

**Neurochemical and Behavioral Analysis of Rodents that Model Cognitive Dysfunction:**

**Application to Chemobrain and ADHD**

By

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**Neurochemical and Behavioral Analysis of Rodents that Model Cognitive Dysfunction:  
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## **Abstract**

In this document, the release and uptake of dopamine, a neurotransmitter involved in numerous aspects of brain function, is investigated in two cognitive disorders: post chemotherapy cognitive impairment (PCCI, ‘chemobrain’) and attention deficit hyperactivity disorder (ADHD). RNA sequencing was also used to investigate transcript expression to determine if chemotherapy or a potential rescue mechanism affect gene transcripts. Throughout these projects, the overall goal of this work aimed to correlate neurochemical and behavioral data with symptoms that are reported or are phenotypically present.

Doxorubicin (Dox) is a chemotherapy agent commonly used to treat multiple types of cancers, including breast cancer, lymphoma, bladder cancer, Kaposi’s sarcoma, and acute lymphocytic leukemia. Patients treated with Dox often suffer from cognitive dysfunction long after the conclusion of treatment, a condition known as chemobrain. In previous reports, we showed that rats treated with carboplatin and 5-fluorouracil, two other commonly used chemotherapy agents, suffer from diminished executive function and impaired release of dopamine and serotonin. In this document, we found, paradoxically, that calcium-dependent dopamine release and reuptake by the dopamine transporter (DAT), measured with fast-scan cyclic voltammetry (FSCV) in brain slices, is increased in rats that had been treated with Dox for two weeks. Pharmacological treatment of brain slices with pramipexole, a D2/D3 agonist, also revealed that Dox treated rats are more sensitive to autoregulation of dopamine release. Additionally, cognitive function, assessed by application of a behavioral paradigm to measure learning acquisition, revealed no difference between Dox-treated and vehicle-treated rats. Collectively, these studies suggest that treatment with Dox for a short time may result in an overactive dopamine system, possibly masking other neural deficiencies that cause chemobrain.

Previous research has shown that treatment with 5-fluorouracil (5-FU) impairs cognitive function, but that concurrent treatment with KU-32, a heat shock protein 90 inhibitor, preserves cognitive function. To elucidate mechanisms underlying these phenomena, mRNA from murine brain tissue was sequenced to examine differential expression (DE) of genes and was subjected to a gene ontology analysis. DE analysis

showed that approximately 1200 genes were differentially expressed between the three groups that were tested. Gene ontology analysis showed that the genes belonged to 57 gene ontology categories that were enriched in mice treated with KU-32+5-FU and depleted in mice treated only with 5-FU. These results suggest that KU-32 may compensate for the genes that are depleted from chemotherapy treatment.

Attention Deficit Hyperactivity Disorder (ADHD) is one of the most common neurodevelopmental disorders in children. There are several defined criteria that must be met to receive a diagnosis according to the Diagnostic and Statistical Manual of Mental Disorders (DSM V). Unfortunately, the mechanism of disorder in ADHD is not fully elucidated. The only treatments for ADHD are pharmaceuticals or behavior therapy. Behavior therapy is becoming the preferred treatment to prevent the substance abuse possibility seen with sustained medication use. Stimulants like amphetamine and methylphenidate are commonly prescribed. This experiment shows how a delay training paradigm is used on an ADHD strain of rat attempting to lower their hyperactive and impulsive behaviors. This behavioral work is complemented by a neurochemical study in which dopamine release and reuptake was measured. The effects of amphetamine on these parameters were also examined. Dopamine is suspected to play a role in ADHD and is highly affected by amphetamine use. The behavior training allowed the rat to overcome a larger delay and appeared to normalized dopamine release so that it was similar to that of the non-ADHD strain. This work supports other studies that suggest behavior therapy can combat the disorder without the use of pharmaceuticals.

Collectively, these projects underly the importance of dopamine system function and illustrate how malfunctions in this system may impact behavior. Moreover, we examine the neurochemical effects that a drug-based intervention (KU-32) and a behavior-based intervention (delay training) can have in models of chemobrain and ADHD. The ultimate goal is to gain clearer understanding of how treatments such as these can be leveraged to improve the conditions of those suffering from these disorders.

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## Chapter 1 : Introduction and Background Information

## **Preface**

This chapter consists of two parts. Part 1 provides information about neurotransmitters and their relation to chemobrain. This includes a review of literature and previous work that has been completed within the field of chemobrain related to neurotransmitters and cognitive function. A section is dedicated to the role of dopamine in cognitive function. Part 2 is focused on electrochemistry and fast-scan cyclic voltammetry, one of the primary methods used throughout this document to measure dopamine.

## **Part 1: Chemobrain and Neurotransmitters**

### Cancer

Cancer is the one of the leading causes of death in the United States.<sup>1, 2</sup> It is estimated that more than 16.9 million Americans have a history of cancer.<sup>3</sup> In 2020 it was estimated that 1,806,590 new cancer cases will be diagnosed.<sup>3</sup> However, the death rate among cancer patients is down 29% since its peak in 1991.<sup>3</sup> This improvement has led to more people surviving cancer than in previous decades. The overall death rate of cancer has decreased by 1.5% per year in the last decade (2008-2017) while other leading causes of death like heart disease and cerebrovascular disease have remained stable over the same period.<sup>3</sup> Over 68% of cancer patients were diagnosed over 5 years ago while around 18% were diagnosed more than 20 years ago.<sup>2</sup> The most common types of cancer are prostate, colon/rectum, and melanoma among men and breast, uterine corpus, and colon/ rectum among females.<sup>4</sup>

There are multiple causes of cancer including successive genetic mutations, lesions in DNA, issues with cell division and missing check points along the cell cycle.<sup>1, 5</sup> Although these changes can originate from many different sources like radiation exposure, smoking, sun exposure, and other environmental factors, some cancers appear due to spontaneous mutations within the genetic code.<sup>1</sup> No matter the cause, the result is uncontrolled and unregulated cell growth and proliferation which is the definition of cancer.<sup>6, 7</sup> These cells can form masses called tumors, some of which have the ability to infiltrate other organs.<sup>7</sup> This migration of the cancerous cells is known as metastasis and can lead to new tumors being found in distantly located from the original cancer site (e.g. melanoma metastasizing to the brain).<sup>7</sup> A majority of cancer

deaths come from cancers that can metastasize, leaving patients with an overall poorer prognosis.<sup>7</sup> Common treatments for all cancers are resection/ removal of affected tissue, radiation, and chemotherapy with many patients receiving adjuvant therapy.<sup>3, 6</sup> Adjuvant therapy is the secondary treatment (commonly chemotherapy or radiation) to kill off any remaining cancer cells that were missed from the primary treatment (usually surgery).<sup>8</sup> This helps to prevent cancer from returning or relapsing.<sup>8</sup>

### Chemotherapy

Chemotherapy is one of the most commonly used treatments for cancer.<sup>1,9</sup> The term was coined in the early 1900s by German chemist Paul Ehrlich who defined it as using chemicals to treat disease.<sup>9</sup> Ehrlich was an immunologist who pioneered early dye techniques to distinguish and classify microorganisms.<sup>10</sup> His work showed that different cells and cellular structures have different affinities for the dyes that he administered.<sup>10</sup> This work paved the way for the identification of the blood brain barrier as the brain and spinal column would not absorb a water-soluble dye like the rest of the body cells tested.<sup>10</sup> He also contributed to immunology with standardizing how immunization of animals was completed and introducing horses to large factories producing antiserum to treat disease.

Ehrlich's major contribution to early chemotherapy work was synthesizing targeted drugs to help treat syphilis.<sup>10</sup> Salvarsan is an arsenic containing compound that showed major success in curing rabbits infected with syphilis.<sup>10</sup> This drug paved the way for translational medicine as it was one of the first to be tested extensively in infected animals for safety and efficacy and then proceed to human testing.<sup>10</sup> With this success, he was interested in treating cancer with aniline dyes or other targeted therapies but did not pursue this further as he thought there was a small chance of success.<sup>9,10</sup> Also, during the time period that he was most active, no cancer causing molecule or structure had been described so there was nothing for his early therapies to target.<sup>10</sup> Nevertheless, Ehrlich's ideas would later evolve into the use of small molecule therapies that target oncogenic kinases and antibody-based therapies that target cell surface structures on cancer cells.<sup>10</sup>

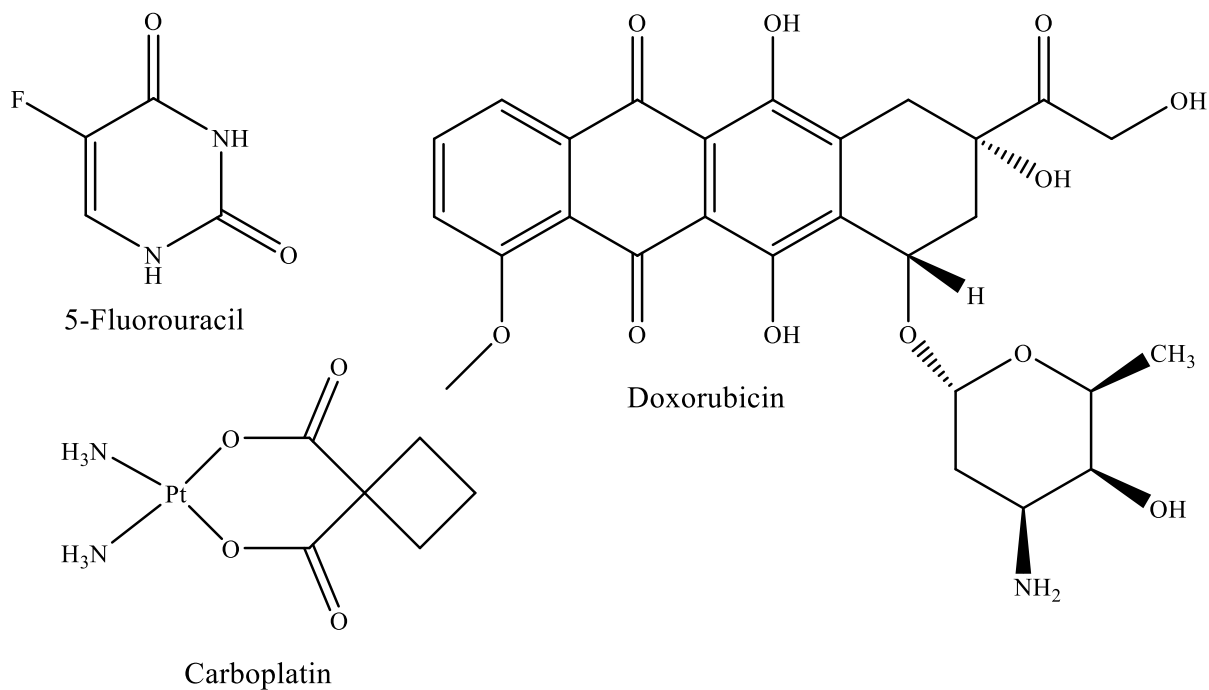
The first class of cancer chemotherapeutics introduced were nitrogen mustards, which attack cells by adding alkyl groups to DNA.<sup>9</sup> The use of sulfur mustards arose from the observation that soldiers exposed to these agents had significantly depleted levels of bone marrow and lymph nodes.<sup>9</sup> One of the first mustard derived chemotherapeutics tested was thiotepa, an alkylating agent, that was used to attack cancer cells which had been released into the system during surgery.<sup>9</sup> This and other studies paved the way for chemotherapy to be widely used in cancer treatment.<sup>6</sup> These drugs were effective in treating hematologic cancers like leukemia and lymphoma, but no drugs had been tested successfully in nonhematologic cancers such as ones with solid tumors.<sup>9</sup> The second group that became popular were the antimetabolites.<sup>6</sup> These drugs act and look like needed parts of DNA and RNA which then are incorporated and prevent cell replication.<sup>9</sup> Adjuvant chemotherapy was not used frequently in cancer treatment until the 1960s when it was used to help treat breast cancers with high incidences of metastasis.<sup>9</sup> This type of therapy targeted the cancerous cells that were left behind after surgery or those that entered the circulatory system during surgery, thereby preventing tumor recurrence or metastasis.<sup>9</sup> Together with radiation and surgery, this chemotherapy regimen created the typical cancer treatment triad.<sup>6,9</sup>

The United States began to consider cancer a public health concern in the 1970's when the National Cancer Act was passed.<sup>9,11</sup> This act greatly increased funding for cancer research and established cancer centers.<sup>11</sup> President Nixon called this act the beginning of the "War on Cancer" as at the time it had risen to be a leading cause of death.<sup>9</sup> By providing new cancer centers and new funding for research, this legislation allowed many chemotherapeutics to be screened and new screening procedures to be established.<sup>9</sup> Through this, and other advances in drug therapy, cancer became a survivable diagnosis.<sup>6</sup>

Alkylating agents and antimetabolites are only a few of the categories of chemotherapy that exist today.<sup>12</sup> There are also platinum- based drugs,<sup>13</sup> antibiotics that have antineoplastic qualities,<sup>14</sup> nitrosoureas,<sup>15</sup> and plant alkaloids.<sup>16</sup> Antimetabolites, platinum- based drugs, and antineoplastic antibiotics will be addressed within the following chapters with the majority of work focused on the antineoplastic antibiotic, doxorubicin. The structures of the 3 compounds of interest are shown in **Figure 1-1**. As

mentioned before, antimetabolites work by looking like necessary pieces of replication hardware and then are incorporated into DNA during replication.<sup>17</sup> When an irreparable mistake is found, DNA replication is arrested, and the cell enters permanent cell cycle arrest (senescence) or dies via apoptosis.<sup>18</sup> This class of drugs was discovered when it was found that rat hepatomas use more uracil than normal cells making uracil a target of interest.<sup>19</sup> One of the commonly used antimetabolite drugs is 5-Fluorouracil (5-FU).<sup>19</sup> 5-FU works by being incorporated into RNA instead of uracil which leads to RNA damage.<sup>19</sup> It also inhibits thymidylate synthase which causes DNA damage.<sup>16</sup>

Another common class of chemotherapeutics are the platinum- based compounds, one of which is second generation drug, carboplatin.<sup>16</sup> It interacts with nucleophilic sites on DNA which creates inter- and intrastrand crosslinks.<sup>20</sup> These crosslinks prevent the two strands of DNA from separating which inhibits replication and transcription.<sup>21</sup> Doxorubicin is an antitumor antibiotic generated from *Streptomyces peucetius*.<sup>21</sup> Doxorubicin acts by intercalating between the strands of DNA.<sup>22</sup> Intercalation is the process of a chemical moiety being inserted between adjacent base pairs of DNA.<sup>23</sup> The planar geometry and aromatic groups of doxorubicin lends itself to this mechanism of action.<sup>21</sup> Doxorubicin also acts by inhibiting topoisomerase-II and its ability to repair DNA.<sup>24</sup> Topoisomerase-II works by creating small breaks within DNA which allow for the unwinding of DNA without creating undue twisting strain on the rest of the DNA strand.<sup>24</sup> When this mechanism is removed, it prevents transcription.<sup>24</sup> These three drugs show that a portion of the wide variety of mechanisms of action of chemotherapeutics. Again, none of these treatments are specific to a cancer cell or tumor and so other healthy cells are affected. When this collateral damage is investigated, the brain becomes an unfortunate casualty.



**Figure 1-1. Structures of 5-FU, Carboplatin and Doxorubicin**

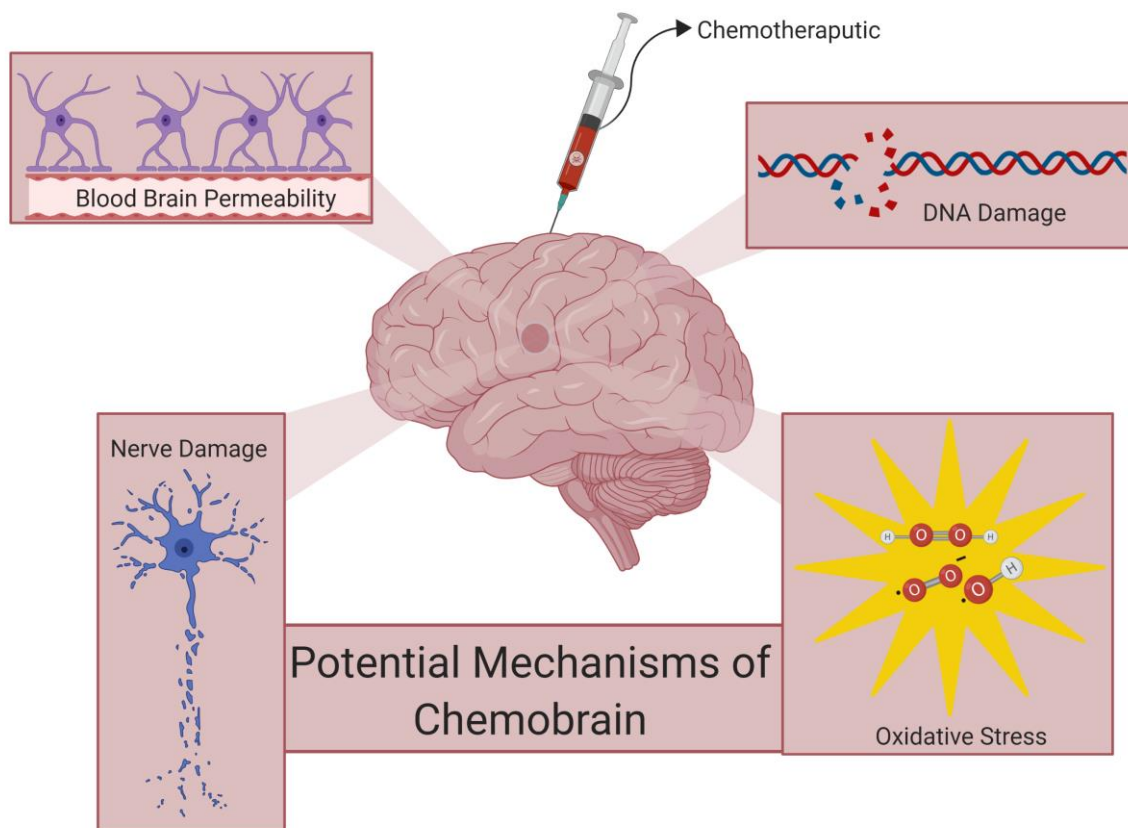
### Chemobrain

The fundamental mechanism of many chemotherapy agents is that they generally target faster reproducing cells over the slower reproducing cells; this means that healthy cells within the body are also affected and may be degraded or die off due to these treatments.<sup>6</sup> Today, with early diagnosis and chemotherapy treatment, the survivability of a cancer diagnosis has increased dramatically.<sup>6, 25</sup> Unfortunately, this attack on healthy tissue negatively affects the overall health of individuals undergoing treatment.<sup>6</sup> As a result, a greater number of people live to experience long-term side effects associated with the toxic nature of these chemotherapeutics.<sup>25</sup> Some of these side effects include fatigue, pain, vomiting, and constipation/diarrhea.<sup>26</sup> Other side effects involve mental deficits, such as weakened cognitive abilities, impaired executive function, problems with information processing, reaction time, and mood changes such as major depressive disorder.<sup>26-29</sup>

These mental symptoms have been classified as a syndrome known as Post Chemotherapy Cognitive Impairment (PCCI) which is commonly referred to as chemobrain or chemofog.<sup>26-29</sup> Within the

following chapters, PCCI will be referred to as chemobrain. Some of the earliest cases of chemobrain symptoms were reported in 1978 in breast cancer patients.<sup>30</sup> Today, symptoms are reported in about half of breast cancer patients and in around 20% of all other cancer patients.<sup>27-29</sup> These numbers are expected to be higher as this syndrome is patient reported and previously not accepted by clinicians.<sup>30, 31</sup> It is estimated that 75% of patients experience chemobrain symptoms during treatment and then 35% of patients continue to experience symptoms after treatment is complete.<sup>30</sup> Some experience these symptoms for years after treatment with some never subsiding.<sup>26</sup>

Unfortunately, there is no confirmed cause of these symptoms. Theories include changes in blood brain permeability, damage to DNA, oxidative stress, and nerve damage (**Figure 1-2**).<sup>16, 27, 28</sup> Other possible causes are listed in **Table 1-1**. 5-FU is known to readily diffuse into the brain and has been implicated in chemobrain.<sup>21</sup> Doxorubicin is known to generate free radicals which then could generate oxidative stress conditions.<sup>21</sup> This is due to the generation of an unstable intermediate that then is converted back to doxorubicin leading to free radical production.<sup>32</sup> This increased oxidative stress results in the upregulation of inflammatory cytokines.<sup>32-34</sup> Cytokines are protein messengers which allow for cells to communicate and interact.<sup>35</sup> One class of these cytokines are pro-inflammatory cytokines which are activated by macrophages creating an inflammatory reaction.<sup>35</sup> One of these pro-inflammatory cytokines, tumor necrosis factor alpha (TNF- $\alpha$ ), crosses the blood brain barrier (BBB) and causes neuronal damage and mitochondrial dysfunction.<sup>33, 34, 36</sup> TNF- $\alpha$  triggers apoptosis and necrosis and is known to increase levels of nitric oxide which produces a neuronally toxic metabolite, peroxynitrite.<sup>26</sup> Investigating chemobrain has proven difficult as the patients also have cancer which causes confounding factors.



**Figure 1-2. Possible Mechanisms of Chemobrain**

Four of the more common theories of chemobrain as described in the literature.

Figure created with Biorender.com

**Table 1-1. List of commonly proposed causes or contributing factors to chemobrain as reported in the literature**

Contributing Factor	References
Oxidative and Nitrosative Stress	26-28, 32, 37
Nerve Damage	26-28, 38
DNA Damage	27, 28, 32
Alteration in Blood Brain Permeability	37
Inflammation	39
Damage to Progenitor and Stem Cells	16, 37
Inadequate Repair Mechanisms	39, 40
Cytokine Release Cascade	41
Additive Effects of Medications used in Cancer Treatment	42
Epigenetics	43
Genetic Predisposition	44
Secondary Effect of Adjuvant Therapy	45
Brain Structure Changes- i.e., changes in brain volume or changes in the stability of white matter tracks	46

## Dysregulation of Neurotransmitter Release and Uptake in Chemobrain

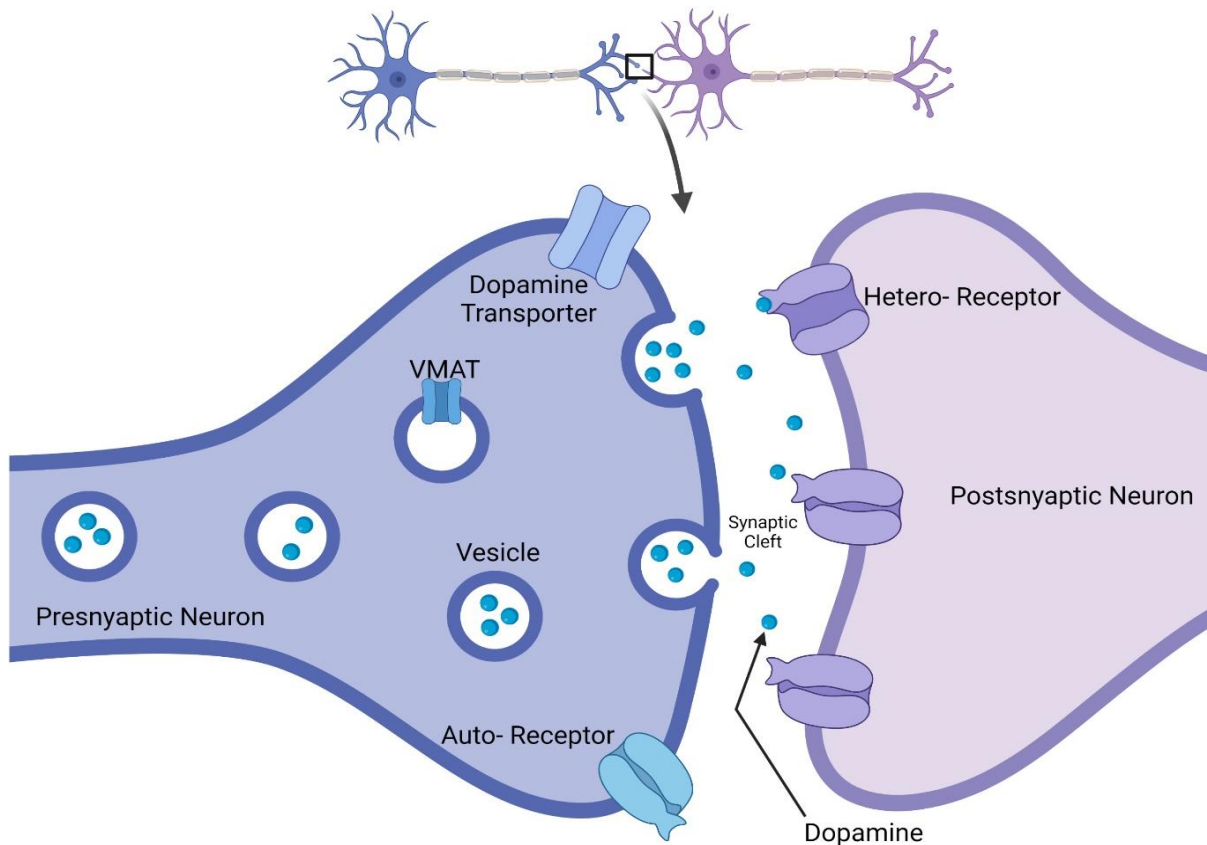
Neurotransmitters mediate chemical communication between neurons and include peptides and small molecules.<sup>46</sup> These compounds are typically small molecules that are synthesized in presynaptic terminals, released into the synaptic cleft, and then received at postsynaptic neuron sites (**Figure 1-3**).<sup>47</sup> Additionally, depending upon the neuronal type, uptake mechanisms, such as the dopamine transporter, and autoregulation mechanisms, such as dopaminergic D2 autoreceptors, further mediate neurotransmission.<sup>48</sup> A sampling of small molecule neurotransmitters that may be important in the expression of chemobrain is shown in **Table 1-2**.

**Table 1-2. List of Neurotransmitters and Functions**

<b>Neurotransmitter</b>	<b>Function</b>	<b>References</b>
Adrenaline	Regulator of blood pressure, vasoconstriction, heart rate, and bronchodilator	<sup>49</sup>
Noradrenaline	Arousal, stress, emotional learning, decision- making, and perception	<sup>50</sup>
Dopamine	Motor function, motivation, reward, cognition, and emotion	<sup>51-53</sup>
Serotonin	Cardiovascular function, bowel motility, temperature, and nociception	<sup>54, 55</sup>
GABA	Inhibitory neurotransmitter, mood, and behavior	<sup>56, 57</sup>
Acetylcholine	Learning, synaptic plasticity, and neuronal development	<sup>56</sup>
Glutamate	Excitatory neurotransmitter, neuronal plasticity and development, learning, and memory	<sup>58, 59</sup>

Neurotransmitter dysfunction can lead to delirious effects on the brain and body.<sup>59</sup> Dysregulation of homeostatic neurotransmitter levels can occur when neurotransmitters are not released in the correct amount, reuptake is too fast or too slow, or the receptors are upregulated or downregulated.<sup>59</sup> These imbalances can result in the alteration of how neurons communicate, and which neuronal pathways are activated.<sup>60</sup> These alterations can be expressed as mental disorders such as anxiety,<sup>61</sup> depression,<sup>62</sup>

schizophrenia,<sup>63</sup> and attention deficit hyperactivity disorder.<sup>62</sup> While many of these conditions do not have defined causes, neurotransmitters are highly implicated in their pathophysiology.<sup>60, 61, 63, 64</sup> Disorders in the brain and body can also occur when the neurons that control the synthesis or release neurotransmitters dysfunction.<sup>65</sup> For example, Parkinson's disease (PD) is a progressive neurological disorder characterized by outward motor and mental deficits.<sup>65</sup> A key contributor to these deficits is the loss of dopaminergic neurons in the substantia nigra pars compacta.<sup>65</sup> The pars compacta is a portion of the substantia nigra and part of the basal ganglia circuit located in the midbrain.<sup>66</sup> The substantia nigra is known to have many dopaminergic neurons which have roles in reward, cognitive planning, and movement.<sup>66</sup> The nigrostriatal pathway, which projects from the substantia nigra to the dorsal striatum, is heavily damaged in Parkinson's Disease.<sup>66</sup> Many of these deficits can be counteracted by agents that increase dopamine levels, such as levodopa.<sup>67</sup> Throughout these chapters, dopamine will be the neurotransmitter of focus and its relationship with chemotherapeutics will be investigated.



**Figure 1-3. Dopamine Neuron with Receptors and Transporter**

Figure adapted from “Synaptic Cleft (Horizontal)”, by BioRender.com (2021). Retrieved from <https://app.biorender.com/biorender-templates>

As described above, normally functioning neurotransmitter systems are essential for the proper working of the brain and the body. Dopamine, a particularly important neurotransmitter, is implicated in movement, reward, and cognition.<sup>68</sup> When dopaminergic neurons are damaged or show dysfunction disorders such as ADHD, autism, mood disorders, and movement disorders may be present.<sup>64, 69, 70</sup> Dopamine has also been reported to increase the efficacy of chemotherapeutics and have a synergistic effect with chemotherapeutic drugs.<sup>71</sup> Dopamine is known to inhibit angiogenesis, normalize tumor vasculature structure and function,<sup>71</sup> and inhibit vascular endothelial growth factor (VEGF).<sup>71, 72</sup> VEGF recruits endothelial cells to the tumor site and allows for vasculature to form providing a stable blood supply for the

tumor.<sup>71</sup> VEGF is also shown to be more abundant in more malignant cancers.<sup>71</sup> Inhibition of this protein allows for the vasculature to be restricted showing a better prognosis for the patient.<sup>71</sup>

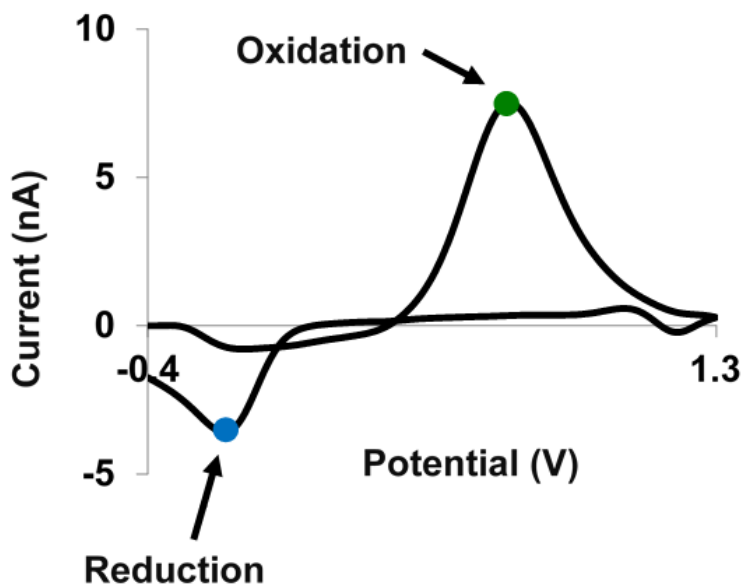
Dopamine is an ideal target as an important neurotransmitter and investigating its correlation with behavioral dysfunction could shed light on chemobrain. Previous work in the Johnson lab has focused on using carboplatin and 5-FU.<sup>67, 72-74</sup> Through this work, it has been found that both carboplatin and 5-FU significantly decrease dopamine release and attenuate cognitive function.<sup>67, 72-74</sup> This work has been done in both rats and zebrafish.<sup>67, 72-74</sup> The primary aim of the chemotherapy chapters in this dissertation was to investigate doxorubicin's effects on the dopamine system and 5-FU's role in differential expression of genes. Doxorubicin is a different class of drug than as previously been used in the lab and investigating it adds to what we know about different chemotherapeutics and their effects on the dopaminergic system. Measurements of dopamine release, uptake, and behavior provide the groundwork for understanding how this drug acts within the dopamine system and cognition. Since this initial data has already been collected with 5-FU, the goal was to investigate the cell transcripts to determine if there are any genes of interest that could provide possible clues for how the rescue mechanism, KU-32, helps to rescue cognitive function in 5-FU treated animals.<sup>73</sup> KU-32 and its mechanism are discussed in detail in chapter 3. We also wanted to determine if there were any genes of interest that are specifically affected by 5-FU that could corroborate the neurotransmitter and cognitive deficits that were previously observed.

