in the brain with high sensitivity is crucial for accurate measurements. Several methods have been developed that attempt to meet these criteria. These methods include both non-sampling and sampling techniques.

#### *Non-sampling methods for measuring DA*

One approach to studying DA in the brain is positron emission tomography (PET). In PET, radiotracers are injected into the subject. The emission of  $\gamma$  rays from the radioactive decay of the radiotracer are monitored.<sup>51,52</sup> The largest advantage with PET is that it is non-invasive.

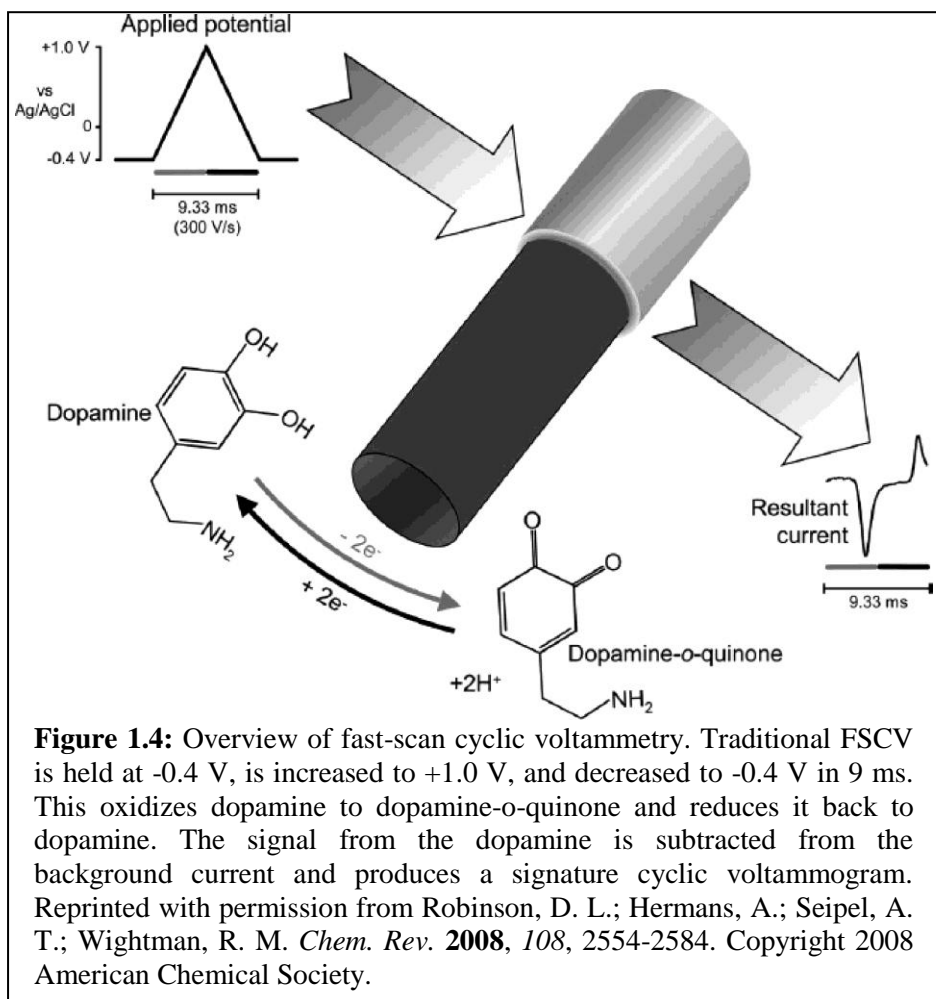
Although DA cannot be directly measured with PET, dopaminergic receptor activity can be monitored. For example, the concentration of extracellular DA in the brain can be correlated to the binding of <sup>11</sup>C-raclopride (D<sub>2</sub> antagonist) to D<sub>2</sub> receptors. This method was used to demonstrate that patients with schizophrenia had a greater decrease in <sup>11</sup>C-raclopride binding than healthy patients after an injection (0.2 mg/kg, i.v.) of amphetamine.<sup>53</sup> Because <sup>11</sup>C-raclopride binding decreased more in schizophrenic patients, then more DA must have been

released. The extra DA occupies more D<sub>2</sub> receptors and less <sup>11</sup>C-raclopride is able to bind to them.

Although a non-invasive approach seems ideal for measuring neurochemical events, PET has many disadvantages. Currently, the radiotracers available are limited. This translates to a low amount of neurochemical systems, namely DA and serotonin (5-HT)<sup>54</sup>, that can be measured with PET. Secondly, the spatial resolution is low. Typical human PET scanners have spatial resolutions of about 4 mm<sup>3</sup>.<sup>52</sup> While acceptable for studies on larger subjects, it is problematic for small rodents. Additionally, PET requires the head to remain still, so PET on rodents is typically done under anesthesia, which precludes correlation with behavior. Further, the anesthesia can affect neurochemistry and receptor-ligand interactions.<sup>55</sup> It should be noted that recent advances have been made in PET technology that are overcoming these limitations. For example, a small, portable PET detector (named RatCAP) has been developed that allows PET scanning of awake, conscious rats.<sup>56</sup> The RatCAP is fixed on the head of the rat and has a counterweight to allow the rat to freely move. Measurements that require higher spatial or temporal resolution often rely on other techniques, such as electrochemical detection (ECD).

ECD is a highly sensitive approach that can be performed on short timescales. DA and other catecholamines are electrochemically active and thus good targets for electrochemical detection. The oxidation of DA to dopamine-o-quinone occurs at about 0.6 V and is reduced back to DA at -0.2 V vs. Ag/AgCl electrode.<sup>57, 58</sup>

Using ECD to measure changes in the brain was pioneered in the 1970s.<sup>59</sup> Efforts have since been made to increase the sensitivity and selectivity of electrochemical detectors.<sup>60, 61</sup> Perhaps the most sensitive detection method with the highest temporal resolution and high spatial resolution is fast-scan cyclic voltammetry (FSCV). In traditional FSCV, voltage is



increased linearly from -0.4 to 1.0 V and back to -0.4 V across a carbon-fiber microelectrode in a total of 9.3 ms (Fig. 1.4). Each voltage is ramp performed 100 ms apart. This high scan rate produces a large charging background current. The current measured from DA is subtracted

from the background current to provide the signature DA cyclic voltammogram.<sup>62</sup> With FSCV, measuring fast neurochemical events such as exocytosis is possible.

FSCV has provided great insight into the neuronal DA system, particularly the reward circuitry of the brain. As an example of the power of FSCV, a previous study demonstrated the effect of DA release during the anticipation and administration of a cocaine injection. This effect was demonstrated by conditioning rats to activate a lever to self-administer cocaine. During these lever presses, DA was measured via FSCV. The authors detected a small increase in DA in the NAc  $7.7 \pm 0.6$  s prior to pressing the lever and receiving cocaine. After the cocaine injection, DA increased even more, producing a 2 phase release of DA.<sup>63</sup> Additionally, when the cocaine and

lever press were associated with an audio-visual cue, DA increased at the presentation of the audio-visual cue without the injection of cocaine. Being able to measure DA release with behavior in this way has provided great information into mechanisms of drugs of abuse. While fast and sensitive, FSCV has a number of disadvantages associated with it. The selectivity of this method is problematic. Interferents, such as ascorbic acid (AA), norepinephrine (NE), DOPAC, and 5-HT, can obscure the signal produced by DA.<sup>64, 65</sup> To overcome this limitation, FSCV electrodes are placed in brain areas thought to have low concentrations of these interferents, such as the NAc.<sup>66</sup> Measured via other methods mentioned later, the concentration of each of these interferents may not be nominal.<sup>67, 68</sup>

Due to multiple interferents and a large charging background, basal concentrations of DA are difficult to obtain with FSCV. Slower electrochemical techniques have estimated basal DA concentrations to be 26 nM.<sup>69</sup> With the use of FSCV, basal DA concentrations have been estimated to range between 6 nM<sup>70</sup> to 2.5  $\mu$ M.<sup>71</sup> Recently, a new approach that averaged DA transients (subsecond fluctuations in DA levels) with FSCV estimated basal DA concentrations to be around 20 nM.<sup>72</sup> These problems with basal concentrations make FSCV an attractive method for measuring differential concentrations, but not absolute differences.

A third problem with FSCV is the inability to measure DA over long periods of time. With problems like electrode fouling and reference electrode drifting, measurements are restricted to about 90 s windows.<sup>72-75</sup> While excellent for measuring short chemical events, these limitations are prohibitive when measuring slow changes over longer periods of time.

FSCV is also limited to measuring mainly DA; however, recent advances have allowed FSCV to become more robust. With modified waveforms, FSCV can measure other neurochemicals such as adenosine<sup>76</sup> and 5-HT.<sup>75, 77</sup> Coating the electrodes with substances such

as Nafion can also be useful in excluding anions from reaching the electrode. Nafion coating has been used to prevent electrode fouling from 5-HIAA, a metabolite of 5-HT that can foul carbon-fiber microelectrodes.<sup>75</sup>

#### *Non-sampling methods for measuring Glu*

Like DA, electrochemical methods can be used for measuring Glu, which is not natively electroactive. To overcome this limitation, electrodes can be coated with L-glutamate oxidase (Glu-Ox).<sup>78</sup> The Glu-Ox produces H<sub>2</sub>O<sub>2</sub> which can be detected at the electrode. These probes sometimes have another electrode void of Glu-Ox called the sentinel electrode.<sup>41</sup> In these probes, the signal produced from the enzyme coated electrode is subtracted from the signal of the sentinel.

These electrodes have been used in several proof-of-concept studies for Glu measurements. The effect of a stressor (tail pinch) on Glu was measured in the striatum and the prefrontal cortex of rats.<sup>79-81</sup> After the tail pinch, Glu increased about 170% basal. These electrodes have shown impressive temporal resolution, which allows Glu transients to be measured. Glu transients were shown to increase in the basolateral amygdala (an area associated with reward seeking behavior) when rats were presented with a food reward after pressing a lever.<sup>82</sup> Additionally, Glu sensors can measure TTX induced decreases in extracellular Glu concentrations. Local application of TTX in the PFC decreased Glu concentration by ~40%.<sup>80</sup> These studies show the ability of Glu sensors to measure fast Glu events that are neuronally derived; however, a number of limitations are associated with these electrodes.

One disadvantage is the ability to only measure 1 neurotransmitter. Measuring multiple analytes can lead to discovering the involvement of unanticipated neurotransmitter systems. Additionally, although the sentinel electrode can help with removing signal from interferents, it



is still possible that electroactive species can cause artificial signals in the electrodes. For example,  $\text{H}_2\text{O}_2$  found in the brain can be an interferent.<sup>83</sup> Ascorbic acid has also been shown to decrease the electrode sensitivity by as much as 50%.<sup>83</sup> Lastly, Glu-Ox is an enzyme, and like all enzymes has different relative activities in different conditions, such as pH.<sup>84</sup> The current design of these microelectrodes cannot account for changes in enzyme activity in biological matrices.

Like the electrodes used to measure DA, controversies arise when comparing basal Glu measurements made by sensors to other techniques. Glu biosensors estimate extracellular Glu concentrations to be in the low  $\mu\text{M}$  levels.<sup>41, 79</sup> In hippocampal brain slices, basal Glu concentrations were estimated to be in the low nanomolar range ( $\sim 25 \text{ nM}$ ).<sup>85, 86</sup> These concentrations were estimated by measuring NMDA receptor currents. The discrepancies between biosensor and brain slices may be due to the damage each of the methods cause, differences in chemical makeup of the brain vs. a perfused buffer, or the presence of different compartments of Glu. In the latter case, each method may be measuring from different compartments.<sup>87</sup> Because of the multiple sources of Glu, the highly regulated system of Glu clearance, and the limitations of measuring techniques, quantifying Glu in the brain is difficult.

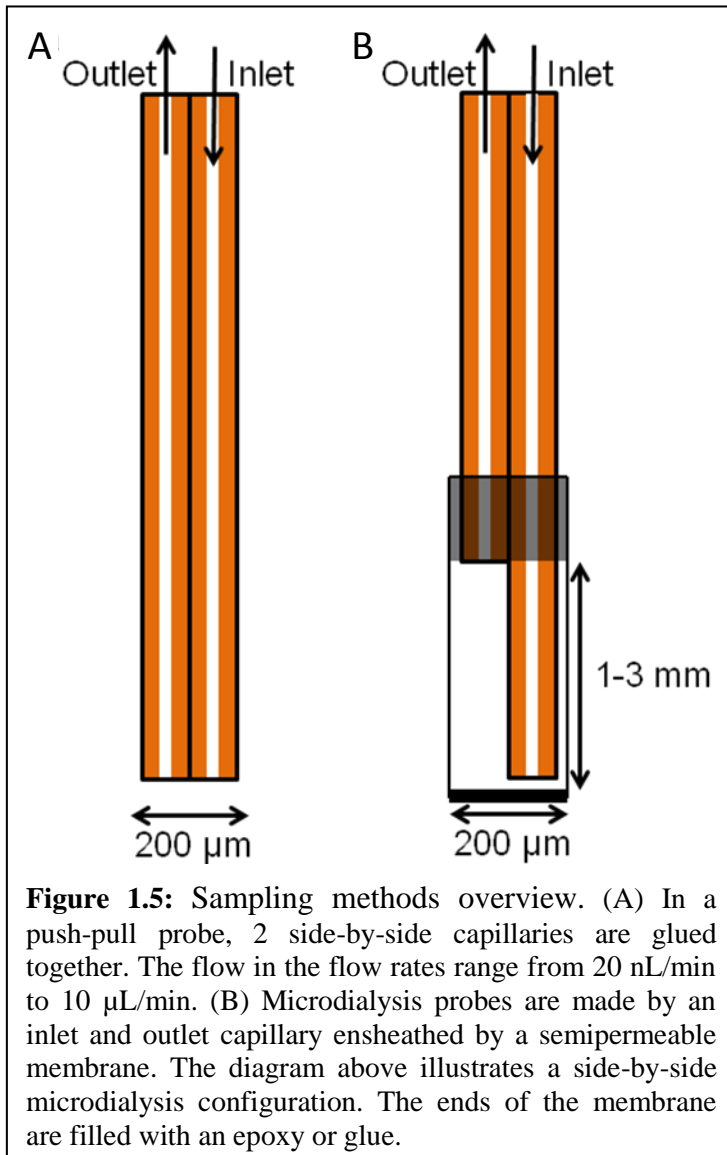
All of the non-sampling methods discussed above can measure only 1 to 2 analytes at a time. Additionally, these methods have difficulty measuring absolute concentrations. To gain complementary information about the central nervous system, sampling methods that do not have these limitations can be used to obtain a more complete picture of the brain.

#### *Sampling methods for measuring DA and Glu*

The main sampling technique for measuring DA and Glu is microdialysis. Microdialysis probes have a semi-permeable membrane which allows chemicals to diffuse down a concentration gradient in or out of the probe.<sup>88</sup> The semi-permeable membrane has a cutoff

weight usually between 20-100 kD<sup>89</sup>. At the lower molecular weight cutoffs, small molecules can diffuse across the membrane while larger proteins cannot (refer to Fig. 1.5).

The relative recovery (RR) of microdialysis is the fraction of analyte that enters the probe relative to the external concentration, which is dependent on the flow rate and size of the probe.



**Figure 1.5:** Sampling methods overview. (A) In a push-pull probe, 2 side-by-side capillaries are glued together. The flow in the flow rates range from 20 nL/min to 10 μL/min. (B) Microdialysis probes are made by an inlet and outlet capillary ensheathed by a semipermeable membrane. The diagram above illustrates a side-by-side microdialysis configuration. The ends of the membrane are filled with an epoxy or glue.

Artificial cerebral spinal fluid (aCSF) is perfused through microdialysis probes at flow rates ranging from 0.2 to 5 μL/min. The lower the flow rate, the higher the RR and the lower the absolute recovery (Fig. 1.6). RR is also affected by the active area of a microdialysis probe. Smaller active areas will recover lower concentrations.

Although not as popular as microdialysis, a second sampling technique for monitoring neurochemical changes is push-pull perfusion (PPP). This method involves directly extracting liquid

from the brain via a vacuum or in-line pump.<sup>90</sup> aCSF is also pumped in an adjacent fluid line to counteract changes in volume associated with removing fluid (Fig. 5). Conventional PPP uses flow rates of ~10 μL.<sup>90,91</sup> The high flow rates of conventional PPP can be problematic with

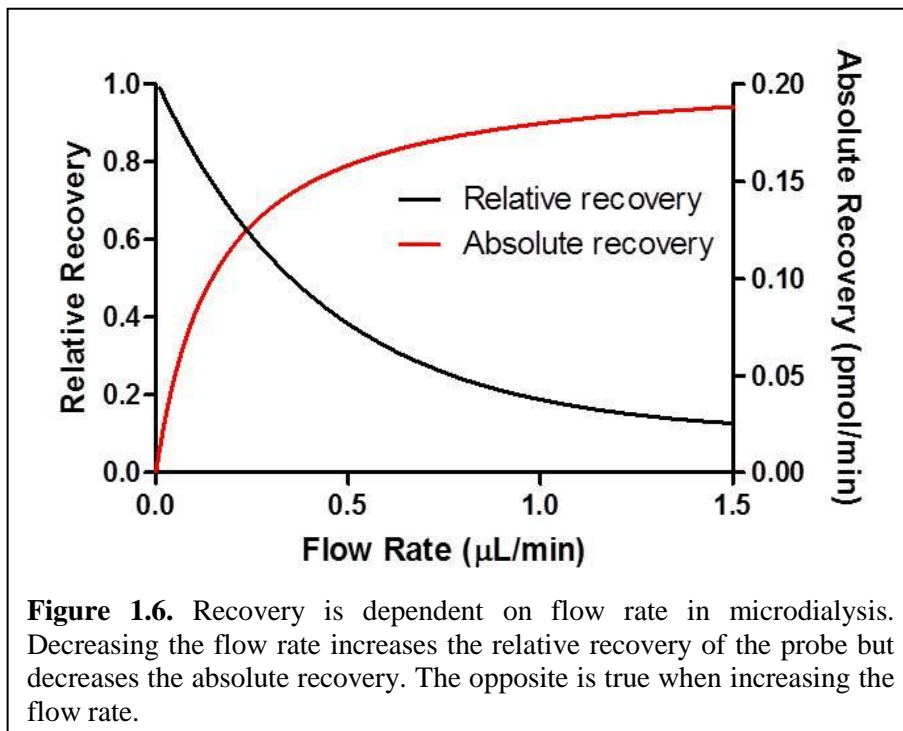
tissue damage. More recently, low-flow (20-50 nL/min) PPP has been implemented to reduce tissue damage.<sup>92, 93</sup> The surface area of a push-pull probe made of 2 side-by-side 40/100 (i.d./o.d.)  $\mu\text{m}$  capillaries is roughly 200 fold lower than a 2 mm x 200  $\mu\text{M}$  dialysis membrane.<sup>94</sup> While low-flow PPP seems attractive, microdialysis dominates the sampling methods for neurochemistry studies due to decreased complexity and greater commercial availability of microdialysis probes. Further discussion on sampling method will be focused on microdialysis.

With microdialysis, collected samples need to be analyzed by a method capable of measuring the neurotransmitters of interest. These analyses can either be performed offline or online.

*Offline analysis for microdialysis*

The most common method for offline analysis for the detection of DA, Glu, and their metabolites is HPLC coupled with fluorescence, electrochemical, or mass spectrometric detection. When using fluorescence, derivatization is required to detect DA and Glu in microdialysis samples.

Common fluorescent tags include o-phthalaldehyde (OPA)<sup>95-98</sup>, naphthalene-2,3-dicarboxyaldehyde (NDA)<sup>99</sup>, and 4-fluoro-7-nitro-2,1,3-benzoxadiazole



(NBD)<sup>100</sup>. These fluorescent tags react with primary amines which are found on both DA and Glu. While fluorescence is a good candidate to measure Glu, measuring DA via fluorescence can be difficult due to the low concentration of DA in the brain. Other disadvantages include the derivatization time required for a fluorescent tags. Some tags require reaction times of a couple min. When looking for high throughput analysis, this derivatization time can be prohibitive. Additionally, stability<sup>101</sup> of the derivatized product and photobleaching<sup>102</sup> can be problematic. When higher sensitivity or stability is required, other detection methods such as ECD may be more suitable than fluorescence.

HPLC coupled to ECD has been mainly used for measuring electroactive neurotransmitters such as DA, its metabolites, 5-HT, and NE.<sup>103</sup> HPLC-ECD can also be used to measure Glu. Because Glu is not electrochemically active, derivatization is required. Glu is commonly derivatized with OPA to make it electroactive.<sup>104, 105</sup> While derivatization allows more neurotransmitters to be detected, the stability and derivatization times of OPA can be problematic with HPLC. The tradeoff between measuring limited electroactive compounds or measuring a greater number of derivatized compounds with decreased stability makes HPLC-ECD an inefficient system. Mass spectrometers (MS), on the other hand, have the potential to detect a broad number of analytes with high sensitivity. HPLC-MS has recently gained popularity as a method for detecting neurotransmitters in dialysate.<sup>106, 107</sup> Dialysate can be problematic in ESI sources due to the high salt content and the high degree of polarity of most neurotransmitters. By derivatizing dialysis samples with a non-polar derivatizing agent, polar neurotransmitters are amenable to reversed phase HPLC. The work in this dissertation uses benzoyl chloride (BzCl) to enhance separation and detection on an HPLC-MS/MS system. With this assay, DA, DOPAC, 3-MT, HVA, Glu, Gln, and 11 other compounds can be measured from

a single dialysis sample.<sup>68</sup> The limits of detection for this method were measured to be on the nM scale.<sup>68</sup> The low limit of detection combined with the large number of neurotransmitters that can be detected in each sample allow monitoring of multiple systems in the brain simultaneously. Additionally, stable-isotope labeled (SIL) neurotransmitters can be differentiated from the endogenous species with MS analysis. Measuring SIL neurotransmitters can lead to information on metabolism and quantification of endogenous neurotransmitters.

Although HPLC is a robust and comprehensive separation method, several drawbacks exist. One large disadvantage is the time required per separation, which is typically several min. A less popular but faster separation method used for offline analysis is capillary electrophoresis (CE).<sup>108, 109</sup> CE separates molecules based on size and charge in an applied electric field. The ions move in the direction of electroosmotic flow. Small, positive ions elute out first, followed by large positive ions, neutral molecules, large negative ions, and then small negative ions. The methods of detection for CE are the same as HPLC, the most common being fluorescence.

Offline analysis has the advantage of being able to collect samples from multiple animals at the same time, which allows for high-throughput analysis. A large disadvantage of offline analysis is the inability to make measurements with high temporal resolution. The number of samples collected increases exponentially with decreasing collection times. When high temporal resolution is required, online analysis is a better suited method.

#### *Online analysis of microdialysis*

Methods for measuring neurotransmitters online include direct infusion into an ESI-MS,<sup>110</sup> CE separation,<sup>40, 111, 112</sup> or enzymatic assay.<sup>93</sup> Direct infusion of dialysate into an ESI-MS has been used to measure acetylcholine (a neurotransmitter)<sup>110</sup> and enkephalins (neuropeptides).<sup>113</sup> The temporal resolution for direct infusion into an ESI-MS has been

measured to be as low as 5 s.<sup>110</sup> This system utilized segmented flow with droplet sizes of 160 nL. The low sample size allows for the high temporal resolution which is hard to achieve with offline analysis which usually require samples on the  $\mu\text{L}$  scale. A main drawback is that the high salt content of microdialysis samples can be problematic for ESI. Another limitation is that the cost of an MS can be prohibitive, although commercial availability of cost efficient systems is increasing. Lastly, the analytes measured with the MS have to be ionized easily.

Another technique used for online analysis is fast CE. This separation technique is useful for monitoring multiple neurotransmitters within each sample.<sup>12, 40, 96, 112, 114-117</sup> Online CE separation can be coupled to a fluorescence detector,<sup>40, 96, 112</sup> ECD,<sup>117, 118</sup> or MS.<sup>119, 120</sup> CE coupled to laser-induced fluorescence (CE-LIF) is the most popular CE method with microdialysis. ECD is limited to electroactive compounds, and coupling CE separation buffers with ESI can be difficult. The temporal resolutions obtained with microdialysis coupled to CE-LIF can reach temporal resolutions on the s timescale. Using OPA as a fluorescent tag, Glu, Gln, and DA along with 5 other neurotransmitters have been separated within 20 s.<sup>112</sup> With overlapped injection on this system, the separation time can be reduced to 14 s.<sup>40, 121</sup> Although DA was separated and detected, the limit of detection for DA was higher than the DA concentration found in the dialysate. Other CE-LIF systems have been developed that are sensitive enough to measure DA in dialysis samples. Using NDA as the fluorescent tag, DA in dialysate was able to be detected in a 90 s separation using micellar electrokinetic chromatography (MEKC)-LIF system.<sup>122</sup> MEKC is CE with micelles in the separation buffer to act as a pseudo-stationary phase. In this system, Glu and 10 other amino acids could be measured. With MEKC-LIF, amphetamine induced increases in DA were greater in the NAc of rats treated with leptin vs. rats treated with a vehicle injection.<sup>123</sup> The increased DA stimulated

release was caused by an increase in DAT activity and an increase in DA release. This work with leptin is expanded upon in chapter 3.

Microdialysis has a large advantage in being a robust technique. The broad range of analytical methods available to measure neurotransmitters in dialysis samples allows the analysis to be tailored to the requirements of an experiment. Like all techniques for measuring neurotransmitters, microdialysis does have several limitations.

