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Anxiolytic effects of propranolol and diphenoxylate on mice and automated stretch-attend posture analysis

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ANXIOLYTIC EFFECTS OF PROPRANOLOL AND DIPHENOXYLATE ON MICE AND AUTOMATED STRETCH-ATTEND POSTURE ANALYSIS

by

Kevin Scott Holly, B.S.

A Dissertation Presented in Partial Fulfillment
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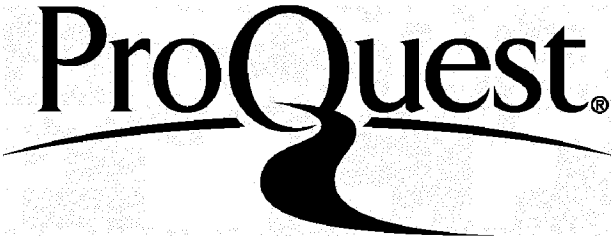

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We hereby recommend that the dissertation prepared under our supervision by

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ABSTRACT

The prevention of social anxiety, performance anxiety, and social phobia via the combination of two generic drugs, diphenoxylate HCl (opioid) plus atropine sulfate (anticholinergic) and propranolol HCl (beta blocker) was evaluated in mice through behavioral studies. A patent published on a September 8, 2011 by Benjamin D. Holly, US 2011/0218215 A1, prompted the research. The drug combination of diphenoxylate and atropine plus propranolol could be an immediate treatment for patients suffering from acute phobic and social anxiety disorders. Demonstrating the anxiolytic effects of the treatment on mice would validate a mouse model for neuroscientist to be used to detect the mechanism of action behind the drug combination.

To detect more sensitive anxiety measures in mice, a MATLAB-based software called MATSAP was developed as a quick, consistent, and open source program that provides objective automated analysis of stretch-attend posture in rodent behavioral experiments. Stretch-attend posture occurs during risk assessment and is prevalent in common rodent behavioral tests. Stretch-attend posture is a more sensitive measure of the effects of anxiolytics than traditional spatiotemporal indices. However, quantifying stretch-attend posture using human observers is time consuming, somewhat subjective, and prone to errors. Unlike human observers, MATSAP is not susceptible to fatigue or subjectivity. MATSAP performance was assessed with videos of male Swiss mice moving in an open field box and in an elevated plus maze. MATSAP reliably detected

stretch-attend posture on par with human observers. This freely-available program can be broadly used by biologists and psychologists to accelerate neurological, pharmacological, and behavioral studies.

To further expand on methods to automate the detection of SAP, EthoStock was developed. This not only can detect SAP, but has the potential to detect other ethological behaviors such as grooming and rearing.

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Author Kain Hall

Date 10-28-2016

DEDICATION

This dissertation is dedicated to all those who have suffered enduring hours manually scoring animal behavior. I hope to relieve some of you with an automated process.

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ACKNOWLEDGMENTS

First off, I will like to thank my advisor, Dr. Teresa Murray, for being a great mentor who has encouraged me throughout my work and set a high standard in leadership to follow. I also thank committee members Thomas Bishop, Mark DeCoster, Bryant Hollins, and Janelle McDaniel. I would like to thank Dr. James Spaulding for taking care of the mice used in this project, for ensuring our safety throughout, and providing access to need pharmaceuticals as animal director, safety inspector, and holder of a FDA approved license. I will also like to thank Mrs. Emily Born, the current animal director and safety inspector. I thank Dr. Ioannis Vlachos for consultations. I thank Todd Cloe for allowing me to use the woodshop to create many behavioral apparatuses. I thank The Thingery for providing means for me to create 3D plastic connectors for various apparatuses. I am grateful for my lab mates Ben Kemp, Chelsea Dressel, Jessica Scoggin, Peace Ibole, and Seona Lee. I thank Casey Orndorff for collaborating. I would like to thank all those whom assisted in my behavioral experiments, which include Mariella Aponte, John Basile, Caroline Bell, Madison Blackwell, Owen Hart, Destiny Hicks, Hartie Spence, Charles (Anthony) Ellis, Eulalie (Tess) Grodner, Hunter Hall, Karly Hooper, Brady Howard, Keith Matthews, Jack Mertens, Jonathan Niemirowski, Suraj Pathak, Anita Percurcika, Kelsey Phelan, Kayla Ponder, TJ Ponder, Daniel Rachal, Daniel Rivera, Aminah Smith, Shyanthony Synigal, Brenna Tull, and Daniel Williams.

CHAPTER 1

INTRODUCTION

1.1 Need for Dissertation Research

It is estimated that 75% of the population has glossophobia, the fear of public speaking [1]. Glossophobia is the number one phobia in the world. Glossophobia even outranks the fear of dying, necrophobia. Glossophobia has the potential to hinder an individual's social influence and career. Patients suffering from glossophobia experience performance anxiety, which includes symptoms of stomach cramping, diarrhea, sudden urinary urges, elevated heart rate, trembling voice, shaky limbs, and confusion. An immediate "on call" treatment for performance anxiety would be a breakthrough for those individuals challenged with stage fright.

Propranolol has often been prescribed by physicians for patients with performance anxiety, even though propranolol has no FDA approval to treat performance anxiety. However, propranolol only addresses somatic anxiety and not psychic. Benzodiazepines, which are used to treat anxiety, are a central nervous system depressant and sedative with the risk of tachyphylaxis, or tolerance. Selective serotonin reuptake inhibitors (SSRI) require 6 weeks of daily oral therapy to have an effect on performance anxiety. The combination of propranolol HCl and diphenoxylate HCl with atropine sulfate could be used to acutely treat performance anxiety that addresses both somatic and psychic symptoms without concern of tolerance or sedation. Verifying the existence of a synergic

effect could perpetuate translation into the marketplace. In an attempt to prove this, a preclinical study was performed with a mouse model to study the effect of the drug combination (see Chapter 2).