## **Part 2: Fast Scan Cyclic Voltammetry**

A variety of analytical techniques have been applied to the study of chemobrain in model rodents. These include microdialysis sampling,<sup>75, 76</sup> selective biosensors,<sup>77</sup> electrophysiology recordings,<sup>76, 78</sup> and select electrochemical methods.<sup>67, 72, 73, 79</sup> As indicated in the previous section, the measurement of neurotransmitter release and uptake can provide unique insight into the underlying mechanisms of neurological disorders such as chemobrain. Therefore, we employed fast-scan cyclic voltammetry (FSCV) at carbon-fiber microelectrodes to measure dopamine release and uptake dynamics in multiple models of chemobrain.

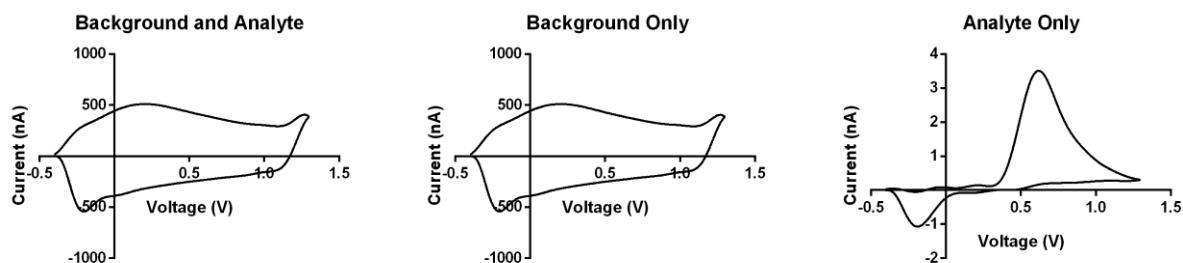
Fast scan cyclic voltammetry is a technique that provides good selectivity and sub-second temporal resolution.<sup>80</sup> To measure dopamine release and uptake with this technique, we apply a holding potential typically at -0.4 V (versus Ag/AgCl reference electrode) and linearly sweep the potential toward +1.3 V at a scan rate of 400 V/s and an acquisition frequency of 10 Hz (one scan per 10 ms).<sup>79</sup> This allows the waveform to be approximately 8 ms in duration.<sup>80</sup> The acquisition frequency is important as it allows for dopamine to adsorb to the electrode surface and essentially preconcentrate.<sup>80</sup> Previous work by Wightman showed that as the acquisition frequency is increase to 100 Hz the sensitivity is decreased by 75%.<sup>81</sup> As the electrode is swept toward the positive potential, dopamine undergoes a two-electron oxidation and a loss of two protons at approximately +0.6 V to form the dopamine ortho-quinone which is shown as the oxidation peak in **Figure 1-4**.<sup>79</sup> The potential is immediately swept back to -0.4 V at 400 V/s and, as it passes through about -0.2 V, the dopamine ortho-quinone undergoes a two-electron reduction and gains two protons to form dopamine which is shown as the reduction peak in **Figure 1-4**.<sup>79</sup> This reduction peak is smaller due to diffusion of the ortho-quinone away from the electrode's surface due to its lowered affinity for the electrode's surface.<sup>80</sup>

A large background current is generated by the non-faradic processes that occur at the electrode surface, creating a large capacitive charging current that is proportional to scan rate.<sup>82</sup> After removing this charging current by subtracting a background cyclic voltammogram (CV) or averaged group of background CVs, the resulting "background-subtracted" CV that serves as a signature for dopamine is formed (**Figures 1-4 and 1-5**).<sup>83</sup> This signature CV is taken at a point of high release and confirms the presence of dopamine. Through calibration of the electrodes against standard dopamine solutions, we can plot dopamine concentration versus time.



**Figure 1-4. Typical Dopamine CV**

Typical Dopamine CV showing the oxidation peak at approximately +0.6 V (green dot) and the reduction peak at approximately -0.2 V (blue dot) versus Ag/AgCl

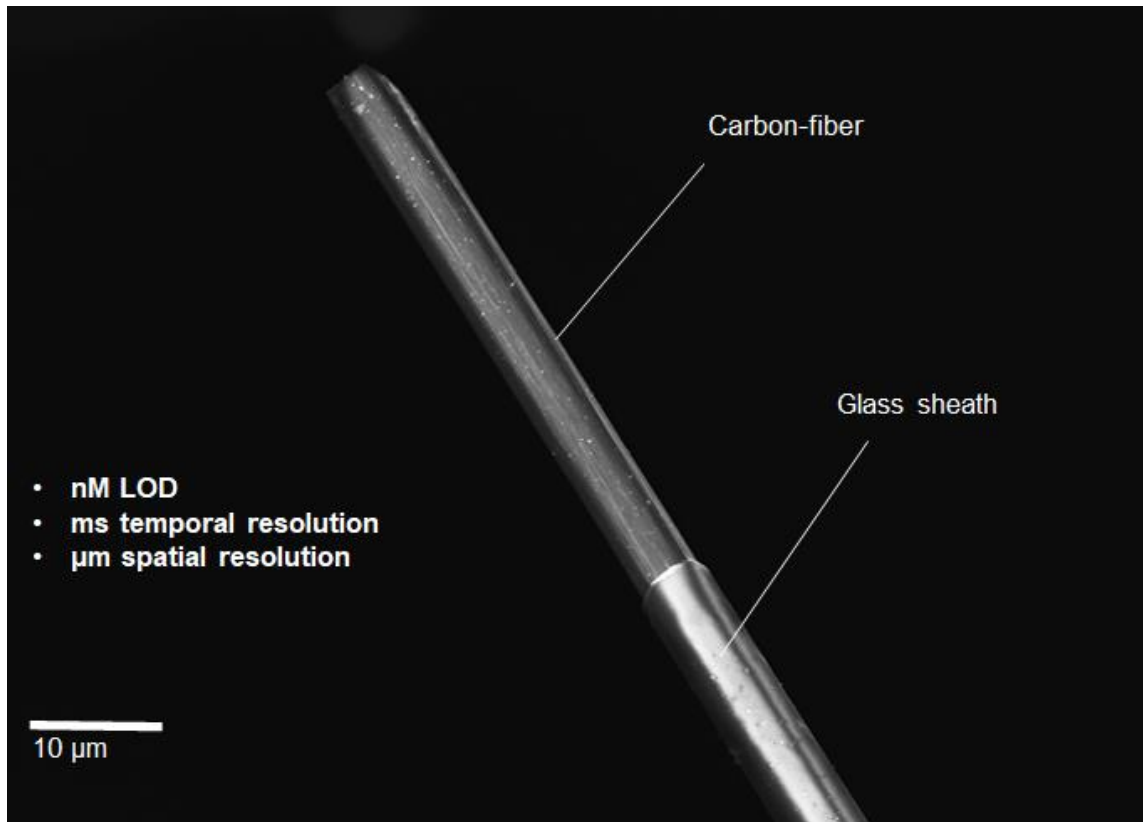


**Figure 1-5. Background vs Analyte CVs**

This series shows how the reductive and oxidative currents associated with the analyte can be completely overpowered by the background, but once the background current is subtracted the faradaic currents corresponding to the analyte can be seen. Background only has no background scans averaged out removing the background subtraction. Background and Analyte has the scan of interest coming from an area before the release event and no background scans subtracted. Analyte has the scan of interest coming from an area of high release with the average of 10 background scans subtracted.

The material and surface diffusional characteristics of carbon-fiber microelectrodes (less than 25 microns in diameter usually) (**Figure 1-6**) make it well-suited for the measurements of neurotransmitter release and uptake in highly resistive brain tissue.<sup>82</sup> When scanning to higher potentials, such as +1.3 V,

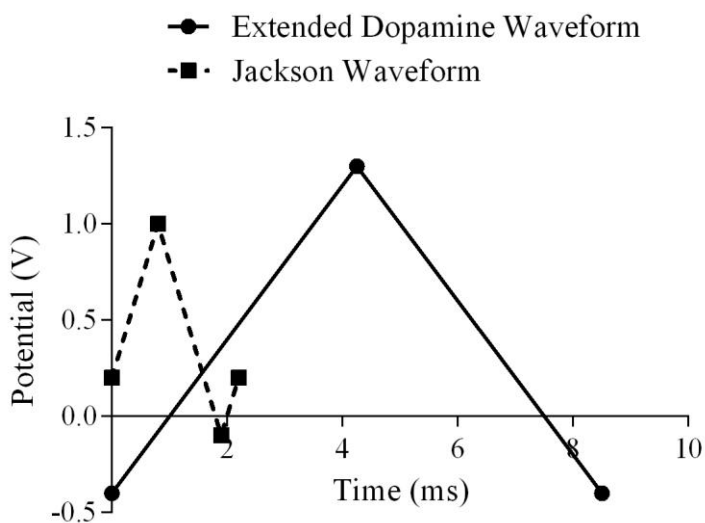
the electrode automatically regenerates, decreasing the degree of electrode fouling.<sup>84</sup> This regeneration occurs from the breaking of carbon-carbon bonds while increasing the number of oxide groups on the surface of the electrode.<sup>84</sup> The small electrode size (5-7  $\mu\text{m}$  diameter as used in our experiments) minimizes tissue disruption and provides good spatial resolution.<sup>82</sup> This small size also allows radial diffusion to occur, facilitating the quick acquisition of steady state faradaic currents.<sup>82</sup> This property allows fast scan rates to be used and sub-second temporal resolution to be achieved.<sup>80</sup> Additionally, IR or ohmic drop, which is the difference in potentials at the working and reference electrodes changes the potential applied to the working electrode and which is greatly amplified in highly resistive environments, such as that found in brain tissue, can be minimized when using microelectrodes because the currents are on the order of nA.<sup>85</sup> This property also eliminates the need for a counter electrode in FSCV, allowing a two-electrode cell to be used.<sup>85</sup> A disadvantage of classically fabricated carbon-fiber microelectrodes (those pulled in a glass capillary) is fragility, so it is imperative that the tip of the fiber touches nothing when installing the electrode onto the headstage. The headstage is a current amplifier that connects the working electrode and reference electrode to the potentiostat.<sup>85</sup> The potentiostat allows for the potentials to be offset so that the software can see the full range of the triangular waveform.<sup>85</sup> It also allows for a gain (usually 100-200 nA/V) to be applied to the current for further amplification.<sup>85</sup> The potentiostat is then connected to a breakout box which allows for the translation of the digital signals from the computer to analog for the potentiostat and vice versa.<sup>85</sup> The computer is then connected with software that can interpret the currents generated from the headstage into plots in the software for analysis.<sup>85</sup>



**Figure 1-6. Scanning Electron Microscope Image of a Carbon Fiber Microelectrode**  
SEM Image collected in Johnson Lab

The ability to tailor the waveform parameters to the analyte of interest enhances the versatility of FSCV and allows the measurement of other electroactive neurotransmitters. For example, the particular waveform employed to measure dopamine release and uptake has been optimized so that the limit of detection is under 50 nM.<sup>86</sup> However, when serotonin is measured under these same conditions, it oxidizes at approximately the same potentials which makes its CV difficult to distinguish from that of dopamine.<sup>86</sup> Additionally, serotonin side products, including 5-hydroxyindoleacetic acid (5-HIAA), are generated through this process. These side products adsorb to the surface of the electrode and obscure the electrochemical signal.<sup>87</sup> Due to this fouling, a new waveform was developed that allowed serotonin to be accurately measured with minimal interference.<sup>88</sup> The “Jackson Waveform” as shown on **Figure 1-7**, scans to different potentials and at a higher scan rate (1000 V/s) than the dopamine waveform.<sup>88</sup> The Jackson waveform is named after Brad P. Jackson, a student in the Wightman Lab, as he optimized the waveform

in 1995.<sup>88</sup> The increased scan rate allows the measured faradaic currents of serotonin oxidation and reduction to outrun the formation of 5-HIAA and other by-products.<sup>84</sup> This waveform allows the limits of detection to be over 800 times lower for serotonin over dopamine.<sup>84</sup> When the optimal waveform is used, limits of detection can be as low as 600 picomolar.<sup>84</sup> The ability to change the waveforms to suit the analyte of interest allows for FSCV to be very versatile and an optimal method for measuring electroactive neurotransmitters.



**Figure 1-7 Dopamine and Serotonin Waveforms**

Waveforms shown on common time and potential (versus Ag/AgCl) axes to show how the scan rate affects the length of each potential cycle (8.5 ms for dopamine and 2.2 ms for the Jackson Waveform). This overlay also shows how different the holding and switching potentials are which lead (along with scan rate) to the specificity of each waveform. The Jackson waveform is shown as a dotted line, while the extended dopamine waveform is a solid line.

### Summary of Chapters in this Dissertation

With nanomolar limits of detection, sub-s temporal resolution, and micron spatial resolution, fast scan cyclic voltammetry with carbon-fiber microelectrodes allows dopamine and other neurotransmitters to be reliably measured *in* and *ex vivo*. In the following chapters, it is shown how different chemotherapeutics affect the brain by measuring dopamine release, reuptake, and performing pharmacology assessments. An RNA sequencing experiment will also be presented as suggested causes of chemobrain are DNA damage or other genetic abnormalities.

Chapter 2 focuses on the administration of doxorubicin in rats. Two dosing periods of 2 and 4 weeks were investigated along with two behavioral paradigms. These paradigms were learning acquisition in the 2-week animals and attention shifting in the 4-week animals. The brain slices generated through both 2- and 4-week administration were subjected to a pramipexole titration to look at doxorubicin effect on the D3 autoreceptor. Dopamine reuptake was mathematically modeled to determine if doxorubicin affects the reuptake of dopamine. Overall, it was shown that the dopamine system is altered in both 2- and 4- week animals with behavior being significantly altered throughout dosing.

Chapter 3 focuses on an RNA sequencing project with C57Bl/6 mice using 5-fluorouracil and KU-32. Mice were treated with 5-fluorouracil and possible rescue compound, KU-32. KU-32 is a heat shock 90 inhibitor that is being used in clinical trials as a remedy for diabetic neuropathy and was developed by Dr. Brian Blagg at the University of Kansas. Previously within the Johnson Lab, it was able to restore cognitive function to animals that were concurrently dosed with 5-FU and KU-32.<sup>73</sup> Differential expression analysis of the RNA transcripts was performed in this project to determine if there are any genes of interest that are over or under expressed that could shed light on how either KU-32 is able to rescue cognitive function<sup>73</sup> or how 5-FU attenuates dopamine release and cognitive function.<sup>72</sup> Through this analysis, it was determined that there were genes over and under expressed with the treatment of KU-32. This helps to provide a genetic rationale for some of the proposed neuroprotective qualities of KU-32 regarding upregulating molecular chaperones and antioxidant genes. Supplementary tables are presented in Appendix 1.

Chapter 4 investigates attention deficit hyperactivity disorder (ADHD) and dopamine release. An ADHD strain of rat was used to model the disorder and a non-ADHD control strain was used as a control. The ADHD animals were then split into two groups and one group was trained to determine if they could overcome the hyperactive and impulsive tendencies common in the strain while the other ADHD animals remained untrained. The goal of the behavior research was to show that behavior therapy is useful in treating ADHD patients without the use of pharmaceuticals. Due to amphetamine being a regularly used pharmaceutical treatment for ADHD, an amphetamine titration was performed to determine if brain slices derived from an untreated ADHD animal responds differently to amphetamine administration than a non-ADHD strain or a behaviorally trained ADHD animal. The dopamine release measurements illustrate the behavior training has an effect neurochemically. Overall, the behavior data support that behavioral therapy can help the animal overcome the impulsivity associated with ADHD as well as make the trained and control strain of animals very similar neurochemically suggesting that this training affects the dopamine system as well.

Chapter 5 presents the future directions of the chemobrain project with rodent models. These include the future of the work in the previous chapters, but also some other ways the chemobrain project and be expanded and changed to continue to be physiologically like humans.

Appendix 1 presents the supplementary tables referenced in chapter 3.

Appendix 2 focuses on foundational work that was done on several projects. These include *in vivo* surgery, doxorubicin treatment in zebrafish, and measuring serotonin in gut tissue within FSCV. The current progress and future directions are also presented.

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## Chapter 2 : Neurochemical Measurements in Doxorubicin-Treated Rats

## **Preface**

This chapter is divided into two sections due to the two different administration times and the associated behavior paradigms that were completed during each administration. Part 1 describes the two-week administration and a learning acquisition experiment and part 2 describes the four-week administration and an attention shifting paradigm. These parts are combined here as the background and methods are essentially the same except for the behavior paradigm and dosing time. The background and methods will be presented in part 1 and the methods that change and the new behavior paradigm will be presented in part 2.

## **Part 1: Two-week Administration**

### Introduction

Post chemotherapy cognitive impairment (PCCI), also known as chemobrain or chemofog, is a syndrome characterized by weakened cognitive abilities, problems with information processing, reaction time delays, lapses in memory, and mood changes.<sup>1-4</sup> Chemobrain symptoms are reported in about half of breast cancer patients and in one out of five patients with other cancers.<sup>1-3, 5</sup> Although the syndrome is well recognized, the underlying causes are not well understood.<sup>1, 2, 4, 5</sup> Current theories include differences in blood brain barrier permeability, DNA damage, decreased cytokine and hormone release, inflammation, and oxidative stress damage.<sup>1, 2, 4, 6, 7</sup>

Alterations in neurotransmitter release properties and cognitive performance have been observed in rats treated with select chemotherapy agents. The treatment of rats with carboplatin, a chemotherapy agent often used to treat cancers of the head, neck, breast, and lung<sup>8</sup>, resulted in diminished dopamine and serotonin release and impaired spatial learning.<sup>8</sup> The neurotransmitter release decreases occurred in a dose dependent manner, with intravenous (i.v.) injections at a dose of 20 mg/kg having a significantly greater effect than a dose of 5 mg/kg.<sup>9</sup> In another study, we found that i.v. injection with 5-Fluorouracil (5-FU), which is used to treat a wide variety of cancers,<sup>10</sup> attenuated dopamine and serotonin release in rats.<sup>10, 11</sup>

Importantly, these treatments negatively affected inhibition and attentional shifting, two key components of executive function that are similarly affected in humans with chemobrain.<sup>8, 10, 11</sup>

The work described in this chapter investigated the effects of doxorubicin administration on dopamine release and reuptake. Doxorubicin (Dox), an anthracycline and antineoplastic antibiotic derived from *Streptomyces peucetius*,<sup>12, 13</sup> is an agent of choice for many cancers, and is used to treat cancers of the bladder, breast, blood, and lymph nodes.<sup>14</sup> It acts by intercalating between base pairs in the DNA helix, thereby inhibiting DNA replication, and by inhibiting topoisomerase II, which prevents the ability to fix mistakes during replication.<sup>13, 15, 16</sup> Dox is known to create oxygen free radicals<sup>12</sup> and is toxic to the heart and the liver.<sup>13, 17, 18</sup>

To investigate the effects of Dox on the dopamine system, we administered two weekly doses (i.v., 2.5 mg/kg) to male Wistar rats. The rats underwent a learning acquisition behavioral paradigm and then *post mortem* neurochemical studies were performed in which we measured extracellular levels of dopamine release and uptake with fast-scan cyclic voltammetry (FSCV). Our results revealed an unexpected increase in dopamine release and uptake and no effect on learning.

## Methods

### *Animals*

All experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Kansas Institutional Animal Care and Use Committee. Twelve male Wistar rats (albino strain of rat commonly used in cancer research), 6 weeks of age, were acquired from Charles River Laboratories, Inc (Wilmington, MA). They were pair housed with food and water available *ad libitum* within the University of Kansas Animal Care Unit. The housing room was maintained at  $70 \pm 2^\circ\text{C}$  and humidity level of  $50 \pm 20\%$  with a 12-hour light/ dark cycle. All injections occurred during the light phase of the day. Six days before euthanasia (approximately 9-10 weeks of age), animals were switched to 22-hour food restriction in preparation for behavioral and neurochemical testing.

### *Drugs*

Pharmaceutical grade drugs and supplies that were purchased and used in these studies are: doxorubicin (Actavis- Parsippany-Troy Hills, NJ, lot: 8LF5061), isoflurane (iso) (VetOne- Fluriso, Boise, Idaho, lot: G129H20A), sterile saline (VetOne, Boise, Idaho, lot: A1909011), heparinized saline (Excelsior Medical, Neptune, NJ- lot: 3136995), and butterfly catheters (Terumo, 23 gauge and 0.2mL volume, lot: 170904B). Pramipexole (lot: 0000016785) was purchased from Sigma (St. Louis, MO).

### *Drug Administration*

Due to the risk of extravasation with Dox, tail vein injections were completed under general anesthesia. Extravasation is the leaking of drugs back out of the vessel into the surrounding tissue usually tissue causing damage like burning or necrosis around injection site.<sup>19</sup> The overall dosing paradigm was 2 doses at 2.5 mg/kg for a cumulative dose of 5 mg/kg or equivalent volume of saline vehicle. The two doses were separated by 7 days. To prevent any bias with the inhalation setup, both Dox- and vehicle-treated rats underwent the same anesthesia procedure as well as the same doses of heparinized saline and the final sterile saline. The animal was anesthetized and maintained on isoflurane via an inhalation machine (Model V3000PK, Parkland Scientific, Coral Springs, FL). Ophthalmic ointment was applied to the eyes to prevent drying out and the rats were placed on a heating pad to maintain body temperature. A butterfly catheter was inserted into a lateral tail vein. If the vein was difficult to see, gentle heat was applied with a heating pad or nitrile glove filled with water (60-80 °C) to dilate the vein. The line was first flushed with 0.2 mL heparinized saline and then Dox or vehicle was administered, and finally the line was flushed with 0.4 mL sterile saline. Blood return is possible while changing syringes and was used as a sign of correct placement in the tail vein. The line was then removed, gauze and pressure were applied to minimize bleeding, and the isoflurane was turned off. Once animals were completely ambulatory, they were returned to their cages with their cage mates and then returned to the housing facility. The rats were checked for signs of necrosis or irritation on their tails periodically over the week between injections and then the week before euthanasia.

### *Electrode Fabrication*

Carbon fiber cylindrical microelectrodes were fabricated as described previously.<sup>10, 20</sup> Briefly, single 7  $\mu\text{m}$  carbon fibers (Goodfellow Cambridge Ltd) were aspirated into glass capillaries (4 in, 1.2 mm OD; A-M Systems, Inc. Carlsborg, WA) and pulled using a heated coil puller (Narishige International USA, East Meadow, NY). Carbon fibers were cut to 50 microns from the glass seal. Electrodes were sealed by dipping into an epoxy mixture of 0.24 g of EPI-CURE 3234 Curing Agent and 2.00 g of EPON Resin 815C. Once the excess was removed using toluene, the electrodes were baked for 1 hour at 100°C. Electrodes were then soaked in isopropyl alcohol for 30 mins before use. To ensure an electrical connection, electrodes were backfilled with 0.5 M potassium acetate. Electrodes were then cycled before measurements in a flow cell and perfusion apparatus for at least 10 mins before use.

### *Behavior Apparatus*

Sessions occurred in commercially available operant conditioning chambers (Med Associates, Inc., Fairfax, VT). The interior dimensions were 30.5 cm long, 24.1 cm wide, 21.0 cm high. Centered on the front wall, 1 cm above the floor grid was a pellet receptacle (3 cm x 4 cm) into which a magazine could dispense grain-based pellets (45 mg; Bio-Serv, Frenchtown, NJ). Retractable levers were positioned on either side of the pellet receptacle (11 cm apart; 5 cm from the floor). A 28-V houselight centered on the back wall (19 cm from the floor) provided general illumination. Chambers were housed in sound-attenuating cubicles with fans to mask extraneous noise. All experimental events were programmed and recorded using MED-PC IV software.

### *Behavior Pre-Training and Testing*

In preparation for behavior pre-training and testing, the day after the second dose of dox or saline, the animals were removed from free feed and placed on 22-hour food restriction. This regimen was maintained for 4 days and then the animal was placed in the Med Associates operant box for magazine training. Magazine training consisted of a pellet being dispensed at a random interval for the rat to become familiar with receiving and retrieving food from the food magazine. Magazine training lasted 30 mins and the

following hour and a half were allocated to feeding time. The following day, the animals were presented with the learning acquisition task. **Table 2-1** illustrates how this schedule worked with the first dose being day 1 for each animal. The goal was to learn to press the lever and a 17.5 second delay to reward was added to increase the difficulty. Once the animal pressed the lever, the trial is initiated and if 17.5 seconds elapse with no response then a food reward was received. This procedure can then be repeated as many times as possible in the 6-hour session time. After the session was complete, animals were returned to their home cages and placed back on free feed and euthanized the following day.

**Table 2-1. Schedule of tasks from injections to euthanasia for rats involved in dox two-week dosing study. Day 1 is established as the day that the first injection is received for each rat.**

Day 1	Day 8	Day 9	Day 13	Day 14	Day 15
First dose of drug or vehicle	Second dose of drug or vehicle	Food Restriction Begins	Magazine Training (30 min session)	Learning Acquisition Experiment (6-hour session)	Euthanasia and neurochemical measurements

#### *Euthanasia and Dissection*

Seven days after receiving their second dose of doxorubicin, animals were anesthetized and euthanized by decapitation. The brain removal process was completed as previous described.<sup>8, 10, 11</sup> In short, the skull was removed, and the brain was then removed and placed in cold (approx. 6°C) and oxygenated (95%:5% Oxygen: Carbon Dioxide) artificial cerebral spinal fluid (ACSF). The ACSF solution consisted of 2.5 mM KCl, 126 mM NaCl, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 2.4 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 20 mM HEPES, 11 mM D-glucose. The pH was adjusted to 7.4. Once chilled, the brain was removed from the ACSF and the cerebellum was removed. The brain was then bisected, and one hemisphere was prepared for slicing using a vibratome (Leica Microsystems, Bannockburn, IL). A 4% agar block was glued using cyanoacrylate glue to provide support for the hemisphere. For coronal slices, the brain was glued cerebellum side down on the stage. Striatal slices, 300µm thick, were made and one was placed in an RC-26 perfusion chamber with the harp used to secure the slice in place (Warner Instruments, Holliston, MA) maintained at 34°C with a Single Inline Solution Heater (SH-27B) (Warner Instruments, Holliston, MA). This chamber allows warmed and oxygenated ACSF to be continuously flowed at 2 mL/min over the brain slice, allowing it to

stay viable to 6-8 hours.<sup>21</sup> Slices were equilibrated in the perfusion chamber for at least 1 h before collecting measurements. Other slices that had large portions of the striatum were preserved by submersion in circulating oxygenated ACSF at room temperature. The second hemisphere was saved in an Eppendorf tube and placed in a -80 °C freezer until needed for other experiments.

#### *Fast Scan Cyclic Voltammetry (FSCV)*

Background subtracted FSCV was performed as previously described using a system in which a potentiostat (Dagan Chem Clamp, Minneapolis, MO) was interfaced with a computer by a breakout box (Knowmad Technologies LLC, Tucson, AZ) and controlled with Wildcat software (Knowmad Technologies LLC, Tucson, AZ).<sup>8, 11, 22</sup> A precalibrated carbon fiber microelectrode was inserted into the brain slice with two stimulus electrodes (A-M Systems Inc., Carlsborg, WA) inserted into the slice on either side. Pre-calibration involves cycling the electrode on the waveform of interest and measuring the current generated when 1 $\mu$ M dopamine is injected into ACSF using a flow cell and a syringe pump (Chemyx Fusion 1, Stafford, TX). This value allows the conversion of current measurements to concentrations. Both electrodes were lowered and positioned using micromanipulators and a stereoscope. The extended dopamine waveform ( -0.4 V to +1.3 V to -0.4 V) was applied versus a silver/silver chloride reference electrode. A scan rate of 400 V/s and a waveform application frequency of 10 Hz were used. A single 4 ms biphasic stimulation pulse of 350  $\mu$ A was applied to evoke dopamine release. Color plots were constructed from the current versus time plots and the cyclic voltammograms (lower part of **Figure 2-1**). Stable dopamine release was achieved by obtaining 3 files where the peak current is within 10 percent of each other. Release in each quadrant of the striatum (**Figure 2-2B**) was then measured in four different locations to account for differences in dopaminergic innervation. Each measurement was collected at least 5 minutes after the previous one. After the 16 release measurements were collected, a pramipexole titration was carried out. Pramipexole was mixed into the ACSF at concentrations of 0, 0.5, 1, 10, 30, 50, 70, 100, and 300 nM and perfused over the slices for 30 minutes. Concentrations were added in ascending order and six measurements were taken during this time. From our experience, more variability in neurochemical

measurements is captured between slices than between animals. Therefore, the n-value for the neurochemical measurements was the number of slices, with 1 to 2 slices per rat being taken from a total of 4 rats per drug condition. Intra-slice variability is accounted for by the random sampling within the quadrant for the 4 measurements. When correlating dopamine release with rat behavior, the average dopamine release per rat was used.

### *Statistical Analysis and Modeling*

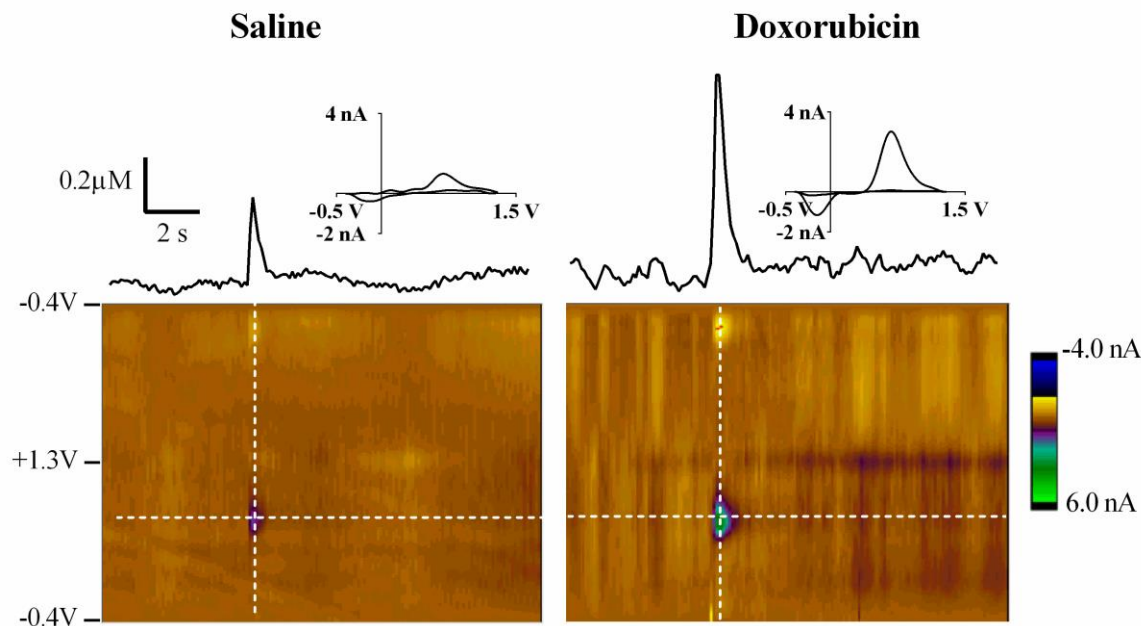
Statistical analysis was completed using GraphPad Prism version 6 (GraphPad Software Inc, La Jolla, CA, USA) and Microsoft Excel. For the behavioral analysis, the n-value is equal to the number of rats. The data was converted from text files and the number of reinforcers earned and the total number of responses were collected for each rat. Modeling to determine dopamine reuptake rate constants and half-lives was performed using GraphPad software as described previously.<sup>23</sup> Briefly, the files were loaded into Wildcat software and the peak current ( $i_{\max}$ ) was determined. The portion of the plot in which current decreases from 80% to 20% of  $i_{\max}$  was modeled to determine the kinetic parameters. The x and y values of the 80% value and the y value for the 20% value were used as constraints in the nonlinear regression- plateau followed by one phase decay model. Data files were excluded from modeling if they did not have a peak current over 0.8 nA and if there were less than 3 points between the 80% and 20% values. Once the modeling was completed, the 1<sup>st</sup> order rate constant (k), and the half-life ( $t_{1/2}$ ), were obtained. The values were compared by quadrant and then compared by treatment group. Modeling for the pramipexole titration was completed using GraphPad and utilized the non-linear model- log(agonist) vs. response software, Find ECanything.