#### *Limitations of microdialysis*

One limitation of microdialysis is the temporal resolution. Most microdialysis experiments have temporal resolution on the min timescale. By using a segmented flow, the highest temporal resolution that has been achieved with microdialysis is 2 s.<sup>109</sup> These values are high compared to electrodes which can reach 100 ms temporal resolution.

One large disadvantage for microdialysis measurements is the difficulty in measuring neuronally derived Glu. Basal Glu levels are insensitive to TTX when measured by microdialysis.<sup>124</sup> The insensitivity of Glu is likely due to the multiple sources of Glu as well as the rapid uptake and regulation of Glu. Astrocytic Glu release has been shown to be a large contributor of basal Glu levels measured by microdialysis. Blocking the cystine-Glu antiporter, found on astrocytes, decreased basal extracellular concentrations of Glu in the striatum.<sup>42</sup> Although not TTX sensitive under basal conditions, microdialysis has been able to measure TTX sensitivity of electrically stimulated Glu<sup>12</sup> and 4-aminopyridine (prolongs opening of K<sup>+</sup> channels) stimulated Glu.<sup>125</sup> These studies give evidence that viable Glu neurons exist within the vicinity of the sampling area of the probe; however, microdialysis studies of Glu are largely hampered by these problems.

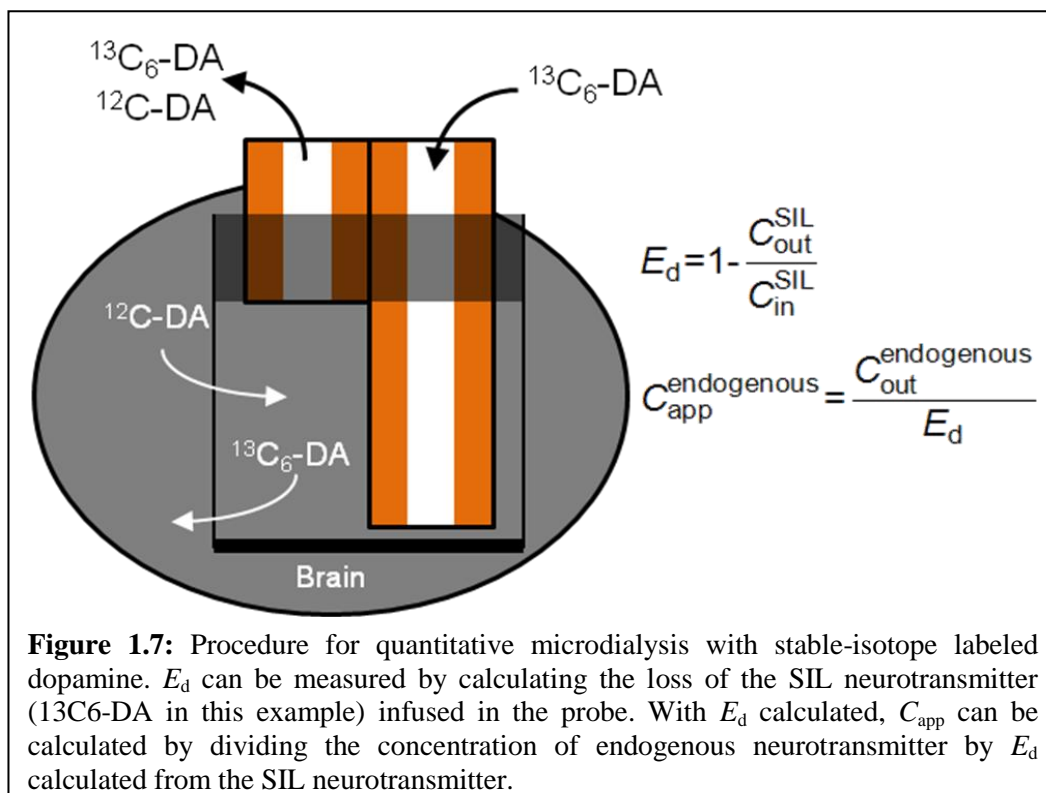
Lastly, making quantitative measurements with microdialysis can be difficult. Active tissue processes, such as uptake, metabolism, synthesis, and release, can alter the recovery of probes *in vivo*.<sup>126</sup> If the recovery is unknown, an accurate measurement of extracellular neurotransmitter concentrations cannot be obtained. Instead, most microdialysis experiments rely on approximations. Some quantitative microdialysis experiments have been developed to account for active tissue processes.

#### *Quantitative microdialysis techniques*

Neurotransmitter concentrations can be approximated by measuring the recovery of the probe *in-vitro* in a well-stirred vial and then applying that recovery to the probe *in vivo*; however, the recovery of the probe *in vitro* may not always match the recovery *in vivo*.<sup>127, 128</sup> Techniques have been developed to measure neurotransmitters quantitatively. These include no-net-flux (NNF), low-flow perfusion, extrapolation to zero flow, and SIL microdialysis. Chapter 2 covers quantitative microdialysis techniques in more detail. Briefly, low-flow perfusion involves decreasing the flow rate of a dialysis probe to the point that recovery approaches 100%.<sup>129</sup> The main disadvantage is the collection times required for slow flow rates. Additionally, the measurements are usually done in steady-state conditions before or after an experiment. This can be problematic if the recovery changes during the experiment. These limitations have hampered the application of low-flow perfusion.

Rather than directly measuring the concentration when recovery approaches 100%, the concentration in the brain (apparent concentration,  $C_{app}$ ) can be quantified by varying the flow rate and extrapolating to a no-flow condition.<sup>129</sup> This approach is time consuming and requires steady-state conditions.





The calibration technique most commonly used is NNF. With this method, varying

concentrations of the analyte of interest are perfused through the probe ( $C_{in}$ ).<sup>130</sup> The concentration that is measured ( $C_{out}$ ) is subtracted from  $C_{in}$  to measure the flux of the analyte. When  $C_{in}-C_{out}$  is plotted vs.  $C_{in}$ , the x-intercept is the point of NNF, where the fraction of analyte recovered matches the fraction of analyte that diffused out of the probe. This point is the  $C_{app}$  of the analyte in the brain. The slope of the line is the extraction fraction ( $E_d$ ), the fraction of analyte that has diffused in and out of the probe.  $E_d$  can be calculated with the following equation:  $E_d=(C_{in}-C_{out})/(C_{in}-C_{app})$ . NNF has been used to show that blocking uptake lowers  $E_d$  of Glu<sup>131</sup> and DA.<sup>126</sup> Inhibiting synthesis, release, and metabolism of DA had no effect on  $E_d$ .<sup>126</sup> NNF is a slow method that requires steady-state conditions for the entirety of the calibration. Thus, changes in  $E_d$  over a short amount of time cannot be accounted for. A second type of NNF, called dynamic NNF (dNNF) has been used to measure  $C_{app}$  and  $E_d$  on a faster timescale. In dNNF, 1 concentration of an analyte is infused through the probe during an entire experiment.

Multiple concentrations are infused through the probes of multiple animals and the results are then pooled. This technique gives higher temporal resolution but requires more animals.<sup>132, 133</sup> Additionally, variations between probes cannot be accounted for.<sup>133</sup> The use of quantitative microdialysis has been hampered by the long time per calibration or the large amount of animals required for faster temporal resolution. These limitations have caused quantitative microdialysis techniques to be used for a small portion of experiments compared to uncalibrated microdialysis.

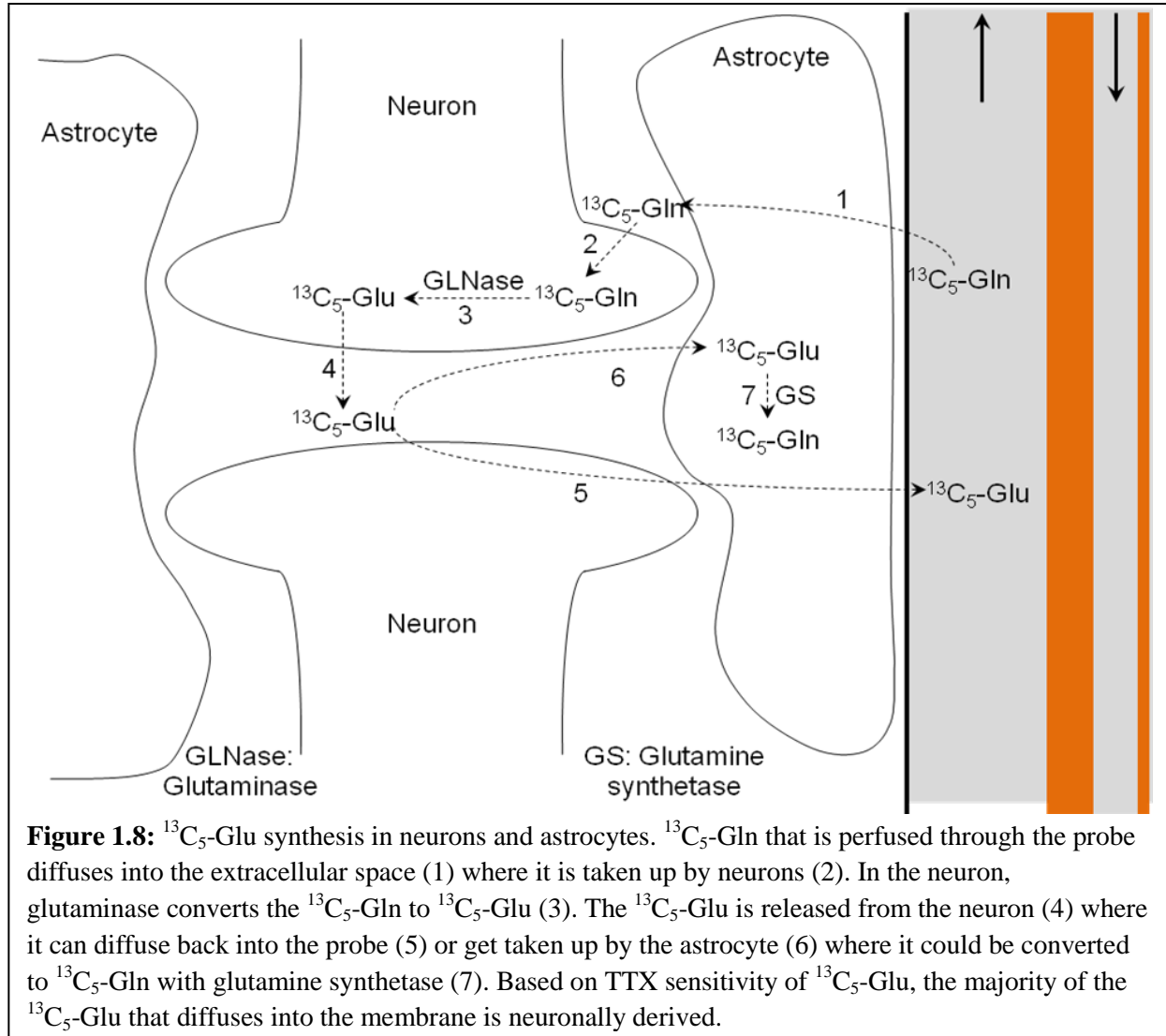
A faster, more efficient quantitative method is reverse microdialysis with a calibrator. With this method, a calibrant is perfused through the probe (Fig. 1.7). The calibrant can either be a homologous, biologically inactive species<sup>134</sup> or a stable-isotope labeled (SIL) standard.<sup>135, 136</sup> SIL analytes are ideal because they will undergo the same processes as the endogenous species.  $C_{app}$  of the SIL analyte in the brain is known to be 0.  $E_d$  can then be measured as the loss of the SIL analyte ( $E_d^{SIL} = 1 - C_{out}^{SIL}/C_{in}^{SIL}$ ). With  $E_d$  measured,  $C_{app}$  for the endogenous species can be calculated with ( $C_{app} = C_{out}^{endogenous}/E_d^{SIL}$ ). This technique has historically been used for measuring pharmacological drugs.<sup>134-137</sup> The application of SIL microdialysis will be discussed in chapter 2.

#### *Evidence for Glu compartmentalization in vivo*

In addition to making quantitative measurements, SIL microdialysis can be used to measure neuronal Glu. As stated above, measuring neuronal Glu release with microdialysis is difficult. In this thesis, we have developed an assay that can be used to measure neuronal Glu release (Fig. 1.8). Neuronal Glu is almost entirely derived from Gln with an enzyme called glutaminase, which is preferentially located within the neuron.<sup>138, 139</sup> By perfusing  $^{13}C_5$ -Gln through the probe, the conversion of  $^{13}C_5$ -Gln to  $^{13}C_5$ -Glu can be monitored with the BzCl derivatization and HPLC-MS/MS described previously (Fig. 7). This newly synthesized Glu

appears to be TTX sensitive, respond to mGluR agonists, and increases with a stressor. These responses indicate that the  $^{13}\text{C}_5\text{-Glu}$  measured is neuronally derived.

The ability to measure neurochemicals accurately in the central nervous system can be a



valuable tool for understanding how the brain works. This thesis focuses on developing microdialysis to become more efficient and accurate. Using SIL neurotransmitters, microdialysis was used quantitatively in studying drugs of abuse and the role of leptin and DA on feeding behavior. Additionally, SIL microdialysis allows for measuring neuronally derived basal Glu.

## Chapter II

### In vivo calibration of microdialysis using infusion of stable-isotope labeled neurotransmitters

#### Introduction

Neurotransmitter concentrations in the extracellular space represent the balance between release (e.g., by exocytosis and reverse transport) and removal (e.g., by reuptake and enzymatic degradation). Measurement of concentration dynamics in this space is valuable for understanding neuronal communication. Microdialysis is a popular approach for such measurements, but its use is hampered by the difficulty of quantifying neurotransmitter concentrations *in vivo*. In this study we report a novel approach to measuring *in vivo* recovery and quantification.

The recovered concentration of an analyte from a microdialysis probe ( $C_{\text{out}}$ ) is a complex function of concentration external to the probe ( $C_{\text{ext}}$ ) and transport into the probe. Relative recovery (RR), defined as  $C_{\text{out}}/C_{\text{ext}}$ , can be directly measured *in vitro* by fixing  $C_{\text{ext}}$  and measuring  $C_{\text{out}}$ . However, it has long been recognized that *in vitro* recovery, which is determined primarily by the probe, dialysis flow rate, analyte, and temperature, is not necessarily accurate *in vivo*.<sup>128, 133, 140</sup> In particular, factors such as tissue permeability, reuptake, and metabolism will affect recovery *in vivo*. For many experiments, only the relative change of neurotransmitter is of interest, and impact of probe recovery is not considered; however, measurement of *in vivo* recovery and quantification, defined as determining the apparent extracellular concentration ( $C_{\text{app}}$ ),<sup>141, 142</sup> can be crucial to obtaining meaningful results.<sup>126, 132, 133, 143-145</sup> Several methods for

*in vivo* calibration have been developed including low flow,<sup>146, 147</sup> no-net-flux (NNF or Lönnroth method)<sup>126, 130</sup> and reverse microdialysis methods.<sup>135-137</sup>

In the low flow method, the dialysis flow rate is reduced so that recovery is ~100%, making it, in principle, independent of external processes. A related alternative is to measure recovery at different flow rates and extrapolate to zero flow.<sup>129</sup> These methods are rarely used because they require long times (to either collect enough sample or to measure at different flow rates), but a recent innovation of using low flow to recover, then higher flow to pass sample to the analytical system overcomes this obstacle.<sup>148</sup>

The most common *in vivo* calibration method is NNF. In this method, several concentrations of analyte are infused into the dialysis probe ( $C_{in}$ ) while recording  $C_{out}$ .<sup>126, 130</sup> The difference ( $C_{in}-C_{out}$ ), which corresponds to flux across the probe membrane, is plotted against  $C_{in}$ , so that the x-intercept is the point where flux is zero. The point of NNF corresponds to  $C_{app}$ . The slope of the NNF line,  $(C_{in}-C_{out})/(C_{in}-C_{app})$ , is extraction fraction ( $E_d$ ), a measure of *in vivo* probe recovery. Providing a value for  $E_d$  is a strong advantage of the NNF method. For example, it has been shown that the  $E_d$  of DA ( $E_d^{DA}$ ) is relatively insensitive to inhibition of release, synthesis, and metabolism but strongly affected by uptake inhibition.<sup>126</sup> This insight allows  $E_d^{DA}$  to be an indirect measure of DA uptake *in vivo*. Knowledge of  $E_d$  can also be helpful in interpreting changes in  $C_{app}$ . If a drug or genetic manipulation evokes a change in  $C_{app}$ , it is not clear if the difference is related to altered release or reuptake; however, knowledge of  $E_d$  can aid interpretation. For example, acute ethanol was shown to increase DA concentration recovered in the nucleus accumbens. After showing no change in  $E_d^{DA}$ , the increase in  $C_{app}^{DA}$  could be attributed to increased DA release.<sup>133</sup>

Despite the appeal of NNF, it suffers from several disadvantages. By requiring infusion of several concentrations it is time consuming. Further, it assumes that recovery is constant over the course of the experiment; however, the recovery of the probe can change under different conditions. For example if uptake is inhibited by a systemic drug injection then uptake and recovery will change as the drug concentration changes.<sup>132</sup> This effect can be accounted for by using dynamic NNF (dNNF) in which one concentration of a neurotransmitter is perfused through the probe for one animal.<sup>132, 133</sup> By infusing different animals with different concentrations and then pooling animals at different concentrations, a NNF trace can be calculated for each time point. dNNF allows for the measurement of  $E_d$  and  $C_{app}$  under transient conditions; however, a large number of animals are required. Further, it assumes minimal probe variability and precludes observation of individual differences.

*In vivo* calibration can also be achieved by using reverse microdialysis. In this case, calibration is achieved before (or after) an experiment by infusing the target compound through the probe *in vivo* while  $C_{app}$  is known to be zero. Measuring  $C_{out}$ , allows a direct measurement of the loss or extraction of analyte by the sample (e.g., brain) and calculation of  $E_d$ . This value can be used to quantify results in that subject during an experiment. The method is readily used for exogenous chemicals, such as drugs; however, it cannot be used for neurotransmitters which cannot be removed from the brain to give a  $C_{app} = 0$ . This method also assumes that recovery is constant over the course of the experiment.

A variation of this method is to continuously infuse a similar but distinguishable form of the compound through the probe. Every fraction collected then allows determination of  $E_d$  of the infused compound (which has  $C_{app} = 0$ ) which can then be used to quantify the  $C_{app}$  for the target analyte. For example, ropivacaine was infused to measure  $E_d$  and then quantify bupivacaine,

which differs by 1 methyl group from ropivacaine.<sup>134</sup> This approach assumes that the modified form behaves identically to the actual analyte. A better standard to infuse is a stable-isotope labeled (SIL) form. In a SIL microdialysis experiment, the concentration of the labeled analyte in the brain ( $C_{app}^{SIL}$ ) = 0 so that  $E_d$  can be calculated directly:

$$E_d = 1 - \frac{C_{out}^{SIL}}{C_{in}^{SIL}} \quad (1)$$

Because  $E_d$  of the analyte is equal to  $E_d^{SIL}$  the apparent concentration can be calculated by:

$$C_{app} = \frac{C_{measured}^{endogenous}}{E_d^{SIL}} \quad (2)$$

where  $C_{measured}^{endogenous}$  is the concentration of endogenous analyte. This approach has been previously used with drugs. For example, d<sub>3</sub>-morphine and <sup>13</sup>C<sub>4</sub>-cortisone have been infused to monitor  $E_d$  of these drugs and quantify them *in vivo*.<sup>135, 136</sup>

Microdialysis with SIL neurotransmitters has not been reported but it would be expected to be especially useful because neurotransmitters are the most frequent target of microdialysis measurements. Reverse microdialysis of SIL neurotransmitters would also allow dynamic changes in  $E_d$  to be recorded. Here we describe the use of this method for DA and glutamate (GLU). <sup>13</sup>C forms of these compounds were infused constantly during experiments allowing calculation of  $E_d$  at every time point. At the same time, the endogenous <sup>12</sup>C form of each neurotransmitter was collected. The different forms of each compound were measured and distinguished using liquid chromatography-mass spectrometry. Comparison to NNF revealed that the SIL microdialysis method yields equivalent results. An important advantage of the SIL method is that it allows *in vivo* correction for *in vivo* extraction of every dialysate sample with no additional measurements in contrast to NNF.

## Methods

### *Chemicals and reagents*

All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Artificial cerebral spinal fluid (aCSF) comprised of 145 mM NaCl, 2.68 mM KCl, 1.01 mM MgSO<sub>4</sub>, 1.22 mM CaCl<sub>2</sub>, 1.55 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.45 mM NaH<sub>2</sub>PO<sub>4</sub> (Fisher Scientific, Pittsburgh, PA). <sup>13</sup>C<sub>6</sub>-DA was purchased from CDN isotopes (Quebec, Canada) and <sup>13</sup>C<sub>5</sub>-GLU was purchased from Cambridge Isotopes (Andover, MA). Mobile phase of the LC column included 10 mM ammonium formate and 0.15% formic acid. Cocaine hydrochloride was purchased from the University of Michigan Hospital (Ann Arbor, MI).

### *Microdialysis probes*

Probes were constructed as previously described.<sup>149</sup> In summary, 40/100 µm (i.d./o.d.) fused silica capillaries (Polymicro Technologies, Phoenix, AZ) were glued side-by-side with a 2 mm offset. The capillaries were ensheathed in a regenerated cellulose membrane with both ends sealed by polyimide sealing resin (Grace, Deerfield, IL). Flow rate of perfusion fluid through the probe was 1 µL/min unless stated otherwise.

### *Sample derivatization and analysis*

Each sample was derivatized with benzoyl chloride and analyzed as described previously.<sup>68</sup> Briefly, 5 µL samples were sequentially mixed with 2.5 µL of 100 mM sodium tetraborate, 2.5 µL of benzoyl chloride (2% in acetonitrile, v/v), and 2.5 µL of internal standard. Internal standard comprised of 10 µM GLU and DA reacted with <sup>13</sup>C<sub>6</sub>-benzoyl chloride (CDN Isotopes) in 100 mM sodium tetraborate. The internal standard was the diluted 1:100 in DMSO and 1% formic acid (v/v). Between each reagent addition, the samples were vortexed. The samples were analyzed on a Waters UPLC system with a Waters HSS T3 column (1 mm x 100 mm, 1.8 µm). A Waters/Micromass Quattro Ultimatriple quadrupole or an Agilent 6410 triple



quadrupole mass spectrometer was used for detection. Mobile phase A was 10 mM ammonium formate and 0.15% (v/v) formic acid in water. Mobile phase B was acetonitrile. The peak areas of each analyte were divided by the area of the internal standard. The limits of detection for DA and GLU were 0.03 nM and 5 nM, respectively.<sup>68</sup> These limits of detection for this method are sufficient for *in vivo* measurements and are comparable to that of electrochemical detection.<sup>65, 79,</sup>

145

#### *Measuring in vitro loss and recovery*

For *in vitro* studies, 200 nM <sup>13</sup>C<sub>6</sub>-DA was perfused through probes at flow rates of 0.2, 0.5, 1.0, and 1.5 μL/min. The probe sampled from a well-stirred vial of 200 nM <sup>12</sup>C-DA. The recovery of the <sup>12</sup>C-DA was compared to the loss of the <sup>13</sup>C<sub>6</sub>-DA at each flow rate. Samples were collected every 5 min with 3 replicates of each flow rate and 3 probes were tested.

#### *Surgery*

Male Sprague-Dawley rats were anesthetized with ketamine (65 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) and dexdomitor (0.25 mg/kg, Pfizer Animal Health, New York, NY) and placed in a stereotaxic frame. A burr hole was drilled where the cannula (Plastics One, Inc., Roanoke, VA) was being implanted (+1.6 A/P, ±1.1 L, cannula aimed to nucleus accumbens).<sup>150</sup> The rats were uni-laterally cannulated. Cannulae were implanted alternately between the left and right side of the brain. Additional burr holes were drilled for skull screws to hold the cap in place. The cannula was lowered 4 mm from the top of the skull and dental cement (A-M Systems, Inc., Sequim, WA) was used to hold the cannula in place. A stylet (Plastics One, Inc.) was inserted into the cannula and the rat was allowed to recover for 5 to 10 days prior to the experiment. All animal procedures were approved by the University Committee for the Use and Care of Animals at the University of Michigan.

The day before the experiment, a microdialysis probe was perfused with aCSF at a flow rate of 1  $\mu\text{L}/\text{min}$ . The rat was lightly anesthetized in an isoflurane drop box. The probe was inserted into the cannula with the active area extending 2 to 4 mm past the cannula to sample from the nucleus accumbens. Probe placements were checked with histology (data not shown). The rat was tethered to a Ratur (Bioanalytical Systems, Inc., West Lafayette, IN). Overnight, the flow rate was lowered to 0.2  $\mu\text{L}/\text{min}$ .

#### *Measuring extraction fraction of DA and GLU*

On the day of the experiment, the flow rate of the probe was increased to 1  $\mu\text{L}/\text{min}$ . Varying concentrations of  $^{13}\text{C}_6\text{-DA}$  (0, 5, 20, 200, 500 nM) were perfused through the probe with and without 5  $\mu\text{M}$  nomifensine. Solutions were switched by hand by disconnecting the fluid line and reattaching another syringe. The time required for the liquid to travel from the syringe to the probe was approximately 10 min. Each concentration of DA was allowed to equilibrate for 10 min prior to sampling, as was performed in previous NNF calibrations.<sup>126</sup> Each fraction was 5  $\mu\text{L}$  and at each concentration 3 samples were collected. The same procedure for DA was performed with GLU.  $^{13}\text{C}_5\text{-GLU}$  at varying concentrations (0, 0.2, 2, 5, and 10  $\mu\text{M}$ ) was perfused through the probe. Although a Ratur was used for all experiments, liquid swivels could be used as well. The internal volume of liquid swivels may be a concern for fast temporal resolution of SIL microdialysis, but no more than that of conventional microdialysis.