Rodent behavioral analysis is often used to assess the effects of pharmaceuticals, implanted devices, or surgical procedures in preclinical research. The development of image analysis tools has enabled researchers to quantitatively assess various rodent behaviors quickly and objectively [2, 3]. However, most automated scoring programs track patterns in spatial locomotor exploration and neglect ethological behaviors, such as head dipping and stretch-attend posture (SAP) [2]. Currently, there is no accurate tracking and scoring software that can directly detect SAP [4] nor any commercially available software that can readily detect SAP. Due to limited funding in some research labs, there is a need for inexpensive software that detects SAP in rodents. To meet this need, a freely available, open source software program with a flexible, user-friendly GUI called MATSAP was successfully developed to detect SAP (see Chapter 3). The program runs in a basic MATLAB installation with the Image Processing Toolbox™. MATSAP allows users to analyze multi-page Tag Image File Format (multi-TIFF, .tif) video files of rodents from an overhead view. However, MATSAP is constrainted to detecting SAP and neglects the detection of other ethological behaviors.

To address this, another MATLAB based software called EthoStock was developed that detects SAP using a different approach that allows for broader future applications in detecting other ethological behaviors such as rearing and grooming (see Chapter 4). Currently, there is no fully automated program to detect these other ethological behaviors and behavioral scientists have been scoring them manually.

After the development of MATSAP, the automated detection of SAP provided opportunities to expand the applications of this underutilized ethological behavior. Currently, the TBI rodent studies that have looked at anxiety have only focused on spatiotemporal measurements found in classical anxiety paradigms. The usefulness of SAP detection was examined in TBI rodent behavioral studies that evaluated anxiety (see Chapter 5).

1.2 Objectives

The dissertation will have two main foci. The first is on the physiological and behavioral effects of the combination of two generic drugs, diphenoxylate HCl (opioid) plus atropine sulfate (anticholinergic) and propranolol HCl (beta blocker) in preventing social anxiety, performance anxiety, and social phobia.

The research is based on a September 8, 2011 patent publication by Benjamin D. Holly, US 2011/0218215 A1. The drug combination of diphenoxylate/atropine plus propranolol could be an immediate treatment for patients suffering from acute phobic and social anxiety disorders. Demonstrating the anxiolytic effects of the treatment on mice would validate a mouse model for neuroscientist to investigate the mechanism of action behind drug combination. For instance, fluorescent microscopy could be used to investigate the co-localization of the targeted receptors to test a hypothesis that a synergic effects of the medication is due to a dimerization of receptors.

The second focus is on developing an automated analysis of the anxiety related rodent behavior, stretch-attend posture (SAP). This tool will provide behavior scientists and neuroscientists with a quick, accurate, and objective method for accessing SAP where time consuming manual scoring has been the norm.

1.2.1 Behavioral Objectives

Perform behavioral studies using mice to evaluate the anxiolytic properties of diphenoxylate/atropine and propranolol. The classical anxiety tests, which are the elevated plus maze test, the light/dark transition test, and the open field test, were used to analyze the exploratory-anxiety conflicts of the mice. Social anxiety was assessed using the 3-chamber social approach test. Phobic anxiety was examined in the rat exposure test.

1.2.2 Automated Detection

Develop automated methods for detecting stretch-attend posture (SAP) in rodents based on videos from an overhead view. First, a program was developed to detect SAP by forming an ellipse around the body of the rodent and using the eccentricity values. Then, another program was developed that uses elliptic Fourier analysis to form Fourier descriptors that represents the silhouette of the rodent. Using a neural network or fuzzy logical along with a clustered databank of these descriptors, SAP was detected [5].

1.3 **Background**

1.3.1 What is Anxiety?

Anxiety is the feelings of nervousness, fear, or apprehension accompanied by symptoms of breathlessness, a choking sensation, palpitations, restlessness, muscular tension, tightness in the chest, giddiness, trembling, and flushing. The nature of anxiety is complex, which leads to a broad range of anxiety disorders that may overlap with depression or fear. DSM-IV classification of clinical anxiety includes generalized anxiety disorder, panic disorder, specific phobia, social phobia, obsessive-compulsive disorder and post-traumatic stress disorder [6].

1.3.2 Neurocircuitry of Anxiety

When anxiety occurs in an animal or person, specific brain regions become more active as shown by electrode and MRI readings. However, these same regions become active during other emotional or cognitive conditions. This implies that the areas activated serve a role in multiple mental states. To understand the exact role of these multifaceted brain regions, scientists have induced lesions or given inhibitory pharmaceuticals at these specific sites in animal models to study the effects on behavior. From these experimentations, it has become apparent that there is a specific electrochemical pathway or circuit that occurs during specific mental states. Models of these neurocircuits have been made to understand emotional or mental states such as anxiety.

The neurocircuitry of anxiety overlaps with other neural pathways that model emotions such as fear and depression. When these emotions occur, signals travel in similar paths through nearly the same brain regions. Before explaining chronologically the entire neural pathway that occurs during anxiety, we will cover the core regions of the circuitry.

The two most studied areas of the brain that are activated during anxiety are the amygdala and the hippocampus. The hippocampus's main function is to store and process memories. The amygdala modulates and regulates other areas of the brain during emotional arousal and stress. These two areas of the brain have a two-way interaction with one another. The amygdala modulates the consolidation of emotional memories in the hippocampus and the hippocampus triggers amygdala activation when emotional memories are recalled [7].

The amygdala, which is stimulated by emotional stimuli, is a main component in facilitating attention toward emotion stimuli. A paradigm called attentional blink has been used to demonstrate that the amygdala creates more attention to arousing stimuli. A damaged amygdala inhibits this emotionally focused attention. During emotional situations, memories may be distortedly focused on the emotional stimuli (which is given higher priority than other surrounding details) and stored in the hippocampus. This is one of the two ways the amygdala modulates emotional memories. The other occurs after the memory is formed and stored in the hippocampus. Memories are strengthened and made long-term through a process called consolidation [8].