## Results and Discussion

### *Striatal Dopamine Release*

**Figure 2-1** shows representative raw data from a saline treated rat and doxorubicin treated rat. The current versus time plots are shown above the color plots with the cyclic voltammograms inset. The color plots are 3-dimensional representations of how current varies with applied potential and scan number, with the color scale representing the magnitude of the current. Cyclic voltammograms were sampled at the

vertical dashed lines and current-time plots were sampled at the horizontal dashed lines. Dopamine release appeared to be greater in slices from Dox-treated rats compared to those treated with vehicle.



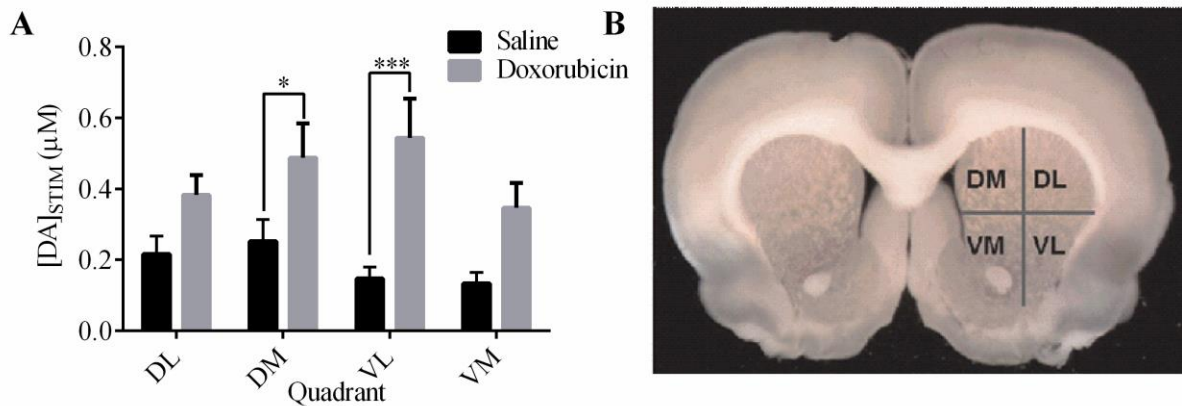
**Figure 2-1. Representative plots of stimulated release from saline- and Dox-treated rats.**

The white vertical and horizontal dashed lines indicate the sampling locations of the CVs (insets) and current-time plots (above the color plots) respectively. The CVs confirm the presence of dopamine. Stimulation occurred at 5 sec on each file and dopamine oxidation is indicated by the green/ purple spot on the color plot. Potentials versus Ag/AgCl reference electrode

The raw data files were analyzed further and the peak concentrations of dopamine within each quadrant and treatment were determined. **Figure 2-2A** shows the peak concentrations of dopamine release in saline- and Dox-treated rats separated by quadrant. **Figure 2-2B** illustrates how the quadrants were demarcated. There was a significant main effect of treatment condition in which dopamine release was greater in Dox-treated rats compared to vehicle-treated rats ( $p < 0.0001$ , two-way ANOVA,  $F[1,56] = 30.94$ , vehicle,  $n = 9$  slices, Dox,  $n = 7$  slices). Sidak *post hoc* analysis showed significant differences in dopamine release between vehicle- and Dox-treated rats measured within the dorsomedial ( $p < 0.05$ ) and ventrolateral ( $p < 0.001$ ) quadrants. There was no significant difference found between quadrants within treatment groups.

The significant increase in dopamine release contrasts sharply with our previous findings in which rats were treated with carboplatin and 5-fluorouracil, both of which cause a significant decrease in release.<sup>8, 10, 11</sup> The cause of this increase in dopamine release could be a response to the lower levels of serotonin<sup>24</sup> and norepinephrine<sup>8</sup> that are associated with doxorubicin treatment. Previous research has shown serotonin release to be decreased with carboplatin treatment in rats.<sup>14</sup> While we have not measured serotonin release in Dox treated rats, there is a literature indication for serotonin to be decreased in the brain.<sup>15, 25</sup> Given that these neurotransmitters are important for cognition and are associated with mood, the increase in dopamine may be a compensation mechanism for the imbalance of other neurotransmitters to stave off symptoms of cognitive decline or mood changes.

It is also possible that dopamine release itself could be causing oxidative damage through its metabolism with monoamine oxidase (MAO), which can act on intracellular dopamine. In this mechanism, MAO catalyzes the conversion of dopamine to dihydroxyphenylacetic acid (DOPAC) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), a reactive oxygen species.<sup>25</sup> This mechanism has also been suggested in methamphetamine neurotoxicity or L-DOPA treated Parkinson's Disease.<sup>25</sup> If Dox induces increased cytosolic levels of dopamine, this mechanism could create additional intracellular oxidative stress.<sup>16, 26</sup> Therefore, future experiments should include quantitating dopamine levels in specific intracellular pools.



**Figure 2-2. Treatment with Dox significantly increased dopamine release.**

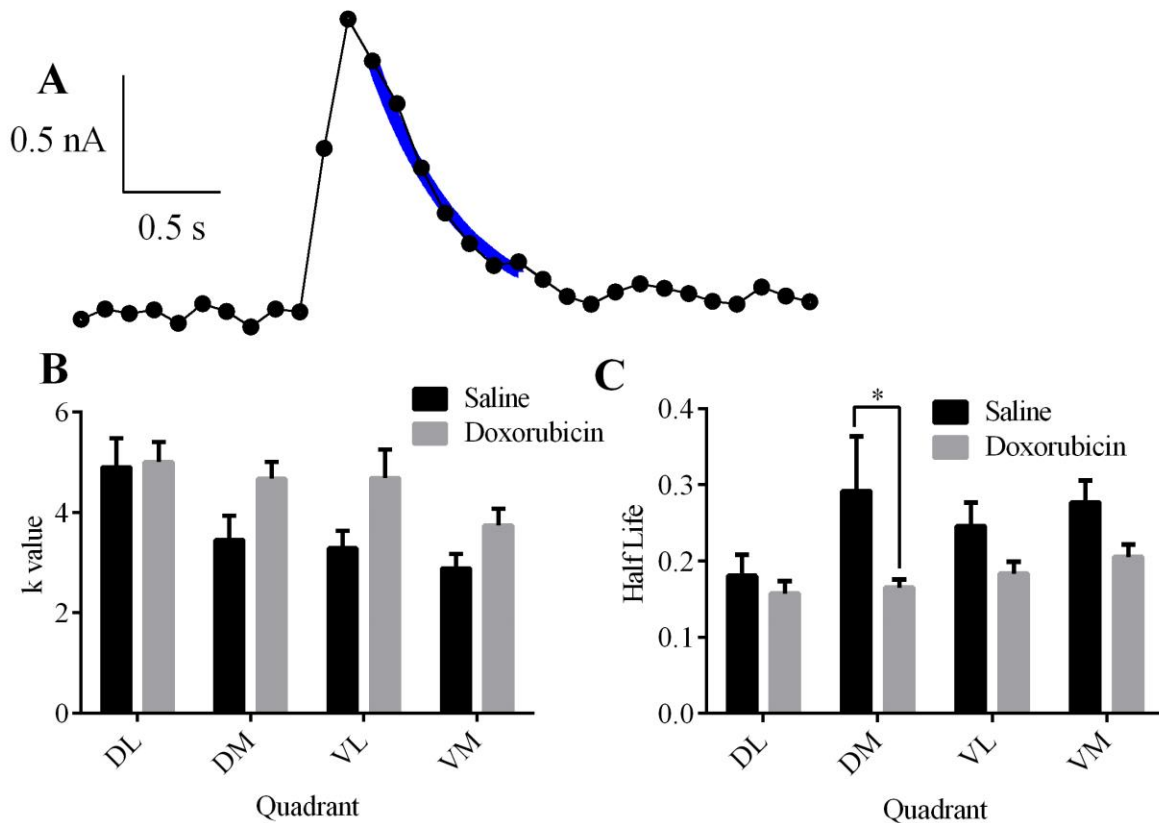
(A) Average release in quadrants of the striatum, defined in (B). There was a significant main effect of treatment condition in which dopamine release was greater in Dox-treated rats compared to vehicle-treated rats ( $p < 0.0001$ , two-way ANOVA,  $F[1,56] = 30.94$ , vehicle,  $n = 9$  slices, Dox,  $n = 7$  slices). Sidak *post hoc* analysis showed significant differences in dopamine release between vehicle- and Dox-treated rats measured within the dorsomedial ( $*p < 0.05$ ) and ventrolateral ( $***p < 0.001$ ) quadrants. There was no significant difference found between quadrants within treatment groups. Abbreviations: DL, dorsolateral; DV, dorsoventral, VL, ventrolateral, VM, ventromedial.

Figure Credit: Kaplan, *et al.* (2016).<sup>8</sup> With permission from the American Chemical Society

### Reuptake

The modeling of the release data allowed us to investigate dopamine reuptake by the DAT. **Figure 2-3A** illustrates the modeling operation in which the first-order rate constant ( $k$ ) and half-life ( $t_{1/2}$ ) was determined for each trace, and then pooled to obtain averages for each quadrant and overall. There were significant overall effects on  $k$  and  $t_{1/2}$ . Compared to vehicle-treated rats,  $k$  was greater in Dox-treated rats ( $p < 0.01$ , two-way ANOVA,  $F[1,132] = 7.083$ , vehicle,  $n = 9$  slices, Dox,  $n = 7$  slices) while  $t_{1/2}$  was less ( $p < 0.001$ ,  $F[1,132] = 12.45$ ). Almost all fits of the model had an  $R^2$  value of greater than 0.95. It was also found that quadrant had a significant main effect on  $k$  with dorsal medial and ventral lateral quadrants varying significantly ( $p < 0.05$ , two-way ANOVA,  $F[3,132] = 3.890$ , vehicle). The ANOVA showed no difference between quadrants in within treatment group. **Figures 2-3B and 2-3C** show these values divided by quadrant and the only significant difference found was in the  $t_{1/2}$  of the dorsomedial quadrant ( $p < 0.05$ , Sidak *post hoc*). Collectively, these data suggest that Dox treatment significantly increases the rate of reuptake.

The relative rate of dopamine uptake, represented by the first order rate constant ( $k$ ), was greater in Dox treated rats compared to controls. Upon release, dopamine is quickly taken back up into neurons by the dopamine transporter (DAT).<sup>27</sup> Differences in DAT expression have been found in Parkinson's Disease<sup>28</sup> and attention deficit hyperactivity disorder<sup>29</sup> patients. It is possible that the increase in the uptake rate found in Dox-treated animals is a compensatory mechanism for the increase in dopamine release, thereby enabling dopamine levels to be maintained at homeostatic levels.



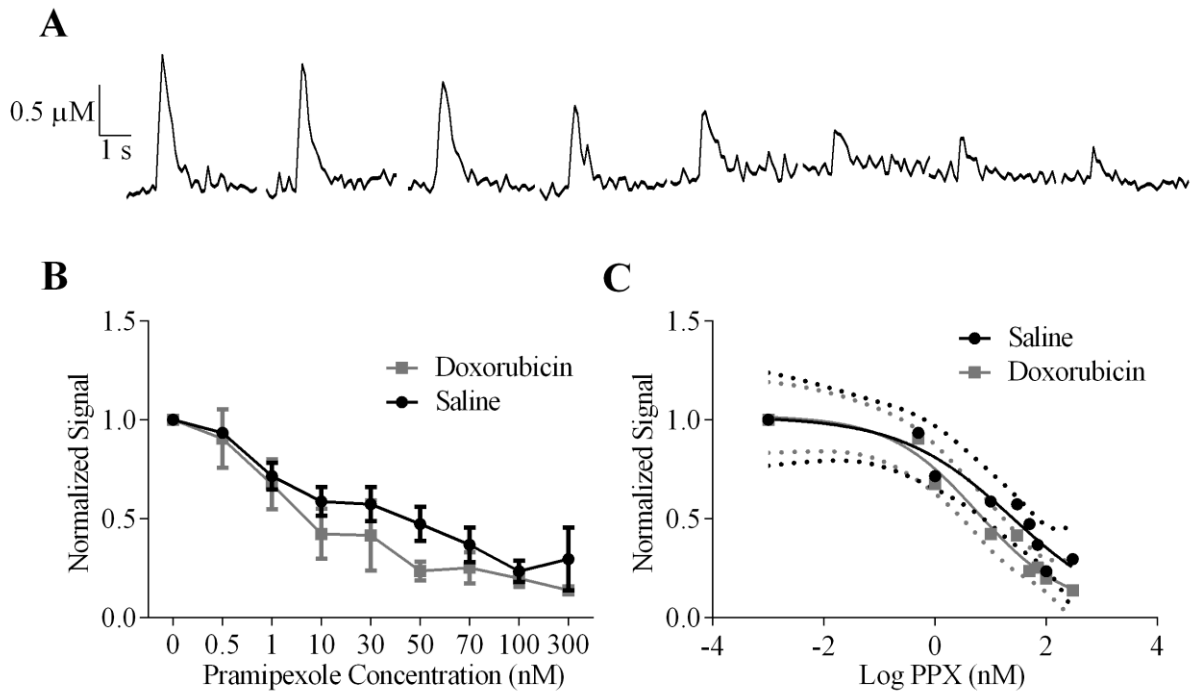
**Figure 2-3. Dopamine Uptake Data**

(A) Sample model of a dopamine reuptake curve. The current vs time graph is shown with black circles for each point while the model is the blue line. This model uses the time point at approximately 80% of maximum and ends at the time point at approximately 20% of maximum. The model gave  $k$ ,  $t_{1/2}$ , and  $R^2$  values. (B) and (C),  $k$  values and  $t_{1/2}$  values, respectively, separated by quadrant of the striatum. (\* $p < 0.05$ , Two-way ANOVA with Sidak Post Hoc, Saline-  $n=7$  slices, Doxorubicin-  $n=8$ )

### *Pramipexole Titration*

Pramipexole is a dopamine receptor agonist that has a high affinity for D3 autoreceptors ( $K_i=0.5$  nmol/L)<sup>23</sup> and lower affinity for D2 autoreceptors ( $K_i=3.9$  nmol/L).<sup>30</sup> To assess possible differences in D3/D2 autoreceptor function in treated versus non-treated brains, we perfused slices with at progressively increasing concentrations of pramipexole. Consistent with previous results with PD 128907,<sup>10</sup> a selective D3 agonist, pramipexole inhibited dopamine release (**Figure 2-4A**). **Figure 2-4B** shows the data

normalized as the fraction of dopamine remaining. The next goal was to determine if there was an overall difference between Dox-treated and vehicle-treated rats. The Find EC anything model in GraphPad was used to determine the concentration of 50% dopamine release inhibition (IC50). Values were normalized against dopamine release current obtained at 0 nM pramipexole. From the modeled lines, plotted in **Figure 2-4C**, the IC50 values were estimated to be 25.1 nM and 5.78 nM for saline treatment and doxorubicin treatment, respectively. The curves are not significantly different, suggesting that Dox treatment does not substantially alter autoreceptor function (Repeated measures Two-Way ANOVA- Sidak Post Hoc  $p = 0.3164$ ).



**Figure 2-4. Pramipexole Titration**

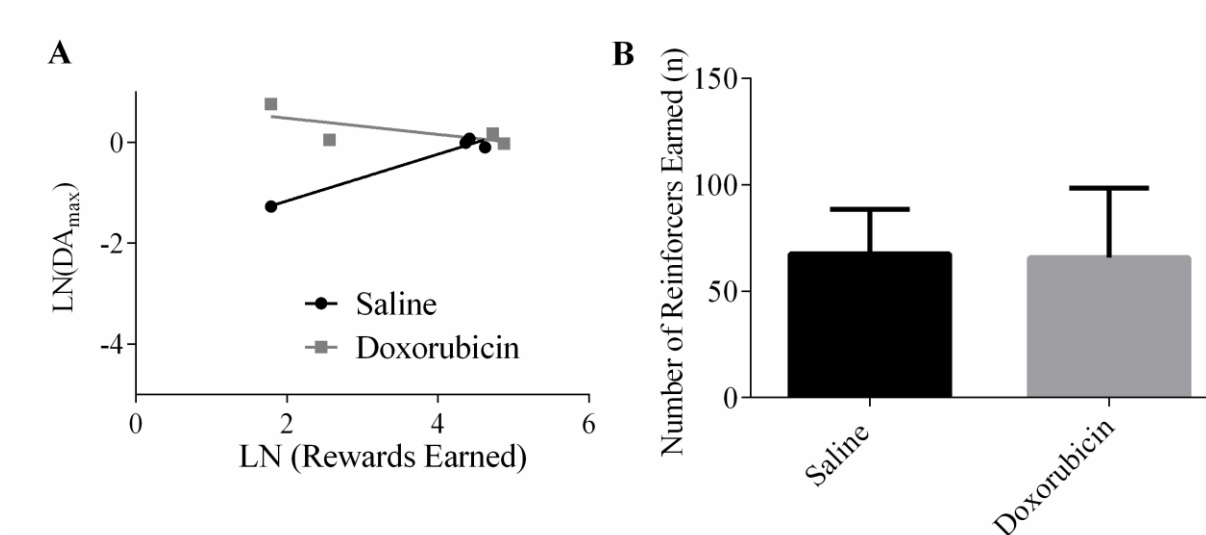
(A) Representative Plot of Dopamine Release throughout Pramipexole (PPX) Titration. Each concentration was held for 30 mins and the plot shown is trace taken towards the end of the titration time. The x-axis shows the time in seconds and the y-axis shows the concentration in micromolar. The scale bars show 0.5  $\mu\text{M}$  vertically and 1 s horizontally. The value at 300 nM is not shown as the plot has an artifact that creates an artificial peak. (B) Scatter plot showing the average release over time over all animals within each treatment group. Error Bars represent  $\pm\text{SEM}$ . These data are normalized as the fraction of the average maximum signal remaining. This was done to eliminate any scale differences among the animals. The first 30 minutes of no drug exposure is averaged to create the maximal release and each sequential point is a fraction of that first point. Each concentration was held for 30 mins, files were taken every 5 mins, and all files were averaged. These data suggest that there is no overall difference in sensitivity to pramipexole with doxorubicin (Repeated measures Two-Way ANOVA- Sidak Post Hoc  $p = 0.3164$ ). (C) Model of curves to determine  $\text{IC}_{50}$ . The points from A were transformed into a Log scale on the x axis and then a decay curve modeled with GraphPad. The  $\text{IC}_{50}$  values were estimated to be 25.1 nM and 5.78 nM for saline treatment and doxorubicin treatment, respectively. The dotted lines are the 95% confidence intervals which show no significance between the lines. The concentration at 0 nM in this plot is estimated by using the value  $1.00 \times 10^{-3}$  nM for the purposes of modeling the curve on the semilogarithmic plot. Saline,  $n = 5$  slices, Doxorubicin,  $n = 7$  slices.

### Behavior

All animals completed a behavioral task in which they pressed a lever and waited 17.5 seconds until responding again. The goal of the study was to investigate how quickly the animals attained proficiency in

the task. As shown in **Figure 2-5A**, the doxorubicin animals and their larger dopamine release is not correlated with a larger amount rewards earned. The fit of the lines was determined to be  $R^2=0.9670$  (saline) and  $R^2=0.4867$  (doxorubicin). **Figure 2-5B** shows that each treatment group received approximately the same number of reinforcers and the error bars show that both groups had some animals that participated and made many responses within the 6 hours while some did not respond as much. From these results, we were not able to identify a difference in responding between treatment groups.

In previous research, it has been shown that dopamine release and rewards earned in executive function tasks are positively correlated. In this instance, treatment did not affect their ability to learn the task more quickly or at a higher rate. Each reinforcer required 1 response and both groups show about the same number of reinforcers earned. There also was no correlation between the larger dopamine release and rewards earned. It is possible that the enhanced release of dopamine masked cognitive deficiencies caused by Dox treatment, making them difficult to ascertain with this paradigm.



**Figure 2-5. Learning Acquisition Data**

(A) Correlation plot showing the natural log of the rewards earned during a behavioral paradigm vs the natural log of maximum dopamine release in doxorubicin treated animals. Saline,  $R^2=0.9670$ , Doxorubicin,  $R^2=0.4867$  (B) Bar graph showing the number of reinforcers earned during a behavior paradigm in doxorubicin treated animals ( $p=0.9656$ , unpaired t-test,  $n=4$  saline rats,  $n=4$  dox rats).

### Conclusion

In this study we measured the effects of Dox treatment on Wistar rats. We found that, in contrast to previous results from us and others, Dox greatly increased stimulated dopamine release and uptake in the striatum. However, we found no difference in the autoregulation of dopamine release or a difference in cognitive function. It is possible that this enhancement in dopamine function and lack of behavioral effect represents a compensatory mechanism induced by changes in other neurotransmitter systems. Nevertheless, more work is needed to understand the underlying molecular mechanisms responsible for these discrepancies. Additionally, as shown by the contrasting pathologies presented here and elsewhere,<sup>8, 11, 31</sup> a “one-size-treats-all” approach may not be appropriate in preventing and understanding chemobrain.

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Figure 2B: from Kaplan, et. al, 2016- <https://pubs.acs.org/doi/10.1021/acchemneuro.5b00029> -further permissions requests should be directed to the ACS<sup>8</sup>

## **Part 2: Four Week Administration**

### Introduction

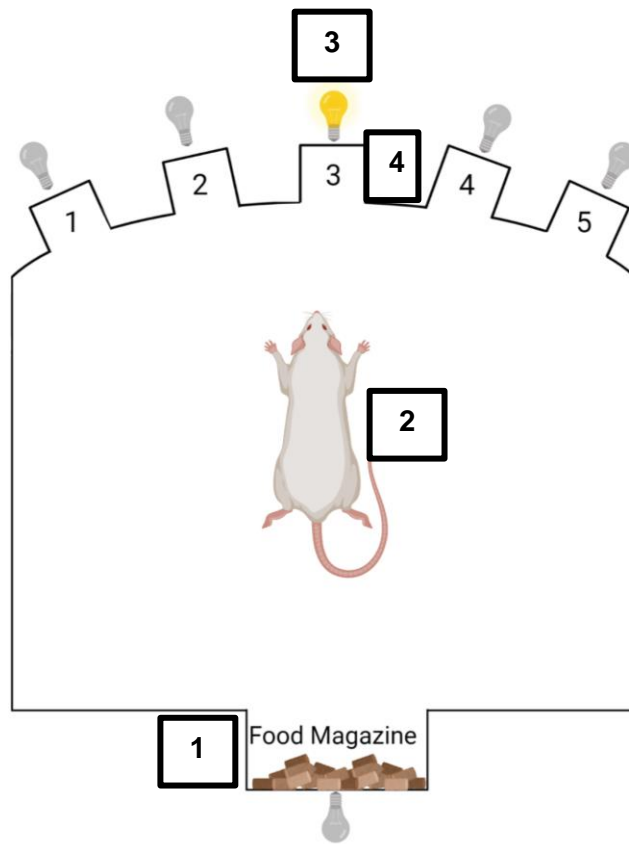
This experiment extended the dosing of Dox to 4 weeks and employed a new behavior paradigm. The dosing time was extended to represent a more physiologically relevant dose to what humans receive in the clinic, increasing the cumulative dose from approximately 12 mg/m<sup>2</sup> to 24 mg/m<sup>2</sup>, where a standard dose in humans is 40 mg/m<sup>2</sup> per month.<sup>32-36</sup> Attention shifting was studied in Dox treated rats because this behavioral component, as one of the contributing components of executive function, is largely processed in the frontal lobe of the brain<sup>37, 38</sup> and is impaired in chemobrain.

Attention shifting is the ability to switch “back and forth between tasks, operations, or mental set”.<sup>39</sup> Of the main components of executive function, this one was selected for investigation as the behavioral test is robust and fairly analogous to human behavior.<sup>37</sup> The effects of frontal lobe damage and attention shifting impairment were observed when Phineas Gage was impaled by a tamping iron leading to a substantial hole in his skull and frontal lobe.<sup>37</sup> His injury led to him becoming an impulsive person with little control over his own behavior.<sup>37</sup> Several mental health disorders also show symptomology including deficits in executive function including ADHD, bipolar disorder, obsessive compulsive disorder, and addiction/substance abuse.<sup>37</sup>

The frontal lobe is innervated with dopamine, norepinephrine, serotonin, and acetylcholine neurons, among others.<sup>37</sup> Dopamine is the neurotransmitter of interest and it has been shown to have large effects on executive function and attention shifting.<sup>37</sup> Studies have been performed in D2/ D3 knockout mice and which showed that the knockout mice performed differently in a behavioral test than the wildtype mice.<sup>40</sup> The mice that were D2 knockouts took longer to figure out the task and the D3 knockouts actually had higher accuracy in their response than the wildtypes.<sup>40</sup> This shows that dopamine and its receptors are essential for proper task discrimination and attention to select the correct answers.<sup>40</sup> The need for dopamine in attention shifting can also be seen when dopamine neurons are damaged in Parkinson’s Disease and patients report problems with higher level cognitive tasks. This is believed to be due to a lack of dopamine

within the PFC. This then manifests what is called cognitive inflexibility as these patients are not able to easily change their behavior based on the presentation of a stimulus. Attention shifting is a main component of these flexibility tests as the patient must switch their attention and behavior based on a certain stimulus. Measuring attention shifting and dopamine levels should shed light on how Dox treatment affects executive function and the dopamine system.

The behavior paradigm that was selected is called the 5-choice serial reaction time task (5-Choice).<sup>39</sup> This is a reliable and robust test that allows for strict control of behavioral contingencies while having a high level of construct validity.<sup>39</sup> Attention is divided in this test between a light over the correct nose poke (5 choices) and the food magazine which required a nose poke to start the trial. At a random interval, the light over the correct target will flash and the rat has to nose poke the correct target within an allotted time (**Figure 2-6**).<sup>39</sup> This experiment allowed for testing throughout all parts of the dosing time, so changes and trends could be tracked over time. Doxorubicin treated animals and their saline counterparts showed differences in behavior. With the longer dosing time, dopamine release was impaired while there were no differences in reuptake in uptake found, which show that with 4 weeks of dosing, the dopamine system is further altered from the 2-week dosing and most likely changing throughout the dosing timeframe.



**Figure 2-6. Schematic of Behavior Chamber for 5-Choice Serial Reaction Time Task**

The steps of the experiment are labeled with the amount of time the rat waits at 2 is increased on probe days. Figure created with Biorender.com

### Methods

Dosing protocol and data analysis were performed in the same way as described above except for dosing continuing for two additional weeks and the age at euthanasia being determined by the behavior training. Animals were euthanized at approximately 31 weeks of age. The neurochemical experiments were performed in the same way as above including the pramipexole titration.

### *Animals*

All experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Kansas Institutional Animal Care and Use Committee. Sixteen male Wistar rats, 6 weeks of age, were acquired from Charles River Laboratories, Inc

(Wilmington, MA). They were pair housed with food and water available *ad libitum* within the University of Kansas Animal Care Unit. The housing room was maintained at  $70 \pm 2^\circ\text{C}$  and humidity level of  $50 \pm 20\%$  with a 12-hour light/ dark cycle. All injections occurred during the light phase of the day. Animals were handled daily during training and dosing. Once animals acclimated to the animal care unit (3 days post arrival), they were placed on 22-hr food restriction for the duration of the behavior experiments.

### *Attention Shifting Paradigm*

Pre-training for this paradigm consisted of several steps. Each step had to be completed before the next could be limited. Pretraining consisted of 2 sessions of fixed ratio of 1 on any nose poke (one nose poke on any target) to receive a reward. The computer then randomly selected nose pokes that were signaled with a cue light located within the nose poke. The animal had to respond once on a randomized nose poke with cue light to receive a reward until 85%, or higher, accuracy was achieved (one nose poke on randomly selected target). A nose poke in the food magazine signaled by a cue light was then added to begin the trial then a randomized nose poke with cue light was required for a reward until 85% or higher accuracy was achieved (one nose poke in food magazine and one nose poke in randomly selected target). The final part was for the time the light in the nose poke was illuminated began decreasing which increased the intertrial interval (5 sec illumination decreased to 3 decreased to 1). The intertrial interval is a blackout period where no behavior is reinforced to equate trial length and access to reinforcers across subjects.

The final test consisted of several steps that allowed the animal to get a food reward

1. A nose poke in food magazine initiated the trial
2. Rat waits an average period of 2 sec (range of 1-3 seconds)
3. A light illuminated in the target nose poke for 1 second and then disappeared
4. The rat then had 5 seconds in which to respond. Early responses or incorrect responses ended the trial.

The selected target remained the same for 3-7 trials, average of 5, and then was randomly assigned to a new target. To further test the animals and present a different test, probe days were included. The probe paradigm

increased time to the light illuminating in nose poke. The rats had to wait and pay attention longer. This means that instead of waiting 1-3 seconds for the light he would have to wait twice as long for the light to appear (2-6 sec).<sup>39</sup>

## Results and Discussion

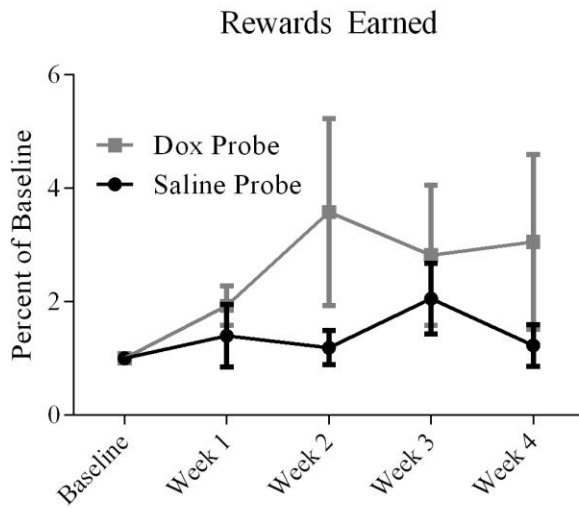
### *Attention Shifting*

Attention shifting was tested via the 5-choice serial reaction time task. The week of treatment and behavioral measurements is indicated on the x-axis, while the percent of baseline values of rewards earned, incorrect answers, and responding without possibility of reward is shown on the y-axis. The comparison of the number of reinforcers earned is shown in the top graph of **Figure 2-7**. This graph shows that after up to 3 doses, the Dox-treated animals trend toward receiving more rewards in the harder trials than their saline counterparts. This means that during these trials, the rats are retaining how to complete the task and are withstanding a larger delay to the stimulus and can pay attention longer. Due to inter-week variability among the Dox animals, there is no statistically significant difference (Repeated Measures 2 Way ANOVA- Sidak Post Hoc -p = 0.2635 Saline n= 8 rats, Doxorubicin- n= 8 rats).