An injection of cocaine (20 mg/kg, i.p.) was administered to the rat to show the effect of a DA uptake inhibitor under transient conditions. The probe was perfused with 200 nM  $^{13}\text{C}_6\text{-DA}$  for the duration of the experiment. 200 nM  $^{13}\text{C}_6\text{-DA}$  was chosen because it produced a large signal so that smaller changes in  $E_d^{\text{DA}}$  could be measured.  $C_{\text{app}}^{\text{DA}}$  and  $E_d^{\text{DA}}$  were measured on every

sample, which were collected every 5 min. As a control, saline (i.p.) was administered to the rat rather than cocaine.

To show the effect of an uptake inhibitor on  $E_d^{GLU}$  under transient conditions, 750  $\mu$ M PDC (Tocris, Bristol, UK) was perfused through the probe along with 2  $\mu$ M  $^{13}C_5$ -GLU.  $E_d^{GLU}$  and  $C_{app}^{GLU}$  were measured during the PDC infusion. The doses and modes of delivery for cocaine and PDC were chosen to replicate doses and modes of delivery of previous studies to show how SIL microdialysis can be used practically.<sup>46, 131, 132</sup>

### *Statistical analysis*

For comparison of NNF to SIL microdialysis, t-tests were performed. Paired t-tests were used for the DA measurements because rats were used as their own control between vehicle and uptake inhibitor perfusion. For GLU studies, unpaired t-tests were used because not all rats were matched with the PDC perfusion. When comparing the non-calibrated vs. calibrated GLU/DA concentrations, a linear mixed model regression was used to test significance. Analysis of variance (ANOVA) could not be used because for most of the experiments described in this study, the same rat was used in multiple groups (e.g. calibrated and not calibrated). The multiple measurements on a single rat violate the assumption of independence between groups; however, a repeated measures ANOVA may have sufficed. The mixed model regression was used because it has less strict assumptions than a repeated measures ANOVA, but provides similar results.<sup>151</sup> SPSS (IBM, Armonk, NY) was used for the mixed model regression. The rat ID was used as the subject, calibration was used as a factor, time was used as a covariate, and the measurement (e.g.  $E_d$ , % basal, concentration) was tested as the dependent variable.

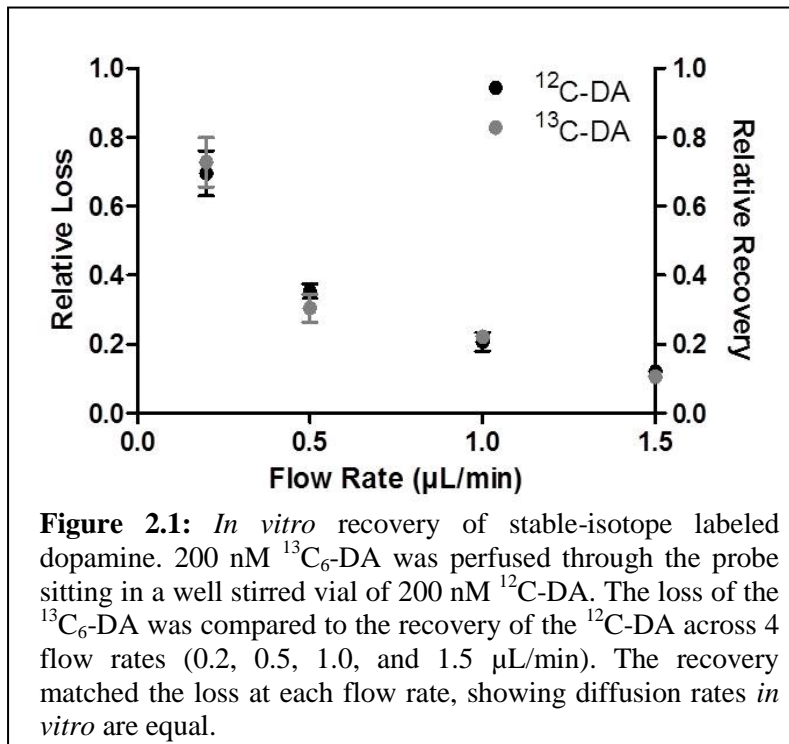
## Results

### *In vitro* SIL microdialysis

In principle, relative loss of  $^{13}\text{C}_6\text{-DA}$  should be the same as relative recovery of  $^{12}\text{C-DA}$  that is being sampled. To test this idea, 200 nM  $^{13}\text{C}_6\text{-DA}$  was perfused through a probe sampling from a well-stirred solution of 200 nM  $^{12}\text{C-DA}$ . The relative loss of  $^{13}\text{C}_6\text{-DA}$  was within 10% of the recovery of  $^{12}\text{C-DA}$  across a range of flow rates showing that transport to and from the probe *in vitro* was equivalent (Fig. 2.1).

### *Effect of infused transmitter on endogenous neurotransmitter*

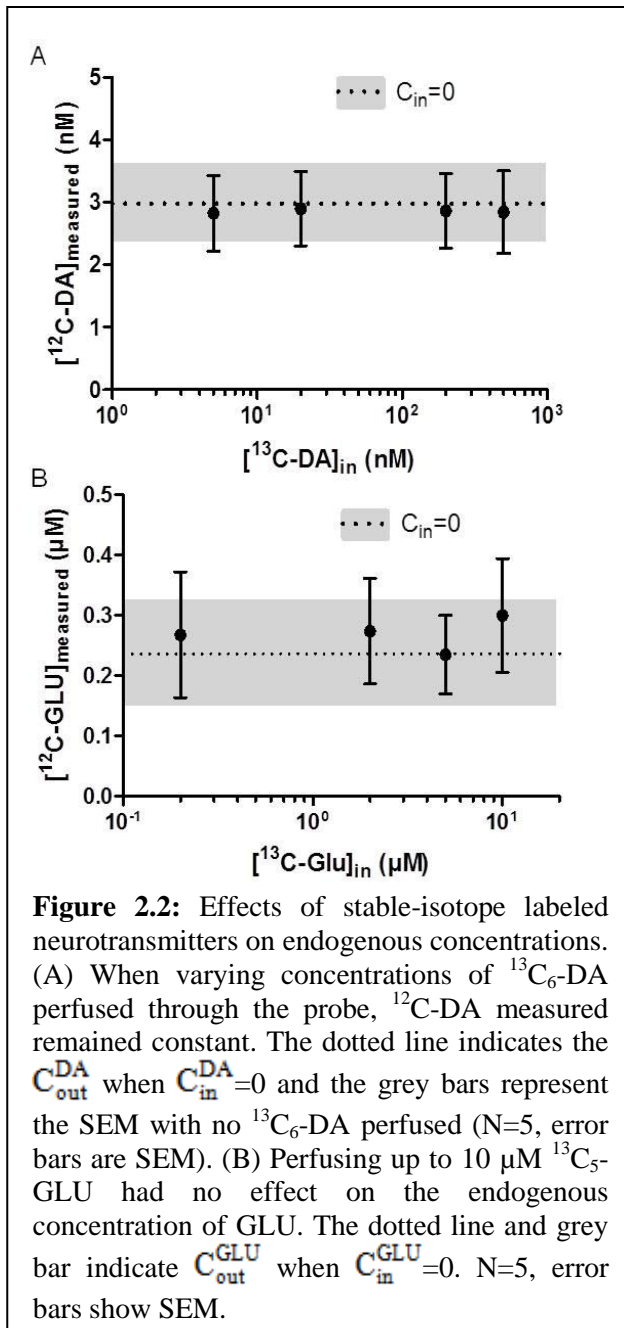
Both NNF and SIL microdialysis rely on infusing a neurotransmitter through the dialysis probe. Such compounds may affect the process being measured by activating receptors (e.g., autoreceptors) or by affecting uptake (e.g. saturating uptake). To determine if infusion of neurotransmitter affected endogenous levels, we infused SIL neurotransmitter while monitoring the endogenous form being collected from the nucleus accumbens. For concentrations typically



used for NNF, the infused SIL form had no effect on the concentration of the  $^{12}\text{C}$  form (e.g., endogenous form) collected for both GLU and DA (see Fig 2.2). This result confirms the conclusion previously reached for lack of an effect by infused DA on DA

during NNF and extends it to GLU.<sup>126</sup>

*In vivo SIL microdialysis*



We next sought to determine if  $E_d$  and  $C_{\text{app}}$  measured by SIL microdialysis of  $^{13}\text{C}$  labeled neurotransmitters would be equivalent to that measured by NNF. For these experiments, the  $^{13}\text{C}$  labeled neurotransmitter was infused at different concentrations and  $E_d$  was calculated using equation 1 for each concentration infused. For NNF,  $C_{\text{in}}$  was the concentration of  $^{13}\text{C}$  labeled neurotransmitter perfused through the probe and  $C_{\text{out}}$  was calculated by summing  $[^{13}\text{C}_6\text{-DA}]$  and  $[^{12}\text{C-DA}]$ . The same method was used for GLU.

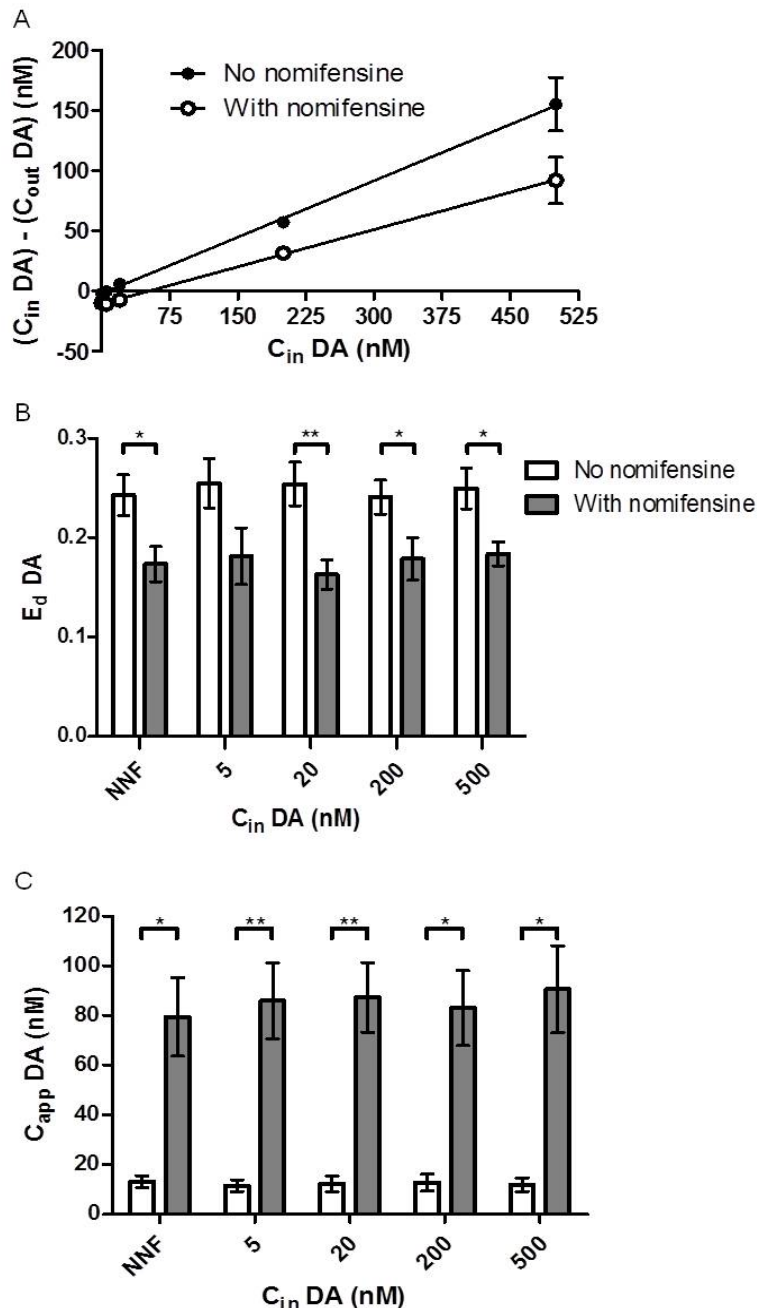
By NNF  $E_d^{\text{DA}} = 0.24 \pm 0.02$  (Fig 2.3A) and  $E_d^{\text{GLU}} = 0.26 \pm 0.02$  (Fig 2.4A). The average  $E_d^{\text{DA}}$  measured by SIL microdialysis was  $0.26 \pm 0.03$ ,  $0.25 \pm 0.02$ ,  $0.24 \pm 0.02$ , and  $0.25 \pm 0.02$  with 5, 20, 200, and 500 nM  $^{13}\text{C}_6\text{-DA}$  infused, respectively (Fig 2.3B). In principle

the highest possible  $E_d$  value is that for a well-stirred solution *in vitro*.<sup>141</sup> It has previously been found that in the nucleus accumbens  $E_d^{\text{DA}}$  approaches the maximal values, likely because of the effects of high uptake rates.<sup>143</sup> In agreement with these previous observations, our *in vivo* values

were not statistically different from the *in vitro* value of  $0.22 \pm 0.003$  at the same flow rate shown in Fig. 2.1. The slight differences are most likely due to the variability inherent in probes. The average  $E_d^{GLU}$  measured by SIL microdialysis was  $0.28 \pm 0.04$ ,  $0.29 \pm 0.01$ ,  $0.27 \pm 0.03$ , and  $0.25 \pm 0.02$  with 0.2, 2, 5, and 10  $\mu\text{M}$   $^{13}\text{C}_5\text{-GLU}$  infused, respectively (Fig 2.4B). The  $E_d$  measured by NNF and SIL microdialysis (at all concentrations) were not statistically different and were on average within 5% of each other. These results show that  $E_d$  was not affected by the concentration infused and SIL microdialysis was equivalent to NNF under these conditions.

With  $E_d$  calculated and the dialysate concentration of neurotransmitter measured,  $C_{app}$  could be calculated. By NNF,  $C_{app}^{DA}$  in the nucleus accumbens was calculated to be  $13 \pm 2$  nM. Using SIL microdialysis (equation 2),  $C_{app}^{DA}$  was measured to be  $11 \pm 2$ ,  $12 \pm 3$ ,  $13 \pm 3$ , and  $12 \pm 3$  nM for 5, 20, 200, and 500 nM  $^{13}\text{C}_6\text{-DA}$  infused, respectively (Fig 2.3C). These concentrations match well with previously reported results for NNF measurements of DA in the nucleus accumbens of awake rats, e.g. 8.8 nM,<sup>133</sup> 11.4 nM,<sup>143</sup> 10 nM,<sup>126</sup> and 5.2 nM.<sup>152</sup> The values also match well the concentration determined by signal averaging DA transients over several min in the nucleus accumbens with fast-scan cyclic voltammetry. By averaging the transients, the extracellular DA concentration was estimated to be  $\sim 20$  nM.<sup>72</sup>

$C_{app}^{GLU}$  was calculated to be  $1.0 \pm 0.4$   $\mu\text{M}$  using NNF. Using SIL microdialysis,  $C_{app}^{GLU}$  was calculated to be  $1.1 \pm 0.5$ ,  $1.0 \pm 0.3$ ,  $1.0 \pm 0.3$ , and  $1.2 \pm 0.4$   $\mu\text{M}$  with 0.2, 2, 5, and 10  $\mu\text{M}$   $C_{in}^{GLU}$  perfused through the probe, respectively (Fig 2.4C).  $C_{app}^{DA}$  and  $C_{app}^{GLU}$  measured with NNF were within 8% that measured by SIL microdialysis and were not statistically different showing that these methods give equivalent results for both neurotransmitters. Our values fell within acceptable ranges compared to previous studies which used uncalibrated microdialysis.<sup>68, 153</sup>



**Figure 2.3:** Quantitative microdialysis with stable-isotope labeled dopamine compared to no-net-flux. (A) A representative NNF curve from one rat shows how perfusing nomifensine through the probe (black open circles), (the slope) reduces  $E_d^{DA}$  and increases  $C_{app}^{DA}$  (x-intercept). (B)  $E_d^{DA}$  was compared between SIL microdialysis calibration and NNF calibration. Nomifensine reduced  $E_d^{DA}$  as measured by both calibration techniques. (C)  $C_{app}^{DA}$  was calculated using equation 2. At all concentrations of  $^{13}C_6$ -DA perfused,  $C_{app}^{DA}$  matched the NNF values. Error bars show SEM (n=5 for each group). Paired t-tests were used for comparison. \*\* indicates p-value < 0.01 and \* indicates p-value < 0.05.

Little work has been performed with quantitative microdialysis of GLU in the nucleus accumbens for direct comparison.

It has previously been reported that  $E_d^{DA}$  is largely governed by reuptake so that uptake inhibition causes  $E_d^{DA}$  to decrease.<sup>126</sup> As a result, if  $E_d^{DA}$  is

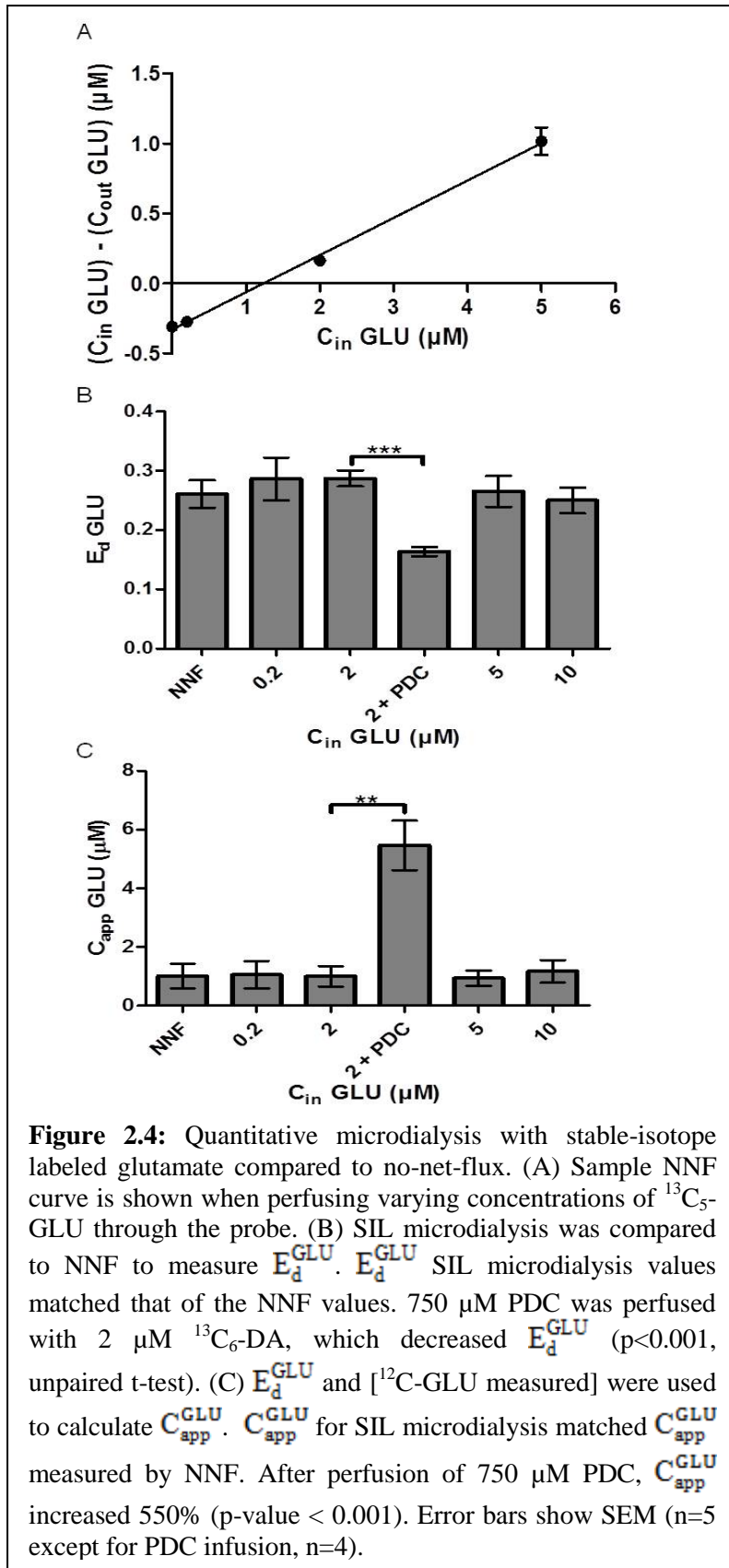
measured prior to uptake inhibition then the concentration would be underestimated either in absolute concentration or by

percent of baseline during uptake inhibition. The effect of uptake inhibition is rationalized by considering that DA is primarily removed from the extracellular space via uptake so that decreasing the uptake will lower the concentration gradient between the extracellular space and the probe. To determine if SIL microdialysis and NNF offered equivalent responses to this perturbation, we blocked uptake by perfusing 5  $\mu\text{M}$  nomifensine through the probe at all  $^{13}\text{C}_6$ -DA concentrations infused. As shown in Fig 2.3, nomifensine decreased  $E_d^{\text{DA}}$  by 30% as measured by both NNF and SIL microdialysis. With nomifensine in the probe,  $E_d^{\text{DA}}$  was calculated to be  $0.17 \pm 0.2$  using NNF. With SIL microdialysis,  $E_d^{\text{DA}}$  was calculated to be  $0.18 \pm 0.03$ ,  $0.16 \pm 0.02$ ,  $0.18 \pm 0.02$ , and  $0.18 \pm 0.01$  with 5, 20, 200, and 500 nM  $^{13}\text{C}_6$ -DA perfused through the probe. As expected,  $C_{\text{app}}^{\text{DA}}$  increased with nomifensine. Using NNF,  $C_{\text{app}}^{\text{DA}}$  was calculated to be  $80 \pm 20$  nM. Using SIL microdialysis,  $C_{\text{app}}^{\text{DA}}$  was calculated to be  $86 \pm 16$ ,  $87 \pm 14$ ,  $83 \pm 15$ ,  $91 \pm 18$  nM for 5, 20, 200, and 500 nM  $^{13}\text{C}_6$ -DA perfused through the probe, respectively.

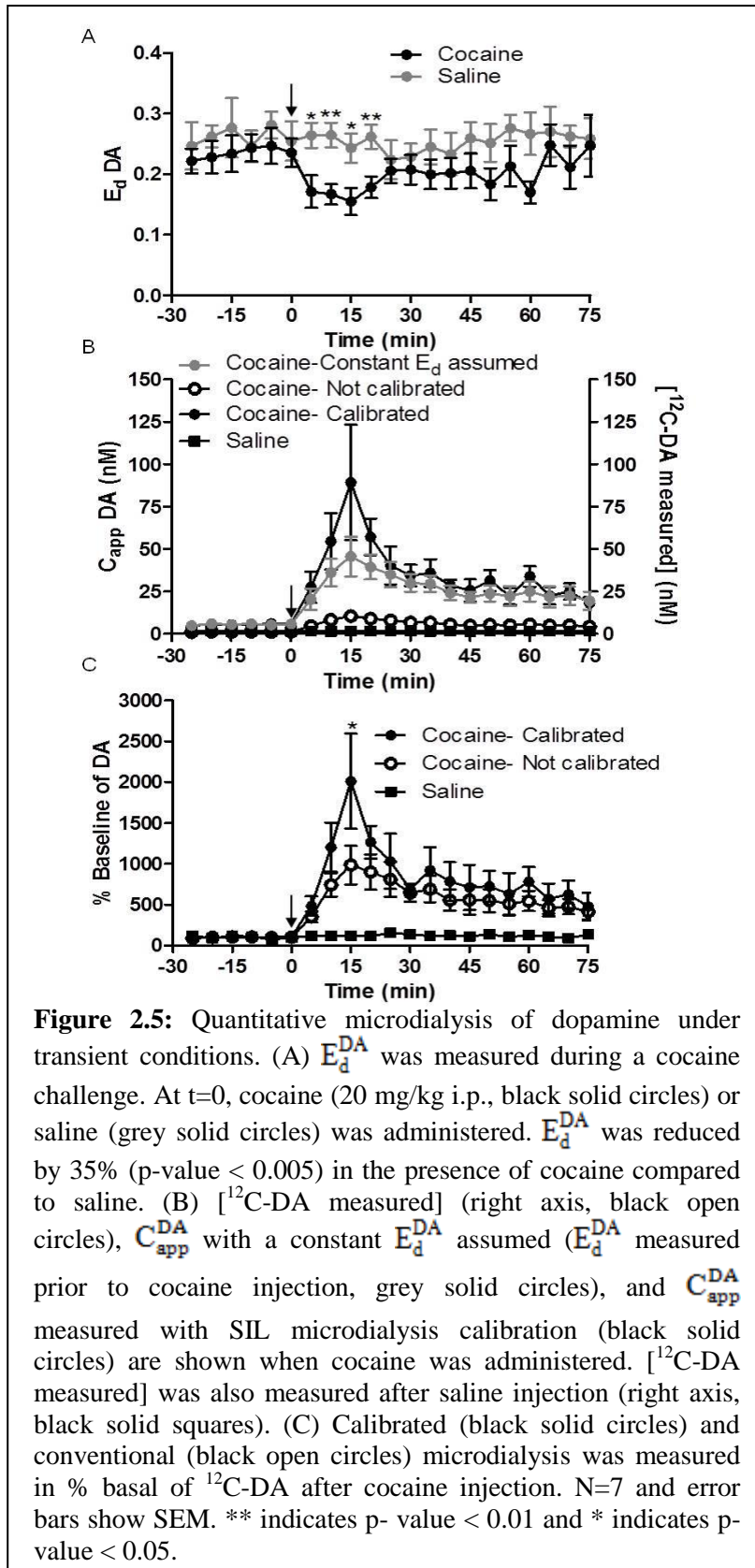
Similar to  $E_d^{\text{DA}}$ ,  $E_d^{\text{GLU}}$  has also been shown to be controlled by reuptake.<sup>131</sup> To determine if a change in  $E_d^{\text{GLU}}$  could be measured by SIL microdialysis during uptake inhibition, we perfused 750  $\mu\text{M}$  of the GLU uptake inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC) through the probe and performed SIL microdialysis using 2  $\mu\text{M}$   $^{13}\text{C}_5$ -GLU. As shown in Figure 2.4B and 2.4C,  $E_d^{\text{GLU}}$  was decreased by 40% as measured by SIL microdialysis ( $p < 0.001$ ) while  $C_{\text{app}}^{\text{GLU}}$  increased 550% ( $p < 0.005$ ). These results show that: 1) SIL microdialysis and NNF provide equivalent results during uptake inhibition; and 2) uptake inhibition decreases  $E_d^{\text{GLU}}$  similar to  $E_d^{\text{DA}}$ .