As shown in **Figure 1-1**, sensory information of the fearful stimuli travels to the amygdala for processing. Audio and somatosensory information enters the amygdala via the dorsal sides of the lateral amygdala (LA), whereas the olfactory information enters the central nucleus amygdala (CEA). The prefrontal cortex, which is a cognitive portion of the brain, provides either excitatory or inhibitory information to the LA. The prelimbic (PL) section of the cortex sends an excitatory signal, which some have viewed as "worry." The PL also talks with the hippocampus, which could represent the conscious recall of fearful memories. The infralimbic (IL) area sends an inhibitory signal. The prefrontal cortex modulates the signals that enter the LA, which then travels to the basolateral amygdala (BLA). From the BLA, the signal travels to the CEA. The CEA sends out a signal to the hypothalamus where it is dispersed through different region of the brain to induce the symptoms of fear (see **Table 1-1**). Similarly, the signal travels to the bed nucleus of the stria terminalis (BNST) where it is sent to the same regions as the hypothalamus to induced symptoms. This forms a parallel circuit that induces the same

symptoms [9]. Michael Davis hypothesized that the signal travelling through the BNST can be thought of as "Anxiety" where the signal leaving the CEA can be viewed as "fear." When the term anxiety is used in this context, Michael Davis described anxiety more as a "sustained fear" [10]. Others have associated the BNST to the experiences of "anticipatory anxiety" [11]. It has been shown that serotonin activity in the amygdala is increased in response to cued conditioned fear, but not unconditioned fear [12].

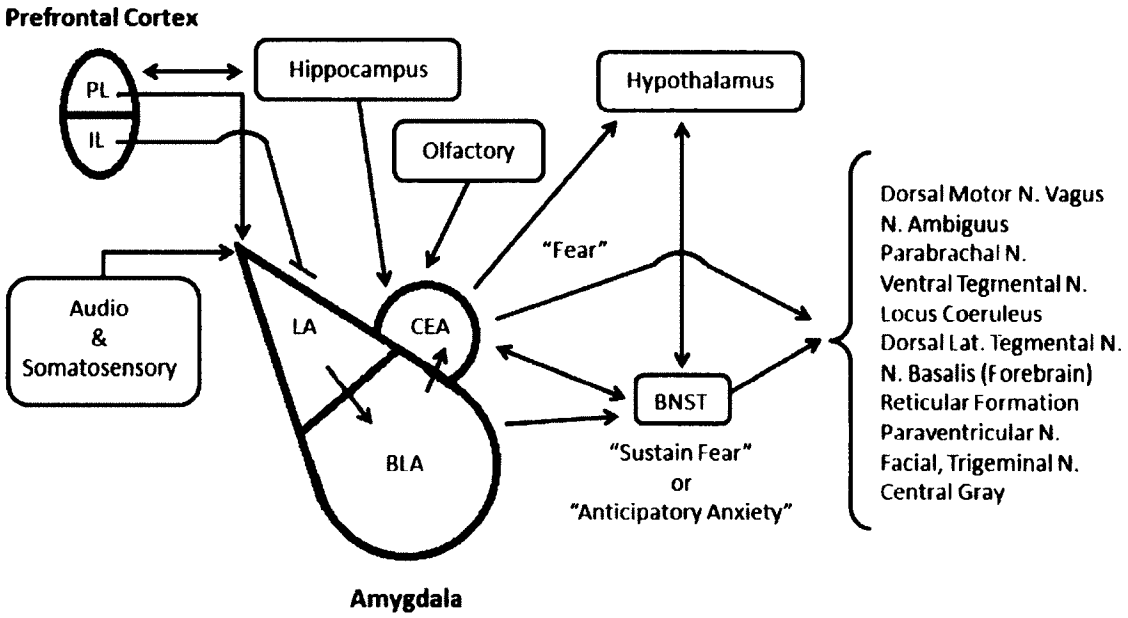

Figure 1-1: Neurocircuitry of anxiety. An overview of the anxiety neurocircuitry in relation to fear as proposed by Michael Davis with a few modification[10, 11]. The pathway includes the lateral amygdala (LA), central nucleus amygdala (CEA), prelimbic (PL) prefrontal cortex, infralimbic (IL) prefrontal cortex, basolateral amygdala (BLA), and the bed of nucleus stria terminalis (BNST), which lead to anatomical sites that trigger symptoms of fear and anxiety (see **Table 1-1** for the associated symptoms for each anatomical site). (Graphic created by Kevin Holly).

Table 1-1: Symptoms of anxiety expressed during activation of anatomical site [13].

Anatomical Site	Symptoms
Lateral Hypothalamus	**Increased heart rate, increased electrodermal activity, increased blood pressure, paleness, and dilated pupils**
Dorsal Motor N. Vagus	Ulcers, urination, defection, and increased heart rate
N. Ambiguus	**Increased blood pressure and vocalization**
Parabrachal N.	Panting and respiratory distress
Ventral Tegmental N.	**Behavioral and EEG Arousal**
Locus Coeruleus	Increased Vigilance
Dorsal Lat. Tegmental N.	**Increased Attention**
N. Basalis (Forebrain)	Increased Motor Responses
Reticular Formation	**Reflex Facilitation**
Paraventricular N.	HPA Axis Activation
Facial, Trigeminal N.	**Facial expression, Open mouth**
Central Gray	Freezing, Hypoalgesia, vocalization

1.3.3 Memory Consolidation, Reconsolidation, and Extinction

The preservation-consolidation hypothesis was first proposed by Müller and Pilzecker in 1900 [14]. It was found that memories of newly learned information were disrupted by learning other information shortly afterwards. This gave birth to the idea that new memories are in a temporary state until they are consolidated over time. Memory is thought to be consolidated through process called long-term potentiation (see section 1.3.2 for more details). Over time memories can be reconsolidated to maintain them for long term memory or they can be erased, which leads to extinction. Extinction has been studied in rodent fear conditioning paradigms. A rodent is conditioned to associate a conditional stimuli (CS) that does not illicit an innate response (a loud noise or bright light) with a fearful stimulus such as a foot shock. After the rodent establishes a fearful association to the CS, the rodent is exposed to the CS alone continually. The process to

remove the fearful association to the CS is known as extinction. It is hypothesized that this process can either occur through the formation of new memories where there is no fearful association with the CS or the erasure of the old memories. The formation of the new memories, which will be addressed as "learning extinction," would require consolidation to take place in order to have long term effects. The erasure of the old fearful memories, which will be addressed as "erasure extinction," would require the inhibition of the reconsolidation process in order to remove these memories assuming they have already been consolidated due to conditioning.