After two doses the dox rats' number of wrong responses (**middle Figure 2-7**) is trending toward being higher than the other animals and stays higher through the rest of dosing. This indicates that they are not paying attention to where the light is or that their spatial awareness is affected throughout treatment. Again, due to inter-week variability among the Dox animals, there is no statistically significant difference (Repeated Measures 2 Way ANOVA- Sidak Post Hoc -p = 0.3766 Saline n= 8 rats, Doxorubicin- n= 8 rats).

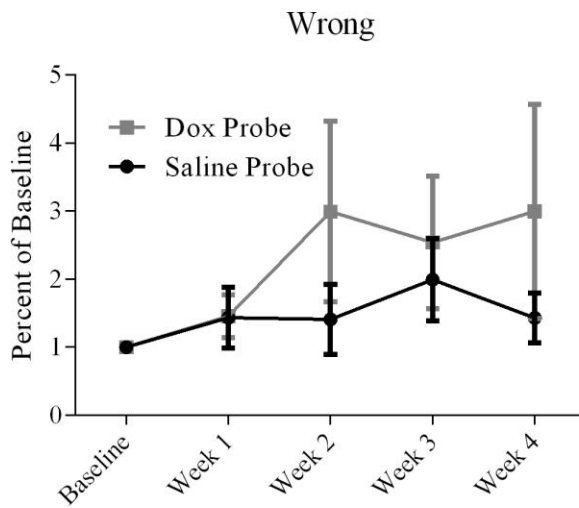
The number of responses during the inter-trial interval (**bottom Figure 2-7**), which is a blackout period to ensure that all trials average to be the same length as the parameters are randomly assigned each trial and each rat, starts to trend toward climbing as soon as dosing starts and then fall substantially during week four. Finally, due to inter-week variability among the Dox animals, there is no statistically significant difference (Repeated Measures 2 Way ANOVA- Sidak Post Hoc -p = 0.1789 Saline n= 8 rats, Doxorubicin- n= 8 rats).

Overall, these three sets of data illustrate that something could be happening in doxorubicin treated animals to affect the rats' cognitive state during dosing which is unfortunately lost to the high variability within the data. Future work would include looking at other parameters of data that could be acquired during the 5-Choice test. It would also be advantageous to determine what, if anything noticeable, is causing such high variability within the Dox animals.

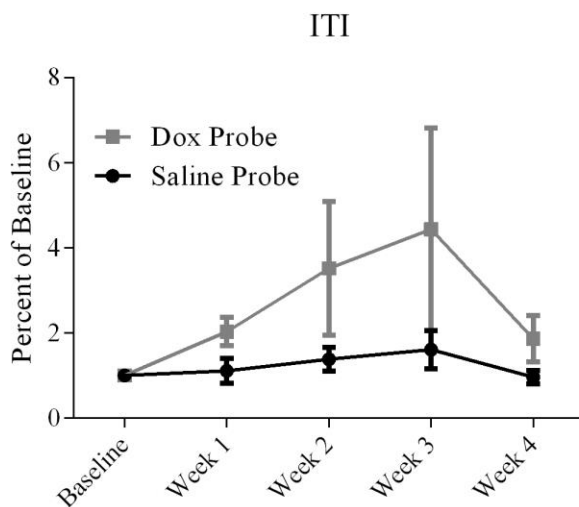


**Figure 2-7. Selected Behavior Data**

Top- Dox probe animals do not vary from saline probe animals- (2 Way ANOVA- Sidak Post Hoc -p = 0.2635 Saline n= 8 rats, Doxorubicin- n= 8 rats).



Middle- Dox probe animals do not vary from saline probe animals- (2 Way ANOVA- Sidak Post Hoc -p = 0.3766 Saline n= 8 rats, Doxorubicin- n= 8 rats).



Bottom- Dox probe animals do not vary from saline probe animals- (2 Way ANOVA- Sidak Post Hoc -p = 0.1789 Saline n= 8 rats, Doxorubicin- n= 8 rats).

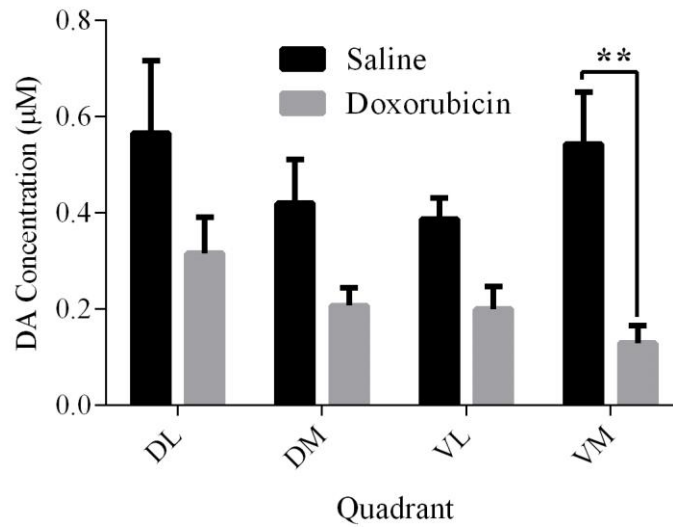
### *Dopamine Release*

After dosing the animals for 4 weeks with doxorubicin, the rats were sacrificed, and striatal dopamine release was measured in the 4 quadrants. Two-Way ANOVA (with Sidak's multiple comparison test) revealed that Dox treatment had significant main effect on dopamine release ( $p < 0.0001$ ). Post hoc analysis showed that release in the ventromedial quadrant is significantly diminished ( $p < 0.01$ ). Repeated measures analysis was not used as each measurement in the striatum was made after the electrode was repositioned. This means that even though each quadrant has four measurements, they are not in the same spot or stimulating the exact same area, so they are not repeated measures.<sup>41, 42</sup>

These data are interesting as it is the opposite of the findings with the 2-week rats which showed an increase in release. This result is similar to those that have previously been observed, with rats treated with carboplatin and 5-FU in which dopamine release was dramatically impaired.<sup>8, 11</sup> It is possible that dopamine release is bimodal over the 4-week dosing period with an increase at the two-week mark and then a decrease at the 4-week mark (**Figure 2-9**). The difference between saline-treated rats (2 weeks versus 4 weeks) may arise from differences in age or the amount of handling (4-week rats handled daily, 2-week rats were only handled to dose), or experimental inconsistencies that occur over time. Handling rats has been shown to improve learning and memory through behavioral testing, but this has not been confirmed neurochemically.<sup>43</sup> Aged rats have been shown to have less striatal dopamine through high-performance liquid chromatography with electrochemical detection (HPLC-ECD).<sup>44</sup> This study wanted to know if dopamine decrease with age correlated with the known age related decrease in D2 in the ventral striatum, but instead showed that dopamine decrease is global and not localized.<sup>44</sup> More work will have to be done to determine if either of these differences could affect dopamine release as there is currently no neurochemical data on handling effects. Our rats were significantly younger at a little over 7 months compared to the 25-27 months of the aged group studied with HPLC-ECD study.<sup>44</sup> Nevertheless, the differences in dopamine release between rats treated with Dox for two and four weeks and their respective

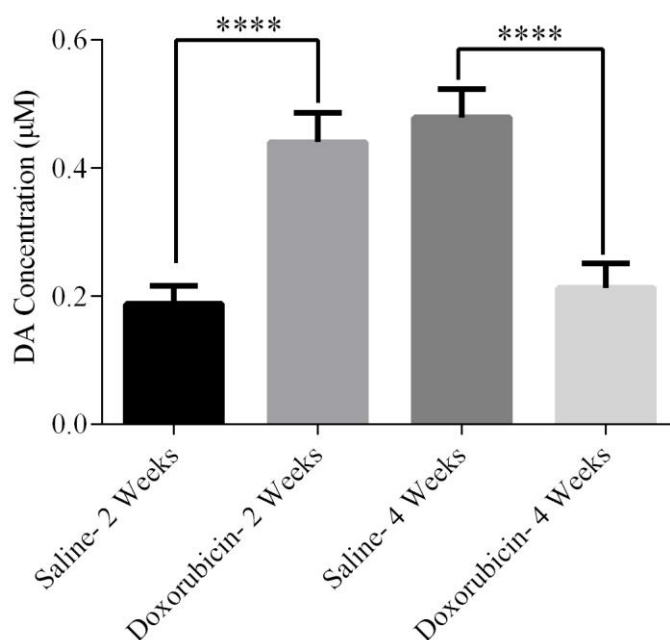
controls is robust, indicating that dopamine significantly increases after two weeks of dosing and significantly decreases after four weeks of dosing with n values of 6-9 slices per treatment group.

Typical doxorubicin dosing in a breast cancer patient is  $40\text{mg}/\text{m}^2$ .<sup>36</sup> This dose is typically given in one shot or infusion, but with rats, such a large bolus injection is not possible. Due to this fact, we relied on cumulative dosing where a  $2.5\text{ mg}/\text{kg}$  dose was given to achieve the  $10\text{ mg}/\text{kg}$  target dose. A cumulative dose of  $10\text{ mg}/\text{kg}$  is closer to an equivalent dose that a human receives at approximately  $24\text{ mg}/\text{m}^2$ .<sup>32-34</sup> Comparison of the behavioral results with the neurochemical results suggests that release of dopamine at 4 weeks trends toward lowering their cognitive state to resemble their non-dosed counterparts. It is unknown what has caused this shift in dopamine release, but it is very interesting that at two weeks (2 doses) the suspected cognitive deviation from the other animals and sessions begins and then at four weeks (4 doses) the dox probe response rate returns to almost baseline. This suggests that the dopamine system is affected by the longer dosing time which correlated with cognitive differences. This change in the dopamine system overtime may arise from a compensatory mechanism to counteract the decrease in other neurotransmitters, such as serotonin.<sup>14</sup> However, after extended exposure, the dopamine system is no longer able to keep up and release is attenuated significantly. This system deficit shows how prolonged administration has different effects from acute administration. Future research should involve determining if dopamine release remains depressed over longer periods of time (months) after treatment has ceased.



**Figure 2-8. Dopamine Release by Quadrant in 4- Week Treated Rats**

Dopamine release by quadrants and then pooled by treatment. Striatal dopamine release is plotted versus the quadrant that it was collected from with 4 measurements being collected per quadrant per rat. The ventral medial quadrant is significantly decreased when compared to the saline treated animal. Overall Main Effect, Treatment decreased dopamine release,  $p < 0.0001$  (Two-Way ANOVA,  $**p < 0.01$ , Sidak post hoc,  $n = 7$  Saline slices,  $n = 6$  Doxorubicin slices).

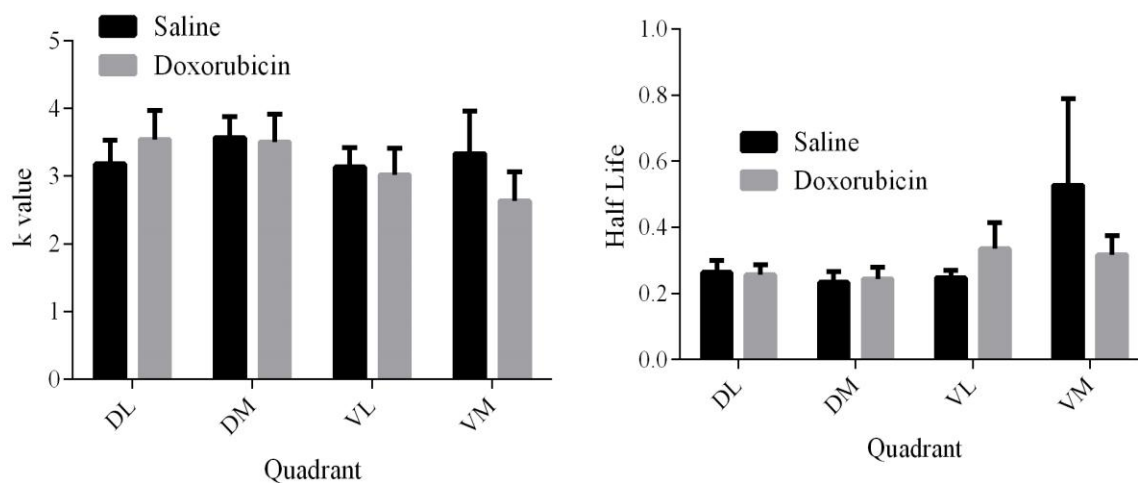


**Figure 2-9. Comparison of 2- and 4-week Dopamine Release Data**

Data is pooled into the average of the average of the quadrant data from each slice. Two Way ANOVA- Tukey Post Hoc \*\*\*\*P value < 0.0001, 2 Weeks- Saline- n=9 slices, Doxorubicin- n=7 slices, 4 Weeks- Saline- n=7 slices, Doxorubicin- n=6 slices

### *Dopamine Uptake*

Dopamine reuptake was modeled the same way as with the previous section. The results showed that there is no difference in reuptake in the 4-week dosed animals versus their counter parts (**Figure 2-10**). In sticking with the theory presented in the previous section, if the increased rate of reuptake was a compensatory mechanism, once the dopamine levels dropped it would no longer need this mechanism and so the rate a reuptake is not different from the rate of the saline animals. The difference in reuptake needs to be further investigated looking at DAT and determining if doxorubicin effects DAT expression or function. This could be done using a DAT inhibitor such as nomifensine.



**Figure 2-10. Dopamine Uptake in 4 week treated animals**

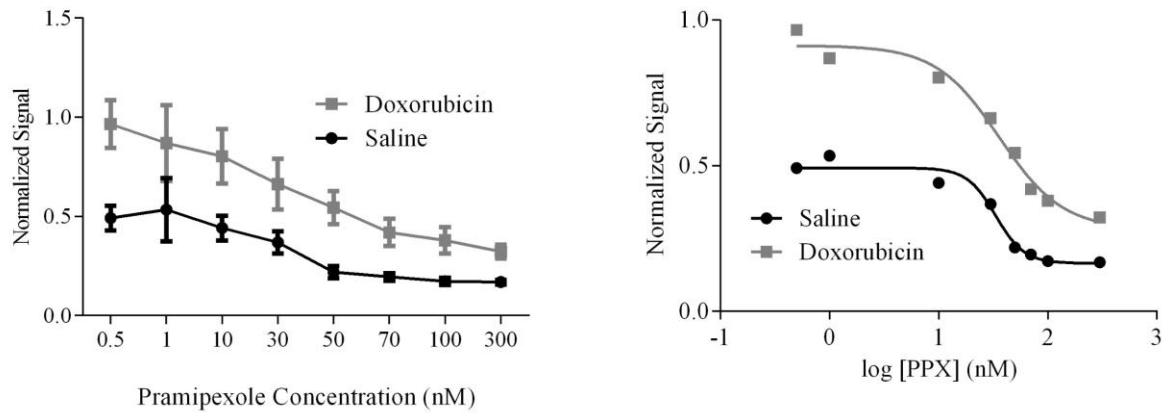
Error bars represent  $\pm$ SEM. Two Way ANOVA with Sidak Post Hoc, Saline, n=6 slices, Doxorubicin, n=6 slices.

#### *Pramipexole Titration*

The pramipexole titration was also performed on the 4-week dox rats and their saline counterparts. Both titrations showed the expected decrease in dopamine throughout the curve with a significant difference in dopamine release between doxorubicin treated animals and saline treated animal ( $p < 0.01$ , Repeated Measures Two Way ANOVA, Sidak Post Hoc) which indicates there is a difference in the D2/D3 receptor system (**Figure 2-11**). Dopamine release is less in the 4-week animals and it is known that agonism of the D3 autoreceptor inhibits dopamine synthesis and increase reuptake. This mechanism could be down regulated as there is a lower amount of dopamine released and the cell is trying to maintain homeostasis with less dopamine.

The Find EC anything model in GraphPad was used to determine the concentration of 50% dopamine release inhibition (IC<sub>50</sub>). Values were normalized against dopamine release current obtained at 0 nM pramipexole. The saline IC<sub>50</sub> is 33.33 nM with an R<sup>2</sup> of 0.9744 while the Dox IC<sub>50</sub> is 36.04 nM with an R<sup>2</sup> value of 0.9804. This shows that while the dopamine release is significantly different at 0.5, the IC<sub>50</sub> values do not differ significantly. It appears that this is due to the modeled lines having very

similar parameters that are generated via the non-linear regression. The magnitude of the two lines shows the treatment differences that are illustrated in the left graph.



**Figure 2-11. 4 Week Dox Treatment Pramipexole Titration**

Left, Scatter plot showing the average release over time over all animals within each treatment group. Dopamine concentrations significantly differ when 0.5 nM PPX is administered (Two-Way ANOVA, Sidak *post hoc*, Dox n = 6 slices/treatment, Saline n = 6 slices/treatment). Right, Model of curves to determine IC50. The pramipexole concentrations were transformed to a Log scale on the x axis and then a decay curve modeled with GraphPad to estimate IC50 at 33.33 nM and 36.04 nM for saline treated and doxorubicin treatment respectively.  $R^2 = 0.9744$  for saline and 0.9804 for dox. Error Bars represent  $\pm$ SEM.

### Conclusion

Animals were treated for 4 weeks with either doxorubicin or saline. After and during this treatment period, there was no statistically significant difference in attention shifting between Dox- and saline-treated rats, as determined by repeated measures ANOVA. Dopamine release was significantly attenuated while dopamine reuptake showed no difference. This suggests that the dopamine system is further being altered by the continued administration of doxorubicin as these results differ from those found at two weeks. The pramipexole titration showed that doxorubicin treated animals have significantly different dopamine release throughout the titration, there was no difference in the IC50 values. Overall, continued administration of doxorubicin to rats, alters their dopamine system from their saline counter parts and from rats that were treated for only two weeks showing that the dopamine system is altered throughout treatment instead of

one effect persisting throughout treatment. Future research would include a longer recovery time to determine if the effects seen at 4 weeks persist once dosing is stopped. The attention shifting paradigm also needs to be repeated to determine if the handling or age of the animals significantly effects dopamine release in the saline animals.

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Chapter 3 : Differential Expression Analysis of C57Bl6/J Mice Treated  
with 5-Fluorouracil and KU-32

## Introduction

Cancer is a leading cause of death worldwide and in the U.S.<sup>1</sup>. Advances in chemotherapeutics have improved survival rates; however, these improved survival rates have also revealed long-term side effects<sup>1-3</sup> that include problems with information processing, mood changes, and memory and attention deficits. Collectively, these cognitive-related effects comprise a syndrome known as Post Chemotherapy Cognitive Impairment (PCCI), commonly known as “chemobrain”.<sup>2-4</sup> Chemobrain afflicts approximately 20-30% of chemotherapy patients; however, this number may be much higher since the symptoms are patient reported.<sup>1-3,5</sup> Mechanisms that may contribute to chemobrain (**Table 1-1**) include inflammation and formation of cytokines as a result of oxidative stress,<sup>2, 3, 6-8</sup> changes to the structure of the brain<sup>1</sup>, and irreparable DNA damage.<sup>2, 3, 9, 10</sup> Many chemotherapeutics inhibit DNA production through mechanisms that include alkylation,<sup>11</sup> intercalation,<sup>12</sup> crosslinking,<sup>13</sup> and synthesis inhibition.<sup>14</sup>

Flurouracil (5-fluorouracil, 5-FU) is a chemotherapy agent associated with chemobrain in humans and has been used to model chemobrain in rodents.<sup>14</sup> It is administered systemically for the treatment of cancers of the colon, esophagus, stomach, pancreas, breast, and cervix and topically for the treatment of skin warts, actinic keratosis, and basal cell carcinoma.<sup>14</sup> Structurally similar to uracil, 5-FU is known as an antimetabolite, with its antiproliferative activity occurring through blocking thymidylate synthase.<sup>15</sup> Although the mechanisms by which 5-FU causes chemobrain are not fully known, we found previously that treatment of rats with 5-FU impairs dopamine release and executive function.<sup>16</sup> KU-32, a novobiocin derivative and heat-shock protein-90 (HSP90) inhibitor<sup>16</sup> prevented cognitive impairment in 5-FU-treated rats.<sup>17</sup> HSP90 is used by cells to prevent apoptosis though assisting in protein trafficking and preventing the buildup of misfolded or non-functional proteins within cells.<sup>18</sup> Unfortunately, this chaperone function overreacts in cancer cells, leading to cell proliferation; thus, inhibiting it is thought to be helpful in cancer patients.<sup>18</sup> The overreacting of HSP90 is damaging to the system because it allows fixing of misfolded proteins within those cancer cells, preventing them from being marked for apoptosis due to misfolded proteins.<sup>18,19</sup> KU-32 was explored as a treatment for diabetic neuropathy as it has protective qualities against neuronal glucotoxicity and is known to repair mitochondrial dysfunction.<sup>15, 16, 19</sup> Given that KU-32 inhibits

HSP90, this allows the correct molecular chaperones and antioxidant proteins to be recruited and used on damaged cells with damaged or misfolded proteins preventing them from replicating.<sup>18</sup>

To determine possible mechanisms underlying how KU-32 prevents cognitive impairment, we employed RNA sequencing (RNAseq) to quantify mRNA gene transcripts.<sup>17</sup> This approach allows determination of which genes are upregulated and downregulated in mice in response to treatment with 5-FU and KU-32,<sup>15</sup> and revealed that KU-32 may compensate for the genes that are depleted from chemotherapy treatment.

## **Materials and Methods**

### Animals

Ten male C57Bl6/J mice (Jackson Laboratories, Bar Harbor, ME) were housed in groups of 2 or 3 with each treatment group having 3-4 mice within the University of Kansas Animal Care Unit. Mice were housed with food and water available *ad libitum*. The housing room was maintained at  $70 \pm 2$  °C and humidity level of  $50 \pm 20\%$  with a 12-hour light/ dark cycle. All injections occurred during the light phase of the day.

### Drug Administration

There were three treatment groups: KU-32+5-FU (three mice), 5-FU (three mice), and saline (four mice). Mice in KU-32+5-FU and 5-FU groups received intraperitoneal (IP) injections of 5-FU (25mg/kg) once per week for two weeks.<sup>20</sup> Mice in the saline group received a saline vehicle injection once per week for two weeks. Additionally, mice in the 5-FU + KU-32 group received KU-32 (20mg/kg, oral gavage) in 40% Captisol (w/w) solution while mice in the 5-FU and saline groups received only 40% Captisol. All animals received 2 IP and 2 oral gavage administrations. For clarity the KU-32+5-FU group will be referred to as KU-32.

### Brain Dissection and Sample Preparation

A week after its final chemotherapy injection, each mouse was anesthetized with isoflurane and decapitated.<sup>15, 20</sup> Brains were removed and placed in cold and oxygenated (95%:5% Oxygen: Carbon Dioxide) artificial cerebral spinal fluid (ACSF).<sup>15, 20</sup> Once chilled, brains were removed from the ACSF,

cerebellums were removed, and brains were bisected.<sup>15, 21</sup> One hemisphere was quartered and placed into cold RNA-later solution for sequencing. Nuclease free pipette tips, microcentrifuge tubes, and instruments were used. A 100 g section was homogenized, and RNA was processed using Qiagen RNeasy Mini spin-columns according to instructions provided by Qiagen. Samples were stored at -80°C until sequencing.

### Sequencing

Once the quality and quantity of the collected RNA was established using an Invitrogen Qubit Fluorometer and Agilent 2200 TapeStation, the KU Genome Sequencing Core constructed and pooled a NEBNext stranded mRNA library for each mouse sample in preparation for RNA sequencing. QPCR was then used to quantify the library the pool of the 10 generated libraries. The pool was run on an NextSeq 550 sequencer (Illumina) using a high-output flowcell, collecting single-end 75 base pair (bp) reads.

### Differential Expression Analysis

Differential expression (DE) analysis was conducted by first performing a quality assessment of raw FASTQ files using FastQC (Babraham Bioinformatics- Version: 0.11.5). Once this assessment was complete, trimming of adapter sequences was performed using Trimmomatic (usadellab.org- Version: 0.36). The quantification of gene expression used two pipelines:

1. HISAT2 (<http://daehwankimlab.github.io/> -Version: 2.1.0) with featureCounts program converting files from HISAT2 to DESeq2 from the Subread package ([subread.sourceforge.net/](http://subread.sourceforge.net/)-Version 1.6.2). R (Version: R: 3.5.0, RStudio: 1.2.717) and DESeq2 with the Wald Test ([bioconductor.org](http://bioconductor.org) -Version: R/DESeq2: 1.20.0) were used to test for differential expression testing.
2. Kallisto ([pachterlab.github.io](http://pachterlab.github.io) -Version: 0.44.0) with differential expression tested with R (Version: R: 3.5.0, RStudio: 1.2.717) and Sleuth ([pachterlab.github.io](http://pachterlab.github.io) -Version: R/Sleuth: 0.30.0)

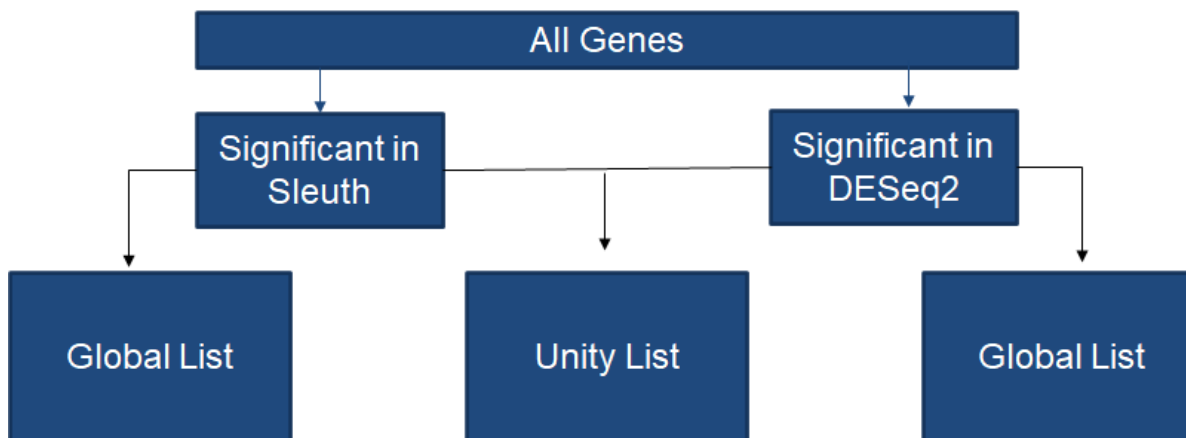
The experiments were conducted in a pairwise fashion where each treatment group was compared individually to each of the other treatment groups. The groups were titled KU32, 5-FU, and Saline. This comparison was completed for each of the pairs of groups yielding a list of differentially expressed genes.

Each of these lists were organized by the Log2 Fold Change (FC) in descending order. Log2FC are a numerical representation of how differentially expressed genes are from the comparison set. The pair names indicate the fold change value is calculated as the ratio of the right term/ left term (ex: 5FU/ Saline). The number of genes found in each list is described in **Table 3-2**. Genes that have a positive Log2FC are considered enriched and those with negative Log2FC are considered depleted. The identifying group number is listed at the bottom of **Table 3-2**.

**Table 3-1. Summarized results from the reorganization of the differentially expressed gene lists**

List	5-FU vs KU-32+5-FU	Saline vs KU-32+5-FU	Saline vs 5-FU
Number of Enriched Genes	481	287	19
Range of Enriched log2FC	0.0768 → 7.2423	0.2076 → 7.2641	0.2338 → 0.5846
Number of Depleted Genes	293	139	28
Range of Depleted log2FC	-4.179 → -0.2063	-3.784 → -0.2161	-4.869 → -0.1857
Total Genes in List	774	426	47
Group Identifier	1	2	3

Due to the overall pipeline having two different methods of determining differential expression, the list that was comprised of genes that are differentially expressed through either pipeline was called the global list. The list of genes that were differentially expressed through both pipelines was called the unity list **Figure 3-1**. The numbers of genes in these lists are shown in **Table 3-4**.



**Figure 3-1. Flow Chart for Grouping Genes Based on Significance**

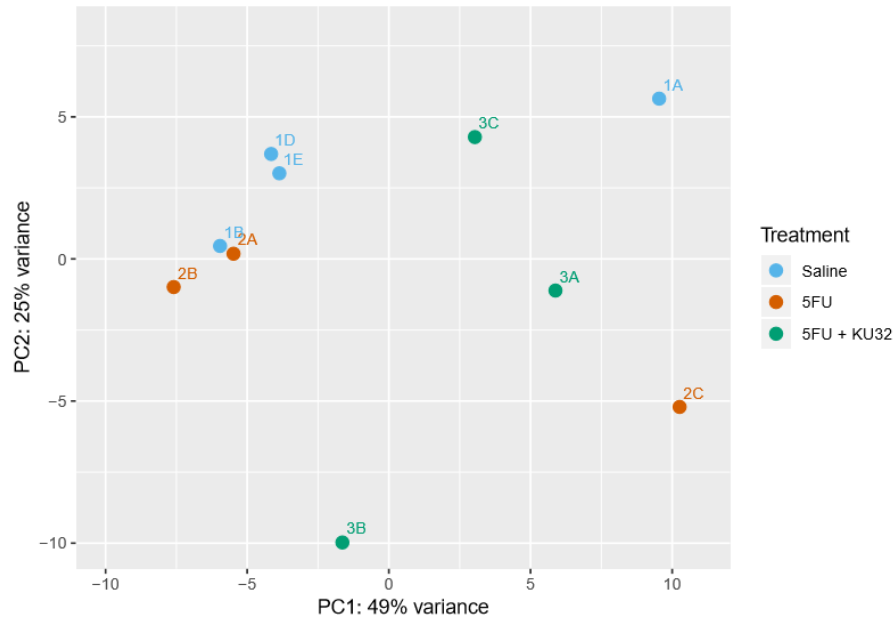
All differentially expressed genes are significant in either Sleuth, DESeq2, or both pipelines. When significant in both, the gene is added to the Unity List. When it is significant in at least one pipeline it is added to the Global List

**Table 3-2. Breakdown of genes on the global and unity lists.**

Group	Number of Genes
5-FU vs KU32- Global- UP	481
5-FU vs KU32- Global- DOWN	293
Saline vs 5-FU- Global- UP	20
Saline vs 5-FU- Global- DOWN	28
5-FU vs KU32- Unity- UP	173
5-FU vs KU32- Unity- DOWN	51
Saline vs 5-FU- Unity- UP	0
Saline vs 5-FU- Unity- DOWN	0

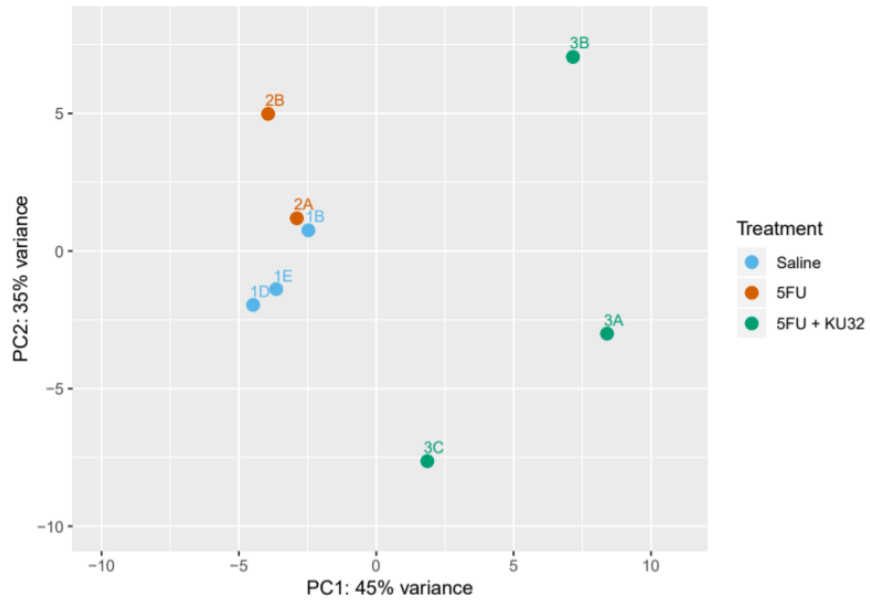
## Results/Discussion

To determine if the treatment groups significantly varied from each other, a Principal Component Analysis (PCA) of the 10 samples was performed (10N analysis) (**Figure 3-2**). Samples 1A (Saline) and 2C (5-FU) were determined to be outliers and removed from further analysis. The new PCA plot of 8 samples (8N analysis) (**Figure 3-3**) showed that the saline and 5-FU treatment groups are close in proximity, with little variation among those groups. The KU-32 group had large variance within its 3 samples as they are spread out on the plot. Future work would have larger n values to prevent the large intragroup variability.