*E<sub>d</sub> changes under transient conditions*





The above results show that uptake inhibition affects  $E_d$  and therefore interpretation of the magnitude of dialysate concentrations that are detected. It is reasonable to expect that uptake inhibition is steady during drug infusion through a probe; however, it will change during a systemic injection due to pharmacokinetics. To demonstrate that SIL microdialysis could monitor dynamic changes in  $E_d$ , we used the method to follow  $C_{app}^{\text{DA}}$  and  $E_d^{\text{DA}}$  during systemic cocaine injection (20 mg/kg, i.p.) while infusing 200 nM  $^{13}\text{C}_6\text{-DA}$  through the probe for the duration of the experiment. As shown in Fig 2.5,  $E_d^{\text{DA}}$  decreased by an average of 35% within 20 min of cocaine injection (p-



value < 0.005 using the mixed model regression) compared to saline injection, similar to previous reports using dNNF.<sup>132</sup> For the first 20 min after cocaine injection  $E_d^{DA}$  was lower than after saline injection (p-values < 0.05, 0.005, 0.05, and 0.005 for the fractions collected at 5, 10, 15, and 20 min after injection using unpaired t-test).

Because  $E_d^{DA}$  was changing following cocaine injection, the SIL microdialysis method reported a higher  $C_{app}^{DA}$  than if it were measured assuming a constant  $E_d^{DA}$  (where  $E_d^{DA}$  was measured prior to cocaine injection) as shown in Figure 5. Using mixed model regression, the SIL

microdialysis  $C_{app}^{DA}$  was measured to be 160% greater on average compared to a constant  $E_d^{DA}$  over the first 20 min of sampling following cocaine injection ( $p < 0.001$ ). In addition, the relative increase in  $C_{app}^{DA}$  was 160% higher when calibrated by SIL microdialysis compared to a constant  $E_d^{DA}$  over the first 20 min of drug ( $p < 0.001$ ). The peak increase as a percent of basal with calibrated DA was measured to be 190% higher than the non-calibrated measurement ( $p$ -value  $< 0.05$ ).

While quantitative microdialysis has been used previously to measure  $C_{app}^{DA}$  under transient conditions, no previous reports have demonstrated such measurements for  $C_{app}^{GLU}$  or  $E_d^{GLU}$ . To show how SIL microdialysis can measure  $E_d^{GLU}$  and  $C_{app}^{GLU}$  under transient conditions, we monitored both as PDC was perfused through the probe. Mixed model regression showed a 45% decrease of  $E_d^{GLU}$  ( $p$ -value  $< 0.001$ ) after PDC was perfused through the probe (Fig 2.6). With calibration at each point by SIL microdialysis,  $C_{app}^{GLU}$  was measured to be 190% higher than if assuming a constant  $E_d^{GLU}$  ( $E_d^{GLU}$  measured with SIL microdialysis prior to PDC perfusion,  $p$ -value  $< 0.001$  using the mixed model regression). Using a paired t-test, 3 of the 4 calibrated microdialysis fractions had statistically higher  $C_{app}^{GLU}$  ( $p$ -value  $< 0.05$ ,  $0.05$ , and  $0.005$  for 10, 15, and 20 min after PDC infusion, respectively). This effect was extended to relative changes as well. Calibrated by SIL microdialysis, the percent basal change was 2-fold larger compared to non-calibrated measurements ( $p$ -value  $< 0.001$  using mixed model regression model, refer to Fig 2.6C). Thus, even relative changes are in error when not using calibration.

The experiments show that SIL microdialysis and NNF provide equivalent  $C_{app}$  and  $E_d$  for two different neurotransmitters across a wide range of conditions. This result is in good agreement with expectation since the techniques both rely on the principle of measuring the

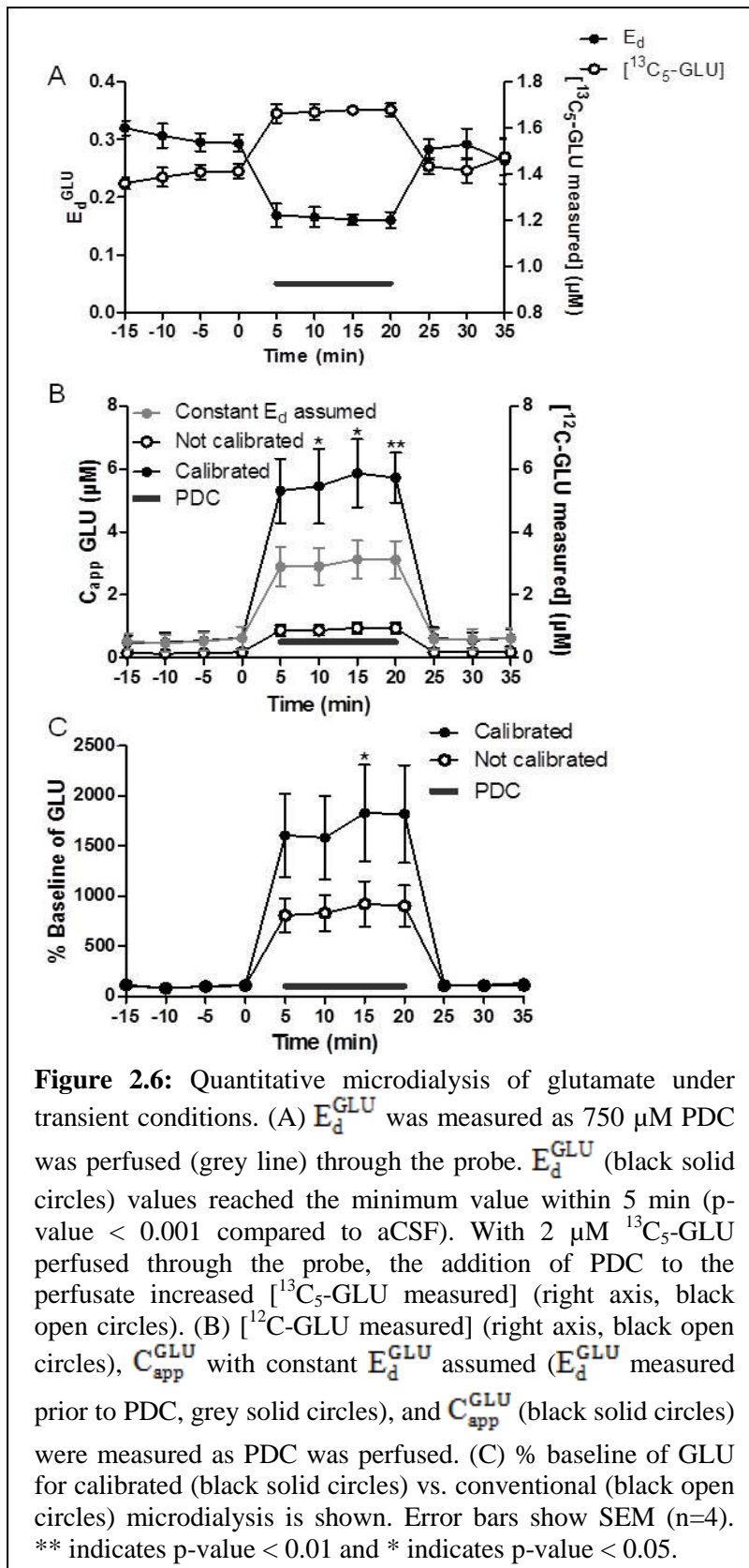
extraction or loss of a molecule of interest from the probe in the *in vivo* environment. Because of this equivalency, the methods not only provide the same information, but are subject to the same limitations. In particular, tissue factors that might influence NNF results would also influence SIL microdialysis.<sup>141, 154, 155</sup>

## Discussion

### *SIL microdialysis is an efficient method*

The largest disadvantage to performing NNF is the added time and expense needed to complete a study. For example, a previous report showing that PDC lowers  $E_d^{GLU}$  required 7 h of fraction collection time when using NNF.<sup>131</sup> With SIL microdialysis of  $^{13}C_5$ -GLU, the same measurement with the same number of replicates required 30 min. If dNNF were to be used to capture the dynamics of  $E_d^{GLU}$ , a minimum of 3 concentrations of perfused GLU would be needed requiring 12 animals assuming 4 replicates for each  $[GLU]_{in}$ . In contrast, the SIL microdialysis method required 4 animals for an equal number of replicates. Using SIL microdialysis is more efficient in terms of time and animal usage than NNF. In addition to time saved for a single analyte, multiple analytes could be used for SIL microdialysis simultaneously saving further time. The benzoyl chloride derivatization and HPLC-MS method described herein allows for quantifying multiple SIL neurotransmitters in a single sample.<sup>68</sup> The ease of use with SIL microdialysis is compromised by the need for a mass spectrometer to perform these measurements; however, as mass spectrometers become more available, this disadvantage will wane.

Apart from the time and work saved, SIL microdialysis with  $^{13}C$  labeled neurotransmitters also allows monitoring for artifacts that may be caused by perfusing neurotransmitter through the probe. By measuring endogenous  $^{12}C$ -DA and  $^{12}C$ -GLU across



multiple perfused concentrations of  $^{13}\text{C}_6\text{-DA}$  and  $^{13}\text{C}_5\text{-GLU}$ , we determined that the concentrations perfused had no measureable effects on endogenous DA and GLU. Thus it is possible to directly show that the calibration method does not perturb the system being sampled.

*Use of  $E_d$  to aid in data interpretation*

The availability of a more efficient method for measuring  $E_d$  *in vivo* may enable quantitative microdialysis studies to be more routinely performed. Such quantification may be significant for many studies. The use of  $E_d$  can help discern the source of a concentration change. For example, one study

showed that  $E_d^{DA}$  was constant for two days allowing concentration changes to be related to endogenous changes rather than artifacts of recovery change.<sup>143</sup> In another study, ethanol was shown to increase DA and GLU in the nucleus accumbens and the hippocampus.<sup>131, 133</sup> By showing that  $E_d$  did not change (requiring 24 animals, 2 h for each animal for DA and 13 animals, 7 h for each animal for GLU), it was possible to demonstrate the change in concentration is induced by release and not a decrease in reuptake. In principle, similar studies by SIL microdialysis could significantly save on animals and time required. Quantification may also be important for determining effects of genetic manipulation. For example, distinguishing the dose-dependent effects of SERT gene on extracellular serotonin concentration required calibrated microdialysis measurements.<sup>145</sup> The heterozygous SERT knockout showed no difference in basal serotonin concentrations in the striatum using uncalibrated microdialysis compared to the wild type; however, when using NNF the heterozygous SERT knockout was shown to have an increased  $C_{app}^{serotonin}$  compared to the wild type. We may expect that other genetic modifications may also result in more subtle changes in neurotransmitter concentration that are best detected by quantification. The availability of SIL microdialysis could aid in such studies.

Quantification may also be important when comparing techniques. The relative DA increase measured in the nucleus accumbens by microdialysis following a 3 mg/kg dose of cocaine I.V. was an order of magnitude lower than that observed by voltammetry.<sup>73, 156</sup> While some of the difference may be attributed to differences in temporal resolution (peak changes may happen quickly and be recorded by voltammetry but are averaged resulting in lower values by microdialysis), some difference must also be due to use of non-calibrated microdialysis probes in these studies. As shown here and elsewhere, lack of calibration can result in substantial

underestimation of extracellular concentrations and relative increases by microdialysis.<sup>126</sup> It is clearly desirable to use *in vivo* calibration if comparing different methods. The use of SIL microdialysis in such studies will reduce the time and animal cost associated with the measurements and therefore enhance the feasibility of such comparisons.

### *Conclusions*

Quantification by *in vivo* calibration is an important tool for microdialysis measurements. A robust and efficient new method for measuring  $E_d$  and  $C_{app}$  of neurotransmitters has been demonstrated using SIL microdialysis. Using SIL microdialysis provides a more accurate neurochemical profile compared to conventional microdialysis, which can aid in interpreting results.

## Chapter III

### ***In vivo* evidence for increases in dopamine uptake and release in the nucleus accumbens after an acute leptin injection**

#### **Introduction**

Leptin is a hormone released by adipocytes that is involved in controlling feeding and energy expenditure.<sup>27, 157, 158</sup> Mice lacking the leptin gene (*ob/ob*) eat excessively and become obese.<sup>27</sup> Leptin treatments have been shown to reverse hyperphagia in multiple species. Chronic injections of leptin caused a 30% reduction in body weight in *ob/ob* mice after 2 weeks and a 12% weight loss in wild-type mice.<sup>30</sup> An injection of leptin over 24 h into the ventral tegmental area (VTA) decreased food intake of rats.<sup>157</sup> Similarly, rats that received chronic intracerebroventricular (i.c.v.) infusions also decreased food intake.<sup>159</sup> In humans, treating a male adolescent that had congenital leptin deficiency with recombinant leptin increased energy expenditure and reduced body fat.<sup>32</sup>

An important question is if leptin modulates feeding behavior through effects on the rewarding or motivating effects of food. Because mesolimbic dopamine (DA) is a critical component of reward pathways,<sup>160-162</sup> considerable attention has focused on the possible modulation of DA in this pathway by leptin.<sup>123, 163, 164</sup> A reasonable hypothesis is that one of leptin's actions is to decrease the rewarding aspects of food by suppressing DA signaling. Several lines of evidence suggest that leptin affects reward pathways. When food deprived rats



underwent sucrose-conditioned place preference training, they showed preference for the compartment where low calorie sucrose pellets were dispensed. With a chronic treatment (7 days, 125 µg/kg/day) of leptin, the food deprived rats no longer displayed a place preference.<sup>165</sup> The authors concluded that leptin decreased the reward value of food. The effect of leptin on reward seeking behavior was also established with a brain stimulation reward (BSR). Food deprived rats with access to a lever that delivered a BSR required a lower frequency BSR to achieve the same number of lever presses as rats fed ad-libitum.<sup>164, 166</sup> The lower the body weight, the lower the stimulation frequency required. Leptin injected into the right lateral cerebral ventricle increased the frequency of BSR required for lever presses in food deprived rats,<sup>164</sup> demonstrating that leptin affected the reward circuitry of the brain.

Direct links of leptin to DA signaling have been found as well. Leptin receptors have been found on dopaminergic neurons in the VTA and the lateral hypothalamus, which innervates the midbrain.<sup>35</sup> The presence of these leptin receptors causes the effects of drugs of abuse to be modulated by leptin. Rats given chronic infusions of leptin (12 µg/day, ICV) had greater amphetamine-induced locomotor activity than rats given vehicle.<sup>159</sup> In *ob/ob* mice, amphetamine induced locomotor activity was lower than that of wild-type mice, but was restored with chronic leptin injections.<sup>163</sup>

Exactly how leptin affects DA in the brain remains unresolved. Brain slices from *ob/ob* mice released less DA during electrical stimulation in the nucleus accumbens (NAc) than wild-type mice.<sup>163</sup> Differences in tyrosine hydroxylase (TH) levels caused by leptin have been observed in areas of the brain associated with reward. For example, *ob/ob* mice have lower TH levels in the NAc than wild type mice.<sup>163</sup> After leptin treatments, the TH levels increased in both the NAc and the VTA.<sup>34, 163</sup> Even with these differences observed *in vitro*, no differences in

extracellular basal DA concentrations in microdialysis samples from the NAc and cocaine induced elevated DA were measured between *ob/ob* and wild-type mice.<sup>167</sup> In agreement with Roseberry, no differences in D<sub>2</sub> binding in brain slices from the same brain region were detected between *ob/ob* and wild-type mice.<sup>168</sup> These studies compared mice that were genetically different. An absence of leptin for the entire lifetime of *ob/ob* mice may affect neurochemical measurements. Additionally, many of the studies were performed in brain slices which can also affect neurochemical measurements.

Performing experiments on intact animals with normal leptin regulation can provide further insights into how leptin affects the DA system. In rats, peripheral leptin altered DA activity in multiple locations of the mesolimbic system. Leptin microinjected into the VTA caused reduced firing of DA neurons in the VTA.<sup>157</sup> Additionally, acute leptin (1 µg, ICV) decreased the extracellular concentration of DA by 40% in the NAc of food deprived rats as measured by microdialysis.<sup>169</sup> This decrease was hypothesized to be due to hyperpolarization of lateral hypothalamic (LH) DA neurons and inhibition of DA release in rat hypothalamic neural endings based on *in vitro* studies; however, leptin has also been shown to depolarize LH neurons,<sup>36</sup> which confounds the cause of decreased extracellular DA. When these food deprived rats were given food, leptin decreased the amount of DA released compared to vehicle treated rats.

In a separate study measuring acute leptin effects on intact rats fed ad-libitum, an acute peripheral leptin injection increased amphetamine induced DA release in the NAc. In this study, TH and DA active transporter (DAT) activity were shown to increase *in vitro* after leptin injection. The effect of amphetamine was attributed to both an increase in DA synthesis and DAT activity (amphetamine reverses DAT therefore more active protein will evoke a greater

DA release into extracellular space).<sup>123</sup> Interestingly, despite apparent increases in TH and DAT, no detectable effect of leptin on basal DA concentrations was measured. This result was surprising because DAT is believed to be a principal controller of extracellular DA concentration. It was concluded that increased TH activity counterbalanced the increased uptake and netted a result of no change in DA basal levels.

To better understand how leptin modulates DA concentrations *in vivo*, we used microdialysis measurements of DA and its metabolites, and pharmacological experiments designed to distinguish effects of increased TH and DAT activity in the NAc of fed Sprague-Dawley rats. In this study, changes in basal DA concentrations were measured with a quantitative microdialysis technique. This method reveals a net decrease in extracellular DA concentration following an acute leptin injection. To determine the mechanism of this decrease, cocaine and raclopride were infused through the probe with or without leptin. These experiments show that leptin both increases uptake and release, which matches results previously measured in *in vitro* studies.<sup>123</sup>

## **Methods**

### *Materials*

All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Artificial cerebral spinal fluid (aCSF) was comprised of 145 mM NaCl, 2.68 mM KCl, 1.01 mM MgSO<sub>4</sub>, 1.22 mM CaCl<sub>2</sub>, 1.55 mM Na<sub>2</sub>HPO<sub>4</sub>, and 0.45 mM NaH<sub>2</sub>PO<sub>4</sub> (Fisher Scientific, Pittsburgh, PA). <sup>13</sup>C<sub>6</sub>-DA was purchased from CDN isotopes (Quebec, Canada). Cocaine hydrochloride was purchased from the University of Michigan Hospital (Ann Arbor, MI).

### *Microdialysis*

Probes were constructed as previously described.<sup>149</sup> Briefly, 40/100  $\mu\text{m}$  (i.d./o.d.) fused silica capillaries (Polymicro Technologies, Phoenix, AZ) were glued side-by-side with a 2 mm offset. The capillaries were ensheathed in a regenerated cellulose membrane with both ends sealed by polyimide sealing resin (Grace, Deerfield, IL). Flow rate of perfusion fluid through the probe was 1  $\mu\text{L}/\text{min}$ .

Male Sprague-Dawley rats were anesthetized with ketamine (65 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) and dexdomitor (0.25 mg/kg, Pfizer Animal Health, New York, NY) and placed in a stereotaxic frame. A burr hole was drilled where the probe was being implanted (+1.6 A/P,  $\pm$ 1.1 L, probe aimed to sample from NAc).<sup>150</sup> The probes were bi-laterally implanted. The measurements for both probes in each animal were averaged. Additional burr holes were drilled for skull screws to hold the cap in place. The probe was lowered 8 mm from the top of the skull and dental cement (A-M Systems, Inc., Sequim, WA) was used to hold the probes in place. Animals were allowed to acclimate in the testing chamber for 24 h prior to testing. All animal procedures were approved by the University Committee for the Use and Care of Animals at the University of Michigan.

The day of the experiment, rats were lightly anesthetized in an isoflurane drop box while the probes were attached to the fluid lines. The microdialysis probe was perfused with aCSF at a flow rate of 1  $\mu\text{L}/\text{min}$  for at least 1 h before the experiments began. Probe placements were checked with histology (data not shown). Rats were tethered to a Ratum (Bioanalytical Systems, Inc., West Lafayette, IN) during testing.

#### *Sample derivatization*

Each sample was derivatized with benzoyl chloride and analyzed as described previously.<sup>68</sup> Briefly, samples were sequentially mixed with, 100 mM sodium tetraborate,

benzoyl chloride (2% in acetonitrile, v/v), and internal standard in a 2:1:1:1 volume ratio.

Internal standard comprised of 10  $\mu\text{M}$  DOPAC, 3-MT, HVA, and DA reacted with  $^{13}\text{C}_6$ -benzoyl chloride (CDN Isotopes) in 100 mM sodium tetraborate. The internal standard was diluted 1:100 in DMSO and 1% formic acid (v/v). Between each reagent addition, the samples were vortexed. Samples were analyzed on a Waters UPLC system with a Waters HSS T3 column (1 mm x 100 mm, 1.8  $\mu\text{m}$ ). An Agilent 6410 triple quadrupole mass spectrometer was used for detection. Mobile phase A was 10 mM ammonium formate and 0.15% (v/v) formic acid in water. Mobile phase B was acetonitrile. The peak areas of each analyte were divided by the area of the internal standard.

#### *Quantitative microdialysis*

For quantitative DA measurements, 200 nM  $^{13}\text{C}_6$ -DA was perfused through probes. The extraction fraction ( $E_d^{\text{DA}}$ ) was calculated with the following equation:

$$E_d = 1 - \frac{C_{\text{out}}^{\text{SIL}}}{C_{\text{in}}^{\text{SIL}}}$$

where  $C_{\text{out}}^{\text{SIL}}$  is the concentration of stable-isotope labeled (SIL) DA that exits the outlet of the probe and  $C_{\text{in}}^{\text{SIL}}$  is the concentration of  $^{13}\text{C}_6$ -DA that is infused into the probe.<sup>126, 143, 170</sup> With  $E_d^{\text{DA}}$  calculated, the apparent concentration ( $C_{\text{app}}^{\text{DA}}$ ) of DA could be calculated with the following equation:

$$C_{\text{app}} = \frac{C_{\text{out}}^{\text{endogenous}}}{E_d^{\text{SIL}}}$$

where  $C_{\text{out}}^{\text{endogenous}}$  is the concentration of endogenous DA measured.  $C_{\text{app}}^{\text{DA}}$  and  $E_d^{\text{DA}}$  were both measured in an awake, conscious rat prior to a leptin or vehicle treatment. Leptin (1 mg/kg, i.p.)

or phosphate buffered saline (PBS, 20 mM phosphate pH 5.2, 0.13 M NaCl) vehicle injection (i.p.) was administered and  $C_{app}^{DA}$  and  $E_d^{DA}$  were each measured again after 4 h.

To determine the rate of production of DA metabolites, the syringe containing aCSF was switched to a syringe with 200 nM  $^{13}C_6$ -DA using a 4-port valve (Valco Instruments, Houston, TX). After 7 min of  $^{13}C_6$ -DA infusion, the 4-port valve was switched back to the syringe containing aCSF. The  $^{13}C_6$ -DA infusion was performed before and 4 h after a leptin (1 mg/kg, i.p.) or PBS vehicle injection (i.p.). The method on the QQQ was modified to scan and measure  $^{13}C_6$ -DOPAC,  $^{13}C_6$ -3-MT, and  $^{13}C_6$ -HVA. Fractions were collected every min and derivatized.

#### *Measuring changes in uptake and release after leptin treatment*

To measure how uptake is affected by leptin, rats were given a single injection of leptin (1 mg/kg, i.p.) or PBS (i.p.) 3.5 h prior to measurements. Basal levels were then collected for 30 min. A 4-port valve was then switched to deliver 100  $\mu$ M raclopride. Samples were collected for an additional 30 min with raclopride infused through the probe. For these experiments, samples were collected every 5 min.

To measure increases in DA release, leptin (1 mg/kg, i.p.) or PBS (i.p.) was administered to the rat. After 3.5 hr, 30 min of baseline samples were collected and derivatized. A 4-port valve was used to switch to a solution of 40  $\mu$ M cocaine. Samples were collected for 30 min with the cocaine infusion. A second switch was made on the 4-port valve to infuse 40  $\mu$ M cocaine and 100  $\mu$ M raclopride through the probe. Samples were collected for another 30 min. Fractions were collected every 3 min.