The strength of a memory can also be enhanced by emotional arousal. During an emotionally arousing situation, the adrenal stress hormones epinephrine and cortisol are released. Epinephrine and cortisol activate adrenergic and glucocorticoid receptors, respectively, to enhance memories. β-adrenergic receptors activated by epinephrine in the amygdala enhances memory formation. Based on animal models, it seems stress hormones trigger the amygdala to modulate memories in the hippocampus [8]. It has also been found that epinephrine activates β-adrenergic receptors found peripherally on vagal afferents projecting to the nucleus of the solitary tract in the brainstem. This triggers signals that influence neuronal activity in other brain regions such as the amygdala.

The consolidation, reconsolidation, and extinction processes play a key role in anxiety and fear. The amygdala consults with the hippocampus before sending out a response to an emotional situation. It also signals to the hippocampus if there is an emotional tie to a particular memory before it is stored. If these processes are interrupted, the anxiolytic and phobic memories may be affected and in turn affect the mental state of the individual or animal.

1.3.4 What is Long-term Potentiation?

Long-term potentiation (LTP) is the strengthening of synaptic connections based on previous activity that last from hours to days or months. LTP was first noted to occur when a repeated high frequency electrical stimulation created an increase in amplitude of the excitatory postsynaptic potentials. This strengthening of the synaptic connection is believed to contribute to learning and memory in certain areas of the brain such as the amygdala.

LTP does not have a specific mechanism of action, but is a term used to describe the outcome of increased signal strength at synaptic connections. There are many mechanisms that can result in a LTP. For example, LTP can occur if there is an increase of certain receptors, increase affinity for certain molecules, or if there is an overall increase in surface area at the synaptic site. The action potential of the presynaptic cells releases neurotransmitters to the postsynaptic cell. If a certain threshold is met, an action potential will be generated in the postsynaptic cell assuming it is a neuron and cause it to fire. This successful retrieval of the signal typically leads to LTP according to Hebbian learning. Donald Hebb's theory was summarized by Carla Shatz as "Cells that wire together fire together."

LTP occurs differently in different synapses. For example, some neocortical synaptic connections will weaken due to correlated firing while the classical hippocampal synapses would strengthen. Anti-correlated firing would weaken the classical hippocampal cell synapses while strengthen some types of neocortical cell synapses. Typically, LTP is produced after the postsynaptic fires an action potential with few milliseconds of an excitatory release by presynaptic cell [15].

The longevity of LTP is made possible through transcription and the translation of new proteins. The postsynaptic cell could have more receptors (which are made of protein) to receive signals from the presynaptic cell. The intrinsic excitability of a neuron could also be changed with new proteins, such as enzymes, to encourage firing from received stimuli. An affinity of a postsynaptic receptor could increase due to phosphorylation induced by an enzyme.

1.3.5 Example of Long-term Potentiation in the Hippocampus

Let's look at an example of a mechanism behind LTP in the hippocampal synapses. This site was chosen as it is the most studied site for LTP. At these synapses, glutamate is released from the axon of a presynaptic neuron to the dendritic spines of the postsynaptic neuron (**Figure 1-2**). The glutamate is released into the synaptic cleft, which is about 20 nm in width [16]. The postsynaptic neuron contains receptors that are about 4 nm. We will focus on the response of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors to the neurotransmitter, glutamate.

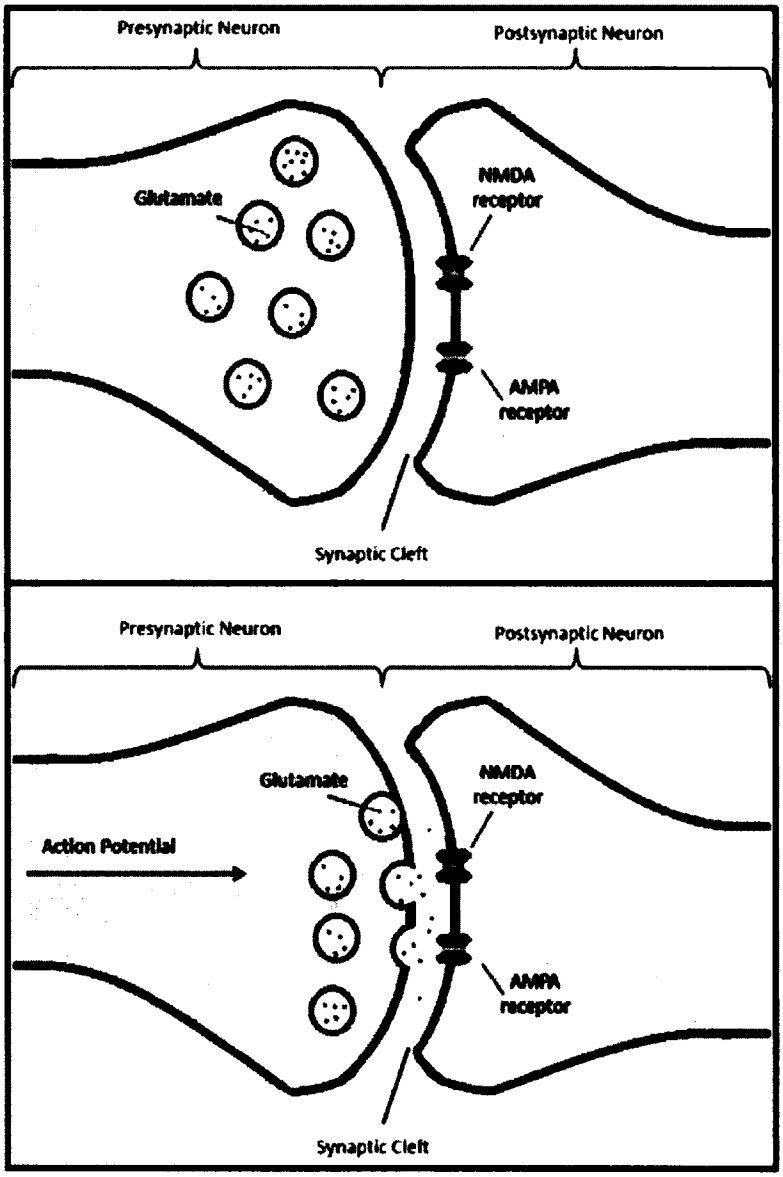

Figure 1-2: The release of glutamate at a synapse (Graphic created by Kevin Holly).