**Figure 3-2. PCA Analysis of 10 samples**

Each dot represents a single mouse with the letter/number code representing the “name of the animal”. Dot colors represent the treatment group of each animal.



**Figure 3-3. PCA Analysis of 8 samples eliminating 1A and 2C as outliers from Figure 3-2**

Each dot represents a single mouse with the letter/number code representing the “name of the animal”. Dot colors represent the treatment group of each animal.

The analysis of the 8N set was conducted with gene numbers representing both the DESeq2 and Sleuth pipelines. A total of 774 genes in the KU-32+5-FU vs 5-FU group, 426 genes in the Saline vs KU-32+5-FU group, and 47 genes in the Saline vs 5-FU group were differentially expressed (**Table 3-2**, full lists in **Supplementary Table 1 (ST1)**). We examined the enrichment and depletion of genes attributed to the effects of KU-32 administration in 5-FU-treated mice (Group 1) and the effects of 5-FU-treatment in drug naïve mice (Group 3). Analysis of the Group 3 results revealed that none of the 47 genes were significantly differentially expressed in both pipelines (**ST2**); thus, there are no genes listed on the unity list. Therefore, we were unable to identify genes with more statistical power in group 3. Given our previous findings in which dopamine release was impaired by 5-FU treatment<sup>22</sup> and KU32 prevented cognitive impairment caused by 5-FU<sup>15</sup>, we focus this discussion primarily on the differential enrichment and depletion of genes that are specifically associated with neuronal function.

Closer inspection of specific genes in the Group 1 unity enriched list (see **ST3**) revealed several that are associated with the enhancement or preservation of neuronal function. A sampling of notable genes include: synaptotagmin-10, synaptotagmin-17, huntingtin-associated protein 1, neuromodulin, Alpha-2A adrenergic receptor, and neuroglobin. Additionally, the depleted list had genes including: Apoptosis-inducing factor 3 and Alpha-1D adrenergic receptor. All of these genes are important to the nervous system and all have implications in the health of the brain.

Synaptotagmin-10 is a synaptic protein that is directly involved with calcium-mediated exocytosis by a pathway that is distinct from other forms of synaptotagmin.<sup>23</sup> This protein assists in vesicle function upon binding with Ca<sup>2+</sup>, thereby release insulin-like growth factor 1 (IGF1).<sup>24</sup> In the central nervous system, IGF1 promotes neuroplasticity and serves as a neuroprotective factor.<sup>24</sup> Although synaptotagmin-17 is not involved directly in exocytosis, it facilitates the trafficking of vesicles from the endoplasmic reticulum, thereby promoting the neuronal regeneration.<sup>25</sup> Synaptotagmin 17 controls neurite outgrowth and synaptic physiology via distinct cellular pathways.<sup>25</sup> Thus, upregulation of these synaptotagmins may be neuroprotective.

Huntingtin-Associated protein 1 (HAP1) is a protein that helps to traffic intracellular molecules through microtubule based transporters and vesicles.<sup>26, 27</sup> It is also known to be a ligand of the Htt gene which, when mutated by expansion of the number of CAG repeat sequences on exon 1, causes Huntington's Disease.<sup>26, 28</sup> HAP1 has also been found to traffic epidermal growth factor receptor (EGFR) which is known to have a role in neuronal survival.<sup>27</sup> Research has shown that when HAP1 is knocked out in mice, their feeding behavior is impaired preventing them from suckling leading to early death.<sup>28</sup> HAP1 is said to bind more tightly to the mutated Htt gene than the wildtype gene.<sup>28</sup> This affinity means HAP1 expression is decreased once bound to the mutant Htt thus leading to lowered EGFR levels and contributing to neuron death.<sup>27</sup> In addition to the brain, Huntingtin-Associated protein 1 also has a function in breast tissue. When overexpressed, it was shown to inhibit the growth of the cancer cells and prevented migration and invasion while inducing apoptosis.<sup>29</sup> While we are using non-cancer bearing animals, it is interesting that this could have an effect with helping to fight cancer and prevent further growth when treated with KU-32.

Alpha 2A and 1D adrenergic receptors are known to contribute to the analgesia and sedation of the central nervous system with norepinephrine and epinephrine acting as agonists.<sup>29, 30</sup> These receptors also have a role in blood pressure.<sup>30, 31</sup> With one receptor being enriched and 1 being depleted, it is possible that they compensate for each other to avoid delirious effects from the overexpression<sup>32</sup> or depletion of these genes<sup>33</sup>.

Neuromodulin is a protein that allows for the growth of neurons and regenerating neurons.<sup>34</sup> When neuromodulin is overexpressed in mice it was found that learning and memory were significantly enhanced.<sup>15</sup> This overexpression could explain the positive cognitive effects that have been in previous work with KU-32.<sup>35</sup>

Neuroglobin is a neuronal analog to hemoglobin that allows for the brain to sequester oxygen during hypoxic events.<sup>35</sup> Increased amounts of neuroglobin have allowed the brain to be protected from oxidative stress related injuries.<sup>1</sup> This could allow the brain to withstand any potential effects of oxidative stress which is a theorized cause of chemobrain.<sup>2, 3, 6, 7, 36</sup>

Apoptosis-inducing factor 3 is a mitochondrial oxidoreductase which participates in programmed cell death or apoptosis.<sup>36</sup> When this protein is depleted, this changes the metabolism of the mitochondria through the elimination of parts of the electron transport chain.<sup>36</sup> This alteration triggers a protective signal to alter the metabolism of the mitochondria to try and preserve the cell.<sup>1</sup> All genes in the Group 1 unity lists are listed in **ST3**.

### **Conclusion**

This experiment was aimed at determining if differences in gene expression are present in mice that are treated with 5-FU versus those treated with saline or those treated with 5-FU and KU-32+5-FU, a potential rescue mechanism. The RNA seq yielded about 1200 differentially expressed genes and genes of interest and the unity lists of Group 1 show that KU-32 may enrich genes that allow growth of the neural system and deplete genes that are associated with its death. While the genes explored within this chapter are differentially expressed, it is hard to make concrete conclusions due to the very small n value. Future work would repeat the experiments with a larger sample size to hopefully have a unity list for the Saline vs 5-FU group to be able to draw conclusions about how 5-FU affects transcript expression. It would also be interesting to test other chemotherapeutics to determine if they also affect the protein expression in similar ways. These results provide a potential reason for why KU-32 helps return cognitive performance to baseline levels with many neuroprotective and neuro-regenerative genes with enriched expression.

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**Chapter 4 : Behavioral Intervention and Neurochemical Work Up of an  
Attention Deficit Hyperactivity Disorder Strain of Rat**

## Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is one of the most common neurodevelopmental disorders in children.<sup>1,2</sup> In 2016, an estimated that 9.4% of US children had been diagnosed with ADHD.<sup>2</sup> There are three presentations of the disease that can appear; primarily inattentive, primarily hyperactive-impulsive, and combined presentation.<sup>1,2</sup> Symptoms must appear before the age of 12, must appear in at least 2 different locations, and are detrimental to daily life.<sup>1,2</sup> The DSM V explains that there must be at least 6 symptoms from either the inattentive or hyperactive-impulsive lists or at least 3 symptoms from each list for the combined presentation.<sup>1,2</sup> Common symptoms of ADHD in children include having trouble holding attention, being easily distracted, being forgetful in daily activities, fidgeting, talking excessively, and having trouble waiting their turn.<sup>1,2</sup> These symptoms must also be inappropriate for the developmental level of the child.<sup>1,3</sup>

It is also becoming more common<sup>2</sup> to be diagnosed with ADHD in adulthood. The symptoms must have been present in childhood and only five are needed for a diagnosis.<sup>3</sup> Also, these symptoms could present differently in adulthood; for example, hyperactivity may appear as extreme restlessness.<sup>3</sup> It has also been found that around two thirds of children will experience ADHD symptoms into adulthood.<sup>4-6</sup> While being prevalent, unfortunately, the cause of ADHD is unknown.<sup>6</sup> Some possible causes include brain injury, alcohol and tobacco use during pregnancy, low birth weight, and prematurity.<sup>6</sup> Others believe that genetic factors and environmental factors such as lead exposure could contribute to ADHD.<sup>5,7</sup> Many believe that the symptoms of ADHD are due to the reduced efficacy of dopaminergic and noradrenergic modulation.<sup>7</sup> This is supported by reduced dopamine efficacy in striatal neurons.<sup>8</sup> Some believe that ADHD patients need an overabundance of dopamine to create “calm” in their brain.<sup>8</sup> Therefore, stimulants help ADHD children who are hyperactive or inattentive.<sup>1</sup>

Even though the cause of ADHD is unknown, treatment for ADHD splits into two different avenues with pharmaceuticals and behavioral therapy.<sup>9</sup> It has been reported that 62% of children with ADHD were taking medication.<sup>10</sup> These medicines include stimulants such as methylphenidate (Ritalin) and

amphetamine (Adderall), and non-stimulants such as Strattera (atomoxetine) which increases norepinephrine, and Intuniv (guanfacine) and Kapvay (clonidine) which are Alpha-2A-adrenoceptor agonists.<sup>1, 11</sup> The stimulant medications are known to be addictive or habit forming, and the goal is to keep children on these medications for as short a time as possible.<sup>11</sup> This is especially important because these medications alter the amount of dopamine in the nucleus accumbens which alters the pathways implicated in addiction.<sup>10</sup> They are also short acting and require many doses to reach a therapeutic level.<sup>1, 10</sup> Stimulants also have bad side effects on the child's ability to sleep and their appetite.<sup>10</sup> Thus, non-stimulant medications may be used in cases where stimulants are not tolerated or patients are nonresponsive to treatment but are not as effective as stimulants.<sup>1, 3</sup>

The second path of treatment for ADHD is behavioral therapy, which includes cognitive behavioral therapy and other psychosocial interventions.<sup>3</sup> About half of patients receive this form of treatment. These behavioral therapeutic approaches offer the possibility of helping patients to cope with the impairments that come with the ADHD.<sup>3</sup> Therapy and medication are often provided together;<sup>9</sup> this approach is becoming the more favored since it may<sup>1, 7</sup> limit the amount of time that patients receive stimulant medications.

The goal of this experiment was to show how delay training can treat a core symptom of ADHD. This experiment used Lewis rats, which are known to exhibit the hyperactivity associated with ADHD.<sup>7</sup> Fischer 344 rats were used as the control for the Lewis rat. We also measured dopamine release, in the absence and presence of amphetamine, in order to understand how training and rat train effect the dopamine system. Amphetamine was investigated because it is commonly prescribed for ADHD and it blocks dopamine uptake and causes dopamine efflux.<sup>12</sup>

## **Methods**

### Animals

All experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Kansas Institutional Animal Care and Use Committee. 16 male Lewis rats and 8 male Fischer 344 rats, 45 days old were acquired from Charles River

Laboratories, Inc (Wilmington, MA). They were pair housed with food and water available ad libitum within the University of Kansas Animal Care Unit. The husbandry room was maintained at  $70 \pm 2$  °C and humidity level of  $50 \pm 20\%$  with a 12-hour light/ dark cycle. After a 14-day acclimation period, delay training was completed by a collaborator's lab and the rats were placed on 22-hr food restriction during this training. All the training occurred during the light part of the cycle and once completed, 15 of the animals were euthanized and dissected for neurochemical experiments.

### Apparatus

All sessions were completed in twelve equally equipped Med Associates (Med-Associates, Georgia, VT) operant chambers which were enclosed in sound attenuated boxes with exhaust fans that provided a masking noise. Each chamber contained two retractable levers with stimuli lights, a house light, and a magazine for pellet delivery. The interior dimensions were 30.5 cm long, 24.1 cm wide, 21.0 cm high. Centered on the front wall, 1 cm above the floor grid was a pellet receptacle (3 cm x 4 cm) into which a magazine could dispense grain-based pellets (45 mg; Bio-Serv, Frenchtown, NJ). Retractable levers were positioned on either side of the pellet receptacle (11 cm apart; 5 cm from the floor). A 28-V houselight centered on the back wall (19 cm from the floor) provided general illumination. Chambers were housed in sound-attenuating cubicles with fans to mask extraneous noise. All experimental events were programmed and recorded using MED-PC IV software.

### Magazine Training

Before the experiment began, all animals were exposed to one/two sessions of magazine training over two consecutive days. During these training sessions, all levers were removed from the chamber, the house light was illuminated, and pellets were delivered according to a variable time (VT) 30s schedule. Magazine training sessions lasted approximately 40-minutes.

## Experimental Procedures

### *Delay Training*

Following magazine training, subjects were split into three different groups, with eight subjects in each group: Lewis Delay, Lewis Control, and Fischer. Lewis rats were randomly assigned to either the delay trained or control group. Each of the 50 delay training sessions lasted six hours. All subjects were exposed to the same amount of training sessions. During delay training with the Lewis delay group, levers were inserted into the chamber to start the trial on a tandem FR1 (fixed ratio), 17.5-s delay schedule. This means that one response started the timer and then 17.5 seconds must elapse with no responding for the animal to receive a reward. If the animal responded before the 17.5 second elapsed, the interval was reset. In the Lewis Control and Fisher groups, they had to respond once and would immediately receive their reward. The lever would then retract for a period and once it reentered it was available to respond again. The interval was based on the average delay time from the Lewis Delay group allowing for the same rate of reinforcement.

### *Delay test Pretraining.*

After all sessions of delay training were completed, subjects were exposed to a fixed ratio schedule, which started at 1 response per reinforcer, and increased until subjects reliably completed a FR5 (5 responses for a reward) schedule. Next rats experienced 2 days on a signaled progressive delay procedure. The rats initiated the trial by performing a defined rule (respond 10 times) and then a light would illuminate through the delay period. Delays to reinforcement doubled after each reinforcer and were signaled with a stimulus light. Once a period of time passed during which the subject did not make a response, the session would end. These are referred to as breakpoints in subsequent sections. Breakpoints were set at 300-s. None of the subjects ever hit breakpoint during the signaled delay condition.

### *Un-signaled delay testing.*

After the signaled delay testing ended, all subjects were moved into a progressive un-signaled delay condition, which was the same as the signaled delay condition except that there were no light signaling the delay. Again, these trials started with a defined rule (respond 10 times) and then there would be a delay to the reward (without the light signal). Success was measured by how many lever presses the animal made per minute and how long they took to start a new trial. Pressing the lever more per minute and having less time before a new trial started showed they were attending to the task and were focused on it. Breakpoints were the same as the signaled delay condition.

### Drugs

d-Amphetamine Sulfate (CAS:51-63-8, lot:065K1894) was purchased from Sigma (St. Louis, MO).

### Electrode Fabrication

Carbon fiber cylindrical microelectrodes were fabricated as described previously.<sup>12-14</sup> In short, single 7 $\mu$ m carbon fibers (Goodfellow Cambridge Ltd) were aspirated into glass capillaries (4 in, 1.2 mm OD; A-M Systems, Inc. Carlsborg, WA) and pulled using a heated coil puller (Narishige International USA, East Meadow, NY). Carbon fibers were cut to 400 microns from the glass seal. An epoxy mixture of 0.24 g of EPI-CURE 3234 Curing Agent and 2.00 g of EPON Resin 815C was used to seal the electrodes. Excess epoxy was removed with toluene and the electrodes were baked for 1 hour at 100°C. Electrodes were backfilled with 0.5M potassium acetate to ensure a connection with the headstage.

### Euthanasia and Dissection

Rats were euthanized by decapitation while under general anesthesia. The brain removal process was completed as previous described.<sup>12-14</sup> In short, the skin and skull were removed, and the brain was placed in cold and oxygenated (95%:5% Oxygen: Carbon Dioxide) artificial cerebral spinal fluid (ACSF). The aCSF solution contained the following concentrations: 2.5 mM KCl, 126 mM NaCl, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 2.4 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 20 mM HEPES, 11 mM D-glucose. The pH was adjusted to 7.4. The chilled brain had the cerebellum removed and then was bisected. One hemisphere was prepared

for slicing using a vibratome (Leica Microsystems, Bannockburn, IL). A 4% agar block was glued using cyanoacrylate glue to provide support for the hemisphere. The hemisphere was then placed on the stage cerebellum side down and secured with glue. Coronal slices (300 $\mu$ m thickness) were then harvested with slices containing the striatum saved. One of the slices containing the striatum was placed on a perfusion chamber allowing for warmed and oxygenated ACSF to be flowed at 2mL/ min continuously. Slices were equilibrated in the perfusion chamber for at least 1 h before collecting measurements. Other saved slices were placed in a slice saver with oxygenated ACSF at room temperature. The second hemisphere was saved in an Eppendorf tube and placed in a -20°C freezer until needed for other experiments.

#### Fast Scan Cyclic Voltammetry (FSCV)

FSCV with background subtraction was performed as previously described using Knowmad/ Wildcat (Knowmad, LLC, Tuscon, AZ).<sup>13-15</sup> A precalibrated carbon fiber electrode was inserted into the brain slice with a micromanipulator. A biphasic stimulating electrode (A-M Systems Inc., Carlsborg, WA) was inserted with leads placed on either side of the working electrode with a micromanipulator. A triangular waveform from -0.4 V to +1.0 V and returning to -0.4 V was applied versus an Ag/AgCl reference electrode. A scan rate of 600 V/s and an update rate of 60Hz were used. A single 4 ms biphasic stimulation pulse of 350  $\mu$ A was applied to evoke dopamine release. Stable dopamine release was found within the dorsal striatum and then an amphetamine curve was started. Increasing concentrations of amphetamine from 3 to 15  $\mu$ M were added to the perfusing ACSF. Files were taken every 5 minutes with total exposure time of 30 minutes for each concentration. Following the last concentration of amphetamine, a washout with fresh ACSF (no amphetamine) was performed for 30 minutes. The electrode was immediately calibrated again after this washout and slice discarded. One slice per animal was analyzed. Analysis of Knowmad files included the color plot and zero phase filters.

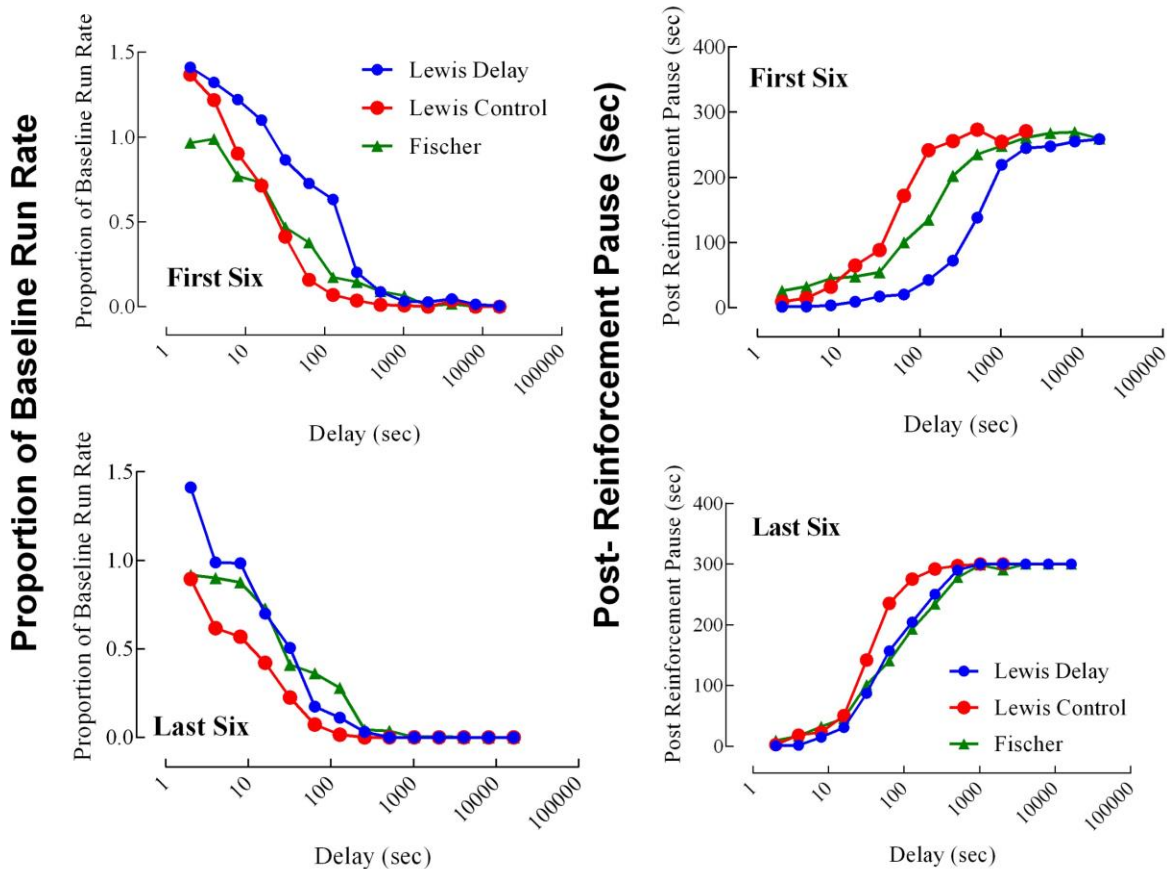
#### Statistical Analysis and Modeling

Statistical analysis was completed using GraphPad Prism version 6 (GraphPad Software Inc, La Jolla, CA, USA) and Microsoft Excel.

## Results and Discussion

Results showed that this training was successful and allowed the ADHD trained rats to resist the delay to reward (**Figure 4-1**). This is seen as the Lewis Delay animals become indistinguishable from the Fischer animals in the last 6 days of the experiment in both run rate and post reinforcement pause). This supports the research that training is useful in the treatment of ADHD and can achieve results without the use of pharmaceuticals.<sup>3</sup>

Data is shown as the first 6 days of the experiment and the last 6 days of the experiment to show progress over time. The first measure of success is proportion of baseline run rate. The data show a higher number of lever presses per minute in the delay trained group (Overall Main Effect- Strain/Training Affect Run Rate, Two Way ANOVA- Tukey Post Hoc, First 6-\*\*\*  $p < 0.001$ , Last 6- \*\* $p$  value  $< 0.01$ . First 6 days- Lewis Delay differ from both groups \*\* $p$  value  $< 0.01$ . Last 6 days- Lewis Control differ from Lewis Delay \*\* $p$  value  $< 0.01$  and Fischer \* $p$  value  $< 0.05$   $n=8$  Rats/Group). This shows how the animals are pressing the lever fast and repeatedly instead of getting distracted after pressing a few times. The other measure of success is post-reinforcement pause, which shows less time before starting a new trial. The pause shows that they are starting a new trial sooner than the others (Overall Main Effect- Strain/Training Affect Post Reinforcement Pause, Two Way ANOVA- Tukey Post Hoc, First 6- \*\*\* $p < 0.001$ , Last 6- \*\* $p$  value  $< 0.01$ . First 6 days Lewis Delay differ from Lewis Control  $p < 0.001$  and Fischer \*\* $p$  value  $< 0.01$ . Last 6 days Lewis Control differ from both groups \* $p$  value  $< 0.05$   $n=8$  Rats/ Group). This is an approximate analogue to attention to a task and/or not getting distracted. This is seen as they are getting a reward and then pressing the lever again, wanting to work more for more food. They know that pressing the lever means the opportunity for food and are focused on that instead of investigating the box or grooming after receiving a reward. The run rate shows higher proportion of responses, so they are working harder or pressing the lever faster. Overall, these data show the delay trained rats are working harder and are more focused than the control Lewis rats. They are more like, if not a little better than the reference group (Fischer).



**Figure 4-1. Delay Training Results**

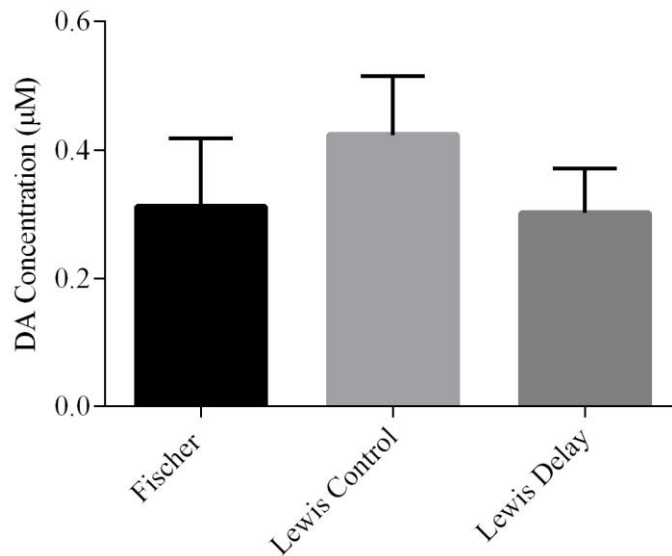
Proportion of baseline run rate shows the amount of lever presses per minute and the post reinforcement pause is the amount of time between each trial.

Overall Main Effect- Strain/ Training Affect Run Rate, Two Way ANOVA- Tukey Post Hoc, First 6- \*\*\*  $p < 0.001$ , Last 6- \*\* $p$  value  $< 0.01$ . First 6 days- Lewis Delay differ from both groups \*\* $p$  value  $< 0.01$ . Last 6 days- Lewis Control differ from Lewis Delay \*\* $p$  value  $< 0.01$  and Fischer \* $p$  value  $< 0.05$   $n=8$  Rats/ Group

Overall Main Effect- Strain/ Training Affect Post Reinforcement Pause, Two Way ANOVA- Tukey Post Hoc, First 6- \*\*\* $p < 0.001$ , Last 6- \*\* $p$  value  $< 0.01$ . First 6 days Lewis Delay differ from Lewis Control  $p < 0.001$  and Fischer \*\* $p$  value  $< 0.01$ . Last 6 days Lewis Control differ from both groups \* $p$  value  $< 0.05$   $n=8$  Rats/ Group

Once the behavior experiments were completed, the animals were euthanized for neurochemical testing. Dopamine release, release during an amphetamine titration, and dopamine uptake were examined. During behavior training and experimentation, Lewis rats were split into two categories: one that received

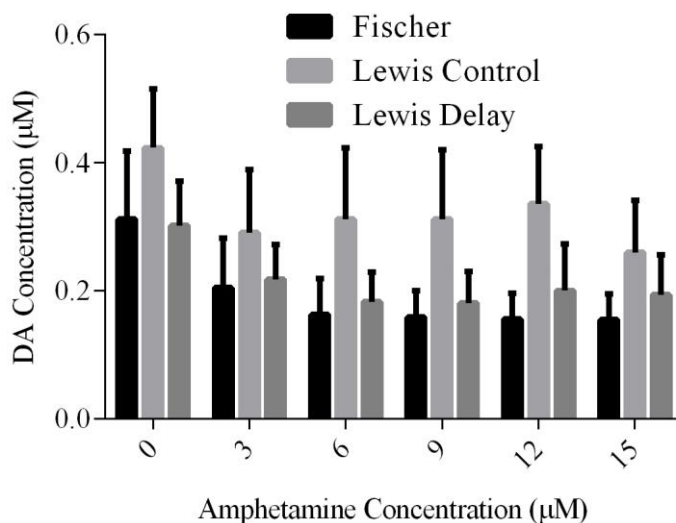
delay training and one that would not. The group that received the delay training are denoted as the Lewis delay group and the group without training is marked as the Lewis control group. Fischer 344 rats were the third group which is a reference or a control strain of rat which shows no symptoms of ADHD. It was found that there was no difference in dopamine release before the titration began (**Figure 4-2**). This indicates that inherently there are not large differences in dopamine release in these animals.



**Figure 4-2. Dopamine release before the amphetamine titration.**

A one-way ANOVA was conducted and no significant difference was found. (One-Way ANOVA with Tukey Post Hoc,  $p=0.5854$   $n=5$  rats/group). Error bars represent  $\pm$ SEM.

An amphetamine titration was then conducted with measurements taken 5 minutes apart during the 30 minute exposure time. **Figure 4-3** shows the dopamine release throughout the titration. Once pooled, the Lewis control rats show a significant increase in dopamine release compared to the Fischer and Lewis Delay rats.



**Figure 4-3. Dopamine Release during amphetamine titration**

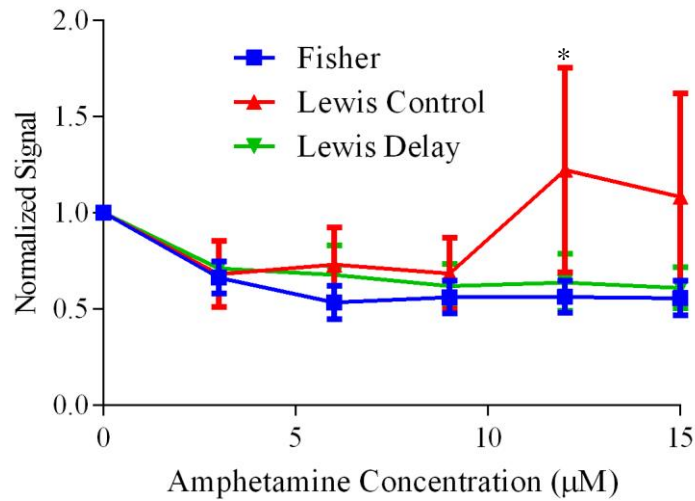
A significant main effect of rat strain/training was observed (Two-Way ANOVA, Tukey Post Hoc, Lewis control versus Fisher,  $p < 0.01$  and Lewis Delay,  $p < 0.05$ ,  $n=5$  rats/group). Error Bars represent  $\pm$ SEM.

The Lewis control rats are the group without training and that showed the hyperactivity symptom phenotype of ADHD. Their responding to the amphetamine at lower concentrations could be indicative of them not receiving treatment. The behavior treatment showed that the training could decrease the signs of ADHD allowing ADHD rats to withstand a delay. It is possible that training could alter how the rats release dopamine, returning their dopamine release to that of the Fischer rats. While there are no significant differences between the groups, once pooled, they show an overall main effect of rat strain/ training on dopamine release. Lewis control animals were significantly different from the Fischer (\*\*) and Lewis Delay (\*) rats. In a previous study, it was found that extracellular dopamine in the nucleus accumbens is equal in both strains of rats.<sup>15</sup> In that study, measurements were sampled from random locations in the striatum and made no distinction between specific locations. Future experiments could use FSCV to determine if dopamine release and uptake are different in the nucleus accumbens.<sup>15</sup> That study also found that D2 receptors and D3 receptors are fewer in the Lewis rats when compared to the Fischer 344 rats using comparative autoradiography.<sup>16</sup>

Amphetamine binds to the dopamine transporter (DAT) thereby inducing reverse transport of dopamine and increasing extracellular dopamine levels while depleting the intracellular dopamine stores. Therefore, a possible interpretation of the results in **Figure 4-3** is that slices from Lewis rats are less affected by amphetamine since their dopamine stores appear not to be depleted. This finding is in line with other studies showing that DAT levels are depleted in Lewis rats.<sup>16</sup> Thus, it is possible that delay training enhances DAT function. Relevant future experiments include treating animals while alive with amphetamine and determining if behaviorally the animals react similarly to humans where the impulsivity is decreased and to determine if the amphetamine treatment affects *post mortem* dopamine release in brain tissue.