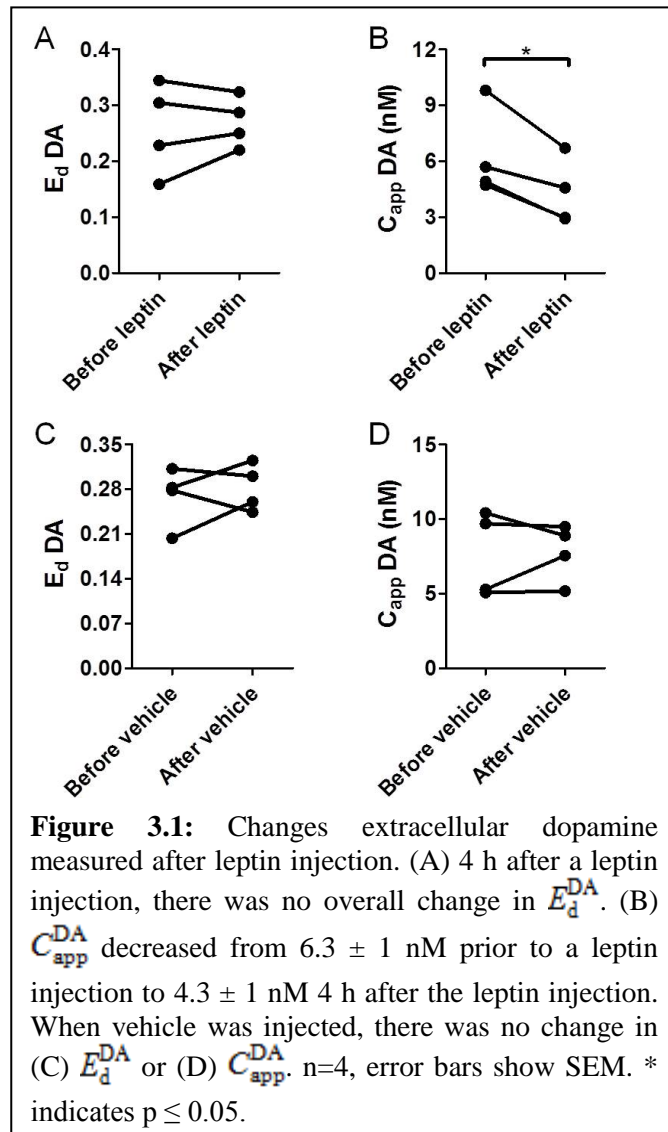
#### *Statistics*

When comparing the average  $E_d^{DA}$  or  $C_{app}^{DA}$ , paired t-tests were used to test for significance. Measurements were taken within the same animal before and after a leptin or PBS injection which warranted a paired vs. an unpaired t-test. For experiments where the relative changes of DA or its metabolites were plotted vs. time, statistical analysis was performed with a 2-way repeated measure analysis of variance (ANOVA) using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). When significance was found in the time treatment interaction, the sequentially rejective Bonferroni test was used to determine significance in individual time points.

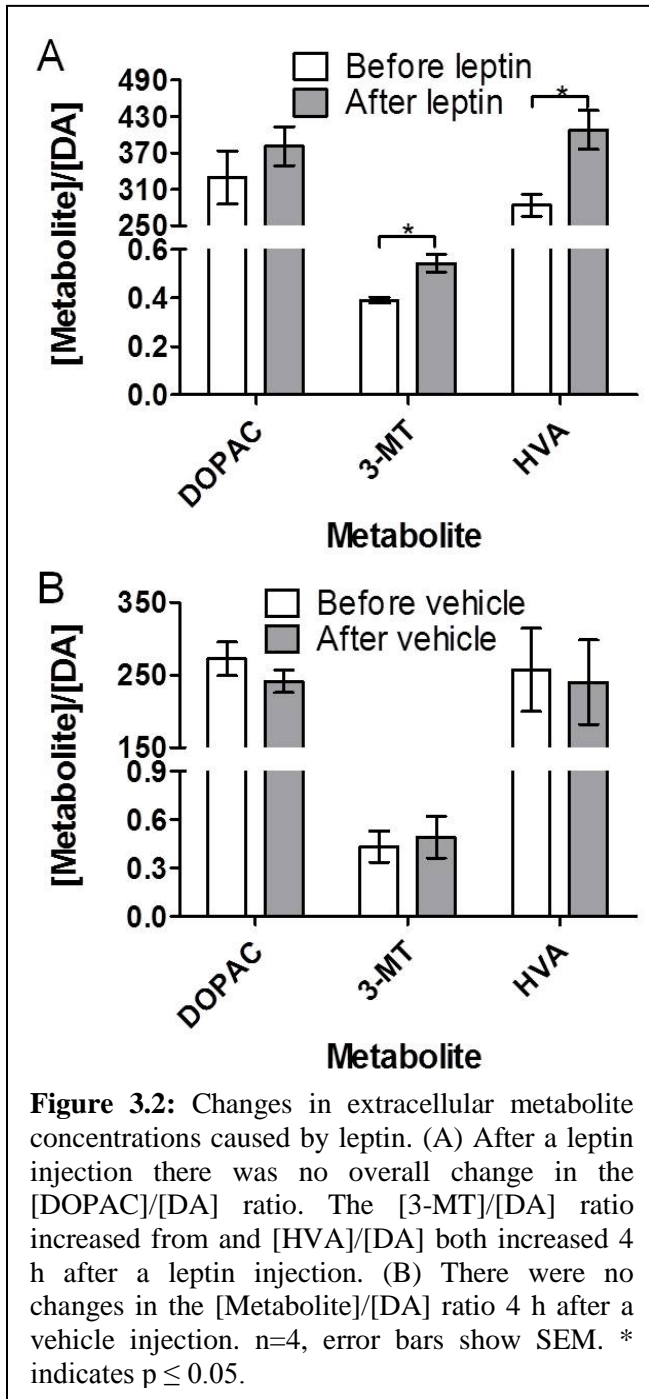
## Results

### *Infusion of $^{13}C_6$ -DA in leptin treated rats*

Acute leptin has been demonstrated to cause increases in both DAT and TH activity *in vitro* without altering basal extracellular DA concentrations between rats.<sup>123</sup> We further investigated how leptin affects basal DA concentrations with a more accurate sampling method (SIL microdialysis) and used rats as their own control. With infusion of 200 nM  $^{13}C_6$ -DA through the microdialysis probe,  $E_d^{DA}$  was calculated to be  $0.26 \pm$



0.04 (n = 4) and  $0.27 \pm 0.02$  before and after a 4 h leptin injection. For the control,  $E_d^{DA}$  was calculated to be  $0.27 \pm 0.02$  and  $0.28 \pm 0.02$  (n = 4, Fig. 3.1) before and 4 h after a vehicle injection, respectively.  $E_d^{DA}$ , which can be used as an indirect measurement of DA uptake, did not change with leptin. DA uptake in the NAc is large enough such that the  $E_d^{DA}$  is limited by

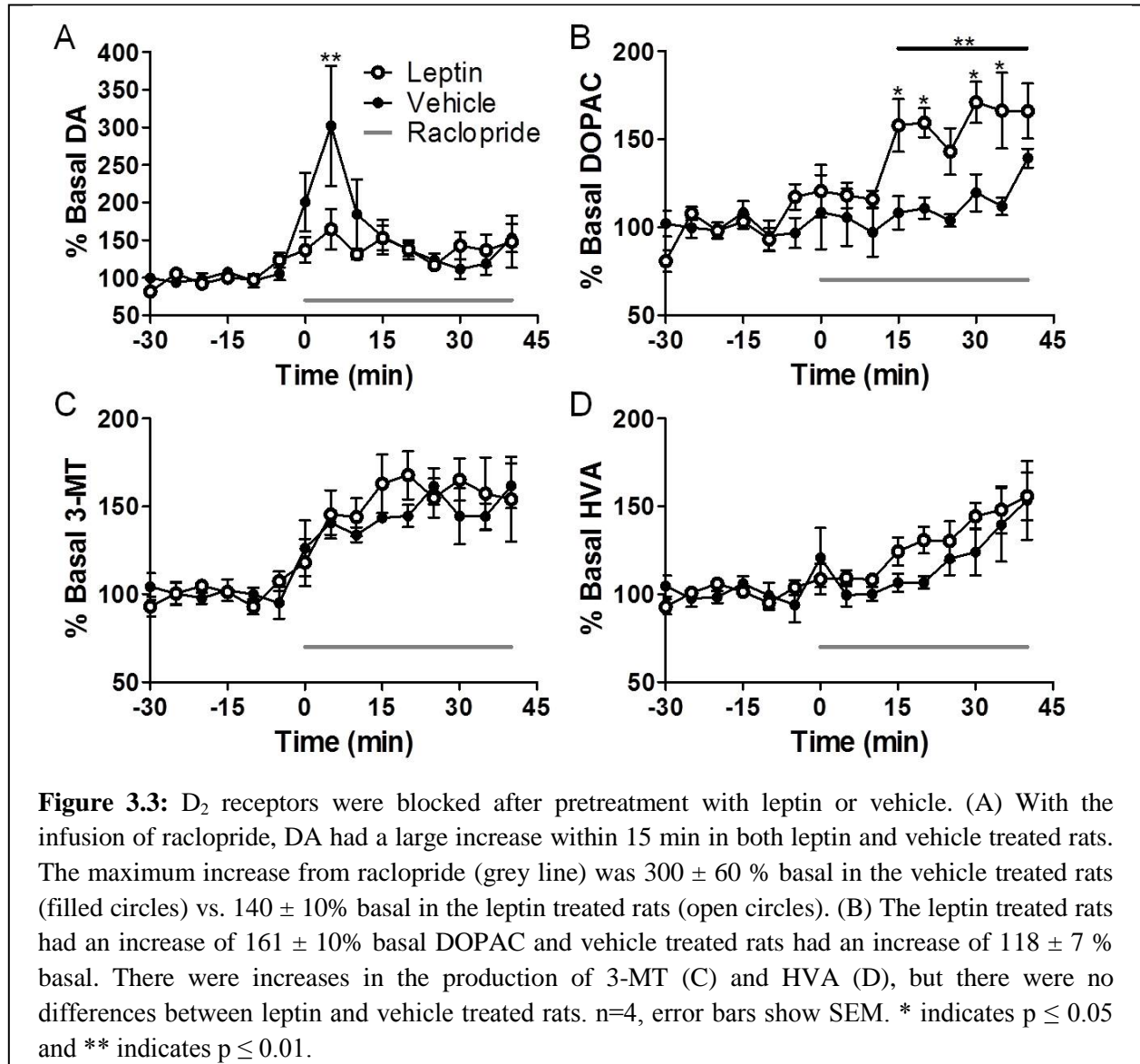


diffusion across the membrane and increases in uptake cannot be measured with  $E_d^{DA}$ .<sup>133, 143, 170</sup>  $E_d^{DA}$  remaining unchanged does not dispute previous studies where leptin was shown to increase uptake.<sup>123</sup>

With the  $E_d^{DA}$  calculated and the extracellular concentration of endogenous DA measured,  $C_{app}^{DA}$  could be calculated. Before the leptin injection,  $C_{app}^{DA}$  was  $6.3 \pm 1$  nM and was reduced to  $4.3 \pm 1$  nM ( $p \leq 0.05$ , paired t-test) 4 h after a leptin injection.  $C_{app}^{DA}$  was calculated to be  $7.6 \pm 1$  nM before the vehicle and  $7.7 \pm 1$  nM 4 h after the vehicle injection. Because  $C_{app}^{DA}$  did not decrease 4 h after a PBS injection, we conclude that the reduction in  $C_{app}^{DA}$  4 h after the leptin treatment was due to the leptin



itself and not to extracellular DA concentrations changing over time. The reduction in basal DA



following a leptin injection was small relative to the variability between animals, but detectable by using quantitative dialysis and within animal controls. Using an unpaired t-test with the same data, the decrease in  $C_{app}^{DA}$  was not statistically significant decrease. The relative standard deviation of basal DA levels between rats and the small decrease in basal DA and not using quantitative microdialysis is most likely the cause of not seeing a decrease in basal DA in a previous study using the same procedure.<sup>123</sup> By using quantitative microdialysis, which takes

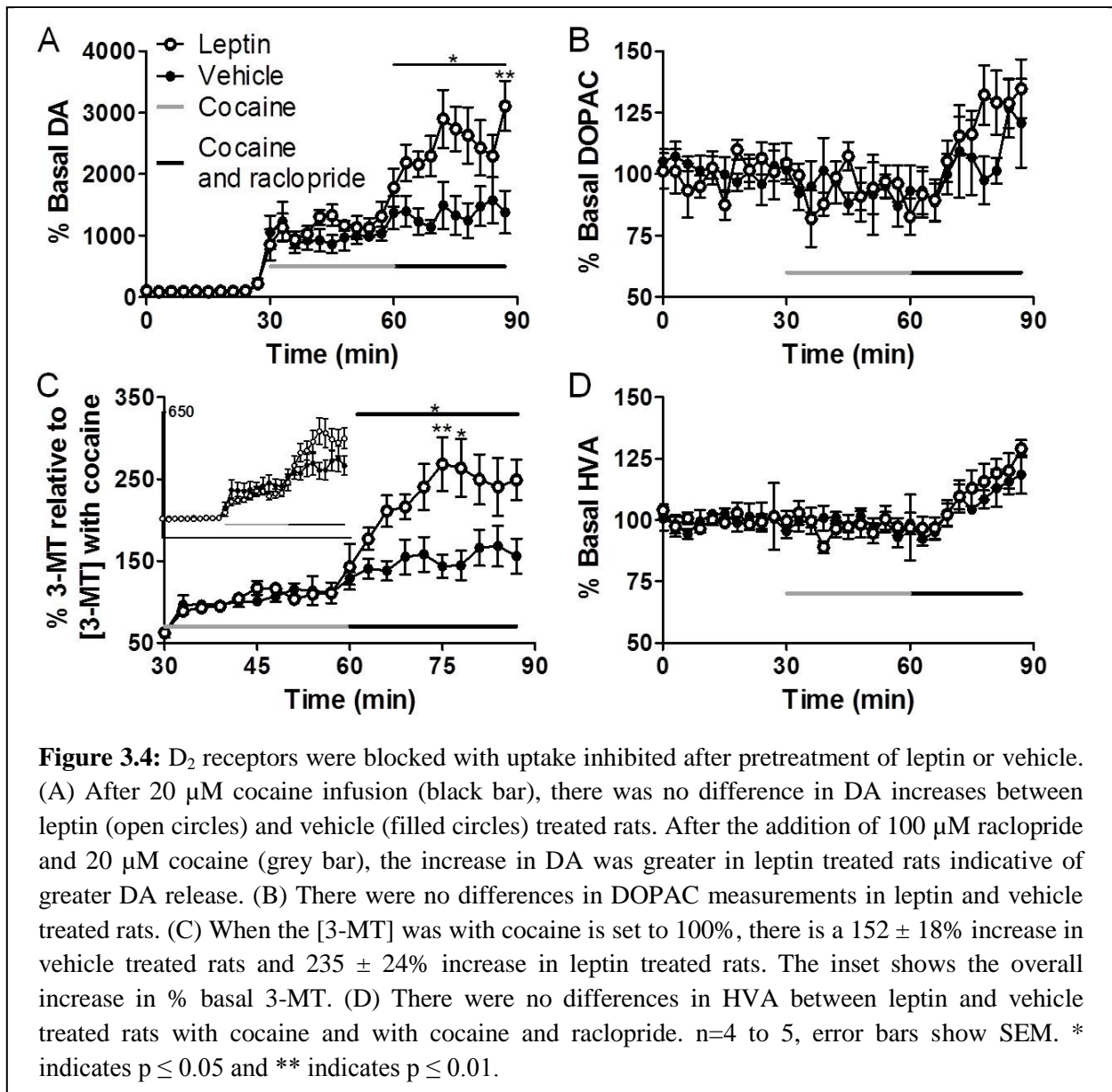
into account  $E_d^{DA}$  and probe variability, as well as using rats as their own control, we were able to measure a decrease in  $C_{app}^{DA}$  within each animal after a leptin treatment.

#### *Endogenous metabolite changes with leptin*

With the UPLC-MS/MS method used for these experiments, the DA metabolites could be measured in addition to endogenous DA. 4 h following the leptin injection, the [3-MT]/[DA] ratio increased from  $0.39 \pm 0.01$  to  $0.54 \pm 0.04$  ( $n = 4$ ,  $p \leq 0.05$ ) 3-MT, which is formed mainly in the extracellular space, has been used as an indicator for DA release.<sup>171-174</sup> The [HVA]/[DA] ratio increased from  $284 \pm 20$  to  $408 \pm 32$  ( $p \leq 0.05$ ) following leptin injection. The [DOPAC]/[DA] ratio increased from  $330 \pm 44$  to  $381 \pm 32$  (Fig. 3.2). While not statistically significant, the leptin treatment showed a trend of increasing the [DOPAC]/[DA] ratio. DOPAC is mainly synthesized inside DA neurons.<sup>175</sup> Thus, measuring extracellular 3-MT and DOPAC can provide a marker for the release and uptake of DA, respectively. The same measurements were taken before and 4 h after a vehicle injection. In these animals, no statistically significant differences in any of the [Metabolite]/[DA] ratios was measured. The increase in [3-MT]/[DA] and [HVA]/[DA] appears to be an effect of leptin rather than a change over time caused by the 4 h time period between measurements.

#### *Raclopride infusion following leptin injection*

To test the effect of autoreceptor regulation on extracellular DA concentrations, dialysis probes were infused with 100  $\mu$ M raclopride, a  $D_2$  antagonist, 4 h after a leptin or vehicle injection. Raclopride evoked an increase in DA that peaked at  $300 \pm 80\%$  ( $n = 5$ , Fig. 3.3) vs.  $164 \pm 30\%$  ( $n = 6$ ) over baseline ( $p \leq 0.01$ ; comparison between increase with and without leptin) after vehicle and leptin injection. After the initial burst in DA, the vehicle treated rats maintained extracellular DA at  $129 \pm 5\%$  of basal concentration and leptin treated rats maintained DA at 136

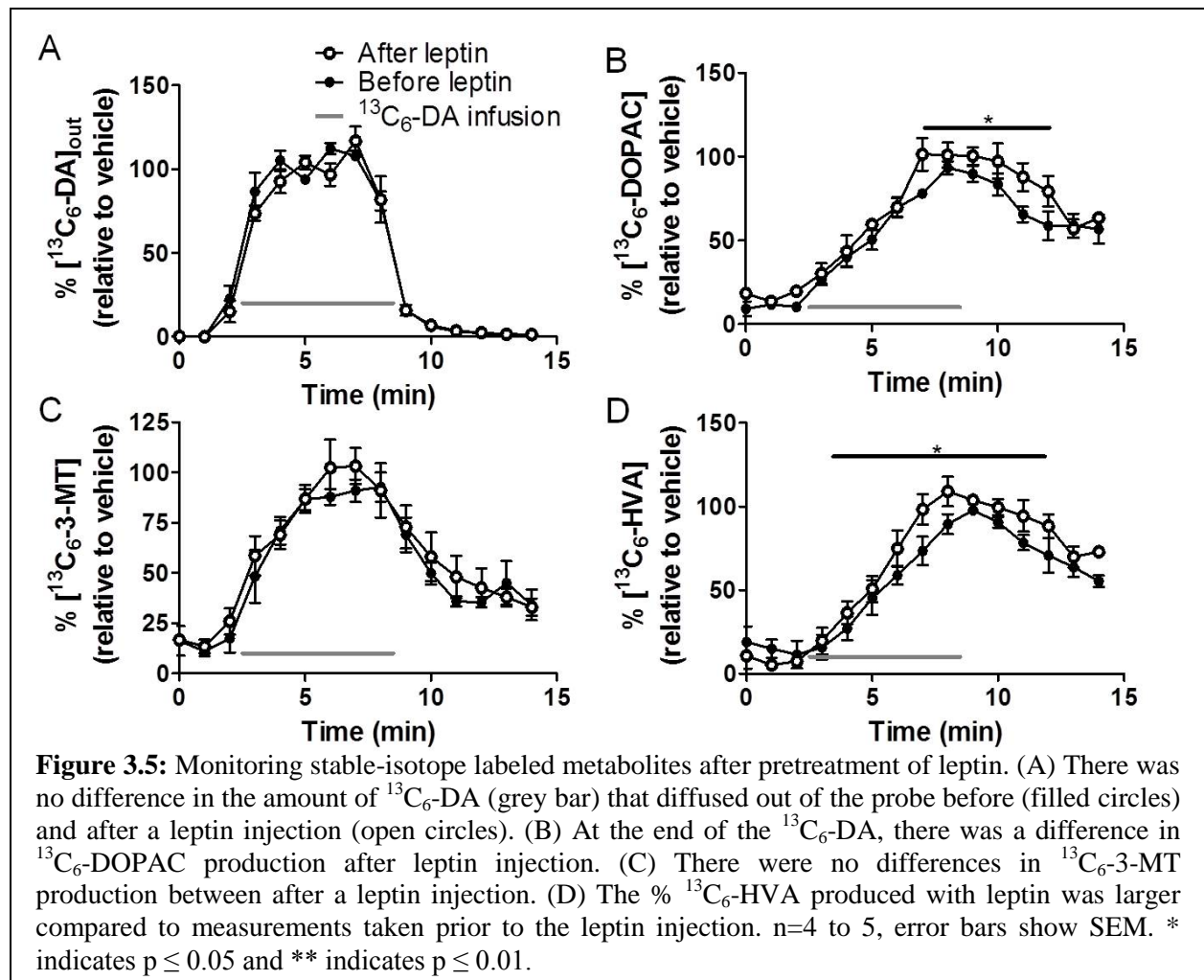


$\pm 15\%$  of basal concentration (no statistically significant difference). The attenuated extracellular DA increase is indicative of a lower tone on  $D_2$  receptors due to lower DA concentration with leptin. DOPAC increased  $146 \pm 7\%$  during raclopride infusion with leptin and  $112 \pm 6\%$  without leptin ( $p \leq 0.01$ ). The stunted release of DA with raclopride and leptin and the increase in DOPAC provides evidence that an increase in DA turnover and uptake is caused by leptin. After the raclopride infusion, 3-MT increased to  $158 \pm 12\%$  basal with leptin and  $148 \pm 6\%$  basal

with PBS. HVA increased slowly and reached a maximum of  $147 \pm 9$  % basal and  $139 \pm 19$  % basal with raclopride after the leptin and PBS injection, respectively. Neither the relative HVA nor relative 3-MT increases were statistically significant.

*Cocaine with raclopride infusion following leptin injection*

Leptin has been shown to increase TH levels in the NAc of *ob/ob* mice<sup>163</sup> and increase TH activity and amphetamine stimulated DA release in the NAc compared to vehicle treated rats.<sup>123</sup> The latter experiment also performed *in vivo* microdialysis and measured no differences in basal extracellular DA concentrations, which was hypothesized to be increased DAT activity and an increase in release due to the increased TH activity offsetting each other. The increase in



the [3-MT]/[DA] ratio we measured was indicative of an increase in release caused by leptin. To further test if DA release increased, cocaine followed by cocaine and raclopride were infused through the probe. Cocaine inhibited DA uptake and raclopride blocked the negative feedback of D<sub>2</sub> receptors. With the cocaine infusion, the leptin treated rats had an increase in DA of  $1130 \pm 150\%$  basal (n = 5) while vehicle treated rats produced a  $990 \pm 150\%$  basal increase (n = 4, Fig. 3.4). No statistically significant differences with the cocaine infusion were measured, which is in agreement with a previous study in *ob/ob* and wild-type mice.<sup>167</sup> Infusion of raclopride with cocaine was then administered to enhance release of DA with uptake blocked by cocaine. The DA increase in vehicle treated rats was  $1390 \pm 300\%$  basal vs. leptin treated rats, which had an increase of  $2450 \pm 270\%$  basal ( $p \leq 0.05$ ; comparison of % basal DA between leptin and vehicle treated rats).

Differences in [3-MT] were also measured between leptin and vehicle treated rats during the cocaine and raclopride infusion. When comparing the change of [3-MT] relative to the [3-MT] with cocaine (basal 3-MT set to 100% during cocaine infusion), the % increase in [3-MT] was  $150 \pm 17\%$  in PBS treated rats vs.  $230 \pm 20\%$  in leptin treated rats ( $p \leq 0.05$ ). No statistically significant difference was measured when comparing the overall increase (e.g. % basal measurements) of 3-MT after cocaine and raclopride infusion. During cocaine infusion, DOPAC decreased slightly to  $96 \pm 5\%$  basal and  $94 \pm 2\%$  basal with and without leptin, respectively. When cocaine was co-infused with raclopride, the DOPAC increased slightly to  $113 \pm 4\%$  basal and  $104 \pm 10\%$  basal with and without leptin, respectively. No differences were detected in % basal HVA between leptin treated and PBS treated animals after a cocaine infusion. With cocaine and raclopride, HVA increased slightly to  $110 \pm 4\%$  basal and  $106 \pm 3\%$

basal with leptin and with PBS treatment, respectively. No statistically significant differences in HVA or DOPAC were measured between leptin treated and PBS treated rats.

#### *Production of $^{13}\text{C}$ metabolites*

To determine if changes in 3-MT, DOPAC, and HVA with leptin were caused by changes in enzyme activity,  $^{13}\text{C}_6$ -DA was infused in the probe before and after a leptin injection and [ $^{13}\text{C}_6$ -3-MT], [ $^{13}\text{C}_6$ -DOPAC], and [ $^{13}\text{C}_6$ -HVA] were monitored. No differences were measured in the amount of  $^{13}\text{C}_6$ -3-MT produced (refer to Fig. 3.5) from  $^{13}\text{C}_6$ -DA prior to and after the leptin injection. The relative amount of  $^{13}\text{C}_6$ -DOPAC and  $^{13}\text{C}_6$ -HVA produced was an average of  $24 \pm 7\%$  ( $p \leq 0.05$ ) and  $30 \pm 8\%$  ( $p \leq 0.05$ ) greater after the leptin injection. The change in the  $^{13}\text{C}_6$ -DOPAC was small but is consistent with greater DA uptake in leptin treated rats.