As illustrated in **Figure 1-3**, the release of glutamate into the synaptic cleft opens the postsynaptic AMPA receptors' ion channels after binding, which allows sodium ions through the membrane and into the postsynaptic neuron. The binding of the glutamate to the postsynaptic NMDA receptors does not allow any ions to pass as they are clogged by magnesium ions. The affinity of NMDA receptors to glutamate when magnesium ions are

bound is also low. The NMDA receptors are voltage-sensitive and require a strong depolarization in the postsynaptic neuron before the magnesium ions are expelled from the pore of the receptor. This depolarization is caused by the influx of sodium ions into the postsynaptic neuron after the AMPA receptors open due to the glutamate binding. This depolarization due to the positive sodium ions causes a voltage change within the postsynaptic neuron from -70 mV to -35 mV. After the magnesium ion are repelled from blocking the channels in the voltage-sensitive NMDA receptors through this process called electrostatic repulsion, sodium and calcium ions pass through the NMDA receptor's channel. There is a 10,000-fold difference in calcium concentration from outside to the inside of the postsynaptic neuron. So once the NMDA receptors are open, the calcium floods quickly into the postsynaptic neuron. This helps propagate an action potential through the postsynaptic neuron. After the magnesium is expelled from the channel pore, the NMDA receptor has a higher affinity for glutamate. So, glutamate release immediately after depolarization is more susceptible to the NMDA receptors. Downstream mechanisms lead to the phosphorylation of the AMPA receptor, which allows more sodium ions to pass through the AMPA receptor. This enables the postsynaptic cell to reach an action potential more easily, thus causing LTP. LTP is further enabled by the phosphorylation of the AMPA receptor subunit GluR1 at the Ser845 and Ser831 sites by CaMKII (calcium-calmodulin–dependent protein kinase II) as well as the generation of new AMPA receptors (**Figure 1-4**) [17]. The phosphorylation of the AMPA receptor opens up the receptor's ion channel and allows more sodium to pass through into the postsynaptic cell. Coupled with the additional AMPA receptors, a lower amount of the neurotransmitter, glutamate is needed to trigger a voltage change to

repel the magnesium from the NMDA receptors' ion channel in order to allow calcium into the postsynaptic cell to generate an action potential.

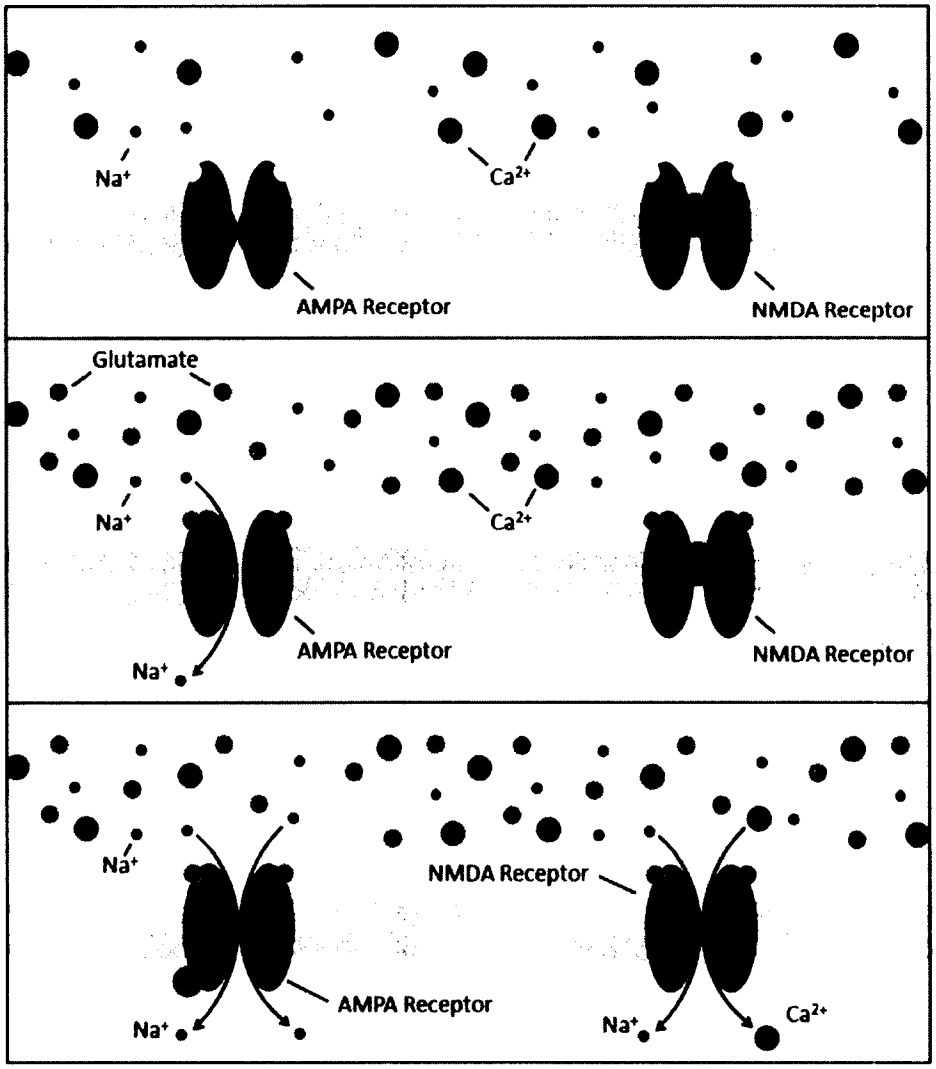

Figure 1-3: The interaction of the postsynaptic neuron's AMPA and NMDA receptors after the release of the neurotransmitter, glutamate, by the presynaptic neuron after a presynaptic action potential. The glutamate opens the AMPA receptor's ion channel which allows sodium ions through the membrane and into the postsynaptic neuron. This causes a voltage change within the postsynaptic neuron from -70 mV to -35 mV, which repels magnesium from blocking the channels in the voltage-sensitive NMDA receptors through a process called electrostatic repulsion. This allows sodium and calcium ions to pass through the NMDA receptor channels. The phosphorylation of the AMPA receptor allows more sodium ions to pass through the AMPA receptor (Graphic created by Kevin Holly).