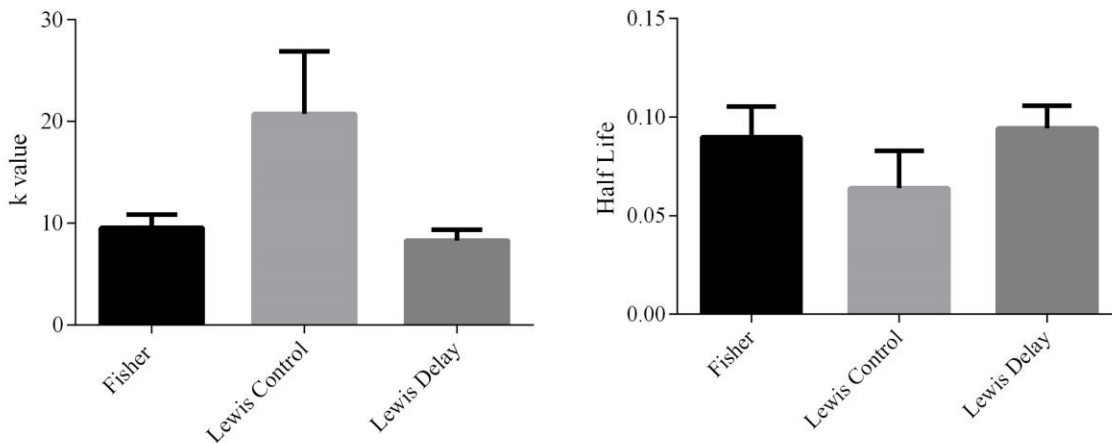
To investigate the amphetamine data further, we normalized each dataset from a given slice against no drug release. These values were then plotted versus the concentrations of amphetamine as shown in **Figure 4-4**. These plots show how the 12 and 15  $\mu\text{M}$  concentrations in the Lewis control group go above 100%. The Lewis delay and Fischer rats are fairly similar and neither goes above 100%. A Two-Way ANOVA shows a significant ( $p < 0.05$ ) difference between the Lewis Control and the Fischer rats at 12  $\mu\text{M}$ . This supports difference shown above with the standard release data between the Lewis Control and the Fischer animals; once normalized, there is no difference between the two Lewis groups.

Amphetamine is a DAT inhibitor and so uptake was also investigated. The data with no amphetamine is shown in **Figure 4-5**. Through a one way ANOVA it was determined that there is no significance between the groups. Thus, this modeling operation was unable to identify difference in uptake between groups that might affect response to amphetamine.



**Figure 4-4. Normalized Dopamine Release throughout the Amphetamine Titration**

Error Bars = SEM n=5 rats/ group. No difference is found except at 12µM. Lewis Control vs Fisher (Two Way ANOVA-Tukey Post Hoc, \* p < 0.05).



**Figure 4-5. k and Half Life Values with No Amphetamine**

Error Bars= SEM n= 5 rats for fisher and Lewis Control groups and 4 rats for Lewis Delay group. (One-Way ANOVA, k- p= 0.0881, half life- 0.3863).

## Conclusions

The behavior data and dopamine release data form a complementary picture that behavior training allows the ADHD animals to physically and neurochemically return to baseline levels of one ADHD animals,

supporting the claim that behavioral therapy can have large impacts on ADHD without the use of pharmaceuticals. The behavior results show that at the end of the experimentation, there is no statistical difference in the performance of the trained rats and the non-ADHD animals. The neurochemical results support this with the Lewis control animals releasing more dopamine during an amphetamine titration. This suggests that training may affect some part of the dopamine system, potentially DAT, to apparently equalize the dopamine release in the training animals. These results illustrate how treating ADHD with behavioral therapy a valid and valuable option.

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## Chapter 5 : Future Directions of Chemobrain Research with Rodents

The use and background of rats in the chemobrain study are reviewed in the previous chapters. There are many ways that this work could continue and there are still many ways that the dopamine system and dox could be investigated. In the future it would be advantageous to repeat the total content and reserve pool tests from the Kaplan paper.<sup>1</sup> This would further elucidate if doxorubicin affects the total amount of dopamine that is made or if the release differences are due to other aspects like vesicle packing. The reserve pool is said to be one of the three pools of dopamine in the brain.<sup>2</sup> The readily releasable pool is the dopamine that is released by the electrical stimulation and the recycling pool fills the vesicles for the next release event.<sup>2</sup> DAT collects the extra dopamine from the extracellular space and allows it to be repackaged and constitute the recycling pool.<sup>2,3</sup> The reserve pool allows dopamine to be released when the other two pools are depleted through inhibition of synthesis or DAT.<sup>4</sup> By investigating the reserve pools, we could determine if doxorubicin affects stored dopamine like it affects the readily releasable dopamine. A DAT inhibitor would also be interesting to look at as the uptake is different in the animals treated with Dox for two weeks. This approach could help in looking at the reserve pool as well due to it preventing uptake with the recycling pool. It would also be interesting to examine RNA and differentially expressed genes from animals treated with dox. This would be less cumbersome than the mice study described previously as this initial study would not need to have a rescue mechanism involved. Looking into the differentially expressed genes could show if there are any dopaminergic or neuronally interesting genes that could shed light on how genetically doxorubicin acts on the body. It would be interesting to look at a vesicular monoamine transporter (VMAT) inhibitor like tetrabenazine as well to look at vesicle packing.<sup>5</sup>

Continuing to investigate the dopamine receptors would further elucidate their effect on the dopamine system as a whole and how doxorubicin affects their action. Closer examination of D1- and D2-like receptors by treatment with select agonists and antagonists may yield more information on how doxorubicin treatment affects dopamine system function.

While exploring the dopamine system with doxorubicin is interesting, it would also be useful to examine the serotonergic system as well. It was noted by Kaplan et al. that serotonin significantly decreased

with carboplatin.<sup>1</sup> Additionally, serotonin is likely an important signaling molecule in the gut-brain axis<sup>6</sup>, given that 90% of it is made in the gut.<sup>7-9</sup> These experiments could improve understanding of how doxorubicin affects gut health and function, and how changes in the gut influence brain function. In the examination of the serotonin system, it would also be advantageous to use selective serotonin reuptake inhibitors (SSRIs) and determine if they have different effects on animals treated with doxorubicin. SSRIs are known to affect mood and mood changes are possible with chemobrain.<sup>10</sup> Again, these studies could determine how dox and chemobrain affect neurotransmitter signaling in other parts of the body.

The last part of this research that could be greatly expanded upon is the behavior paradigms that are tested. The only robust test of behavior that has been investigated is attention shifting. There are aspects of executive function like memory and inhibition, which have established testing protocols, that have not been investigated in rats treated with Dox.<sup>11, 12</sup> It would also be interesting to further explore learning and create a paradigm that allows the measurement of the acquisition of knowledge but have more than one day of testing or have a baseline established. This would allow the problems encountered with the two-week dox experiment to be avoided. While this approach seems contradictory, it would be useful to develop a robust behavioral assay for learning given its association with dopamine and serotonin signaling.

While all of these points suggest where this work could go based on previous experiments, it would also be useful to treat animals in a way similar to that of children that receive Dox to treat cancer.<sup>13</sup> Children receive doxorubicin for a number of cancers including leukemias and lymphomas among other common cancers seen in children.<sup>13</sup> This treatment carries risks of cardiac disease and other side effects during and after treatment.<sup>13</sup> This study would involve the use of pups and raising them to adulthood. Behavioral and cognitive testing and periodic health checks would be used to assess growth and cognition, which would replicate a real-life situation of a child growing up after Dox treatment. This experiment also could represent an avenue to explore learning and determine if the rats have trouble understanding novel tasks at the level of their untreated peers. Gene sequencing could also be used in this experiment to determine how the genome is changed with early exposure to chemotherapy and then growing up as normal. Practical

considerations like size, growth, and mood/social behavior could be assessed over time. This set of experiments could shed light on how dopamine and cognition is affected through early exposure to chemotherapeutics. Another important aspect of this approach would be to determine if the neurochemical or cognitive effects of the drug persist over time and the animal must adapt to life with those differences.

All the experiments described above show how this work and could be expanded and continued. This work is still in progress; nevertheless, it has been fascinating to determine how parts of the dopamine system are affected by doxorubicin. It has also been useful to establish a new protocol for administering dox using the inhalation anesthesia system. This itself has some ways to be further optimized to decrease the amount of people that are needed to complete the procedure as it currently requires at least two, but it has significantly impacted how the animals fair during the long dosing times. Throughout the experiment that required rats to receive four injections of Dox over four weeks, there were only 3 animals that showed detrimental effects of the Dox injections on the tails. Of these animals, only one required treatment with a topical anesthetic and disinfectant. Importantly, the use of meloxicam, which could alter the results of the studies, was avoided. It is highly advised that this dosing method is continued when working with Dox in the future. There are still many parts of the dopamine system to be investigated and ways to expand the doxorubicin work that has its foundations in the work described within this dissertation.

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## Appendix 1: Supplementary Tables from Chapter 3

**Supplementary Table 1. Log2FC of All Genes**

Log2 Fold Change of all differentially expressed genes

<b>Log2FC of All Genes</b>					
<b>5FU vs KU32</b>		<b>Saline vs KU32</b>		<b>Saline vs 5FU</b>	
<b>Gene Name</b>	<b>log2FC</b>	<b>Gene Name</b>	<b>log2FC</b>	<b>Gene Name</b>	<b>log2FC</b>
Slc6a3	7.242269	Hcrt	7.264078	Dkk1l	0.584618
Hcrt	7.106177	Slc6a3	7.068851	Epop	0.465864
Pmch	6.787118	Trim54	7.044427	Kcnv1	0.44121
Calcr	5.984632	Pmch	6.990955	Adra1d	0.410025
Trdn	4.478013	Alox12e	4.69743	Npas4	0.38462
Otp	4.468016	Sim1	4.527266	Kcnmb4	0.375412
Barhl2	3.889322	Gm19757	3.99262	Nrgn	0.352451
C130021I20Rik	3.783073	Trdn	3.493749	Rgs4	0.348937
Krt2	3.679425	Dio3	3.115457	Kcnh3	0.339602
Dsc3	3.674542	Gm12031	2.830873	Kctd1	0.327832
A730046J19Rik	3.644134	AA414992	2.820221	Gabrd	0.296972
Dlk1	3.493034	Ccdc42	2.730031	Cdk5r2	0.296949
AA414992	3.175137	Th	2.60381	Osbp1a	0.294431
Arhgap36	3.133903	6430628N08Rik	2.553445	Arhgap33	0.293959
Gpr101	3.03547	Scn5a	2.496398	Stk32c	0.293081
Th	2.856472	Rtp1	2.467766	Stk32c	0.293081
Col10a1	2.840038	Prss30	2.348422	Pdp1	0.283454
Irs4	2.763355	A730046J19Rik	2.222291	Pamr1	0.283243
Cdhr1	2.71385	Scn10a	2.19027	Dlgap3	0.265849
Gal	2.700913	Casr	2.16443	Dmtn	0.233831
Rtl1	2.685639	Rtl1	2.081618	Col25a1	-0.18572
Frdm7	2.683416	Pomc	2.070662	Smg1	-0.19259
Gm34466	2.655199	Pappa2	1.969781	Ptpnb	-0.21972
Casr	2.640662	Trhr	1.953164	Csmd3	-0.28431
Ndst4	2.639731	Gm44812	1.924806	Gprasp2	-0.29069
Igbp1b	2.588291	Frdm7	1.902258	Padi2	-0.31978
Scn5a	2.526567	Ecel1	1.867768	Nhs1l	-0.37604
Trhr	2.435534	Atp6v1c2	1.854254	Aebp1	-0.39442
AW551984	2.418318	Irs4	1.852412	Lfng	-0.39788
Chrm5	2.417306	Trh	1.846171	Colla2	-0.40028
Cryga	2.363134	Esyt3	1.798722	Aqp4	-0.42525
Dio3	2.34772	Nmbr	1.681605	Slc13a3	-0.45983
Gabrq	2.34469	Gm11639	1.591175	Amigo2	-0.51413
G630018N14Rik	2.306287	Krt77	1.546381	Magee2	-0.55326
Fat2	2.303925	Lefty2	1.52774	Tnxb	-0.59451
Lnp1	2.296328	Arhgap6	1.514394	Gfap	-0.61419
Gpr50	2.273915	Esr1	1.494057	Fgl2	-0.61555

Nts	2.267707	Plcz1	1.489825	Nnat	-0.63795
Baiap3	2.212186	Cd36	1.469474	Spp1	-0.6391
Pomc	2.198214	Optc	1.436915	Dcn	-0.66488
Sec14l3	2.185286	Col23a1	1.417019	Cdhr1	-0.99225
Ecel1	2.165993	6530403H02Rik	1.414567	Lhx9	-1.01416
Tfap2d	2.09754	Otof	1.405644	Cubn	-1.11216
Optc	2.021409	Gm48898	1.40146	Ndst4	-1.38745
Itgbl1	1.939559	Oxtr	1.372475	Cryga	-1.73287
Chst9	1.935739	Crb1	1.328002	Uncx	-2.47956
Nmbr	1.932783	Scml4	1.32562	Dsc3	-2.89704
Dbh	1.906292	4930426D05Rik	1.254103	Pou4f2	-4.86922
Syt14	1.876593	Ndst4	1.25228		
Zan	1.864332	Nr2f2	1.228136		
Arhgap6	1.847152	Gm16702	1.2057		
Enpp3	1.819778	Lypd1	1.203004		
Gm10710	1.814947	Hpcal1	1.193627		
Plcz1	1.782635	Itgbl1	1.164073		
Trh	1.777057	Sec14l3	1.158228		
Atp6v1c2	1.76859	Dchs2	1.131349		
Acp7	1.762596	Tnfrsf8	1.130318		
Dchs2	1.752877	Ptpn14	1.129264		
Dcn	1.740607	Trpc7	1.117525		
Nnat	1.734262	Ptpro	1.115583		
Gpx3	1.723948	Ankfn1	1.115347		
Pappa2	1.712407	Cyp26b1	1.112347		
Gm20394	1.635008	Nnat	1.09631		
Col23a1	1.634201	Gpx3	1.092439		
Magel2	1.6181	Gm32444	1.083433		
Pdzd3	1.617348	Alas2	1.077291		
6530403H02Rik	1.612466	Dcn	1.075722		
Hpcal1	1.596205	Filip1	1.067952		
Cdh23	1.579487	Syt10	1.065121		
Slc47a1	1.570452	Cdh23	1.058357		
Zim1	1.569441	Hap1	1.052233		
Cpne7	1.52675	Cdh4	1.047319		
Cd36	1.52417	Kcng1	1.031778		
Cyp26b1	1.515571	Amz1	1.021943		
Esy3	1.491244	9530059O14Rik	1.021308		
Nr2f2	1.486832	Hrk	1.003754		
Scn9a	1.481785	Ngb	0.992918		
Olfm4	1.478653	Cartpt	0.984897		
Hap1	1.478068	C130026L21Rik	0.97851		
Oxtr	1.477499	Scn9a	0.977071		

Esr1	1.464413	Tmem132e	0.976857
Ptpro	1.45064	Hbb-bs	0.965347
Crb1	1.437963	Myo1h	0.954704
Krt77	1.435902	Nek10	0.952645
Mpzl2	1.423214	Gxylt2	0.93096
Scml4	1.422461	Myh7	0.922384
Fgl2	1.420353	Syt17	0.91397
Syt15	1.400653	Vegfd	0.906593
Nkain3	1.399203	Marcks11	0.902073
Ngb	1.377767	Col6a1	0.901011
Ptpn14	1.372588	Hbb-bt	0.891224
Zcchc12	1.370127	Qrfpr	0.863513
Zfp618	1.344897	Cmb1	0.859526
Npbwr1	1.344609	Hes5	0.858959
Nxph4	1.341087	Megf6	0.850662
Mirt1	1.333551	D630023F18Rik	0.839806
Fam196b	1.319009	Gm37928	0.838476
Omd	1.311266	Gng4	0.836713
Sgcd	1.306835	Tox3	0.831562
Gm16702	1.284166	Camk2d	0.826449
Otof	1.281985	Col6a2	0.8172
Angpt1	1.278779	Mpzl2	0.815586
Tmem255a	1.276218	Gpc3	0.810854
Gm38372	1.272086	Fgl2	0.804803
Filip1	1.264429	Zcchc12	0.803199
Rxfp3	1.263851	Dpyd	0.799852
Syt10	1.259458	Pgam2	0.79539
Cd44	1.254943	Zfp618	0.786372
Tmem132e	1.247017	Meis1	0.774404
Cdh1	1.2428	Wfs1	0.774285
Eya2	1.235476	Dlx5	0.756371
Spp1	1.219961	Pdzn4	0.751759
Gla3	1.216493	Nos1	0.751482
Tacr1	1.211822	Atp2b4	0.748817
Crabp2	1.210122	Hba-a2	0.744648
Nek10	1.208972	Angpt1	0.738679
Htr2c	1.204088	Lmo3	0.734362
Smoc1	1.202906	Nrp2	0.72849
Trpc7	1.199123	Sla	0.728152
Pde11a	1.198253	Acvr2a	0.726763
9530026P05Rik	1.186629	Oprk1	0.726199
Lypd1	1.182097	Fndc1	0.725981
Piezo2	1.178869	Gm10605	0.721442

Fibin	1.177455	Tmem255a	0.718675
Tex15	1.170221	Cntnap3	0.716857
Stpg1	1.164637	Smoc1	0.716454
Adamts13	1.154299	Ypel1	0.715562
Nos1	1.149368	Rbm20	0.709896
Cdh4	1.145825	Ndnf	0.705516
Ankfn1	1.139916	Apaf1	0.698733
Dpyd	1.113531	Mrap2	0.686964
Nrp2	1.108554	Slc22a6	0.6849
Gpc3	1.085474	Igfbp3	0.683743
Gm10605	1.075655	Ly6h	0.681088
Cd74	1.07194	Ntsr1	0.680722
Gm32444	1.061451	Cntn5	0.678767
Gdpd2	1.054167	C2cd4c	0.677376
Kcna5	1.041702	Gnb4	0.676032
Cntnap5c	1.034257	Efnb2	0.674798
Fmod	1.032708	Fxyd6	0.67439
Slc9a2	1.031981	Rgs12	0.670017
Pgam2	1.028227	Grin3a	0.659033
Mrap2	1.006828	Cgref1	0.657424
Fndc1	1.00159	Trp53i11	0.651741
Cacna1s	1.000766	Fmod	0.648147
Ogn	0.999004	2900052N01Rik	0.637147
Adamts9	0.989612	Grem2	0.635435
Lgr5	0.983232	Glra3	0.633916
Vegfd	0.971459	Cpne2	0.632521
Cartpt	0.970697	Glra2	0.631932
Cmb1	0.969448	Hba-a1	0.624731
Slco5a1	0.960655	Adra2a	0.623087
Gm33533	0.959073	Wdr6	0.621353
Adra2a	0.956218	Pnma1	0.620678
Thbs1	0.955326	Gm38413	0.618822
Wdr6	0.954987	Plcx3	0.615096
D630023F18Rik	0.952819	Tenm1	0.614511
Megf6	0.949427	Samd14	0.612973
Camk2d	0.949114	Gap43	0.61222
Adgrg2	0.945425	Foxo6	0.602534
Marcks11	0.944949	Tnfaip813	0.601196
Gxylt2	0.93409	Mapk3	0.5969
Gm47171	0.928501	Wdr66	0.596014
Pbx3	0.926948	Il17rd	0.593545
Arsj	0.925722	Ybx2	0.592732
Aldh1a2	0.92227	Gm47135	0.592237

Syt17	0.921205	Gm48408	0.58787
Samd14	0.917963	Cdk18	0.580777
Igsf10	0.914406	Hpcal4	0.577633
Myo1h	0.913568	Rreb1	0.569268
Meis1	0.90999	Plxnc1	0.566837
Rab27a	0.908515	Arhgap33	0.566103
Hrk	0.904271	Sstr1	0.563617
Pdzrn4	0.89239	Rcn1	0.556578
Gng4	0.884761	Ppfibp1	0.551425
Adamts6	0.88464	Pcdh19	0.547628
Col1a1	0.879926	Pcdh8	0.544853
Gpr182	0.875288	Mylk	0.544779
Bmp5	0.874349	Pbx3	0.542712
Hes5	0.872752	Rsph9	0.539476
Wfs1	0.868571	D430019H16Rik	0.536716
Gm37928	0.866936	Reln	0.529963
Plcx3	0.86682	Tmem35a	0.52942
Col5a2	0.86593	Wnt7b	0.529087
Fabp7	0.862724	Cntnap5a	0.528624
Pfce1	0.858244	Olfml2a	0.527657
Grin3a	0.856909	Cdh13	0.521569
Ndnf	0.85686	Pnck	0.520567
Fat4	0.852121	Plagl1	0.520463
Aloxe3	0.851624	Timp2	0.515859
Slc44a5	0.845123	Phactr2	0.507849
Acvr2a	0.843693	Dner	0.505151
Apaf1	0.841536	Ehbp111	0.501721
Slc22a6	0.83498	Gmip	0.499785
Slc6a13	0.827989	Cfap54	0.499524
Kcng1	0.827098	Tiam1	0.499501
Ptgis	0.82347	Nrarp	0.499127
Pde3a	0.821834	Rps4l	0.497824
Tmem91	0.816796	Ptgds	0.492117
Ptgds	0.814576	Crtac1	0.488652
Rbm20	0.797076	Fmo1	0.488312
Slc13a3	0.796385	Prkg2	0.475464
Necab2	0.788462	Stum	0.474187
Crym	0.78771	Bmp7	0.47266
Amz1	0.777667	Kirrel3	0.470736
Lmo3	0.775586	Ccdc148	0.469178
Tll1	0.775256	Fras1	0.466909
Tenm1	0.769659	Kcnj16	0.466789
Itga4	0.769226	Fstl5	0.465825

2900052N01Rik	0.767564	Lin28b	0.463467
Ppl	0.766407	Pid1	0.46185
Myh7	0.764446	Adamts6	0.459938
Magee2	0.764024	Fabp7	0.446664
Pcdh19	0.761914	Cadm1	0.444731
Nr4a2	0.76024	Arhgef28	0.442854
Aebp1	0.751364	Gm35339	0.438232
Cpne6	0.750938	Calb1	0.433029
Olfml2a	0.736373	Celf6	0.431973
Mgp	0.736173	Vstm2b	0.428441
N4bp2	0.732592	Slc6a7	0.428438
Colla2	0.724262	Ptprf	0.426607
Gm42851	0.722104	Kcnn3	0.424654
Adam12	0.720631	Fhl1	0.423937
Pnck	0.718602	Gpsm1	0.423617
Rps4l	0.716608	Trim62	0.423573
Fstl5	0.715266	Pgrmc1	0.418557
Cntnap3	0.711529	Mov10	0.4173
Ly6h	0.711072	Efemp1	0.414217
Wdfy4	0.710173	Ankrd6	0.4137
Reln	0.705568	Syn2	0.412758
Tekt5	0.703763	Sst	0.400409
Pcdh18	0.702034	Ehd4	0.39886
Sema3d	0.700863	Klhdc8b	0.398241
Gm35339	0.694465	Nexmif	0.397083
Gabrg1	0.694261	Slc7a11	0.396064
C1ql1	0.69417	Rac3	0.387049
Cntn5	0.683429	Rab3c	0.386758
Grem2	0.681069	Ripor2	0.38658
Myoc	0.679574	Fam102b	0.378502
Rhbdl3	0.67901	Slc22a8	0.376585
Hfm1	0.676465	Caln1	0.374627
Cpne2	0.67227	Ninj1	0.373446
Il17rd	0.664946	Sh3bp4	0.370647
Rreb1	0.664391	Slc6a20a	0.369669
Igfbp3	0.660698	Rnf165	0.367777
Colec12	0.657887	Celf4	0.367401
Fgf11	0.656774	Pde9a	0.364755
Dcc	0.65395	Cd200	0.364039
Pcdh8	0.652807	Zscan18	0.358485
Wnk4	0.650821	Chd3	0.357053
Mylk	0.650631	Aebp1	0.356947
Phactr2	0.64776	Radil	0.356869

Sox11	0.647703	Ltbp4	0.356584
Cdk18	0.645135	Auts2	0.350634
Mdga1	0.645114	Mgat5b	0.349088
Bmp7	0.642891	Plppr3	0.343106
Timp2	0.639859	Ahi1	0.339383
Crtac1	0.639587	Pgap1	0.338083
Csmn3	0.637713	Smardc3	0.337935
Myof	0.634728	Slc13a3	0.336555
Nhs	0.63449	Slc30a10	0.334136
Serping1	0.631737	Cxadr	0.329591
Tnfaip813	0.631358	Slc39a6	0.31787
Myo5b	0.631195	Fgd4	0.317741
Il13ra1	0.630401	Thra	0.314233
Nsun7	0.629199	St3gal1	0.313676
Rfx3	0.627981	Mtfp1	0.312935
Fam19a2	0.623137	Pea15a	0.304976
Klhdc8a	0.619012	Nav1	0.304912
Npsr1	0.618364	Pxdn	0.302933
Lama1	0.61771	Ano6	0.300736
Kcnn3	0.617111	Abca8b	0.298658
Eln	0.613019	Nsg2	0.297673
Kcnj16	0.610636	A230057D06Rik	0.29657
Cgref1	0.610192	Sdc3	0.295846
Mrc2	0.608883	3110039I08Rik	0.295088
Dner	0.606803	Zmym3	0.283017
Gjb2	0.603311	Dcaf12l1	0.277462
Ranbp3l	0.601201	Tubb2a	0.273671
Efemp1	0.599421	Caly	0.267115
Fbln1	0.598323	Sesn3	0.26103
Gap43	0.598186	Uchl1	0.255034
Gira2	0.596457	Cacna1c	0.249164
Fmo1	0.592253	Adcyap1r1	0.242614
Acvr2b	0.592019	Ypel5	0.23642
Tfpi	0.592005	Zwint	0.214825
Dpysl5	0.590861	Evl	0.207614
A230077H06Rik	0.589698	Fam57b	-0.21611
Tmem35a	0.58544	Nefl	-0.23484
Oprk1	0.585168	Raly	-0.24593
Foxo6	0.582623	Osbpl6	-0.265
Trp53i11	0.582513	Pou3f3	-0.28351
Xkr4	0.581632	Gpd1	-0.28371
Cntnap5a	0.581381	Sertad4	-0.28584
Ahi1	0.579253	Kcnk1	-0.28652

Nrsn2	0.578077	Tspan17	-0.28678
Trpc5	0.576469	Kcnab2	-0.29359
Gdf10	0.572233	Slc30a4	-0.3019
Arhgef28	0.571823	Fut9	-0.30272
Col6a2	0.570441	Atp2a2	-0.30338
Cacng5	0.568327	Il11ra1	-0.31039
Rgs12	0.56406	Ctsz	-0.31148
Cntnap5b	0.563324	Cdh11	-0.32203
Rcn1	0.561957	Aifm3	-0.32921
Gpc4	0.561312	Asap2	-0.33068
Slc13a4	0.560899	Arhgef10l	-0.34554
Sall3	0.560065	Dusp6	-0.35769
Slc7a11	0.55975	Map6d1	-0.36012
Ninj1	0.554596	Sema6c	-0.36757
Gm48408	0.554405	Atp2b1	-0.36867
Bgn	0.55369	Tle4	-0.37325
Dlg5	0.552205	Wipf3	-0.38785
Gpsm1	0.550173	Cdh8	-0.38918
Galns	0.544746	Jph1	-0.39489
Slc6a20a	0.543649	Syt12	-0.40014
Col25a1	0.541874	Frmd5	-0.40279
Bche	0.541165	Cds1	-0.41065
Megf11	0.537257	Evc	-0.41452
Kirrel3	0.535606	Ell2	-0.42566
Wnt5a	0.534087	Nos1ap	-0.43007
Fkbp10	0.53387	Clstn2	-0.43065
Nrarp	0.533415	Lynx1	-0.43868
Efcab1	0.529792	AI593442	-0.43928
Fxyd6	0.529752	Fam43a	-0.45303
D430019H16Rik	0.526869	Hhatl	-0.45342
Slc26a2	0.523501	Skida1	-0.45385
Fn1	0.52266	Fbxo32	-0.45527
Lin28b	0.520748	Crem	-0.45983
Wnt7b	0.517545	Gls2	-0.46076
Grb10	0.515286	Dusp5	-0.46909
Mapk3	0.511993	Cdh12	-0.48007
Nexmif	0.511954	Syndig1	-0.49248
Cdh13	0.508269	Syt12	-0.49501
Mrgpre	0.506857	Abcc8	-0.49663
Slc30a10	0.505776	Slc24a2	-0.51384
Rab3c	0.504496	Nfix	-0.5157
Plxnc1	0.503703	H2-T23	-0.51625
Sstr1	0.502025	Hapln4	-0.51813

Col16a1	0.500496	Spsb1	-0.52748
Plagl1	0.499872	Epha4	-0.53013
Tmem130	0.496884	Dusp4	-0.54006
Aqp4	0.494231	Lrrc55	-0.5401
Ptpre	0.491331	Plxdc1	-0.54456
Thbd	0.491232	Pigz	-0.54653
Rcan3	0.482167	Fam78a	-0.54992
Kit	0.481573	Satb1	-0.55039
Trim62	0.481168	Hpca	-0.55109
Pid1	0.479102	Otx1	-0.55488
Fhl1	0.477537	Klhl33	-0.55631
Zkscan16	0.476566	Neurod6	-0.58016
Rsph9	0.474938	Sema3e	-0.58764
Carmil3	0.474217	Ephb2	-0.58974
Tiam1	0.470174	Itga7	-0.59052
Gpr17	0.468354	Ier5	-0.5956
Adgrg1	0.466399	Adcy1	-0.59595
Pnma3	0.464507	Hr	-0.60059
Plxna3	0.463492	Sema7a	-0.61626
Gm16008	0.461325	Pou3f1	-0.61723
Erich3	0.44817	Rell1	-0.62952
Prmt2	0.442064	Dnajc21	-0.63205
Fat1	0.441867	Zbtb16	-0.64072
Pgap1	0.440828	Zfpm2	-0.65649
Cfh	0.439439	Adra1d	-0.66298
Cadm1	0.438632	Gm3294	-0.67705
Pgrmc1	0.436022	Stac2	-0.68074
Slc22a8	0.435507	Gm34583	-0.68865
Slc6a7	0.435364	Ccbe1	-0.70226
Ttc39c	0.434256	Dkk3	-0.71531
Zfp462	0.431442	Igfbp4	-0.71855
Fbxo10	0.430574	Hunk	-0.71915
Caln1	0.430098	Cd34	-0.72064
Ptprz1	0.429642	Anxa11	-0.72255
Scn3b	0.429443	1700001O22Rik	-0.73532
Fras1	0.425502	Cfap43	-0.73917
Mafk	0.42429	Iqgap2	-0.76619
Nav1	0.424185	Ttc16	-0.76725
Slc7a2	0.423871	Nrep	-0.7826
Ehd4	0.423866	Tnfrsf12a	-0.81302
Rac3	0.423778	Gm15721	-0.81615
Prkg2	0.422181	Gm40518	-0.8206
Zscan18	0.418382	Npc111	-0.82186