## **Discussion**

### *Leptin increases DA uptake*

Several lines of evidence obtained here and elsewhere support the idea that acute leptin can decrease the extracellular DA concentration in the brain.<sup>169</sup> Further, these studies suggest that this effect is caused by increased DAT activity rather than decreases in release or enhanced metabolism.

The raclopride infusion revealed a lower increase in extracellular DA in leptin treated rats vs. vehicle treated rats. These changes could be due to a lower tone on  $\text{D}_2$  receptors, which may have caused a smaller increase in release. Another possibility is the increased uptake cleared the raclopride stimulated DA quicker in leptin treated rats. DOPAC, on the other hand, increased more in leptin treated rats vs. vehicle treated rats with raclopride. Additionally, the rats produced more  $^{13}\text{C}_6$ -DOPAC from  $^{13}\text{C}_6$ -DA following a leptin injection. Measuring DOPAC provides insight into the metabolic fate of DA. The DA that is taken up into the neuron is converted to

DOPAC or repackaged in vesicles. The lower release in DA after raclopride and the increase in DOPAC provided evidence that uptake increased due to a systemic leptin injection.

The decrease in extracellular DA concentrations in the NAc may be one mechanism for how leptin modulates feeding behavior. Previous work has shown that increasing extracellular DA in this brain region increases the incentive salience of sucrose.<sup>26</sup> In food deprived rats, basal extracellular DA concentrations decreased and feeding stimulated DA was attenuated in the NAc in rats that received leptin vs. vehicle.<sup>169</sup> If leptin can decrease extracellular DA and decrease the amount of DA released during feeding, the incentive salience of food should be decreased. Our study used a more accurate method than uncalibrated microdialysis and further showed that extracellular DA concentrations decreased in rats fed ad libitum after a leptin treatment. The cause of this decrease was demonstrated to be likely due to an increase in DAT activity. Leptin increasing DA uptake may cause the overall DA tone to decrease along with food induced increases in extracellular DA. Leptin would then restrict feeding by decreasing the reward value of food.

#### *Leptin increases DA release*

Although leptin reduces the DA concentration, our group and others have shown that it can increase TH activity. We sought to better understand how this might affect DA concentration and dynamics. The increased [3-MT]/[DA] ratio after a leptin injection with no difference in <sup>13</sup>C<sub>6</sub>-3-MT production from <sup>13</sup>C<sub>6</sub>-DA provided evidence that a greater amount of DA was being released with no change in COMT activity. Additionally, the infusion of raclopride with uptake blocked by cocaine caused a larger increase in extracellular DA in leptin treated rats vs. vehicle treated rats. These experiments provide evidence that DA release was increased in the NAc following a leptin injection.

The increased DA release caused by leptin appears to be minor. The cocaine infusion on its own produced no differences between leptin and vehicle treated rats. Our results are in agreement with a previous study with *ob/ob* and wild-type mice where no differences were detected in the cocaine induced DA release in the NAc,<sup>167</sup> even with more TH present in wild-type mice.<sup>163</sup> The small increase in release was only measured in our study with raclopride + cocaine infusion following cocaine alone. The small effect on release with the greater effect on uptake netted a decreased  $C_{app}^{DA}$  of DA in the NAc. Nevertheless, leptin does appear to play a role in affecting multiple processes in the DA system which ultimately allows it to modulate feeding behavior.

## **Conclusion**

Changes in basal extracellular DA concentrations caused by leptin in rats fed ad libitum has not been previously reported. Our study used a quantitative microdialysis technique with rats as their own control to demonstrate the net effects of leptin on DA in the NAc. By infusing raclopride through the probe, the cause of the net decrease was shown to be an increase in uptake. The infusion of cocaine and raclopride revealed an increase in DA release caused by leptin, although it appears to be minor. The ability to understand how leptin modulates the DA system in the brain may elucidate causes and possible treatments for feeding disorders such as obesity.



## Chapter IV

### Evidence for compartmentalization of extracellular glutamate in vivo

#### Introduction

Glutamate (Glu) is the primary excitatory neurotransmitter in the brain, yet its acceptance as a neurotransmitter was slowed by the observation that it is ubiquitous, seeming to preclude its ability to act as a selective messenger molecule. It is now thought that highly active reuptake and possibly compartmentalization, e.g. of the synapse from the rest of the brain extracellular space, facilitates Glu signaling. Understanding of Glu signaling and operation of different compartments has continued to evolve based on improved images of synapses, modeling, and direct chemical measurements. The role of glial cells in particular seems to be important shaping Glu signaling. A common view is that Glu neurotransmission is facilitated by a tripartite synapse consisting of presynaptic neuron, postsynaptic neuron, and astrocyte.<sup>45, 176, 177</sup> In this view, neurons release neurotransmitter into a synaptic cleft raising the concentration to 1.1 mM in a few ms.<sup>178</sup> The concentration is rapidly reduced by diffusion away and reuptake by excitatory amino-acid transporters (EAAT). Astrocytes are critical in this regard because they provide barriers to diffusion and contain ~90% of the EAAT.<sup>179</sup> Spill-over is common, but on a small scale (1-10%). The astrocytes may also release Glu and other neuromodulators by a variety of mechanisms. Such release may be to maintain Glu tone, modulate neurotransmission, or send signals to more distant synapses.<sup>42, 45, 180</sup> Astrocytes also support neurons by providing a source of Glu via the Glu-glutamine (Gln) shuttle. Glu that is taken up by glia<sup>181</sup> can be converted to

Gln by glutamine synthase. This Gln is released and can be taken back up by neurons where it is converted to Glu by glutaminase, an enzyme localized primarily in neurons. In this way it is thought that glial cells maintain a supply of neuronal Glu for signaling.<sup>182-186</sup> Astrocytes may also help couple neurotransmission to metabolism by connection to blood vessels.<sup>187, 188</sup>

Within this framework a number of controversial issues have arisen. One relates to extracellular Glu concentration and its primary sources. Electrophysiological measurements in hippocampal slices yield estimates of ~25 nM for Glu.<sup>85, 189</sup> On the other hand, *in vivo* measurements in the extracellular space by microdialysis show a higher concentration of 1-10  $\mu$ M.<sup>68, 170</sup> This “basal” concentration appears to contain little Glu that was directly released by neurons. This conclusion is based on the finding that it is unaffected by blocking sodium channels with TTX.<sup>190, 191</sup> Rather, evidence suggests that this extracellular Glu is released from astrocytes by a variety of mechanisms including Cys-Glu antiporter,<sup>192</sup> and vesicular release by activation of voltage-dependent calcium channel.<sup>44</sup> It is concluded from these observations that little Glu escapes the synapse under basal conditions.<sup>41, 124</sup> Studies using another invasive probe technique, enzyme electrode sensors, have reported somewhat higher concentrations that can be reduced by ~40% by microinjection of TTX near the electrode.<sup>79, 80</sup> An interpretation of these observations is that Glu does escape the synapse at appreciable concentrations to enable detection.

While the basal Glu measured by microdialysis is not regulated as expected for neuronal release, under some conditions a change in extracellular Glu can be elicited that appears to directly relate to neuronal activity.<sup>193-195</sup> Electrical stimulation evoked release that was blocked by TTX and regulated by autoreceptors<sup>193</sup> and stress has increased glutamate in the cortex that is TTX sensitive.<sup>194, 195</sup> Combined, these results indicate that at least in some brain regions under

some conditions neuronal release can be detected in extracellular space. While such release may be direct spillover from the synapse, it has also been proposed that they represent active release by astrocytes in response to being stimulated by neuronal Glu.<sup>196</sup>

A number of explanations have been put forth to explain the discrepancies in concentrations and sources of extracellular Glu. Experimental artifacts may contribute to these differences. It has been suggested that tissue damage caused by invasive probes results in falsely high concentrations.<sup>85, 189</sup> This argument is bolstered by the observation that if the concentration was 1-30  $\mu\text{M}$ , then the concentration would be close to excitotoxic levels and receptors would be desensitized. Tissue damage has also been invoked to explain why electrochemical sensors give a higher concentration than dialysis probes.<sup>197</sup> Another view that has been proposed is that compartmentalization between the synapse, perisynapse, and extracellular space, enforced by spatial distribution of release and reuptake sites, allows for differences in concentration among these regions *in vivo*.<sup>198</sup> According to this view, the different techniques are measuring from different pools based on the nature of the technique. This view accommodates roles for neuronal and astrocytic release in Glu transmission. While attractive, evidence that multiple extracellular pools or compartments actually exist *in vivo* is lacking. One issue has been the difficulty of using a single technique that allows distinction of a neuronal pool separate from an astrocytic pool.

Another issue relates to the necessity and timing of the Glu-Gln shuttle for maintaining Glu signaling. The Glu-Gln shuttle is well-established. Its significance has been bolstered by the observation that effects of Glu transmission can be blocked by inhibiting the conversion of Gln to Glu.<sup>182-184, 186, 199</sup> The recent observation of rapid fluctuations in Gln during neuronal activity also seem to support the importance of Gln for moment to moment Glu signaling.<sup>200</sup> At the same time, modulation of Glu neurotransmission by Gln has been difficult to observe. For example, in

rat hippocampus slices, Gln applied at high concentrations (4 mM) had no effect on the quantal amplitude or frequency of somatic whole-cell voltage-clamp recordings for up to 4 h.<sup>201</sup>

In this report, we use microdialysis and stable isotope metabolic labeling to distinguish different pools of Glu *in vivo*. We reason that if <sup>13</sup>C<sub>5</sub>-Gln was delivered to the brain, it would form <sup>13</sup>C<sub>5</sub>-Glu mainly in neurons. Neuronal release and collection of this <sup>13</sup>C<sub>5</sub>-Glu would in turn allow measurement of neuronally derived Glu despite a high background of Glu from non-neuronal sources normally detected. We find that the <sup>13</sup>C<sub>5</sub>-Glu is detected and is regulated differently from the endogenous or background <sup>12</sup>C-Glu. These results suggest two different compartments, one is primarily neuronal while the other is not, can be accessed *in vivo*. The results also suggest that spill-over can occur leading to potential for neuronal communication to astrocytes. Finally, the results support the idea that that Gln-Glu cycling is rapid with newly formed Glu being readily released by neurons.

## Methods

### *Chemicals and reagents*

All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Artificial cerebral spinal fluid (aCSF) comprised of 145 mM NaCl, 2.68 mM KCl, 1.01 mM MgSO<sub>4</sub>, 1.22 mM CaCl<sub>2</sub>, 1.55 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.45 mM NaH<sub>2</sub>PO<sub>4</sub> (Fisher Scientific, Pittsburgh, PA). <sup>13</sup>C<sub>5</sub>-Glu was purchased from Cambridge Isotopes (Andover, MA). Mobile phase of the LC column included 10 mM ammonium formate and 0.15% formic acid.

### *Microdialysis probes*

Probes were constructed as previously described.<sup>149</sup> Briefly, 40/100 μm (i.d./o.d.) fused silica capillaries (Polymicro Technologies, Phoenix, AZ) were glued side-by-side with a 2 mm

offset. The capillaries were ensheathed in a regenerated cellulose membrane with both ends sealed by polyimide sealing resin (Grace, Deerfield, IL). The flow rate through the probe was set to 1  $\mu$ L/min.

#### *Sample derivatization and analysis*

Each sample was derivatized with benzoyl chloride and analyzed as described previously.<sup>68</sup> Briefly, samples were sequentially mixed with 100 mM sodium tetraborate, benzoyl chloride (2% in acetonitrile, v/v), and internal standard in a 2:1:1:1 ratio. Internal standard comprised of standards reacted with <sup>13</sup>C<sub>6</sub>-benzoyl chloride in 100 mM sodium tetraborate. The internal standard was the diluted 1:100 in DMSO and 1% formic acid (v/v). The samples were analyzed on a Waters UPLC system with a Waters HSS T3 column (1 mm x 100 mm, 1.8  $\mu$ m). A Waters/Micromass Quattro Ultimatriple quadrupole or an Agilent 6410 triple quadrupole mass spectrometer was used for detection. Mobile phase A was 10 mM ammonium formate and 0.15% (v/v) formic acid in water. Mobile phase B was acetonitrile. The peak areas of each analyte were divided by the area of the internal standard.

#### *Surgery*

Male Sprague-Dawley rats were anesthetized with ketamine (65 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) and dexdomitor (0.25 mg/kg, Pfizer Animal Health, New York, NY) and placed in a stereotaxic frame. A burr hole was drilled where the probe was being implanted (+0.2 A/P,  $\pm$ 2.3 L, aimed at cortex).<sup>150</sup> The rats were bi-laterally implanted. Additional burr holes were drilled for skull screws to hold the cap in place. The probes were lowered 3 mm from the top of the skull and dental cement (A-M Systems, Inc., Sequim, WA) was used to hold the probes in place. All animal procedures were approved by the University Committee for the Use and Care of Animals at the University of Michigan.

The day of the experiment, rats were lightly antethetized in an isofluorane drop box. The microdialysis probes were attached to the syringes and perfused with aCSF at a flow rate of 1  $\mu\text{L}/\text{min}$ . The rat was tethered to a Ratum (Bioanalytical Systems, Inc., West Lafayette, IN).

#### *Measuring $^{13}\text{C}_5\text{-Glu}$ production with 14 min plug of $^{13}\text{C}_5\text{-Gln}$*

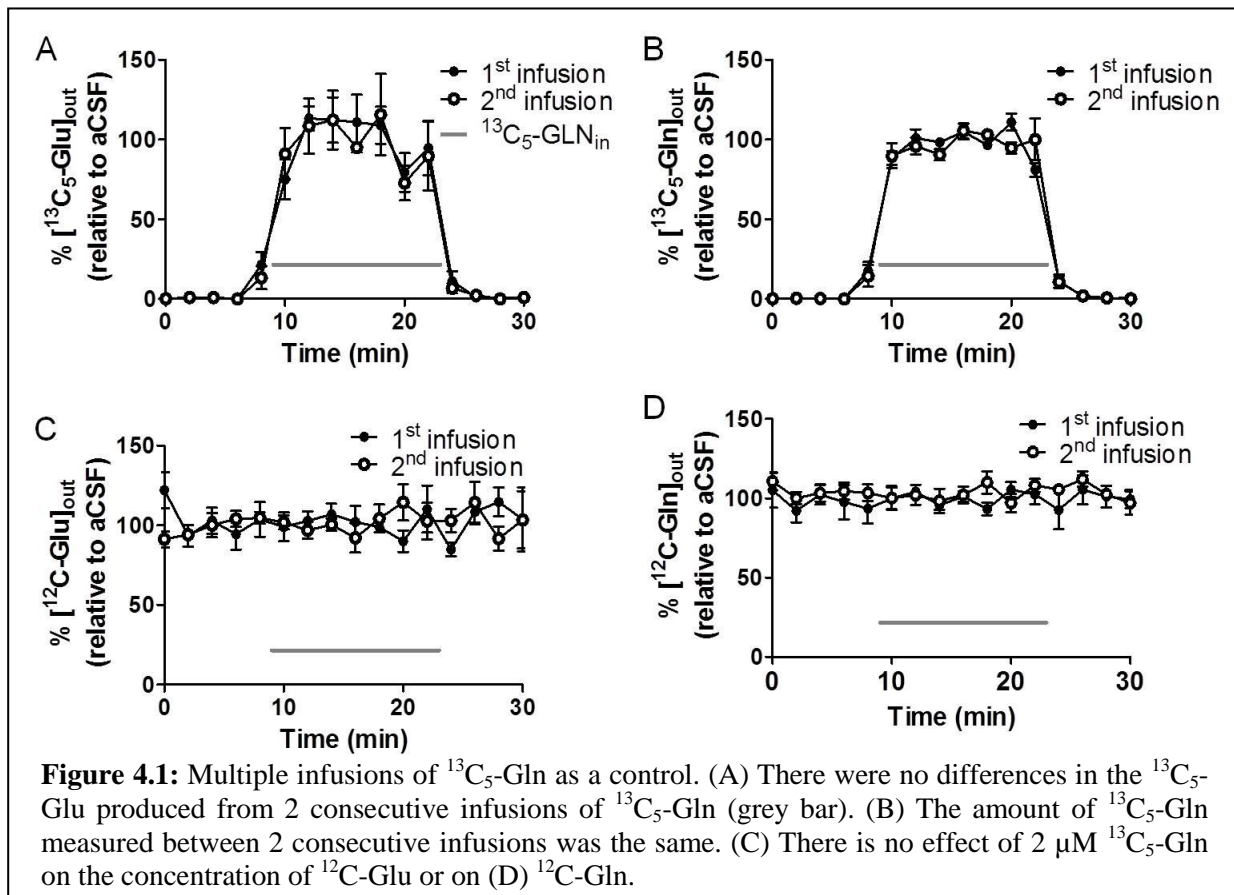
Syringes containing aCSF or a pharmacological agent were switched to a syringe containing 2.5  $\mu\text{M}$   $^{13}\text{C}_5\text{-Gln}$  and aCSF or a pharmacological agent using a 4-port valve (Valco Instruments, Houston, TX). The pharmacological agents administered were 2  $\mu\text{M}$  tetrodotoxin (TTX), 200  $\mu\text{M}$  (2*R*,4*R*)-4-Aminopyrrolidine-2,4-dicarboxylate (ACPD), or 200  $\mu\text{M}$  (*RS*)- $\alpha$ -Methyl-4-carboxyphenylglycine (MCPG, Tocris, Bristol, UK). If TTX, ACPD, or MCPG were tested, they were perfused for 15 min prior to the  $^{13}\text{C}_5\text{-Gln}$  switch and were also present during the  $^{13}\text{C}_5\text{-Gln}$  infusion. For the 1 mM L-*trans*-Pyrrolidine-2,4-dicarboxylic acid (PDC, Tocris) aCSF was perfused and the PDC was perfused only during the  $^{13}\text{C}_5\text{-Gln}$  perfusion. Samples were collected every 2 min and derivatized.

#### *Measuring effect of longer infusion of $^{13}\text{C}_5\text{-Gln}$*

For the tail pinch, a syringe containing 2.5  $\mu\text{M}$   $^{13}\text{C}_5\text{-Gln}$  with and without 2  $\mu\text{M}$  TTX was switched to the probe for 10 min prior to the baseline collection. After 3 baseline fractions, a binder clip was attached to the rat for 10 min. The clip was removed and collections continued for an additional 14 min. Fractions were collected every 2 min. To test the TTX sensitivity of the  $^{13}\text{C}_5\text{-Glu}$  over a longer period, 2.5  $\mu\text{M}$   $^{13}\text{C}_5\text{-Gln}$  was infused through the probe for 60 min. At 60 min, the syringes were switched with the 4-port valve to a solution containing 2.5  $\mu\text{M}$   $^{13}\text{C}_5\text{-Gln}$  and 2  $\mu\text{M}$  TTX. Sample fractions were collected every 5 min for an additional 60 min.

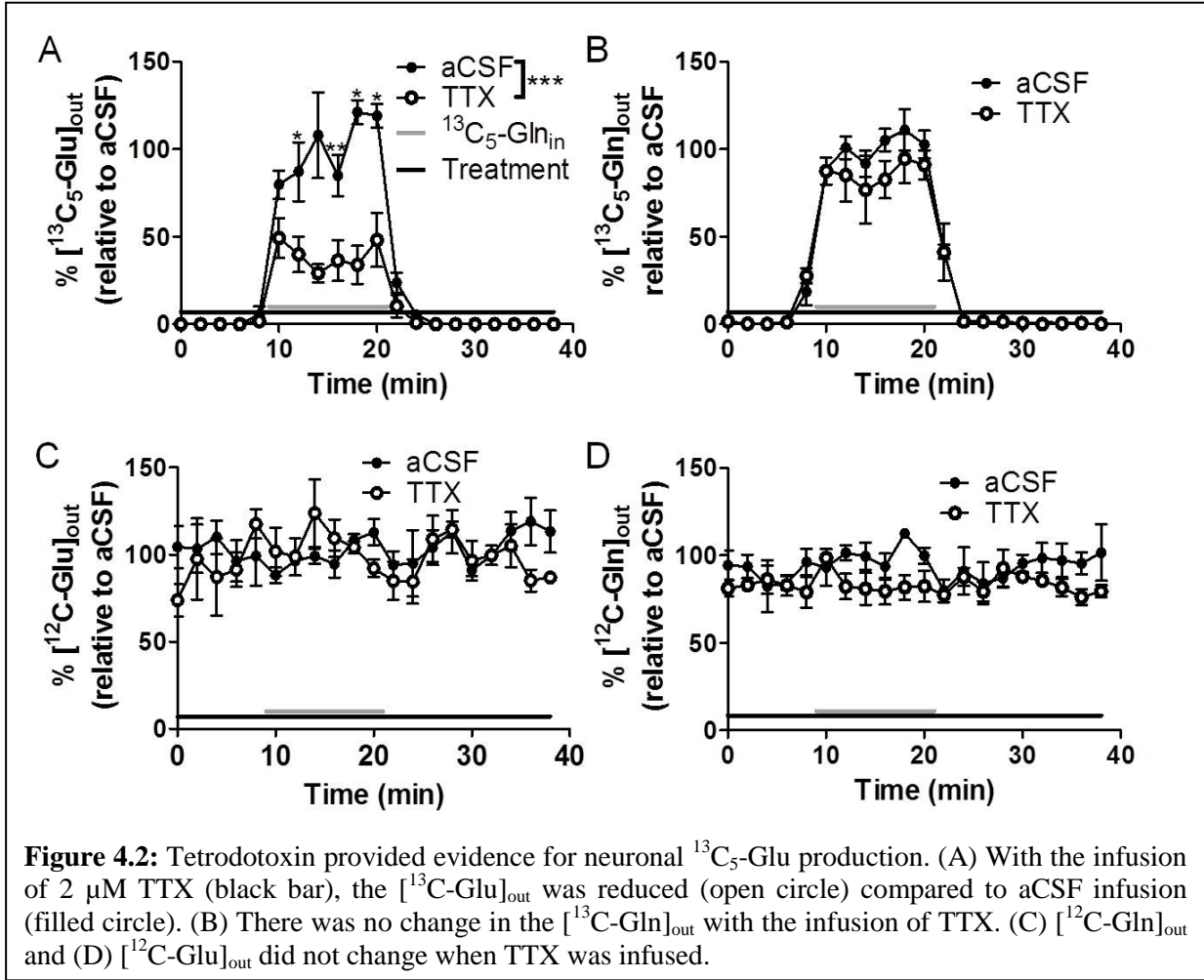
#### *Statistical analysis*

For comparison of individual time points for  $^{13}\text{C}_5\text{-Glu}$  produced with different drug treatments, paired t-tests were performed. When comparing the  $^{13}\text{C}_5\text{-Glu}$  production with aCSF vs. drug treatment, a linear mixed model regression was used to test significance. SPSS (IBM, Armonk, NY) was used for the mixed model regression. The rat ID was used as the subject, drug treatment was used as a factor, time was used as a covariate, and the measurement (e.g. % basal) was tested as the dependent variable.