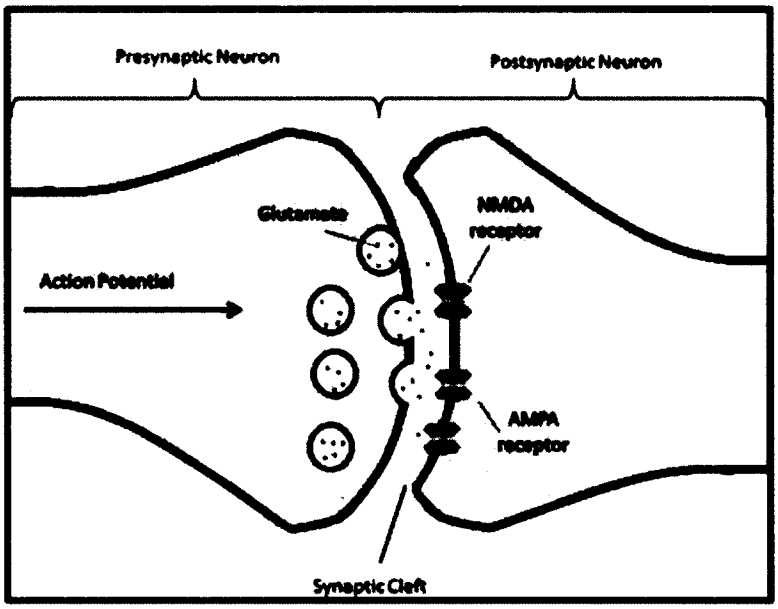

Figure 1-4: LTP in the hippocampal synapses is partially caused by an increase of AMPA receptors on the postsynaptic neuron (Graphic create by Kevin Holly).

1.3.6 What is Long-term Depression?

Long term depression (LTD) is the opposite effect of LTP where there is a weakening of a synaptic connection that last from hours to days or months [15]. LTD mostly occurs due to a lack of receptors on the postsynaptic neuron. LTD is needed process to prevent synapses overly strengthening, which would inhibit the encoding of new information. LTD is also important for erasure extinction of memories.

1.3.7 What are G Protein-coupled Receptors?

G protein-coupled receptors (GPCRs) are structured with seven-transmembrane α-helix resulting in a receptor without a channel. To communicate with the inside of the cell, ligands bind to the extracellular side of the receptor and cause the receptor to undergo a conformational change. After this shape change, the receptor's associated G protein can bind to it intracellularly by phosphorylating its bound GDP to GTP. The G

protein then breaks down into different subunits (α, β, and γ), which then triggers a set of intracellular signal cascades.

Two GPCRs that are of importance in this dissertation are adrenergic and opioid receptors. Adrenergic receptors can be found in cells throughout the body and are activated by the stress hormones norepinephrine and epinephrine. Opioid receptors are found mostly in the brain and also in the spinal cord and digestive tract. In our study, we were particularly interested in μ-opioid receptors which are mostly activated by the endogenous opioids, endorphins and endomorphins.

1.3.8 What is Stretch-attend Posture?

Stretch-attend posture, an anxiety-related behavior, occurs when the rodent lowers its back, elongates its body, and is either standing still or moving forward very slowly [18]. SAP is a naturally occurring behavior found in hamsters, guinea pigs, mice, and rats that can be reliably intensified by certain experimental paradigms, such as placing the rodent in an open area [18, 19]. In mice, the SAP behavior occurs when the mouse is undergoing risk-assessment specifically due to an internal exploratory-anxiety conflict. It can also occur under fearful risk-assessment where SAP would be an ambivalent element reflecting an approach-avoidance tendency [18, 20]. When SAP is present during a passive avoidance situation, mice approach or avoid the object at nearly equal rates, which confirms they are undergoing risk-assessment during an approach-avoidance conflict [19, 20]. SAP is a good identifier for conflict behavior in mice and can be used to evaluate the effects of drugs at reducing these internal conflicts [21]. During exploratory-anxiety conflicting situations, SAP can be used as a valid measure of anxiety as anxiolytic drugs have successfully reduced SAP [19, 21-23].

SAP has been found to be more sensitive to the effects of classical and atypical anxiolytics than traditional spatiotemporal indices in the murine plus-maze [24, 25]. For example, SAP is especially sensitive to the effects of ligands acting on 5-HT1A receptors [24, 25]. It is hypothesized that SAP can be related more to the cognitively oriented aspects of anxiety [24]. Inclusion of ethological measurements such as SAP provides a more comprehensive profile on the anxiolytic or anxiogenic effects of a treatment [23, 25, 26]. SAP can also help differentiate between anxiogenesis and sedation effects of drugs [2, 27]. Despite finding risk assessment measurements to be more sensitive to anxiety modulating drugs than traditional indices, Carobrez *et al.* found that only a quarter of studies have adopted them [27].

SAP has also been evaluated in open field (OF) [28], rat exposure test [29], and canopy stretch attend posture test [22]. The increase in SAP behavior along the border of the canopy further supported that SAP was a risk assessment behavior, which paralleled the increased SAP behavior of rodents near the entrance of the open arms in EPM [22, 30].

CHAPTER 2

ANXIOLYTIC EFFECTS OF PROPRANOLOL AND DIPHENOXYLATE ON MICE

2.1 Introduction

2.1.1 Treatment of Acute Social Anxiety

In the early 1960s, the discovery of benzodiazepines led to the first pharmaceutical treatment of anxiety [31]. The benzodiazepines increased the efficacy of the neurotransmitter, gamma-aminobutyric acid (GABA), to the $GABA_A$ receptor, which led to hypnotic, anxiolytic, and muscle relaxant properties. Benzodiazepines have been effective in the treatment of acute anxiety [32]. Unfortunately, long term use of benzodiazepines can cause tolerance and physical dependence [33].