Dgkg	0.417991	Gm37795	-0.82357
Stum	0.414094	Scnn1a	-0.8245
Gfra1	0.413405	9930014A18Rik	-0.82495
Sh3bp4	0.413169	Cd109	-0.82549
Pdgfra	0.41231	Wnt2b	-0.82789
Pnmal2	0.411415	Gm26673	-0.85591
Scn3a	0.41056	Gcnt4	-0.85602
Cd200	0.409255	Mycbpap	-0.86576
Abca8b	0.407269	Arc	-0.87865
Zkscan2	0.405679	Fap	-0.89141
Plppr3	0.405004	Adgrd1	-0.90136
Hs6st2	0.402835	Fam84b	-0.90763
Optn	0.402178	Ighm	-0.92295
Fgd4	0.400022	Olfml2b	-0.94293
Chd3	0.398466	Serinc2	-0.96585
Rimklb	0.397944	Tmem232	-0.97402
Rrp1b	0.39519	Npnt	-0.97494
Kctd12	0.393569	Lrrc36	-1.0622
Ankrd6	0.392261	Mylk3	-1.06442
Sdc3	0.391885	C1ra	-1.1125
Pde9a	0.383759	Rxfp2	-1.16241
Prkdc	0.383429	Rnf39	-1.19737
Slc16a11	0.38107	Rspo1	-1.20471
Blcap	0.380647	Hfe2	-1.23885
Slc39a6	0.378642	Lrrc74b	-1.26741
Arxes1	0.37845	Iqca	-1.29885
Usp11	0.3782	Ak7	-1.32154
Radil	0.376487	Cdhr4	-1.32995
Smardc3	0.374838	Trbc2	-1.3551
Ptprg	0.370496	Krt80	-1.36381
Ptpn13	0.368426	2310002F09Rik	-1.36653
Entpd6	0.367809	Il20rb	-1.41146
Mdn1	0.367286	H2-T-ps	-1.42942
Syn2	0.364715	Sebox	-1.45021
Adcyap1r1	0.363686	Thbs4	-1.47115
Sorcs2	0.360054	Gm22389	-1.5294
St3gal1	0.356879	Lrrc71	-1.68402
Ripor2	0.356468	Gm10635	-1.7073
A230057D06Rik	0.356123	Cldn22	-1.7345
Tubb2a	0.356048	C230072F16Rik	-1.74864
Tubb2b	0.355538	6330420H09Rik	-2.18032
Grip1	0.354862	Pla2g2f	-2.40322
Fam102b	0.353665	C630031E19Rik	-2.53205

Lyrm9	0.350169
Chl1	0.34944
Mmab	0.348646
Pea15a	0.347078
Vstm2b	0.345986
Fam118a	0.343362
Donson	0.341131
Wsb1	0.340517
Rapgef6	0.332494
Pcdh15	0.331776
Rev3l	0.331041
3110039I08Rik	0.326449
Gabrb1	0.325812
Grik2	0.319336
Ano6	0.318742
Cacna1e	0.312669
Cacna1c	0.311699
9330132A10Rik	0.310294
Mtfp1	0.308772
Kcnd3	0.308624
Tro	0.308401
Mmd2	0.308047
Cpsf4	0.303226
Izumo4	0.302342
Igsf11	0.301971
Zmym3	0.297841
Ltbp4	0.296496
Slc7a14	0.294586
Armex4	0.293488
Lrrn1	0.291307
Nsg2	0.279965
Smyd2	0.277885
Asx13	0.276272
Zfp941	0.269003
Smg1	0.265871
Nup205	0.265252
Grm5	0.261862
Firre	0.259934
Med12l	0.257113
Fam184a	0.256815
Wnk3	0.251167
Pak3	0.24953
Cbl	0.248543

Lct	-2.58678
Igkc	-3.78466

Kcnk9	0.245922
Shisa9	0.243569
Dcaf12l1	0.243476
Prrc2c	0.23664
Cald1	0.225511
Luzp2	0.205571
Ubn2	0.20357
Golgb1	0.201522
Map1b	0.188865
Tnik	0.18627
Tenm4	0.183308
Ptprb	0.173227
Atp8a2	0.158028
Taok1	0.076811
Map1lc3b	-0.20625
Scn2b	-0.2141
Anp32e	-0.22179
Coro2b	-0.22426
Fam57b	-0.22546
Smim10l1	-0.2309
Rmnd5a	-0.24166
Abcf2	-0.24266
Kif5a	-0.24567
Ctsb	-0.24769
Ccni	-0.24985
Ubl3	-0.24986
Cep19	-0.25013
Ckb	-0.25429
Ank	-0.2551
H2afz	-0.26549
Nkiras1	-0.26687
Adam9	-0.27155
Nefl	-0.27191
Fbxo33	-0.27337
Elmod1	-0.2734
Ccdc92b	-0.27733
Kdm7a	-0.27878
Iqsec1	-0.28502
Rtkn	-0.28688
Zdhhc9	-0.28793
D17Wsu92e	-0.28877
Polr2c	-0.29144
Klhdc3	-0.29271

Tmem151b	-0.29538
Dhx16	-0.29543
Uap1	-0.29562
B230217C12Rik	-0.29736
Ddx41	-0.30072
Hsph1	-0.30229
Paqr9	-0.30347
Epdr1	-0.30639
Wipf3	-0.30701
Fgfr1op2	-0.3141
Dhrs7	-0.31775
St6galnac4	-0.31807
Kcnab2	-0.31983
Spred1	-0.3201
Kcnk1	-0.3208
Pou3f3	-0.32426
Vmp1	-0.32488
Cacna2d3	-0.32641
Frmf5	-0.32833
Dpy19l1	-0.33048
D130017N08Rik	-0.33094
Zkscan17	-0.33368
Ube2g1	-0.33425
Fut9	-0.33471
Stim2	-0.33778
Raly	-0.34021
Nr3c1	-0.34032
Sertad4	-0.34475
Ppm1h	-0.34653
Tspan17	-0.3473
Klc2	-0.34776
Gmeb2	-0.3486
Wee1	-0.3494
Myrip	-0.35048
Il11ra1	-0.35083
Napa	-0.35291
4933439C10Rik	-0.35425
Acvr1	-0.35496
Lurap1	-0.35594
Dusp7	-0.35961
Rgs7bp	-0.36316
Cyb5r4	-0.36531
Magi2	-0.36747

Pik3r1	-0.36767
Ppp1r16b	-0.36976
Hrasls	-0.37297
Vsnl1	-0.373
Synj2	-0.37706
Nudt4	-0.37931
Camkk1	-0.38544
Nat8l	-0.38642
Ndfip2	-0.38881
Arhgef10l	-0.39013
Cdh11	-0.39018
Tle4	-0.39468
Kcnk3	-0.39554
Cxxc5	-0.39624
Lzts3	-0.39807
Zfp180	-0.39854
Ezr	-0.40121
Ppp3ca	-0.40173
Cdk19	-0.40315
Aifm3	-0.40404
Gpd1	-0.40416
Map6d1	-0.40451
Fam53b	-0.40683
Rapgef4	-0.40732
Deptor	-0.40859
Clic5	-0.40894
Acvr1c	-0.41053
Rph3a	-0.41099
Tox	-0.41137
P2rx4	-0.41466
Etv6	-0.41566
Camk1g	-0.41575
Arap2	-0.41839
Frrs1l	-0.4203
Atp2a2	-0.4218
Siae	-0.4219
Akt2	-0.42531
Cdk2ap1	-0.42556
Herc3	-0.42803
Tmem218	-0.43342
Rnd1	-0.43709
Cacng2	-0.43853
Ptk2	-0.44001

Nfic	-0.44014
H6pd	-0.44122
Ccng1	-0.44141
Khdrbs3	-0.44304
Adora1	-0.44624
Slc30a4	-0.44714
Thrb	-0.44867
Fam107a	-0.44974
Atp2b1	-0.45219
Gfod1	-0.45397
Srl	-0.45449
Nptx1	-0.45707
Hecw1	-0.46016
Sh3bp1	-0.46092
Them6	-0.46151
Crem	-0.46152
Msrb3	-0.4629
Pou3f2	-0.4682
Asap2	-0.47003
Dusp3	-0.47246
Tmem56	-0.47264
Klf9	-0.48012
Kcnj11	-0.48364
Plcl2	-0.48711
Scn4b	-0.48751
Ppme1	-0.49389
Fbxo32	-0.49451
Igsf9b	-0.49918
Chn2	-0.4997
D1Ert622e	-0.51414
Sorl1	-0.51429
Dagla	-0.52018
Scn1b	-0.52349
Cds1	-0.52487
Pdp1	-0.52535
Cacnb4	-0.52622
Zbtb18	-0.52649
Tbc1d30	-0.52947
Slc24a2	-0.53205
Cdh12	-0.53796
Hpca	-0.53806
Afap111	-0.54268
Abcd2	-0.54471

Gabrd	-0.54811
Mapk11	-0.54903
Lynx1	-0.55205
Rangrf	-0.55266
Otx1	-0.55751
Epha4	-0.56036
Atf6	-0.56429
Abcc8	-0.5675
Slc26a10	-0.56804
Zbtb16	-0.56842
Ell2	-0.56914
Hivep1	-0.57132
Hapln4	-0.57285
Spsb1	-0.57383
Hlf	-0.57548
Pou3f1	-0.57923
Ccl27a	-0.58123
Ephb2	-0.58301
Slco4c1	-0.58688
Pigz	-0.5885
Fam43a	-0.59124
Dusp10	-0.59159
Lrrc55	-0.59658
Neurod6	-0.59979
Galnt9	-0.5999
Foxp1	-0.60136
1110008P14Rik	-0.60327
Etv5	-0.6037
Efhd2	-0.60505
Diras2	-0.60559
Fam78a	-0.60894
Ubtd2	-0.61203
Atp2b2	-0.61571
Syt12	-0.61639
Zfpm1	-0.62353
Nfix	-0.6284
Trhde	-0.63239
Sel1l3	-0.63622
Rell1	-0.658
AI593442	-0.66103
Rgs4	-0.66745
Adcy1	-0.67816
Efna5	-0.68268

Sytl2	-0.68857
Wnt2b	-0.6948
Nos1ap	-0.69491
Osbpl1a	-0.69632
Plxdc1	-0.70134
Ccbe1	-0.70379
Sowahb	-0.70403
Fhad1	-0.70779
Adra1b	-0.70854
Iqgap2	-0.71058
Rims3	-0.71213
Psrc1	-0.71218
Syndig1	-0.71519
Nrep	-0.71614
Gm47155	-0.72221
Mical2	-0.72318
Hr	-0.72808
Satb1	-0.73089
H2-T23	-0.73623
Gm37795	-0.74007
Tmem178	-0.76073
Gm34583	-0.76099
Gm3294	-0.76993
Rassf3	-0.77137
Gfra2	-0.77938
Egr1	-0.78746
Ier5	-0.78817
Osbpl3	-0.79321
Teddm2	-0.79351
Herc6	-0.79373
Plau	-0.794
Gm45606	-0.79479
Ephb6	-0.79568
Cyp11a1	-0.79722
Homer1	-0.79822
Anxa11	-0.79999
Sema7a	-0.80572
Stac2	-0.81854
Gm26673	-0.82642
Kcns1	-0.84429
Dnajc21	-0.85724
Scnn1a	-0.85955
Ddit4l	-0.86039

Gm48932	-0.86162
1700001O22Rik	-0.86697
9930014A18Rik	-0.87685
Ntn5	-0.88426
Ttc16	-0.89399
Dkk3	-0.92092
Egr3	-0.93081
Mkx	-0.9327
Inhba	-0.94055
1110032F04Rik	-0.9432
Dalir	-0.95726
Npc111	-1.00768
Arhgap25	-1.01079
Igfbp4	-1.01139
Gcnt4	-1.03928
Fap	-1.04411
Adra1d	-1.07301
Cd34	-1.08051
Ighm	-1.08742
Adgrd1	-1.09316
Ovol2	-1.09875
Lrrc74b	-1.10361
Npnt	-1.10952
Serinc2	-1.13269
Tmem232	-1.1425
Gm30731	-1.14684
Myl4	-1.14976
Gm15721	-1.15234
Rnf225	-1.15716
Gm40518	-1.22737
G530011O06Rik	-1.25413
Rxfp2	-1.26826
Iqca	-1.32004
Rnf39	-1.36566
C1ra	-1.41029
Gm30648	-1.45874
H2-T-ps	-1.52777
Mylk3	-1.53679
Thbs4	-1.54397
Gm13306	-1.57084
Gm43684	-1.57593
Rspo1	-1.58144
Sebox	-1.6023

Krt80	-1.7025
Cd7	-1.85473
Trbc2	-1.86677
Lct	-2.15511
Gm10635	-2.50065
mt-Nd3	-4.17915

**Supplementary Table 2. Lists of Global and Unity Up and Down Differentially Expressed Genes**

Group 1		Group 3		Group 1		Group 3	
Global- Up	Global- Down	Global- Up	Global- Down	Unity- Up	Unity- Down	Unity- Up	Unity- Down
Slc6a3	Map1lc3b	Dkk1l	Col25a1	Col23a1	Adra1d		
Hcrt	Scn2b	Epop	Smg1	Hpcal1	Igfbp4		
Pmch	Anp32e	Kcnv1	Ptprb	Ndst4	Nfix		
Calcr	Coro2b	Adra1d	Csmc3	Cyp26b1	Syndig1		
Trdn	Fam57b	Npas4	Gprasp2	Nnat	Dnajc21		
Otp	Smim10l1	Kcnmb4	Padi2	Dcn	Lct		
Barhl2	Rmnd5a	Nrgn	Nhs1l	Camk2d	H2-T23		
C130021I20							
Rik	Abcf2	Rgs4	Aebp1	Trhr	Thbs4		
Krt2	Kif5a	Kenh3	Lfng	Hap1	Hpca		
Dsc3	Ctsb	Kctd1	Col1a2	Adra2a	Anxa1l		
A730046J1							
9Rik	Ccni	Gabrd	Aqp4	Angpt1	Sebox		
Dlk1	Ubl3	Cdk5r2	Slc13a3	Slc13a3	Trbc2		
AA414992	Cep19	Osbp1a	Amigo2	Smoc1	Iqgap2		
Arhgap36	Ckb	Arhgap33	Magee2	Scn5a	Gent4		
Gpr101	Ank	Stk32c	Tnxb	Arhgap6	Stac2		
Th	H2afz	Stk32c	Gfap	Itgb1l	Tle4		
Col10a1	Nkiras1	Pdp1	Fgl2	Ptpn14	AI593442		
Irs4	Adam9	Pamr1	Nnat	Wdr6	Rspo1		
Cdhr1	Nefl	Dlgap3	Spp1	Phactr2	Nos1ap		
Gal	Fbxo33	Dmtn	Dcn	Gira3	Sytl2		
Rtl1	Elmod1		Cdhr1	Lypd1	Slc30a4		
Frmf7	Ccdc92b		Lhx9	Ptpro	1110032F04Rik		
Gm34466	Kdm7a		Cubn	Ptgds	Fam43a		
Casr	Iqsec1		Ndst4	Aebp1	C1ra		
Ndst4	Rtkn		Cryga	Zcchc12	Pou3f1		
Igfbp1b	Zdhhc9		Uncx	Grin3a	Asap2		
Scn5a	D17Wsu92e		Dsc3	Cmb1	Atp2b1		
Trhr	Polr2c		Pou4f2	Ogn	Galnt9		
AW551984	Klhdc3			Nrp2	Sorl1		
Chrm5	Tmem151b			Cdh4	Pik3r1		
Cryga	Dhx16			Tmem132e	Dusp3		
Dio3	Uap1			Nr2f2	Raly		
Gabrg	B230217C12Rik			Tmem255a	Atp2a2		
G630018N14Rik	Ddx41			Slc7a11	Kcnab2		
Fat2	Hsph1			Gap43	Ndfip2		

Lnp1	Paqr9
Gpr50	Epd1
Nts	Wipf3
Baiap3	Fgfr1op2
Pomc	Dhrs7
Sec1413	St6galnac4
Ecel1	Kcnab2
Tfap2d	Spred1
Optc	Kcnk1
Itgb11	Pou3f3
Chst9	Vmp1
Nmbr	Cacna2d3
Dbh	Frmd5
Syt14	Dpy1911
Zan	D130017N0 8Rik
Arhgap6	Zkscan17
Enpp3	Ube2g1
Gm10710	Fut9
Plcz1	Stim2
Trh	Raly
Atp6v1c2	Nr3c1
Acp7	Sertad4
Dchs2	Ppm1h
Dcn	Tspan17
Nnat	Klc2
Gpx3	Gmeb2
Pappa2	Wee1
Gm20394	Myrip
Col23a1	Il11ra1
Magel2	Napa
Pdzd3	4933439C1 0Rik
6530403H0 2Rik	Acvr1
Hpcal1	Lurap1
Cdh23	Dusp7
Slc47a1	Rgs7bp
Zim1	Cyb5r4
Cpne7	Magi2
Cd36	Pik3r1
Cyp26b1	Ppp1r16b
Esyt3	Hrasls

Fabp7	Cacng2
Ngb	Synj2
Dpyd	Aifm3
Gpc3	Satb1
Apaf1	Them6
Fhl1	Tmem56
Ly6h	Npc111
Pgap1	Kcnk1
Kcnj16	Slc26a10
Samd14	Ubt2
Col1a2	Dhrs7
Fgl2	Spred1
Tenm1	Klhdc3
Timp2	Uap1
Dchs2	Nat8l
Mpzl2	Dkk3
Krt77	
Caln1	
Dner	
Marcks11	
Acvr2a	
Wfs1	
Kirrel3	
Ankrd6	
Nmbr	
Necab2	
Cntnap5b	
Colec12	
Fmod	
Sec1413	
Cdh23	
Adcyap1r1	
Nav1	
Gm35339	
Pgrmc1	
Crb1	
1700001O2 2Rik	
Syt10	
Myoc	
Gla2	

Nr2f2	Vsn11
Scn9a	Synj2
Olfm4	Nudt4
Hap1	Camkk1
Oxtr	Nat8l
Esr1	Ndfip2
Ptpro	Arhgef10l
Crb1	Cdh11
Krt77	Tle4
Mpzl2	Kcnk3
Scml4	Cxxc5
Fgl2	Lzts3
Syt15	Zfp180
Nkain3	Ezr
Ngb	Ppp3ca
Ptpn14	Cdk19
Zcchc12	Aifm3
Zfp618	Gpd1
Npbwr1	Map6d1
Nxph4	Fam53b
Mirt1	Rapgef4
Fam196b	Deptor
Omd	Clic5
Sgcd	Acvr1c
Gm16702	Rph3a
Otof	Tox
Angpt1	P2rx4
Tmem255a	Etv6
Gm38372	Camk1g
Filip1	Arap2
Rxfp3	Frrs11
Syt10	Atp2a2
Cd44	Siae
Tmem132e	Akt2
Cdh1	Cdk2ap1
Eya2	Here3
Spp1	Tmem218
Glra3	Rnd1
Tacr1	Cacng2
Crabp2	Ptk2
Nek10	Nfic
Htr2c	H6pd
Smoc1	Ccng1

Slc6a20a
Col1a1
Fat4
Cpne6
Mrap2
Csmd3
Ndnf
Cntn5
Hes5
Chrm5
Syt17
Nexmif
Cd200
Cntnap3
Ptprz1
Mdga1
Mgp
Rreb1
Scn9a
Bmp7
Barhl2
Pcdh19
Cgref1
Rab3c
Sox11
Aldh1a2
Sema3d
Mapk3
Eya2
Efemp1
Cdk18
Arhgef28
Abca8b
Cd74
Dpysl5
Kcnn3
Slc22a6
Slc7a2
Slc6a7
Cadm1
Tubb2a
Slc7a14
Mylk

Trpc7	Khdrbs3
Pde11a	Adora1
9530026P0 5Rik	Slc30a4
Lypd1	Thrb
Piezo2	Fam107a
Fibin	Atp2b1
Tex15	Gfod1
Stpg1	Srl
Adamts13	Nptx1
Nos1	Hecw1
Cdh4	Sh3bp1
Ankfn1	Them6
Dpyd	Crem
Nrp2	Msrb3
Gpc3	Pou3f2
Gm10605	Asap2
Cd74	Dusp3
Gm32444	Tmem56
Gdpd2	Klf9
Kcna5	Kcnj11
Cntnap5c	Plcl2
Fmod	Scn4b
Slc9a2	Ppme1
Pgam2	Fbxo32
Mrap2	Igsf9b
Fndc1	Chn2
Cacna1s	D1Erttd622e
Ogn	Sorl1
Adamts9	Dagla
Lgr5	Scn1b
Vegfd	Cds1
Cartpt	Pdp1
Cmb1	Cacnb4
Slco5a1	Zbtb18
Gm33533	Tbc1d30
Adra2a	Slc24a2
Thbs1	Cdh12
Wdr6	Hpca
D630023F1 8Rik	Afap111
Megf6	Abcd2
Camk2d	Gabrd
Adgrg2	Mapk11

Plppr3
Cdh13
Fbxo10
Crtac1
Ahi1
Meis1
Slc44a5
Rac3
Slc16a11
Omd
Col5a2
Nhs
Megf11
Xkr4
Ttc39c
Chd3
Pnmal2
Wipf3
Pea15a
Klc2
Entpd6
Serping1
Mdn1
Blcap
Fbln1
Kit
Sstr1
Sh3bp4
Zkscan16
Vegfd
Ptpre
Tacr1
Plxnc1
Plxna3
Acvr2b
Cd44
Fn1
Tubb2b
Plcx3
Slc39a6
Vstm2b
Smarcd3

Marcks11	Lynx1
Gxylt2	Rangrf
Gm47171	Otx1
Pbx3	Epha4
Arsj	Atf6
Aldh1a2	Abcc8
Syt17	Slc26a10
Samd14	Zbtb16
Igsf10	Ell2
Myo1h	Hivep1
Meis1	Hapln4
Rab27a	Spsb1
Hrk	Hlf
Pdzn4	Pou3f1
Gng4	Ccl27a
Adamts6	Ephb2
Col1a1	Slco4c1
Gpr182	Pigz
Bmp5	Fam43a
Hes5	Dusp10
Wfs1	Lrrc55
Gm37928	Neurod6
Plcx3	Galnt9
Col5a2	Foxp1
Fabp7	1110008P1 4Rik
Plce1	Etv5
Grin3a	Efhd2
Ndnf	Diras2
Fat4	Fam78a
Aloxe3	Ubt2
Slc44a5	Atp2b2
Acvr2a	Syt12
Apaf1	Zfpm1
Slc22a6	Nfix
Slc6a13	Trhde
Kcng1	Sel1l3
Ptgis	Rell1
Pde3a	AI593442
Tmem91	Rgs4
Ptgds	Adcy1
Rbm20	Efna5
Slc13a3	Syt12

Pcdh18
Usp11
Gdf10
Armex4
Fras1
Lnp1
Mmab
Fxyd6
Grm5
Cfh
Lama1
Gpr50
Mmd2

Necab2	Wnt2b
Crym	Nos1ap
Amz1	Osbp11a
Lmo3	Plxdc1
Tll1	Ccbe1
Tenm1	Sowahb
Itga4	Fhad1
2900052N0 1Rik	Adra1b
Ppl	Iqgap2
Myh7	Rims3
Magee2	Psrc1
Pcdh19	Syndig1
Nr4a2	Nrep
Aebp1	Gm47155
Cpne6	Mical2
Olfml2a	Hr
Mgp	Satb1
N4bp2	H2-T23
Col1a2	Gm37795
Gm42851	Tmem178
Adam12	Gm34583
Pnck	Gm3294
Rps4l	Rassf3
Fstl5	Gfra2
Cntnap3	Egr1
Ly6h	Ier5
Wdfy4	Osbp13
Reln	Teddm2
Tekt5	Herc6
Pcdh18	Plau
Sema3d	Gm45606
Gm35339	Ephb6
Gabrg1	Cyp11a1
C1ql1	Homer1
Cntn5	Anxa11
Grem2	Sema7a
Myoc	Stac2
Rhbdl3	Gm26673
Hfm1	Kcns1
Cpne2	Dnajc21
Il17rd	Scnn1a
Rreb1	Ddit4l

Igfbp3	Gm48932
Colec12	1700001O2 2Rik
Fgf11	9930014A1 8Rik
Dcc	Ntn5
Pcdh8	Ttc16
Wnk4	Dkk3
Mylk	Egr3
Phactr2	Mkx
Sox11	Inhba
Cdk18	1110032F0 4Rik
Mdga1	Dalir
Bmp7	Npc111
Timp2	Arhgap25
Crtac1	Igfbp4
Csmd3	Gcnt4
Myof	Fap
Nhs	Adra1d
Serping1	Cd34
Tnfaip813	Ighm
Myo5b	Adgrd1
Il13ra1	Ovol2
Nsun7	Lrrc74b
Rfx3	Npnt
Fam19a2	Serinc2
Klhdc8a	Tmem232
Npsr1	Gm30731
Lama1	Myl4
Kcnn3	Gm15721
Eln	Rnf225
Kcnj16	Gm40518
Cgref1	G530011O0 6Rik
Mrc2	Rxfp2
Dner	Iqca
Gjb2	Rnf39
Ranbp31	C1ra
Efemp1	Gm30648
Fbln1	H2-T-ps
Gap43	Mylk3
Glra2	Thbs4
Fmo1	Gm13306

Acvr2b	Gm43684
Tfpi	Rspo1
Dpysl5	Sebox
A230077H0 6Rik	Krt80
Tmem35a	Cd7
Oprk1	Trbc2
Foxo6	Lct
Trp53i11	Gm10635
Xkr4	mt-Nd3
Cntnap5a	
Ahi1	
Nrsn2	
Trpc5	
Gdf10	
Arhgef28	
Col6a2	
Cacng5	
Rgs12	
Cntnap5b	
Rcn1	
Gpc4	
Slc13a4	
Sall3	
Slc7a11	
Ninj1	
Gm48408	
Bgn	
Dlg5	
Gpsm1	
Galns	
Slc6a20a	
Col25a1	
Bche	
Megf11	
Kirrel3	
Wnt5a	
Fkbp10	
Nrarp	
Efcab1	
Fxyd6	
D430019H1 6Rik	
Slc26a2	

Fn1
Lin28b
Wnt7b
Grb10
Mapk3
Nexmif
Cdh13
Mrgpre
Slc30a10
Rab3c
Plxnc1
Sstr1
Col16a1
Plagl1
Tmem130
Aqp4
Ptpre
Thbd
Rcan3
Kit
Trim62
Pid1
Fhl1
Zkscan16
Rsph9
Carmil3
Tiam1
Gpr17
Adgrg1
Pnma3
Plxna3
Gm16008
Erich3
Prmt2
Fat1
Pgap1
Cfh
Cadm1
Pgrmc1
Slc22a8
Slc6a7
Ttc39c
Zfp462

Fbxo10
Caln1
Ptprz1
Scn3b
Fras1
Mafk
Nav1
Slc7a2
Ehd4
Rac3
Prkg2
Zscan18
Dgkg
Stum
Gfra1
Sh3bp4
Pdgfra
Pnmal2
Scn3a
Cd200
Abca8b
Zkscan2
Plppr3
Hs6st2
Optn
Fgd4
Chd3
Rimklb
Rrp1b
Kctd12
Ankrd6
Sdc3
Pde9a
Prkdc
Slc16a11
Blcap
Slc39a6
Arxes1
Usp11
Radil
Smarcd3
Ptprg
Ptpn13

Entpd6
Mdn1
Syn2
Adcyap1r1
Sorcs2
St3gal1
Ripor2
A230057D0 6Rik
Tubb2a
Tubb2b
Grip1
Fam102b
Lym9
Ch11
Mmab
Pea15a
Vstm2b
Fam118a
Donson
Wsb1
Rapgef6
Pcdh15
Rev3l
3110039I08 Rik
Gabrb1
Grik2
Ano6
Cacna1e
Cacna1c
9330132A1 0Rik
Mtfp1
Kcnd3
Tro
Mmd2
Cpsf4
Izumo4
Igsf11
Zmym3
Ltbp4
Slc7a14
Armxc4

Lrrn1
Nsg2
Smyd2
Asx13
Zfp941
Smg1
Nup205
Grm5
Firre
Med12l
Fam184a
Wnk3
Pak3
Cbl
Kcnk9
Shisa9
Dcaf12l1
Prrc2c
Cald1
Luzp2
Ubn2
Golgb1
Map1b
Tnik
Tenm4
Ptprb
Atp8a2
Taok1

**Supplementary Table 3. Genes with Gene Names of the Group 1 Unity List**

<b>Group 1</b>			
<b>Unity-Up</b>	<b>Gene Names</b>	<b>Unity-Down</b>	<b>Gene Names</b>
Col23a1	Collagen alpha-1(XXIII) chain	Adra1d	Alpha-1D adrenergic receptor
Hpcal1	Hippocalcin-like protein 1	Igfbp4	Insulin-like growth factor-binding protein 4
Ndst4	Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 4	Nfix	Nuclear factor 1 X-type
Cyp26b1	Cytochrome P450 26B1	Syndig1	Synapse differentiation-inducing gene protein 1
Nnat	Neuronatin	Dnajc21	DnaJ homolog subfamily C member 21
Dcn	Decorin	Lct	Lactase-phlorizin hydrolase
Camk2d	calcium/calmodulin-dependent protein kinase II, delta	H2-T23	H-2 class I histocompatibility antigen, D-37 alpha chain
Trhr	Thyrotropin releasing hormone receptor	Thbs4	Thrombospondin-4
Hap1	huntingtin-associated protein 1	Hpca	Neuron-specific calcium-binding protein hippocalin
Adra2a	Alpha-2A adrenergic receptor	Anxa11	Annexin A11
Angpt1	Angiopoietin-1	Sebox	Homeobox protein SEBOX
Slc13a3	Solute carrier family 13 member 3	Trbc2	T cell receptor alpha chain MC.7.G5
Smoc1	SPARC-related modular calcium-binding protein 1	Iqgap2	Ras GTPase-activating-like protein IQGAP2
Scn5a	Sodium channel protein type 5 subunit alpha	Gcnt4	Beta-1,3-galactosyl-O-glycosylglycoprotein beta-1,6-N-acetylglucosaminyltransferase 4
Arhgap6	Rho GTPase-activating protein 6	Stac2	SH3 and cysteine-rich domain-containing protein 2
Itgb11	Integrin beta-like protein 1	Tle4	Transducin-like enhancer protein 4
Ptpn14	Tyrosine-protein phosphatase non-receptor type 14	AI593442	Uncharacterized protein C11orf87 homolog
Wdr6	WD repeat-containing protein 6	Rspo1	R-spondin-1
Phactr2	Phosphatase and actin regulator 2	Nos1ap	Carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase protein
Gla3	Glycine receptor subunit alpha-3	Syt12	Synaptotagmin-like protein 2
Lypd1	Ly6/PLAUR domain-containing protein 1	Slc30a4	Zinc transporter 4
Ptpro	Receptor-type tyrosine-protein phosphatase O	1110032F04Rik	Uncharacterized membrane protein C3orf80 homolog
Ptgds	Prostaglandin-H2 D-isomerase	Fam43a	Protein FAM43A
Aebp1	Adipocyte enhancer-binding protein 1	C1ra	Complement C1r-A subcomponent