## Results.

*Concentration of Glu and Gln in the brain extracellular space.*



*In vivo* calibration of a microdialysis probe is useful to correct for probe recovery and effects of brain tissue on transport within the brain to the microdialysis probe. One method of *in vivo* calibration is to infuse a stable isotope labeled (SIL) form of a compound and measure its loss from the microdialysis probe.<sup>135, 136, 170</sup> This loss is used to calculate the extraction fraction ( $E_d$ ), which can be used to calculate the recovery of the probe *in vivo*, with the following equation:

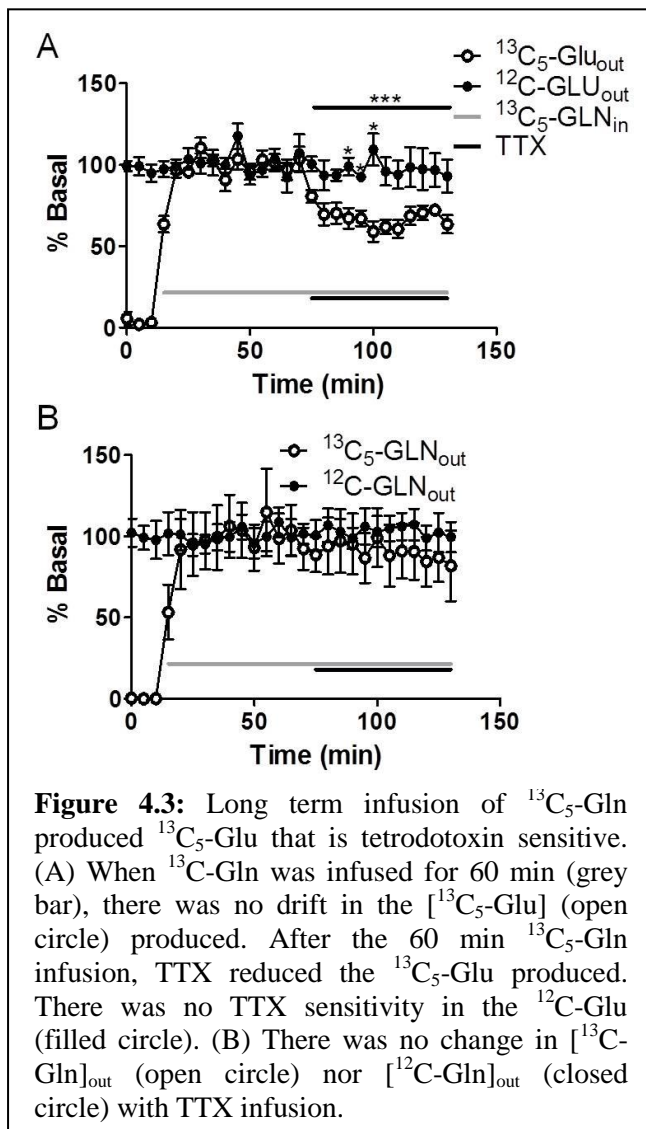
$$E_d = 1 - \frac{C_{\text{out}}^{\text{SIL}}}{C_{\text{in}}^{\text{SIL}}}$$



In the above equation,  $C_{out}^{SIL}$  and  $C_{in}^{SIL}$  are the concentration of SIL compound that is measured at the outlet of the probe infused into the probe, respectively. The apparent concentration ( $C_{app}$ ) of the compound in the brain can be measured with the following equation:

$$C_{app} = 1 - \frac{C_{out}^{endogenous}}{E_d^{SIL}}$$

where ( $C_{out}^{endogenous}$ ) is the measured concentration of endogenous compound in dialysate. Using



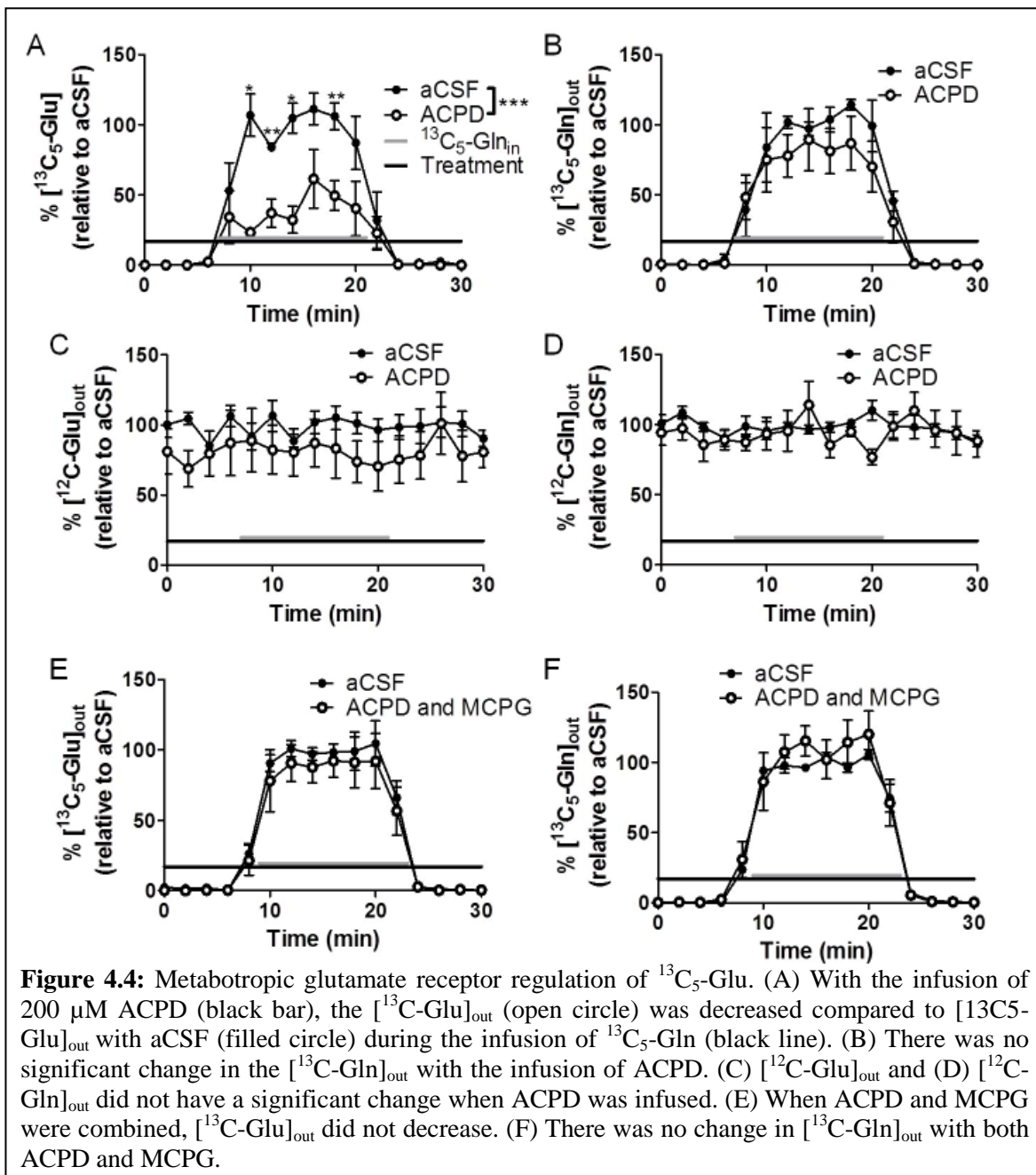
this method, we calculated that probe recovery was  $0.30 \pm 0.03$  for Gln and  $0.29 \pm 0.01$  for Glu while infusing  $2.5 \mu M$  and  $5.0 \mu M$  of Gln and Glu respectively. The  $C_{app}$  of Gln =  $179 \pm 20 \mu M$  and Glu =  $9.4 + 0.6 \mu M$  ( $n=11$ ). These values were in good agreement with previous microdialysis and electrode sensor estimates in other areas of the cortex.<sup>41, 153</sup>

*Gln is rapidly converted to Glu in the brain*

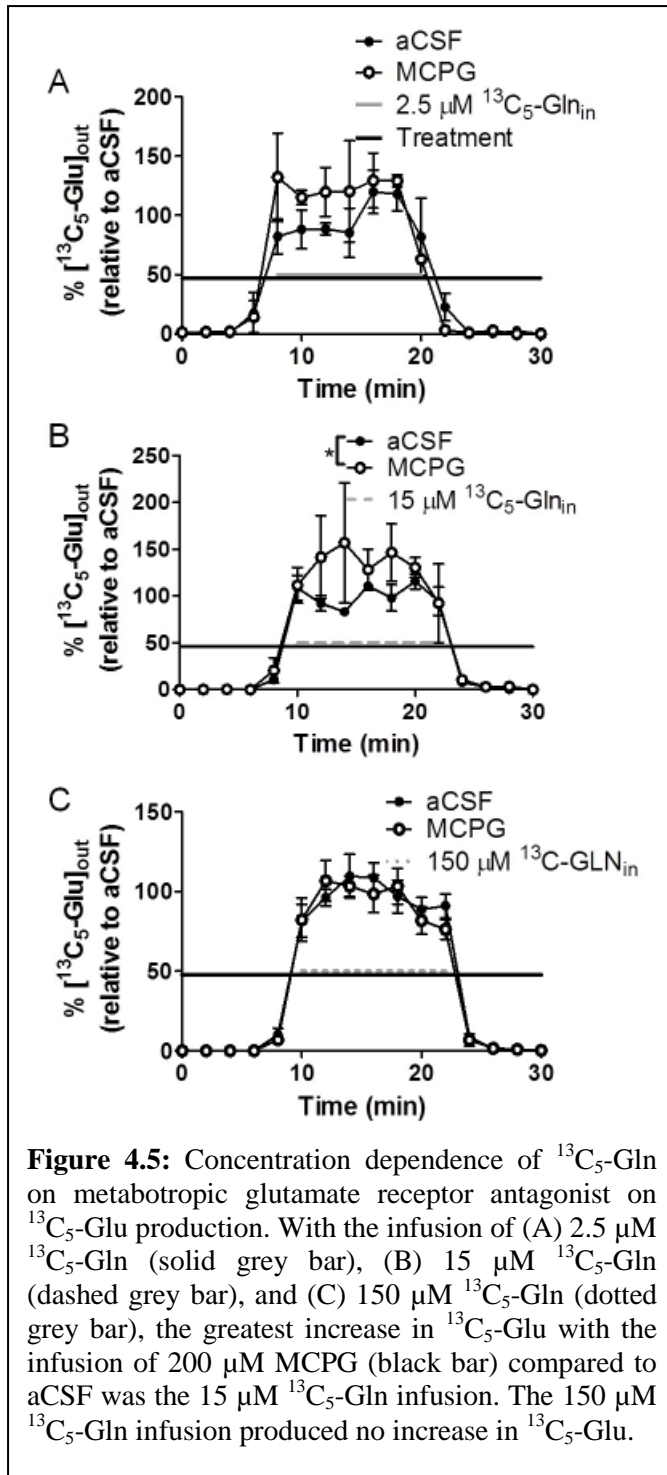
Infusing  $2.5 \mu M$   $^{13}C_5$ -Gln through the probe resulted in a loss of  $^{13}C_5$ -Gln due to diffusion into the brain so that the concentration in dialysate exiting the probe was  $1.74 \pm 0.07 \mu M$  ( $n = 11$ ). During infusion of  $^{13}C_5$ -Gln, we also found that

$^{13}C_5$ -Glu was collected. The  $^{13}C_5$ -Glu reached a steady state concentration within 1 min of

starting  $^{13}\text{C}_5\text{-Gln}$  perfusion (data not shown). The recovered  $^{13}\text{C}_5\text{-Glu}$  was  $60 \pm 7$  nM ( $n = 9$  animals) and stayed stable for up to 60 min, the longest time tested. Once  $^{13}\text{C}_5\text{-Gln}$  was removed from the probe, the  $^{13}\text{C}_5\text{-Glu}$  signal declined to near baseline in the next fraction suggesting rapid dissipation, either by diffusion or metabolism or both, of the labeled Glu. The concentration of  $^{13}\text{C}_5\text{-Gln}$  added to brain and  $^{13}\text{C}_5\text{-Glu}$  recovered were low, less than 1%, compared to their



**Figure 4.4:** Metabotropic glutamate receptor regulation of  $^{13}\text{C}_5\text{-Glu}$ . (A) With the infusion of 200  $\mu\text{M}$  ACPD (black bar), the  $^{13}\text{C-Glu}_{\text{out}}$  (open circle) was decreased compared to  $^{13}\text{C}_5\text{-Glu}_{\text{out}}$  with aCSF (filled circle) during the infusion of  $^{13}\text{C}_5\text{-Gln}$  (black line). (B) There was no significant change in the  $^{13}\text{C-Gln}_{\text{out}}$  with the infusion of ACPD. (C)  $^{12}\text{C-Glu}_{\text{out}}$  and (D)  $^{12}\text{C-Gln}_{\text{out}}$  did not have a significant change when ACPD was infused. (E) When ACPD and MCPG were combined,  $^{13}\text{C-Glu}_{\text{out}}$  did not decrease. (F) There was no change in  $^{13}\text{C-Gln}_{\text{out}}$  with both ACPD and MCPG.



estimated *in vivo* concentrations. This concentration appeared to be low enough to not cause irreversible or unexpected effects. For example, infusion of Gln did not affect endogenous concentrations of Gln or Glu and the concentration of  $^{13}\text{C}_5\text{-Glu}$  produced from  $^{13}\text{C}_5\text{-Gln}$  was unchanged between multiple infusions in the same rat (Fig. 4.1).

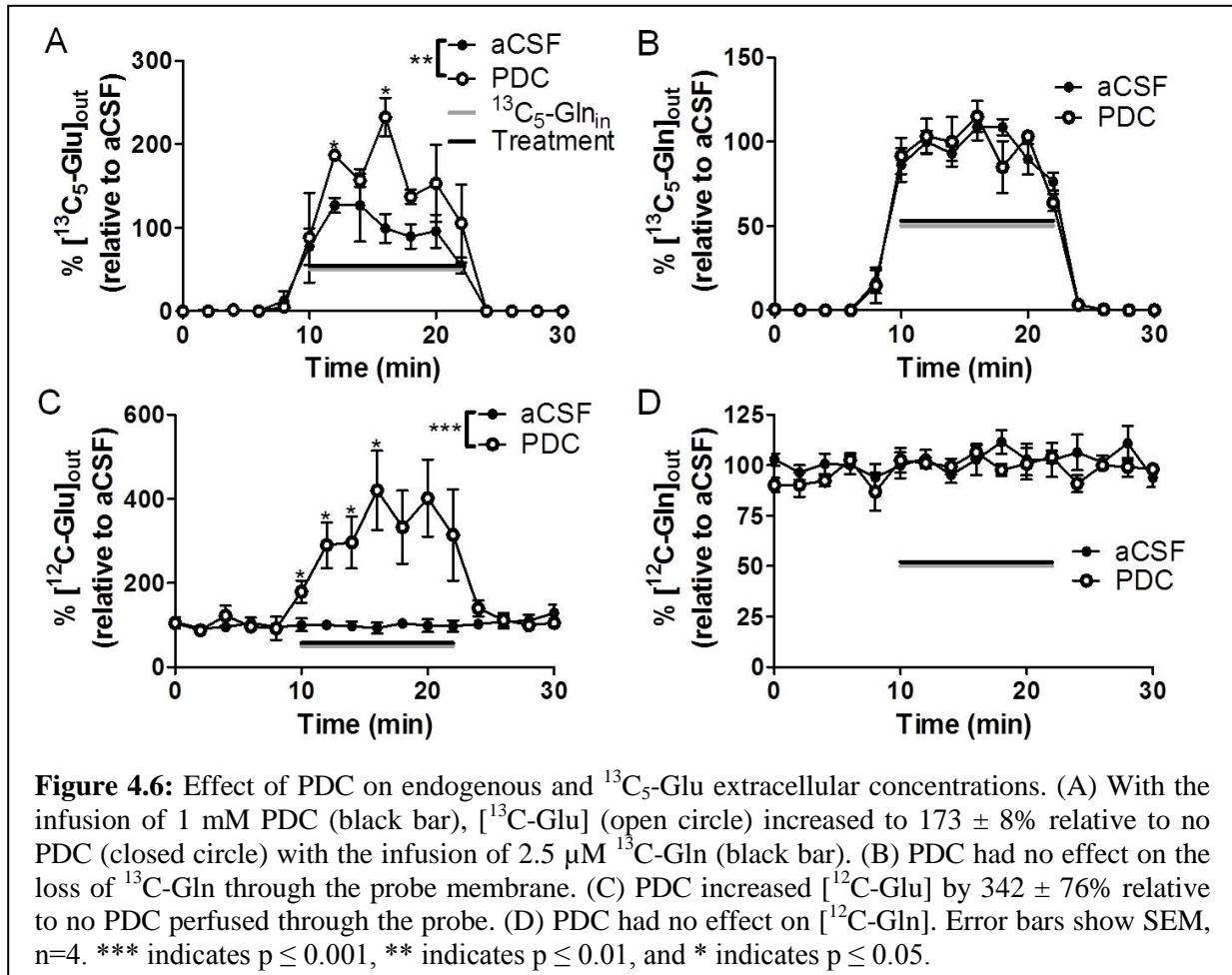
*$^{13}\text{C}_5\text{-Glu}$  concentration is regulated by TTX and autoreceptors*

Gln is mainly converted to Glu in neurons; therefore to reach the probe  $^{13}\text{C}_5\text{-Glu}$  must have been released by neurons. We asked if the  $^{13}\text{C}_5\text{-Glu}$  that was detected was modified by treatments that would be expected to modify neuronal release.

Infusion of  $2\ \mu\text{M}$  TTX prior to and during  $^{13}\text{C}_5\text{-Gln}$  infusion (Fig. 4.2) suppressed release of  $^{13}\text{C}_5\text{-Glu}$  by  $62 \pm 7\%$  ( $p \leq 0.001$ ). Use of a higher concentration of

TTX ( $50\ \mu\text{M}$ ) did not further reduce  $^{13}\text{C}_5\text{-Glu}$  concentration. If TTX was applied 60 min after beginning  $^{13}\text{C}_5\text{-Gln}$  infusion (Fig. 4.3), the  $^{13}\text{C}_5\text{-Glu}$  was still reduced but by just  $34 \pm 3\%$  ( $p \leq$

0.001). No statistically significant difference was measured between the 62% reduction of the short  $^{13}\text{C}_6\text{-Gln}$  infusion vs. the 34% reduction in the longer  $^{13}\text{C}_6\text{-Gln}$  infusion. Unlike  $^{13}\text{C}_5\text{-Glu}$ , the endogenous  $^{12}\text{C-Glu}$  was unchanged by TTX treatment (Fig. 4.2). This latter result is in agreement with numerous studies that have found little effect or even increases in dialysate Glu with local TTX treatment.<sup>124, 202</sup>



Neuronal Glu release is also modulated by mGluR. In previous studies, group II mGluR agonists have decreased Glu in the hippocampus<sup>203</sup> and nucleus accumbens when infused through the probe;<sup>204</sup> however, other studies have shown that infusion of group I and II mGluR agonists and antagonists infused through microdialysis probes have no effect on basal Glu measured by microdialysis<sup>12, 205, 206</sup>. We found no effect on basal concentrations of endogenous

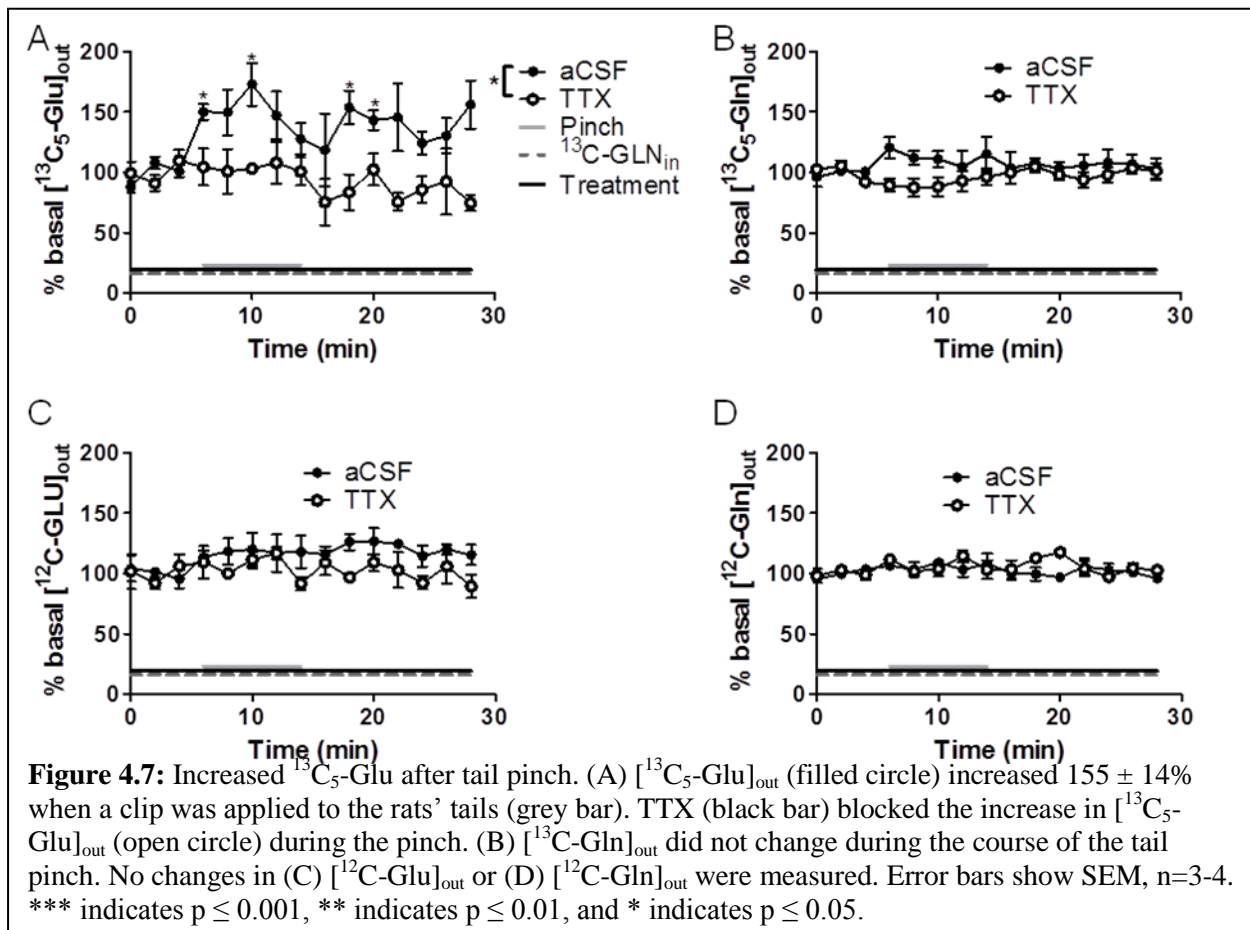
Glu (and Gln) following infusion of 200  $\mu\text{M}$  ACPD (group I and II mGluR agonist) and 200  $\mu\text{M}$  MCPG (group I and II mGluR antagonist). In contrast, the  $^{13}\text{C}_5\text{-Glu}$  detected was reduced by  $59 \pm 9\%$  ( $p \leq 0.001$ ) by ACPD (Fig. 4.4). This effect was blocked by simultaneous infusion of 200  $\mu\text{M}$  MCPG with the ACPD. MCPG infusion alone increased  $^{13}\text{C}_5\text{-Glu}$  to  $124 \pm 17\%$  of baseline when 2.5  $\mu\text{M}$   $^{13}\text{C}_5\text{-Gln}$  ( $n = 3$ ) was infused through the probe. To determine if the small increase was due to the [ $^{13}\text{C}_5\text{-Glu}$ ] not being large enough to activate the group I and II mGluR autoreceptors, 15 and 150  $\mu\text{M}$   $^{13}\text{C}_5\text{-Gln}$  was infused into the probe with and without 200  $\mu\text{M}$  MCPG (Fig. 4.5). The infusion of 15  $\mu\text{M}$   $^{13}\text{C}_5\text{-Gln}$  produced 690 nM  $^{13}\text{C}_5\text{-Glu}$  in the dialysate which was increased  $140 \pm 30\%$  in the presence of MCPG ( $p \leq 0.05$ ,  $n = 3$ ). These results are consistent with autoreceptor regulation of the  $^{13}\text{C}_5\text{-Glu}$  that is collected by the dialysis probe.  $^{13}\text{C}_5\text{-Glu}$  produced from 150  $\mu\text{M}$   $^{13}\text{C}_5\text{-Gln}$  infused through the probe did not increase with MCPG ( $n=4$ ).

#### *Effect of Gln Concentration on Glu*

While most experiments infused 2.5  $\mu\text{M}$  Gln, we also evaluated the dependency of Glu on the concentration of Gln infused. Infusing Glu up to 150  $\mu\text{M}$  produced a linear concentration of  $^{13}\text{C}_5\text{-Glu}$ . At a higher concentration (50  $\mu\text{M}$   $^{13}\text{C}_5\text{-Gln}_{\text{in}}$ , 14 min infusion), the  $^{13}\text{C}_5\text{-Glu}$  Glu was not TTX sensitive.

#### *Uptake inhibition*

PDC is a transportable Glu uptake inhibitor that significantly raises extracellular Glu concentration by both blocking reuptake and by being exchanged with intracellular Glu.<sup>131, 207</sup> Addition of 1 mM PDC to the dialysis perfusion fluid while  $^{13}\text{C}_5\text{-Gln}$  was also infused increased  $^{12}\text{C-Glu}$   $342 \pm 76\%$  and  $^{13}\text{C}_5\text{-Glu}$   $173 \pm 8\%$  ( $p \leq 0.01$ ) relative to that without PDC (Fig. 4.6). PDC did not affect  $^{13}\text{C}_5\text{-Gln}$  or endogenous Gln.



### Stimulated release of Glu

Stressors such as tail pinch have previously been reported to increase extracellular Glu in the cortex as measured by microdialysis and sensors.<sup>79, 80, 194</sup> Interestingly, this observation has not been widely reported and others have found that a stress response is highly variable.<sup>190</sup> In our hands, tail pinch had no effect on endogenous  $^{12}\text{C-Glu}$  (Fig. 4.7C) or  $^{12}\text{C-Gln}$ . However, tail pinch did increase  $^{13}\text{C}_5\text{-Glu}$  following infusion of  $^{13}\text{C}_5\text{-Gln}$  by  $155 \pm 14\%$  ( $p \leq 0.05$ ) (Fig. 4.7A). This increase was completely abolished by TTX infusion ( $p \leq 0.05$ ).

### Discussion

*$^{13}\text{C}_5\text{-Glu}$  is from neurons*

Our objective in perfusing  $^{13}\text{C}_5\text{-Gln}$  was to use the Glu-Gln shuttle to isolate a neuronal component of Glu in the extracellular space that could be monitored by endogenous measurements alone. Here we uncover a TTX-sensitive extracellular compartment of Glu that is apparent under “basal conditions”, e.g. without specific behavioral, drug, or electrical activation of neurons. The Glu is derived from  $^{13}\text{C}_5\text{-Gln}$  added exogenously at low concentrations. Because neurons preferentially convert Gln to Glu in the brain, a majority of this  $^{13}\text{C}_5\text{-Glu}$  must be released from neurons. Supporting the idea that it derives from neurons, we find that it is strongly inhibited by activation of group I and II mGluR, an effect blocked by antagonists, and increased by antagonists. We interpret this as modest neuronal overflow under basal conditions under some control by autoreceptors. In contrast, the endogenous  $^{12}\text{C-Glu}$  is completely unaffected by these treatments. We conclude, like others, that the endogenous  $^{12}\text{C-Glu}$  must have a non-neuronal source. Thus, the  $^{13}\text{C}$  and  $^{12}\text{C}$  glu are coming from different extracellular sources, one primarily neuronal ( $^{13}\text{C}$ ) and the other primarily non-neuronal ( $^{12}\text{C}$ ). These results show that the use of  $^{13}\text{C}_5\text{-Gln}$  allows effective probing of neuronally derived Glu while the endogenous Glu measured is primarily from other sources, likely astrocytic.