Propranolol, which was created by Sir James Black in the early 1960s, is a non-selective beta adrenergic blocker initially prescribed to treat angina pectoris [34]. Propranolol lowers heart rate and cardiac output [32] and received approved from the United Sates Food and Drug Administration in 1968 for the treatment of tachyarrhythmia. In subsequent years, propranolol received approval for the treatment of ischemic heart disease, hypertension, and myocardial infarction.

The use of beta adrenergic receptor blocking agents to treat anxiety was first suggested by Granville-Grossman and Turner in 1966 [35]. A double blind study was conducted to study the effects of propranolol on anxiety. Half of the patients were given 20 mg of propranolol four times day for one week and given a placebo the following week. The other patients received the same treatment but were given the placebo the first week and the propranolol the second week. After each week, an investigator who was blind to the study interviewed the patient. During each session the investigator rated the anxiety levels of the patient on a five-point scale and the patient rated each of his symptoms on a five-point scale. At the end of the second week, the investigator decided which treatment had the greater benefit to the patient. After 15 patients were examined, propranolol was shown to be significantly more effective than the placebo. For six of the patients, propranolol was as effective as the placebo, but for nine of the patients the propranolol made them less anxious. It was concluded from the rating scores of the patients' symptoms that propranolol was more effective than the placebo only in relieving autonomic symptoms.

In 1974, Tyrer and Lader further investigated the effects of propranolol when treating 12 patients with anxiety. Six of the patients suffered mainly from somatic anxiety, where the focus was on their bodily symptoms. The other six patients suffered mainly from psychic anxiety, where the bodily symptoms were viewed as secondary features. The patients were given three bottles of identical white capsules that either contained either propranolol (40 mg), diazepam (2 mg), or placebo. Each week, the patients would take 3-9 pills daily from one of the bottles for three weeks. After this time period, investigators who were blind to the contents of the bottles asked the patients to

rank the bottles in order of efficacy. When looking at both the somatic and psychic anxiety groups, diazepam was the favored medication and propranolol appeared to be no better than the placebo. However, propranolol was preferred over the placebo in the somatic group. It was even close to being as effective as diazepam in the somatic group, but it was substantially less effective in the psychic group. Tyrer and Lader conclude that propranolol would be a better treatment than diazepam in patients with somatic anxiety because it is safe, is not prone to abuse, and rarely produces sedation.

There are well documented concerns over withdraw and dependence of benzodiazepines during long term treatment [33]. Propranolol would be more favorable than benzodiazepines in cases where the person needs to be awake, such as performance anxiety, or when an individual has built up a tolerance to benzodiazepines [32].

Introduced for treating anxiety in the 1980s, the daily administration of selective serotonin reuptake inhibitors (SSRI) antidepressant was shown useful in the treatment of an array of anxiety disorders including obsessive-compulsive disorder, post-traumatic stress disorder, panic disorder, social phobia, and general anxiety disorder [36]. Antidepressants for the most part have been safe and effective in both long and short term cases leading to SSRIs becoming the most prescribed treatment for anxiety disorders [6].

In 2011, Benjamin Holly had a patent published that included the combination of propranolol HCl and diphenoxylate HCl with atropine sulfate to treat social anxiety [37]. Disclosed in the patent is an open clinical trial aimed specifically at treating performance anxiety. Wherein, subject 2 ingested 5.0 mg of diphenoxylate HCl with 0.05 mg of atropine sulfate 90 minutes prior to public speaking and reported a trembling voice and pounding heart with no urinary or fecal urgency as expected of the anti-diarrheal

compound. Subject 2 later ingested 40 mg of propranolol HCl 90 minutes prior to public speaking and reported no tachycardia or trembling voice, but reported fecal urgency and a less confident presentation. When subject 2 took the combination of 40 mg propranolol HCl and 5.0 mg of diphenoxylate HCl with 0.05 mg of atropine sulfate 90 minutes prior to public speaking on five separate occasions, there were no signs of tachycardia, trembling voice, or urinary or fecal urgency. In addition, the subject felt more confident and had no fear. This suggests that the combination has a synergic effect that not only alleviates the somatic symptoms, but also addresses psychic anxiety. Further research is needed to ensure the psychic effects are not due to the relief of the somatic symptoms as Tyrer and Lader found to be the case when treating patients in the somatic anxiety group with propranolol HCl [38].

2.1.2 Metabolic Properties of Diphenoxylate and Propranolol

Diphenoxylate is a μ-opioid agonist with a half-life between 12-14 hours and is metabolized by the liver into diphenoxylic acid, also known as difenoxine [39].

Propranolol is a lipophilic alkaline compound with a half-life of approximately 4 hours and is metabolized mainly in the liver. In first pass metabolism through the liver, around 60-70% of propranolol is metabolized [32]. Cytochrome P450 1A2, a liver enzyme, converts propranolol into N-desisopropylpropranolol. Cytochrome P450 2D6 breaks propranolol into 4'-hydroxypropanolol. The majority of the metabolites can be found in urine [39].

2.1.3 Animal Models

The classical mouse anxiety tests (elevated plus maze test, the light/dark transition test, and the open field test) utilize the exploratory-anxiety conflict within mice. Mice are

scavengers and are apt to explore their surroundings. However, our hypothesis states anxiety hinders the mice from exploring well-lit, high, and open areas. Models that address this conflict became known as 'approach-avoidance' tasks [6].

These anxiety models were created mainly to detect the anxiolytic effects of benzodiazepines, as they were the only successful marketed anxiolytic agents at the time [31]. The limitations of these models led to problems in detecting other anxiolytics such as buspirone and SSRI [6, 31, 36]. Animal models of anxiety such as the elevated plus maze, light-dark transition, and social interaction tests are not effective in detecting the anxiolytic-like effects of antidepressants [36]. There is concern that these classical anxiety tests will not detect novel anxiolytic medications [6].