Zcchc12	Zinc finger CCHC domain-containing protein 12	Pou3f1	POU domain, class 3, transcription factor 1
Grin3a	Glutamate receptor ionotropic, NMDA 3A	Asap2	Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 2
Cmb1	Carboxymethylenebutenolidase homolog	Atp2b1	Plasma membrane calcium-transporting ATPase 1
Ogn	Mimecan	Galnt9	Polypeptide N-acetylgalactosaminyltransferase 9
Nrp2	Neuropilin-2	Sor11	Sortilin-related receptor
Cdh4	Cadherin-4	Pik3r1	Phosphatidylinositol 3-kinase regulatory subunit alpha
Tmem132e	Transmembrane protein 132E	Dusp3	Dual specificity protein phosphatase 3
Nr2f2	COUP transcription factor 2	Raly	RNA-binding protein Raly
Tmem255a	Transmembrane protein 255A	Atp2a2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2
Slc7a11	Cystine/glutamate transporter	Kcnab2	Voltage-gated potassium channel subunit beta-2
Gap43	Neuromodulin	Ndfip2	NEDD4 family-interacting protein 2
Fabp7	Fatty acid-binding protein, brain	Cacng2	Voltage-dependent calcium channel gamma-2 subunit
Ngb	Neuroglobin	Synj2	Synaptojanin-2
Dpyd	Dihydropyrimidine dehydrogenase [NADP(+)]	Aifm3	Apoptosis-inducing factor 3
Gpc3	Glypican-3	Satb1	DNA-binding protein SATB1
Apaf1	Apoptotic protease-activating factor 1	Them6	Protein THEM6
Fhl1	Four and a half LIM domains protein 1	Tmem56	TLC domain-containing protein 4
Ly6h	Lymphocyte antigen 6H	Npc111	NPC1-like intracellular cholesterol transporter 1
Pgap1	GPI inositol-deacylase	Kcnk1	Potassium channel subfamily K member 1
Kcnj16	Inward rectifier potassium channel 16	Slc26a10	Solute carrier family 26 member 10
Samd14	Sterile alpha motif domain-containing protein 14	Ubt2	Ubiquitin domain-containing protein 2
Col1a2	Collagen alpha-2(I) chain	Dhrs7	Dehydrogenase/reductase SDR family member 7
Fgl2	Fibroleukin	Spred1	Sprouty-related, EVH1 domain-containing protein 1
Tenm1	Teneurin-1	Klhdc3	Kelch domain-containing protein 3
Timp2	Metalloproteinase inhibitor 2	Uap1	UDP-N-acetylhexosamine pyrophosphorylase
Dchs2	Dachsous cadherin-related 2	Nat8l	N-acetylaspartate synthetase
Mpz12	Myelin protein zero-like protein 2	Dkk3	Dickkopf-related protein 3

Krt77	Keratin, type II cytoskeletal 1b
Caln1	Calcium-binding protein 8
Dner	Delta and Notch-like epidermal growth factor-related receptor
Marcks1	MARCKS-related protein
Acvr2a	Activin receptor type-2A
Wfs1	Wolframin
Kirrel3	Kin of IRRE-like protein 3
Ankrd6	Ankyrin repeat domain-containing protein 6
Nmbr	Neuromedin-B receptor
Necab2	N-terminal EF-hand calcium-binding protein 2
Cntnap5b	Contactin-associated protein like 5-2
Colec12	Collectin-12
Fmod	Fibromodulin
Sec14l3	SEC14-like protein 3
Cdh23	Cadherin-23
Adcyap1r1	Pituitary adenylate cyclase-activating polypeptide type I receptor
Nav1	Pituitary adenylate cyclase-activating polypeptide type I receptor
Gm35339	Predicted gene, 35339
Pgrmc1	Membrane-associated progesterone receptor component 1
Crb1	Protein crumbs homolog 1
170001O22Rik	DUF4685 domain-containing protein
Syt10	Synaptotagmin-10
Myoc	Myocilin
Gla2	Glycine receptor subunit alpha-2
Slc6a20a	Sodium- and chloride-dependent transporter XTRP3A
Col1a1	Collagen alpha-1(I) chain
Fat4	Protocadherin Fat 4
Cpne6	Copine-6
Mrap2	Melanocortin-2 receptor accessory protein 2
Csmd3	CUB and sushi domain-containing protein 3

Ndnf	Protein NDNF
Cntn5	Contactin-5
Hes5	Transcription factor HES-5
Chrm5	Muscarinic acetylcholine receptor M5
Syt17	Synaptotagmin-17
Nexmf	Neurite extension and migration factor
Cd200	Cell surface glycoprotein CD200 receptor 3
Cntnap3	Contactin-associated protein-like 3
Ptprz1	Receptor-type tyrosine-protein phosphatase zeta
Mdga1	MAM domain-containing glycosylphosphatidylinositol anchor protein 1
Mgp	Matrix Gla protein
Rreb1	Ras-responsive element-binding protein 1
Scn9a	Sodium channel protein type 9 subunit alpha
Bmp7	Bone morphogenetic protein 7
Barhl2	BarH-like 2 homeobox protein
Pcdh19	Protocadherin-19
Cgref1	Cell growth regulator with EF hand domain protein 1
Rab3c	Ras-related protein Rab-3C
Sox11	Transcription factor SOX-11
Aldh1a2	Retinal dehydrogenase 2
Sema3d	Semaphorin-3D
Mapk3	Mitogen-activated protein kinase 3
Eya2	Eyes absent homolog 2
Efemp1	EGF-containing fibulin-like extracellular matrix protein 1
Cdk18	Cyclin-dependent kinase 18
Arhgef28	Rho guanine nucleotide exchange factor 28
Abca8b	ABC-type organic anion transporter ABCA8B
Cd74	H-2 class II histocompatibility antigen gamma chain
Dpysl5	Dihydropyrimidinase-related protein 5
Kcnn3	Small conductance calcium-activated potassium channel protein 3

Slc22a6	Solute carrier family 22 member 6
Slc7a2	Cationic amino acid transporter 2
Slc6a7	Sodium-dependent proline transporter
Cadm1	Cell adhesion molecule 1
Tubb2a	Tubulin beta-2A chain
Slc7a14	Probable cationic amino acid transporter
Mylk	Myosin light chain kinase, smooth muscle
Plppr3	Phospholipid phosphatase-related protein type 3
Cdh13	Cadherin-13
Fbxo10	F-box only protein 10
Crtac1	Cartilage acidic protein 1
Ahi1	Jouberin
Meis1	Homeobox protein Meis1
Slc44a5	Choline transporter-like protein 5
Rac3	Ras-related C3 botulinum toxin substrate 3
Slc16a11	Monocarboxylate transporter 11
Omd	Osteomodulin
Col5a2	Collagen alpha-2(V) chain
Nhs	Nance-Horan syndrome protein
Megf11	Multiple epidermal growth factor-like domains protein 11
Xkr4	XK-related protein 4
Ttc39c	Tetratricopeptide repeat protein 39C
Chd3	Chromodomain-helicase-DNA-binding protein 3
Pnmal2	Paraneoplastic antigen-like protein 8B
Wipf3	WAS/WASL-interacting protein family member 3
Pea15a	Astrocytic phosphoprotein PEA-15
Klc2	Kinesin light chain 2
Entpd6	Ectonucleoside triphosphate diphosphohydrolase 6
Serpinc1	Plasma protease C1 inhibitor
Mdn1	Midasin
Blcap	Bladder cancer-associated protein
Fbln1	Fibulin-1
Kit	Mast/stem cell growth factor receptor Kit

Sstr1	Somatostatin receptor type 1
Sh3bp4	SH3 domain-binding protein 4
Zkscan16	Zinc finger protein 483
Vegfd	Vascular endothelial growth factor D
Ptpre	Receptor-type tyrosine-protein phosphatase epsilon
Tacr1	Substance-P receptor
Plxnc1	Plexin-C1
Plxna3	Plexin-A3
Acvr2b	Activin receptor type-2B
Cd44	CD44 antigen
Fn1	Fibronectin
Tubb2b	Tubulin beta-2B chain
Plcx3	PI-PLC X domain-containing protein 3
Slc39a6	Zinc transporter ZIP6
Vstm2b	V-set and transmembrane domain-containing protein 2B
Smard3	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 3
Pcdh18	Protocadherin 18
Usp11	Ubiquitin carboxyl-terminal hydrolase 11
Gdf10	Growth/differentiation factor 10
Armxc4	Armadillo repeat-containing X-linked protein 4
Fras1	Extracellular matrix organizing protein FRAS1
Lnp1	Endoplasmic reticulum junction formation protein lunapark
Mmab	Corrinoid adenosyltransferase
Fxyd6	FXFD domain-containing ion transport regulator 6
Grm5	Metabotropic glutamate receptor 5
Cfh	Complement factor H
Lama1	Laminin subunit alpha-1
Gpr50	Melatonin-related receptor
Mmd2	Monocyte to macrophage differentiation factor 2

## Appendix 2: Preliminary Data and Future Directions

## **Preface**

This appendix is aimed at providing some preliminary data that was able to be collected. These projects have promise as avenues in the lab and so the preliminary results are presented with proposed future directions and experiments. Projects that will be presented are *in vivo* surgery, Doxorubicin treatment in zebrafish, and very early work with investigating the gut brain axis.

## **In Vivo Surgery**

### Background

*Ex vivo* experiments are useful for investigating how drugs and other treatments affect the brain and neurotransmitter release.<sup>1-3</sup> Unfortunately, a rat's whole brain cannot be kept alive on perfusion systems as it is too thick for perfused oxygen and nutrients to diffuse to the center of the brain from the surface.<sup>4</sup> Due to this, brain slices are commonly used to perform *ex vivo* experiments.<sup>4</sup> *Ex vivo* experiments provide a large amount of information, but they do not maintain the native structure of the brain as the BBB is removed, the metabolism is different, and the pathways are no longer completely intact.<sup>5</sup> In order to avoid these problems and take measurements in a whole brain, *in vivo* applications are used.<sup>6,7</sup> This allows the system to be kept intact and means that transient neurotransmitter release is able to be seen.<sup>8,9</sup> This also allows for continuous monitoring of analytes of interest via applications like microdialysis or electrochemistry. Our lab is interested in performing survival surgery to replicate experiments that were previously conducted using rat brain slices. Doing a similar study *in vivo* would allow for behavioral data and neurochemical measurements to occur simultaneously.<sup>9</sup> This allows for a more thorough picture of how the behavior affects the dopamine release. Other sampling techniques like microdialysis are also available to increase the amount of data that can be collected from animals while awake and moving.<sup>10</sup> Eventually, it is hoped that this work could include chemotherapy treatment to provide a more complete picture of the brain during treatment. Terminal surgery or ones where the animals is anesthetized with urethane or other anesthetic that does not allow the animal to wake up can also be performed and is used for longer experiments or ones where behavior is not needed.<sup>11</sup> The updated freely moving surgical procedure is

provided below after much trial and error. This trial and error included determining that the ideal size for a surgical animal is between 300-500 grams, different order or amounts of drugs to have the best sedation with the lowest risks to the animal. The higher weight the animal was, the longer it took them to wake up as anesthesia is sequestered in the fat and then slowly leaks back out. This means that it takes more drug to achieve a proper level of sedation, but then takes significantly longer for them to wake up from anesthesia (usually more than 10 hours). It was also observed that many animals treated with buprenorphine do show pica symptoms and the time in which buprenorphine is administered plays a large role in the recovery time of the animal. Observing the animals through recovery also shed light on how some of them will just not recover while others will act like animals without surgery within a few days. This guided how medication was given and different signs to look for in terms of distress and pain.

#### Updated Procedure

The rat was fully anesthetized with ketamine/xylazine and given buprenorphine or meloxicam analgesic and fluids. The fur was removed with shears. The rat was then mounted onto a high precision stereotaxic frame and the site was treated with betadine and isopropyl alcohol or 200 proof ethanol, applied in alternating applications by swab three times. A longitudinal incision was created down the center to expose the skull along the midline of the skull. Stereotaxic coordinates in relation to bregma were used to identify proper locations for drilling small holes to implant guide cannula and electrodes. Two access holes were drilled: one above the striatum for insertion of the guide cannula and working electrode into region rich in compound of interest and one hole contralateral to the working electrode for a Ag/AgCl reference electrode. Screws used for securing the electrodes to the skull and bases of the electrodes were secured by dental cement. The stimulating electrode was wrapped around one of the screws prior to applying the cement to allow a better anchoring point for the leads. Prior to waking up, rats were given meloxicam or buprenorphine as an analgesic and fluids subcutaneously. Yohimbine was also used to reverse the xylazine. Once the rat was awake and ambulatory, it was returned to the animal care unit and allowed to recover.

The lab worked with ACU vet staff to optimize a drug protocol. The current procedure is listed below.

1. Meloxicam (SC)
2. Ketamine/ Xylazine (IP) or induction and sustained inhaled isoflurane
3. Saline (SC)
4. Surgery- if injectable booster needed- Ketamine (IP)
5. Buprenorphine (SC)
6. Yohimbine (IP)- if injectable anesthesia is used (none if on isoflurane)
7. Saline (SC)
8. 8 Hours post-surgery- Buprenorphine (SC)
9. Next morning- Meloxicam and fluids as needed for at least 48 hours (Buprenorphine if animal appears to be in significant pain)
  - a. Lidocaine topical as needed if animal shows signs of pain or irritation around wound
  - b. 8-12 hours later- meloxicam or buprenorphine dosing for at least 48 hours
10. Meloxicam and fluids as needed throughout 5-day recovery period

### Progress

Through using this procedure, which is adapted from the Roitman procedure<sup>12</sup>, mainly differing in the drug order, drug amounts and the which drugs given to the animals with our procedure including yohimbine and topicals like lidocaine. With this optimized procedure the animal woke up easily from surgery and was able to be returned to the ACU sooner than without the reversal agent. Unfortunately, this complete procedure was only completed once. Future surgeries would include further testing the drug paradigm listed above to determine if it is truly optimal or if there is a better way to dose without opioids or the risk of pica. Pica is a known side effect of buprenorphine administration and means that the rats ingests anything that he can get in his mouth.<sup>13</sup> This is less than ideal as bedding and anything besides his food could cause an obstruction and make the rat sick.<sup>13</sup> This rat did recover well from surgery and was able to perform the task of pressing the lever. There were a few days of acclimation to the chamber and one day of performing the full three-hour experiment. During this last day of testing with the leads attached, the

animal fell asleep and only responded about 30 times. However, when he was placed in the chamber without the leads attached to the head cap the following day, he responded over 300 times. It is undetermined if this is an effect of having the leads attached or if it is because the animal was more comfortable with working and moving in the chamber the following day. Future work would also include determining if animals are able to acclimate to the leads attached to the headcap allowing the animal to perform similarly to when the leads are not attached. It is also to be determined if the surgery affects the animal's ability to learn how to perform the task. It would be worthwhile to investigate if training before surgery could be recalled and then animals have minimal post-surgery training before testing.

Terminal surgery was conducted to attempt to replicate some results from another professor. The method for this surgery is very similar to freely moving except the anesthesia is urethane instead of ketamine and xylazine and is considered terminal as the animal does not wake up from the urethane. While completing these experiments, it was difficult to find a reliable dopamine signal. It is undetermined if this was due to coordinates being incorrect, electrode issues, or hardware issues. Dopamine was able to be measured during a calibration in a flow cell on the electrodes before implantation, but once in the brain, a reliable signal was not found. Freely moving work had a similar problem where a clean dopamine signal was not able to be found. Those electrodes were not able to be precalibrated due to their fragility and flow cell problems and a stimulating electrode was not used. The lack of stimulating electrode was due to not wanting to damage the brain through the stimulation before the animal had a chance to recover from the surgery. Early surgeries were more concerned with the animal surviving surgery and the recovery process than the electrochemical data. There is debate about the best way to calibrate electrodes for *in vivo* work, as calibrations in a flow cell allow for dopamine to be seen, but do not replicate the brain environment. The other side of the debate is if the electrodes are calibrated within the tissue, each time an electrode is placed, and each stimulation creates damage in the brain tissue. The next step in the freely moving surgery would be to incorporate the stimulating electrode and checking placement before the animal wakes up. During the terminal experiments, a lesion device was used to try and determine placement of the electrodes at the end

of a recording session and the stimulating and reference electrode spots were easily seen. The lesion from the working was not easy to see in a macro dissection. It may be possible to see these marks using a cryostat to slice the brain very thinly. For future work, this step may need to be incorporated to determine if the coordinates are the problem in not being able to see dopamine.

Another issue that was encountered in the freely moving surgery was that the Med Associates software and Knowmad software were not able to have their recordings start at the same time. This was worked on through phone calls and adding different wires to the bus in the breakout box and an external lever that could be pressed to start the med associates recording to try and get them to communicate, but to no avail. The issue was that one needed an interruption in current to register a signal while the other needed a current to be emitted to register a signal. Through much trial and error, this was worked on and some work arounds were investigated. Future work with the Med Associates and/or Pine Instruments needs to occur for the systems to communicate and sync their recordings. This is essential to allow them to be synced with each other and allows the times to be matched to a tenth of a second. This would allow for trends like dopamine release occurring before a reward is earned to be seen easily.<sup>14</sup>

The noise seen on the Knowmad software made seeing any dopamine basically impossible. The noise was worked on by attempting to have the rat not touch anything made with metal as it was found that the animal would ground itself through contact with the metal bars of the floor or metal walls of the operant box. The carbon fiber of the microelectrodes was sealed in the glass using epoxy to prevent movement of the fiber within the electrode microdrive. Unfortunately, as when the rat moved, the noise increased. Grooming and other behaviors were able to be seen as noise spikes on the FSCV software. When the rat fell asleep, the background looked very acceptable and had low levels of noise which was a promising start, but no detectable dopamine was able to be measured. Future work needs to determine what caused the noise in the system and develop ways to reduce the noise.

The *in vivo* surgery has many ways to be improved such as working on the surgery protocol, modifying the behavior box set up to prevent noise, and other optimization to allow for electrodes to be

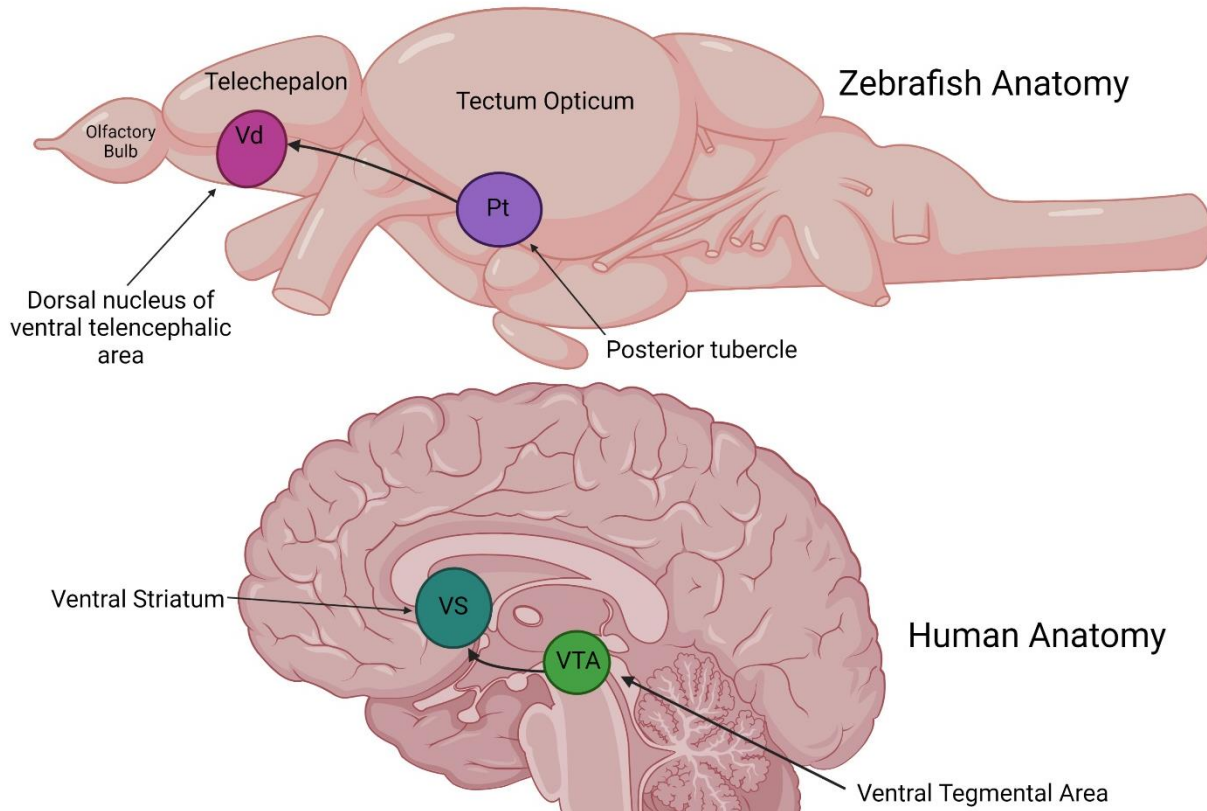
stable and dopamine be reliably measured. After these problems are addressed, this project could be expanded to microdialysis or serotonin monitoring and adding chemotherapy treatment to the process. This would also allow for a better understanding of how chemotherapy affects the system as a whole instead of seeing the effects just in brain slices.

## **Doxorubicin Treated Zebrafish**

### Background

All the research presented thus far has involved rodent models. While this allows for more in depth behavioral measurements, they also have several disadvantages. These includes the cost of acquisition and care<sup>15</sup>, how long experiments take with behavior training and dosing, 2-4 weeks for dosing and then up to years for behavior depending on the paradigm. Rats also are not high throughput due to wanting to limit the number of animals used with n values usually under 8 for each group in a study. Due to these concerns, the Johnson Lab has begun working with zebrafish and working to validate them as a neurochemical model.<sup>16</sup> Zebrafish (*Danio rerio*) are a widely used model throughout biology.<sup>17-19</sup> Their main highlight is that they have a fully sequenced genome and are easy to genetically modify.<sup>20</sup> The larvae are also transparent which allow for imaging and other studies to easily be performed during the larval stage.<sup>20</sup> When compared to rodents, they are both vertebrates and have many analogous neural pathways to the mammalian brain.<sup>21</sup> The mesolimbic pathway and its projections to the striatum are said to be analogous to the posterior tubercle projecting to the dorsal nucleus of the ventral telencephalon in zebrafish (**Figure 5-1**).<sup>21</sup> Zebrafish also allow for higher throughput than rodents making biological and toxicology experiments common.<sup>22</sup> Their dosing time and training time is about half of that of rodents. Another advantage is the whole brain can be used for measurements and does not need to be sliced like a rodent brain.<sup>16</sup> Investigating chemotherapy and its effects on the brain has been a focus of the Johnson Lab. In rodents, as previously stated, it has been found that 5-FU and carboplatin significantly deplete dopamine and serotonin levels in the striatum.<sup>3, 23, 24</sup> Wanting to continue this work, food and water administration of 5-FU and carboplatin was completed.<sup>1</sup> It was found that while food administration decreases dopamine release quicker, both food

and water administration of carboplatin and food treatment of 5-FU lead to the significant decreases in dopamine in the telencephalon.<sup>1</sup>



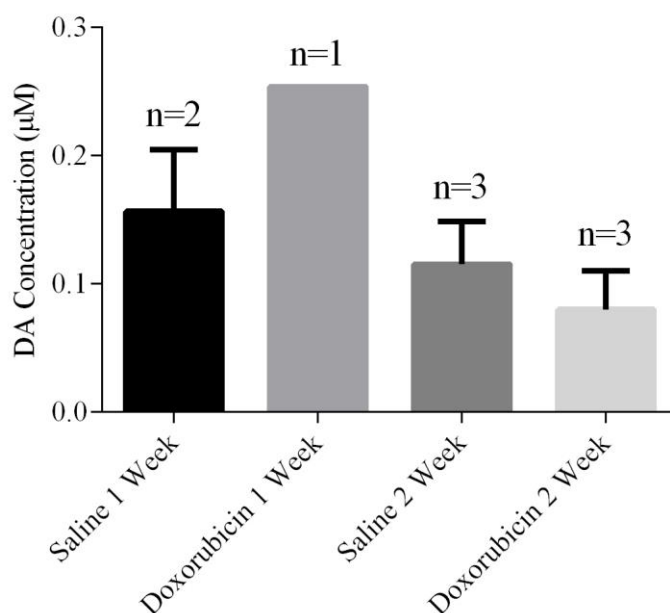
**Figure 0-1. Anatomy of Fish and Human Pathway of Interest**

Ascending dopaminergic system to the zebrafish telencephalon showing how the pathway is like that of the mesolimbic pathway to the striatum in rodents. Top- Fish Pathway (Adapted from Rink, 2002) Bottom- Human Pathway. Figure created with BioRender.com

### Progress

Due to previous results with zebrafish<sup>1</sup> and the results that were found in the doxorubicin study (Chapter 2), it was proposed to dose zebrafish with doxorubicin and see if they would have decreased dopamine release similar to 5-FU and carboplatin or if they would have an increase in dopamine similarly to the rats. The experimental setup included a one week and two-week trial and 7 fish per group totaling 14 fish per treatment. 1- and 2-week administration were investigated due to the water treatment previously having effects after 7 days and 2 weeks to determine if doubling the exposure time had different effects as seen in the rats (2 vs 4 weeks). Fish included in these experiments were removed from the aquatic system and had

their water changed daily. Dox was dosed at 10  $\mu\text{M}$  which is 10 times less than previously used with carboplatin and 5-FU. This is also mirroring the rats in that a typical dose of 5-FU was 25mg/kg and dox was dosed at 2.5mg/kg. The change with the fish allowed for the drug to be conserved while achieving results. After 7 or 14 days of exposure, the water was changed to clean water for the day and the following day (day 9 or 16) neurochemical experiments were completed. Stimulated dopamine release was found in the telencephalon using the same dopamine waveform as the rats (-0.4 to +1.3 to -0.4V and 10 Hz update rate). The stimulation parameters used were 350  $\mu\text{A}$ , 25 pulses, 60 Hz, 2 ms width, and 89.50 stim to scan delay. Six measurements were taken from each fish in an area of high release with 10 minutes in between each measurement. Once all the measurements were taken, the data was worked up in Excel and GraphPad version 6. The results showed that after one week of dox treatment, dopamine release was increased and at two weeks it was decreased (**Figure 5-2**). Unfortunately, due to low n-values, the one-week data was not able to be statistically tested and the two-week data was not significantly different. The trends of the data with the 1-week data increasing and the two-week data decreasing mirror the trends that are seen in the rats. Due to low n-value however, this conclusion has no statistical power. The n-values are low due to not being able to find dopamine or the brain being lost to dissection. Dissection issues are possible with the zebrafish as their brains are small and delicate. This was the first experiment that I had completed with the fish and due to this inexperience, positioning the electrode and finding dopamine proved to be rather difficult.



**Figure 0-2. Zebrafish Dopamine Release**

Stimulated dopamine release with doxorubicin and saline treatment having one and two weeks of drug exposure

If the experiment is repeated, it appears that effects of the drug are present within one week of water treatment and 10 µM is an adequate drug dose. With replication to get a larger n-value, it is hoped that there will be statistically significant results. It also would be interesting to determine if dosing the food has a different effect than dosing the water. There is a possibility that the fish will not eat the food as it is an acidic drug which could possibly affect the digestive system due to its ability to extravasate. It is the hope that this effect does not happen if ingested, but it is known to cause damage to vessels through exposure. While these things should be kept in mind, the expansion of this project has some promising avenues with the continued use of zebrafish.

## **Chemo Gut**

### Background

The brain and the gut both exert effects throughout the body. The gut which is comprised of the stomach, small and large intestine, and the microbiome of bacteria present in those organs is sometimes

referred to as a second brain due to it having such huge effects on the overall health of the body.<sup>25</sup> The tenth cranial nerve, or the vagus nerve, connects the brain to the gut allowing them to communicate via a pathway referred to as the gut-brain axis.<sup>26</sup> Investigating this pathway has been done extensively in mood disorders like anxiety and depression as they often have comorbid gut issues.<sup>27</sup> The second part of the gut that is very important is the microbiome.<sup>27</sup> The microbiome is comprised of the essential bacteria that work throughout the gut to help digest food and absorb nutrients.<sup>27</sup> Different medications can affect the microbiome in turn affecting the overall health of the body.<sup>28</sup> Through this pathway, several neurotransmitters are produced with over 90% of serotonin being produced in the gut.<sup>26, 29, 30</sup> This connection of the brain and gut should be explored to determine if the effects of the chemotherapeutics affect the gut's level of neurotransmitters as well. Serotonin has been found to be decreased with carboplatin treatment in the brain<sup>23</sup>, but does this trend continue in the gut. To begin this investigation, a piece of small intestine was to be dissected out and kept alive on the perfusion system in a similar way to brain slices. Electrodes then would be placed in the mucosal layer to detect serotonin release. Through this, it was proposed that we would be able to see the effects of chemotherapy on gut serotonin.

### Progress

The gut dissection was able to be tested and pieces of the small intestine were able to be removed. It is important that these are removed very soon after euthanasia so that decomposition can be limited, and the gut does not begin to smell. If completing a brain slice experiment, this will require two people to ensure that the brain receives the attention it needs, and the gut receives its attention, so all of the tissue remains viable. Slices of the small intestine close to the stomach and further away from the stomach were able to be collected. These pieces are placed in cold, oxygenated Krebs buffer (117 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub> and 11 mM glucose).<sup>31</sup> Once cooled, these pieces were cut so the tube became a slab. Another way of doing this is to cut rings of the intestine after cooling. These rings are left whole and not cut in half. The mucosal layer was then placed up with the slabs and it stayed on the inside of the rings. Both were added to the perfusion chamber with Krebs buffer flowing

with oxygen and heat applied. It appears that these tissues were able to be kept viable on the station, but this is very difficult to determine as no serotonin or norepinephrine was seen using FSCV. The tissue also visibly puffed up and became engorged with liquid. It is thought that this is due to more exposure of water in the perfusion chamber than in the body causing the tissue to absorb it to try and reach equilibrium. Papers have been published with Krebs buffer being used with an organ bath.<sup>31</sup> An organ bath is a commonly used apparatus to hold excised tissue in a vat of heated and oxygenated liquid. Because our lab does not have an organ bath, the perfusion chamber was used. The main difference appears to be the physical volume of the bath and how the tissue is held. Within the bath there is much more liquid, but also a structure that securely holds the tissue. Within the perfusion chamber there is a small harp that is placed on the tissue to stabilize it. This means as the tissue puffed up, the harp physically moved and eventually broke as the strings were under so much pressure from the expanding tissue. The electrode placement was also not able to be precise as there were no distinguishing anatomical landmarks on the tissue. The top of the slabs and the inside of the rings were aimed for as this is where the mucosal layer should be. Again, due to the puffing up, the electrode placement changed as time passed. More work needs to be done on the best way to keep the tissue viable for FSCV. It also needs to be determined if the stimulated release of serotonin is possible in the gut or if either transient or sustained release occur. Sustained release would eliminate FSCV as a measuring method as it is a background subtracted method and this subtraction would eliminate the signal.

Once measurements can be made, it will be interesting to investigate how serotonin is released from the tissue under different conditions such as SSRI or chemotherapy administration. Due to the vagus nerve being the connection point of the gut brain axis,<sup>26</sup> work could be done to stimulate that nerve or other parts of the brain while measurements are performed in the gut. This would become an *in vivo* project with having to measure this pathway. It would also be worthwhile to sever the vagus nerve<sup>32</sup> or use vagus nerve stimulation to determine how this affects the system.<sup>33</sup> Vagus nerve stimulation is used as a way to control certain type of epilepsy and other disorders<sup>34</sup>, but it is undermined how this affects the gut or neurotransmitter release. This project has use in the chemobrain project as it is known that the gut is severely

affected when dopamine is altered in the brain.<sup>35, 36</sup> Two of the best examples of these are IBS and Parkinson's disease. IBS is a series of gut issues that occur in times of stress and is common in people with anxiety or depression.<sup>35</sup> Parkinson's Disease is the loss of dopamine neurons in the substantia nigra pars compacta and is known to cause many problems in the gut.<sup>36</sup> Measuring in the gut could further illustrate how chemotherapeutics affect the whole body and different rescue mechanisms like SSRI treatment could be tried as this is proposed to help the gut.<sup>37</sup> The microbiome is also a huge area that could be investigated as without it, the body does not function normally.<sup>28</sup> This testing could show if drugs like chemotherapeutics (like dox which is an antibiotic) affect the bacteria that makes up the microbiome. Overall, the gut brain axis has a lot of promise to add to the research that the lab has done and to the chemobrain project.

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