#### *Detection of $^{13}\text{C}_5\text{-Glu}$*

The significant dichotomy between  $^{13}\text{C}_5\text{-Glu}$  and  $^{12}\text{C-Glu}$  found here is perhaps surprising. If Glu can normally escape from the synapse, then the basal concentration of endogenous,  $^{12}\text{C-glu}$  should be at least partially TTX/ACPD sensitive; however, it is not.

We believe that this discrepancy may be because the  $^{13}\text{C}_5\text{-Glu}$  that is detected is formed recently.  $^{13}\text{C}_5\text{-Glu}$  appears no more than 60 s after the  $^{13}\text{C}_5\text{-Gln}$  is added and is eliminated as soon as the  $^{13}\text{C}_5\text{-Gln}$  is removed. The rapid clearance of  $^{13}\text{C}_5\text{-Glu}$  from the extracellular space means that the  $^{13}\text{C}_5\text{-Glu}$  detected in any fraction was recently formed and therefore unlikely to be

significantly mixed with other compartments. In effect, the rapid flux of Glu allows the  $^{13}\text{C}$ -labeled form to fill an “empty” pool making it readily detected. In contrast,  $^{12}\text{C}$ -Glu detected could be coming from multiple sources. As a result, the percentage that is coming directly from neurons must be lower. Judging by the insensitivity to TTX, only a very low percentage is directly from a neuron.

A second reason is spatial distribution of the possible sources of Glu. The  $^{13}\text{C}_5$ -Glu detected must have been formed primarily near the probe surface and therefore has a minimal distance to diffuse to the probe. This would lessen the likelihood of being captured by transporters or other elimination mechanisms. In contrast,  $^{12}\text{C}$ -Glu may be formed from both close and farther away allowing for the possibility that astrocytic release or other homeostatic mechanisms would dominate.

When  $50\ \mu\text{M}$   $^{13}\text{C}_5$ -Gln was infused through the probe for 14 min,  $^{13}\text{C}_5$ -Glu did not decrease with the infusion of TTX. While a 64% decrease was measured with a 14 min infusion of  $2.5\ \mu\text{M}$   $^{13}\text{C}_5$ -Gln, longer infusion times of  $2.5\ \mu\text{M}$   $^{13}\text{C}_5$ -Gln caused less TTX sensitivity. Higher concentrations of  $^{13}\text{C}_5$ -Gln infused and longer infusions may cause the more concentrated  $^{13}\text{C}_5$ -Glu to reach compartments available to the astrocytes, which may cycle into the glutamate pool normally measured by microdialysis. We do not believe that the detection of  $^{13}\text{C}_5$ -Glu is an artifact due to altering or saturating normal Gln/Glu processing. The  $^{13}\text{C}_5$ -Gln concentration exiting the probe was  $760 \pm 70\ \text{nM}$ . This concentration would be the maximal possible added to the brain and therefore much lower than the  $50 \pm 4\ \mu\text{M}$  endogenous that we measured. Similarly, the concentration of  $^{13}\text{C}_5$ -Glu recovered was  $60 \pm 7\ \text{nM}$ , much less than the endogenous Glu measured. We have previously shown that infusing up to  $10\ \mu\text{M}$  Glu had no effect on the recovered [ $^{12}\text{C}$ -Glu].<sup>170</sup> The situation for Glu is different from most other neurotransmitters.



Neuronal Glu is dependent on a supply of Gln and even moment-to-moment changes in Gln are believed to occur<sup>200</sup>. This strong metabolic dependency of Glu makes it more susceptible to disturbance by depletion than other neurotransmitters. Interestingly, our observation is in contrast to that observed in hippocampal slices where it was found that exogenous Gln did not affect Glu signaling until 4 h after exposure leading to the conclusion that in that environment the neurons do not depend upon astrocytic Gln, at least for moment to moment signaling.<sup>201</sup>

#### *Differentiation of stimuli*

The use of the <sup>13</sup>C<sub>5</sub>-Gln allows differentiation of how perturbations modulate different sources of Glu. Our results show that a stress stimulus, tail-pinch, evokes release of <sup>13</sup>C<sub>5</sub>-Glu and that the release is blocked by TTX. In contrast the <sup>12</sup>C-Glu was relatively unaffected. We conclude that the Glu released is due to neuronal activation with little response or modulation by astrocytes. A few previous studies have shown that this stimulus can evoke release of endogenous Glu although we could not replicate that finding.<sup>79, 80, 194, 208</sup> One of the studies was based on electrochemical sensing, which may have a different ability to detect neuronal Glu. The microdialysis study showed that Glu release is dependent on conditions, for example a second tail pinch gives much reduced release.<sup>208</sup> Therefore the state and prior stress of the animal may influence detection of Glu. Also, technical issues may influence ability to recover a neuronally derived Glu signal.

PDC is a transportable inhibitor and therefore could affect extracellular Glu in two ways. One is to allow spread of Glu released from neurons or glial cells. A second action is release of Glu due to exchange of drug for intracellular Glu. Since Glu is primarily taken up by glial cells, we would expect that the exchange mechanism would cause glial cell release. In agreement with

this expectation we saw considerable increase in both  $^{13}\text{C}_5\text{-Glu}$  and  $^{12}\text{C-Glu}$  with PDC treatment. The  $^{12}\text{C-Glu}$  increased  $342 \pm 76\%$  baseline and  $^{13}\text{C}_5\text{-Glu}$  increased  $173 \pm 8\%$  compared to aCSF.

### *Implications*

These results provide strong evidence of at least two, differentially regulated sources for glu within the brain extracellular space. One source is detected as endogenous Glu and is likely due primarily to astrocytic release. The other source is detected as  $^{13}\text{C}_5\text{-Glu}$  derived from Gln and appears to be released from neurons. Detection of  $^{13}\text{C}_5\text{-Glu}$  suggests that it can “leak” from synapses and therefore must intermingle with the other pool in the extracellular space where the endogenous  $^{12}\text{C-Glu}$  is found. The clear distinction in sources and regulation of Glu supports the concept of a compartmentalized Glu. Glu from neurons is released from synapses and possibly ectopic synapses generating a concentration that dependent upon the local environment. The other source, for example astrocytes, is releasing Glu with a different regulation and in a different space.

The ability to neuronal release of Glu without any stimulation by merely adding a small amount of Gln also supports the idea that Glu neurons in the vicinity of the probe are functional in the sense that they take up Gln, convert it to Glu, and release it in a TTX-sensitive and mGluR modulated manner. Thus, despite the physical trauma of probe insertion, the probe is able to monitor regulated Glu neurotransmitter release as established for other neurotransmitters.

The relatively high concentration of Glu detected by microdialysis and sensors has been suggested to be an artifact<sup>85</sup>, despite identification of reasonable sources such as the Cys-Glu antiporter<sup>42</sup>. Our results show that despite the concentration measured, autoreceptors are not normally activated since MCPG did not affect release by itself. These receptors are functional because their activation suppressed the neuronal component and this effect was blocked by

MCPG. This result also supports compartmentalization since the receptor regulation for the different sources is different.

The results also support the idea that Glu-neurons are dependent upon the Glu-Gln shuttle to maintain a supply of Glu for release<sup>49, 50</sup>. Indeed, the <sup>13</sup>C<sub>5</sub>-Glu was released within 1 min of supplying <sup>13</sup>C<sub>5</sub>-Gln showing that this new Glu rapidly put to use by neurons for signaling. The ready manipulation of Glu levels by Gln suggests that this may be a route for astrocytes to influence neuronal signaling. That is, changes in metabolic conversion of Glu to Gln or release of Gln would manipulate Glu signaling.

Released Glu is known to be taken up by glial cells. Interesting, once the Gln is removed, the <sup>13</sup>C<sub>5</sub>-Glu was immediately depleted. Therefore, multiple fates for glu taken up by glial cells must exist. These include metabolic conversion to  $\alpha$ -ketoglutarate<sup>188</sup> or converted back to <sup>13</sup>C<sub>5</sub>-Gln. Due to the large concentration of <sup>12</sup>C-Gln in the extracellular space vs. the low concentrations of <sup>13</sup>C<sub>5</sub>-Glu produced in these experiments, the converted <sup>13</sup>C<sub>5</sub>-Gln would be released by the astrocyte at relatively low concentrations.

In this study, we have successfully shown evidence for the compartmentalization of Glu production limited mainly to neurons. With <sup>13</sup>C<sub>5</sub>-Gln, we showed evidence through activating mGluR 2/3 receptors and using TTX that the production of <sup>13</sup>C<sub>5</sub>-Glu was neuronally derived. We also showed that increases in <sup>13</sup>C<sub>5</sub>-Glu can be measured through a stress test and through blocking uptake.

## Chapter V

### Conclusions and future directions

Measuring neurochemical changes from an intact brain can provide insight into many neural functions including memory, learning, mental diseases, and cognition. Microdialysis is the workhorse of sampling methods to study the central nervous system. The small size of the probes and the large amount of analytical techniques available to analyze dialysate samples makes microdialysis a versatile, robust method. A large drawback to this method is the lack of quantification with uncalibrated microdialysis. Without *in vivo* calibration, only relative changes can be measured and not absolute concentrations. Absolute concentrations may be important for setting the tone or endogenous exposure of receptors to neurotransmitter under basal conditions. Further, *in vivo* calibration of microdialysis provides the *in vivo* recovery which provides additional information on the *in vivo* environment. In particular, *in vivo* recovery and extraction fraction can be useful in discerning how neurotransmitter mass transport is affected by active processes such as reuptake and metabolism. In this work, a novel approach to quantitative microdialysis was developed based on infusion of stable isotope labeled neurotransmitter and then applied to the measurement of DA and Glu under different conditions. It is shown that the calibrated method is efficient relative to other calibration methods and provides novel insights into DA and Glu regulation that are not possible without calibration.

*Quantitative microdialysis conclusion*

Uncalibrated microdialysis is only able to provide a subset of information available with quantitative microdialysis. For example, decreased uptake caused a decrease in  $E_d$  for both DA and Glu when measured using NNF.<sup>126, 131</sup> By measuring  $E_d$  and monitoring changes in DA or Glu concentrations, it was possible to determine the source of increases (increased release or decreased uptake) in extracellular DA and Glu concentrations.<sup>133</sup> In addition to DA and Glu studies, accounting for differences in uptake has proven useful in serotonin transporter (SERT) knockout mice. Uncalibrated microdialysis showed no differences in  $C_{app}$  between the wild-type and heterozygous knockout, which had a 50% reduction in SERT gene expression. After quantitative microdialysis, basal [5-HT] was indeed higher in the heterozygous knockout.<sup>145</sup>

The main problem with employing quantitative microdialysis techniques is the large amount of time or animals required for each calibrated measurement. NNF measurements can take up to 7 hr per subject to obtain calibrated measurements.<sup>131</sup> dNNF requires less time per animal but requires at least 3x more animals for each experiment.<sup>133</sup>

The SIL microdialysis technique demonstrated in this work can be performed on the same timescale and with the same number of subjects as uncalibrated microdialysis because extraction fraction is measured on every sample of every animal. In our study, SIL microdialysis measurements of  $C_{app}$  and  $E_d$  for Glu and DA matched the same values within 10% when measured with NNF. With uptake blockers,  $E_d^{DA}$  was shown to decrease by 30% and 35% with the local infusion of 5  $\mu$ M nomifensine and systemic injection of cocaine (20 mg/kg, i.p.), respectively. With the local infusion of Glu uptake inhibitor PDC,  $E_d^{Glu}$  decreased by 40%. These decreases in  $E_d$  effectively caused  $C_{app}$  for both DA and Glu to be undervalued by as much as 50%. These results illustrate the potential of the SIL method to provide information on tissue

effects (e.g. changes in uptake) and avoid artifacts due to such changes during dynamic measurements.

In addition to the calibrated concentration and tissue effects measured with SIL microdialysis, the SIL method also provides the opportunity to monitor metabolism and flux of neurotransmitters by recovering and measuring SIL metabolites. Measuring the metabolism of SIL neurotransmitters is especially useful for measuring neuronally derived Glu. Because Glu is released from both neurons and astrocytes, trying to differentiate the two sources is difficult. By infusing  $^{13}\text{C}_5\text{-Gln}$  through the probe, newly formed  $^{13}\text{C}_5\text{-Glu}$  can be measured. The enzyme responsible for the conversion of Gln to Glu, glutaminase, is preferentially located within the neuron. The newly formed  $^{13}\text{C}_5\text{-Glu}$  is ACPD and TTX sensitive and increases with a tail pinch. These findings suggest that it is neuronally derived. TTX sensitivity of basal Glu has been measured with electrodes<sup>41, 80, 209</sup>, but has not previously been measured with microdialysis.<sup>124</sup> Measuring neuronally derived extracellular Glu with SIL microdialysis can be used to the Glu system in models for stress, disease, and addiction, which has been severely limited with uncalibrated microdialysis.

Along with monitoring metabolism in the Glu-Gln cycle, SIL microdialysis can be used to measure the metabolism of DA. DA can be metabolized into 2 different products: 3-MT and DOPAC. Both of these metabolites can be further metabolized to HVA. By infusing  $^{13}\text{C}_6\text{-DA}$ , the  $^{13}\text{C}_6$  version of each metabolite can also be monitored. These metabolic measurements could prove useful for probing the effects of substances that alter DA turnover.

SIL microdialysis can also be used to monitor small changes in the DA system otherwise unseen in uncalibrated microdialysis. For example, previous studies have shown that leptin increases DA uptake and TH activity, but has no effect on extracellular basal DA concentrations

in the NAc of rats fed ad-libitum when measured with uncalibrated microdialysis.<sup>123, 163</sup> Using the increased accuracy afforded by SIL microdialysis, we sought to further examine the effects that leptin has on extracellular DA concentrations, uptake, and release in the NAc.

### *Leptin conclusions*

Leptin is a hormone that is associated with food satiety with receptors present on dopaminergic neurons in multiple areas of the brain including the lateral hypothalamus, the ventral tegmental area, and the midbrain.<sup>35</sup> Because leptin receptors are on DA neurons in areas of the brain associated with reward and feeding, studying the interaction between leptin and feeding can reveal a facet of the neurochemical cause of obesity. With SIL microdialysis, we were able to show that leptin (1 mg/kg i.p.) reduced the basal concentration of DA by  $32 \pm 5\%$ . This effect was further investigated by a series of pharmacological studies. The decrease in DA concentration affected receptor occupancy because when the D2 antagonist raclopride was infused through the probe, the increase in DA was greater in vehicle treated rats vs. leptin treated rats. This result was interpreted as the effect of lower tone on D<sub>2</sub> receptors with leptin causing lower release with raclopride. This effect was attributed to an increase in uptake. Indeed, our study was able to corroborate previous observations and further illustrate that uptake activity was greater with leptin. Extracellular DOPAC (produced in the neuron) concentrations increased more following the raclopride infusion in leptin treated rats vs. vehicle treated rats. The extracellular DOPAC increase could show that more DA is entering the neuron. The lower increase in extracellular DA and increase in DOPAC is indicative that leptin increased uptake.

Monitoring the metabolites of DA with SIL microdialysis offers further insight into DA turnover with a leptin treatment. After leptin, the small increase in <sup>13</sup>C<sub>6</sub>-DOPAC produced from <sup>13</sup>C<sub>6</sub>-DA could be caused by more <sup>13</sup>C<sub>6</sub>-DA being able to enter the neuron. While the endogenous

[3-MT]/[DA] ratio increased with leptin, the  $^{13}\text{C}_6$ -3-MT produced from  $^{13}\text{C}_6$ -DA was not affected by leptin. The increase in [3-MT]/[DA] was not due to an increase in COMT activity and may provide evidence of greater DA release.

Because previous studies had shown an increase in TH activity<sup>123</sup> along with our observations of increased [3-MT]/[DA] ratio, we hypothesized that DA release might be increased. This effect was presumably masked in the basal concentration measurement by the increased DAT activity. To determine if increased DA release could be detected, cocaine and raclopride were infused through the probe. With uptake blocked, the DA in leptin treated rats increased more when compared to vehicle treated rats. The contribution of increased DA release seems to play a minor role compared to the increased uptake as these two opposing factors yielded a net decrease in basal DA.

The increased DA uptake may be one of the processes used by leptin to decrease feeding. In a previous study, extracellular DA in the NAc increased more in vehicle treated vs. leptin treated food deprived rats when they received free access to food.<sup>169</sup> Greater DA uptake may have been the cause for the attenuated increase. In a separate study, increasing DA in the NAc has been shown to increase the incentive salience of food.<sup>161</sup> With leptin decreasing extracellular DA levels associated with feeding, the food becomes less rewarding. We propose that the modulation of DA by leptin through increased DAT activity is one of the mechanisms that leptin utilizes to promote food satiety.

This thesis demonstrates the potential that SIL microdialysis has for making measurements in the central nervous system. The accuracy and information output is greater than uncalibrated microdialysis but requires the same amount of time and animals. The application of



SIL microdialysis should be pushed further to provide greater insight into the active processes and metabolic pathways that affect neurotransmitters other than DA and Glu.

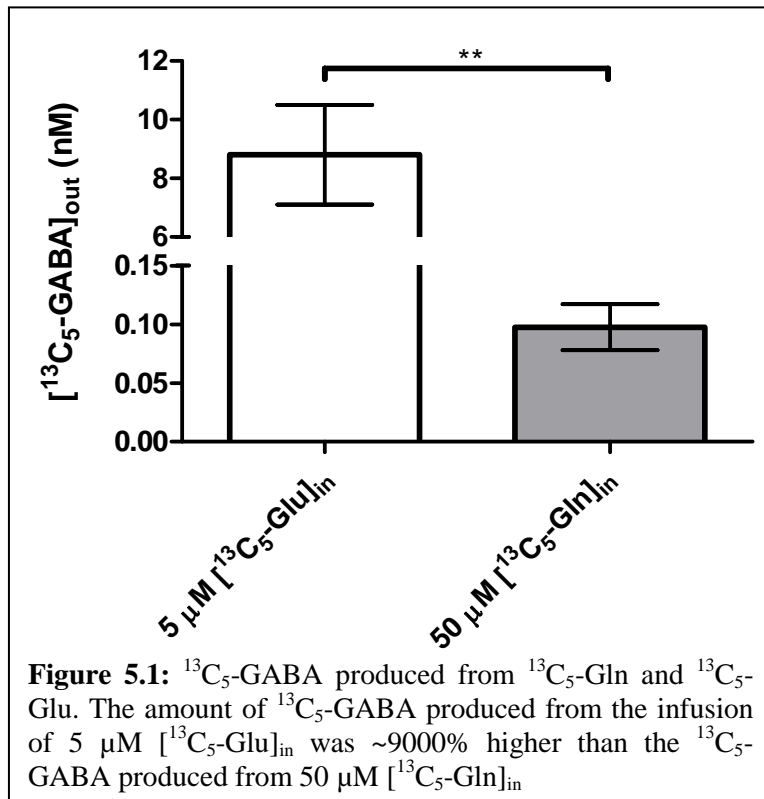
#### *Future directions for SIL microdialysis*

A useful direction for this work would be to evaluate how active processes in tissue modulate *in vivo* recovery by microdialysis. In previous studies of DA, the metabolism, synthesis, and release of DA were shown to have no effect on  $E_d^{DA}$ .<sup>126</sup> Only changes in uptake affected  $E_d^{DA}$ . The rate of metabolism of DA is relatively slow compared to the uptake, which is why uptake controls  $E_d^{DA}$  and decreasing metabolism does not.<sup>126</sup> No studies like this have been performed with Glu. Glu is regulated differently than DA so inhibiting other processes like metabolism may affect  $E_d^{Glu}$ . Glu is toxic at higher concentrations so the brain rapidly removes and metabolizes it. Inhibiting uptake of Glu decreases  $E_d^{Glu}$  but how metabolism of Glu affects  $E_d$  remains unknown. The  $E_d$  of other neurotransmitters, such as acetylcholine, may be controlled entirely by metabolism (acetylcholinesterase is the main method for clearing acetylcholine). By determining which tissue effects can control  $E_d$ , greater insight can be gained into how different pharmacological treatments affect neurotransmitter processing.

Studying how different processes affect  $E_d$  can provide a method for determining the source of neurochemical changes. For example, when rats were given ethanol, an increase in  $C_{app}^{DA}$  was measured in the hippocampus with no change in  $E_d^{DA}$ . The increased  $C_{app}^{DA}$  was determined to be from increased release and not decreased uptake.<sup>143</sup> Because the active processes that affect  $E_d$  in other neurotransmitters remain unknown, determining the source of increases and decreases in extracellular neurochemical concentrations remains challenging. Additionally,  $E_d$  may provide a tool for measuring possible drug efficacy. In a previous study, infusions of a glutamine synthetase inhibitor into the hippocampus induced seizures in rats.<sup>210</sup> If

$E_d^{Gln}$  was known to be dependent on the metabolism of Gln, the extent to which antiepileptic drugs affected  $E_d^{Gln}$  in the hippocampus might be correlated to the ability of the drug to reduce seizures. Characterization of SIL microdialysis using other neurotransmitters can lead to an understanding of which processes affect  $E_d$  for each neurotransmitter, which would provide measurements on tissue effects not observable with uncalibrated microdialysis.

Additionally, SIL microdialysis of  $^{13}C_5$ -Glu or  $^{13}C_5$ -Gln could be used to probe the source of  $^{13}C_5$ -GABA in the brain. GABA neurons do not have the ability to form GABA from glucose so GABA is derived from Gln. The Gln enters the neuron, is converted to Glu, and is then



converted to GABA by glutamate decarboxylase. In a preliminary experiment, we infused  $50 \mu\text{M } ^{13}C_5$ -Gln through the probe and found that  $0.10 \pm 0.02 \text{ nM } ^{13}C_5$ -GABA was recovered by the probe. If  $5 \mu\text{M } ^{13}C_5$ -Glu (Fig. 5.1) was infused through the probe, then  $8.8 \pm 2 \text{ nM } ^{13}C_5$ -GABA was recovered. The  $^{13}C_5$ -GABA produced from  $5 \mu\text{M } ^{13}C_5$ -Glu is 9000% greater than from  $50 \mu\text{M } ^{13}C_5$ -Gln, suggesting that extracellular Glu may play a larger role as a precursor to GABA than Gln.

t Experiments testing TTX, Glu uptake inhibitors, and Glu decarboxylase inhibitors may shed light onto how GABA is synthesized.

SIL microdialysis has been used to measure absolute concentrations, tissue effects, and metabolic flux. Our study of the effects of leptin on the DA system in the NAc can be expanded further into other areas of the brain. The measurements made in our study were downstream of leptin receptor containing neurons. Measuring the direct effect of leptin on the DA in the midbrain may yield additional information on feeding and obesity.

#### *Future directions for leptin study*

In our study, a decrease in  $C_{app}^{DA}$  was detected along with evidence for increases in uptake and release (most likely due to increase TH activity) in the NAc. With the use of benzoyl chloride and liquid chromatography coupled to a mass spectrometer, the sensitivity of our system is sufficient to measure DA with probes as small as 300  $\mu\text{m}$ . This probe size is also acceptable for sampling from the lateral hypothalamus and the VTA, where leptin receptors are present. A future study could use SIL microdialysis to monitor how leptin alters the DA system in these areas. Leptin causes neuronal depolarization on DA neurons that contain leptin receptors.<sup>36</sup> Additionally, leptin has been shown to increase the amount of TH in both the VTA and NAc but not in the mediobasal hypothalamus of *ob/ob* mice after a leptin treatment.<sup>163</sup> With the increased TH caused by leptin in the VTA, increased DA release should be observable after a leptin injection. DAT activity and changes in extracellular DA concentrations have not been measured in the VTA or the LH. Leptin may increase DAT activity in the LH and VTA, causing a decrease in extracellular DA. If not, an increase in the extracellular DA concentration in the VTA may be due to the increase in TH. Amphetamine and  $D_2$  antagonists could be injected or perfused through the probe to see the effect of leptin on these areas much like the studies performed in the NAc in this work and in other studies.<sup>123,169</sup> Monitoring the changes in the DA system can demonstrate the direct effects of leptin on brain areas that contain leptin receptors.

Performing these measurements could provide insight into other mechanisms in which leptin regulates feeding. Microinjecting leptin directly into the VTA has been shown to decrease neuronal firing rates in anesthetized rats and decrease feeding in awake rats for up to 24 h.<sup>157</sup> The VTA is a central site in the reward circuit in the brain. While our study provides evidence for changes in DA caused by leptin in the NAc, the mechanism of leptin regulating feed is not exclusive to the NAc. Measuring changes in the VTA and LH could provide additional information into obesity, rewarding effects of food, and how the interaction of leptin and DA play on each.

### *Conclusion*

The complexity and heterogeneity of the brain require analytical techniques with a high sensitivity, selectivity, and temporal resolution. This thesis provided a tool to improve upon microdialysis. With SIL neurotransmitters as a calibrant, microdialysis can be a quantitative technique that is able to indirectly measure tissue effects. SIL microdialysis also adds further information by providing a tool to measure metabolism. Monitoring SIL metabolites can provide information on enzymatic activity and can be used as an assay for measuring neuronal Glu release. No additional time is required for SIL microdialysis over uncalibrated microdialysis which makes microdialysis a more efficient and comprehensive tool for neurotransmitter measurements.

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