2.2 **Methods**

2.2.1 Mice Living Conditions

80 white wild-type mice (43 male and 37 female) were used in this study. The mice were bred and raised at Louisiana Tech University from either Jackson Laboratories or Mutant Mouse Resource Center. The mice were housed in a 12 hour day/night cycle where food was administered *ad libitum*. Administration of drugs and behavioral test procedures were approved by the Louisiana Tech University Institutional Care and Use Committee and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996).

2.2.2 Drug Administration

The following drug administration techniques have been approved by the university's IACUC. 4 mg/mL propranolol HCl oral solution (strawberry-mint flavored) and 0.5 mg/mL diphenoxylate HCl with 0.005 mg/mL atropine sulfate oral solution

(cherry flavored) was administered via oral gavage using a flexible feeding tube. The mice were either given water, propranolol HCl, diphenoxylate HCl plus atropine sulfate, or both propranolol and diphenoxylate HCl plus atropine sulfate. The human equivalent dose (HED) of 40 mg propranolol HCl and the diphenoxylate HCl plus atropine sulfate HED of 5 mg and 0.05 mg, respectively, were given to the mice. The control group was administered water of equal volume via oral gavage. The body surface area (BSA) normalization method will be used to calculate the mouse dose from the HED by using **Eq. 2-1** [40].

$$\textbf{HED}\left(\frac{\text{mg}}{\text{kg}}\right) = \textbf{Mouse dose}\left(\frac{\text{mg}}{\text{kg}}\right) \times \frac{\textbf{Mouse km factor}}{\textbf{Human km factor}} \qquad \textbf{Eq. 2-1}$$

A table constructed by Reagan-Shaw *et al.* (2008) based upon data from the FDA Draft Guidelines will be used for the mouse and human (adult) km factors and for the weight of the human adult (**Table 2-1**).

$$\textbf{km factor} = \frac{\textbf{Body Weight (kg)}}{\textbf{Body Surface Area } (\text{m}^2)}$$

Table 2-1: Values based on data from FDA draft guidelines [40, 41].

Species	Weight (kg)	BSA (m^2)	*km* factor
Human			
Adult	60	1.6	37
Child	20	0.8	25
Baboon	12	0.6	20
Dog	10	0.5	20
Monkey	3	0.24	12
Rabbit	1.8	0.15	12
Guinea pig	0.4	0.05	8
Rat	0.15	0.025	6
Hamster	0.08	0.02	5
Mouse	0.02	0.007	3

To begin the administration of the oral solutions, a syringe was filled up with the appropriate amount of oral solution. Then a flexible disposable feeding tube was attached onto the end of a syringe filled with the solution. Any excess solution was wiped from the cannula. The length required for the feeding tube to reach the stomach was measured on the outside of the mouse by locating the last rib. The mouse was held firmly by the scruff to ensure that there was no head movement and in an upright position. The tube was then gently inserted into the mouth along the upper palate and down the esophagus to the stomach. The tubing was not be forced down. If the mouse struggled or there was any sign of respiratory distress, the tubing was immediately removed. To minimize reflux from the stomach, the solution was administered slowly. After dosing, the tubing was gently pulled until it was removed [42, 43]. The mouse was then placed into its home cage unless stated otherwise.

2.2.2.1 *HED calculation example.* To calculate the human equivalent dose for 40 mg of propranolol:

From Table 1, adult human = 60 kg, adult mouse Km = 3, adult human Km = 37

$$\text{HED}\left(\frac{40\text{ mg}}{60\text{ kg}}\right) = \text{Animal dose}\left(\frac{\text{mg}}{\text{kg}}\right) \times \frac{3}{37}$$

$$\text{Animal dose} = \left(\frac{40\text{ mg}}{60\text{ kg}}\right) \times \frac{37}{3} = 8.23\frac{\text{mg}}{\text{kg}}$$

Assuming mouse weight of 0.02 kg:

$$\text{Animal dose} = \left(\frac{40\ mg}{60\ kg}\right) \times \frac{37}{3} \times 0.02\ kg = 0.164\text{ mg}$$

Therefore, 41 μL of 4 mg/mL propranolol, or 41 μL of water for the control, would be administered to the mouse.

2.2.3 Behavior Tests

To evaluate the anxiety levels of the mice, six behavioral tests (the elevated plus maze test, the light/dark transition test, the open field test, 1-chamber social interaction test, 3-chamber social approach test, and rat exposure test) were performed. All mice were transported to the room at least 30 minutes prior to the experiment. The experiment was conducted 1 hour after the doses were administered to mice, unless stated otherwise. All behavioral tests have been approved by the university's IACUC.

2.2.3.1 *Elevated plus maze.* The apparatus that was used for the elevated plus maze test had a flat platform in the shape of a plus sign that was 50 cm off of the ground (Figure 2-1). Two opposing arms of the plus sign were enclosed with a 25 cm wall, while the other two arms were open. To begin the test, a mouse was placed in the center area of the maze with its head directed toward the north closed arm. The mouse was allowed to move freely about the maze for 10 min. The movement of the mouse was then tracked and recorded with a camera mounted to the ceiling. The platform of the maze was black to provide color contrast in order to track the white wild-type mice. The public domain ImageJ 1.47t program developed by Wayne Rasband at the National Institute of Mental Health along with plugin called ImageEP developed by Tsuyoshi Miyakawa was utilized to perform video analysis. The distance traveled, the number of entries into each arm, the time spent in each arm, and the percent of entries into the open arms are calculated by the ImageEP program as well as the generation of traces of the mouse's movement (Figure 2-2 and Figure 2-3).The distance traveled measurement served to detect if locomotion

was reduced due to the medication, thus skewing results. The percentage of time spent in the open arm and the percent of entries into the open arms was used to determine the anxiety levels of the mice. The results of the experimental groups were compared to the sham mouse and any significant differences were noted. Based on the exploratory-anxiety conflict, the more anxious the mouse, the lower the percentage of time the mouse will spend in the open arm and lower the amount of entries the mouse will make in the open arm. Between trials, all arms and the center area was cleaned with super hypochlorous water to remove odors left by the previous mouse [3]. Mice ranging in age from 6-9 weeks were used.

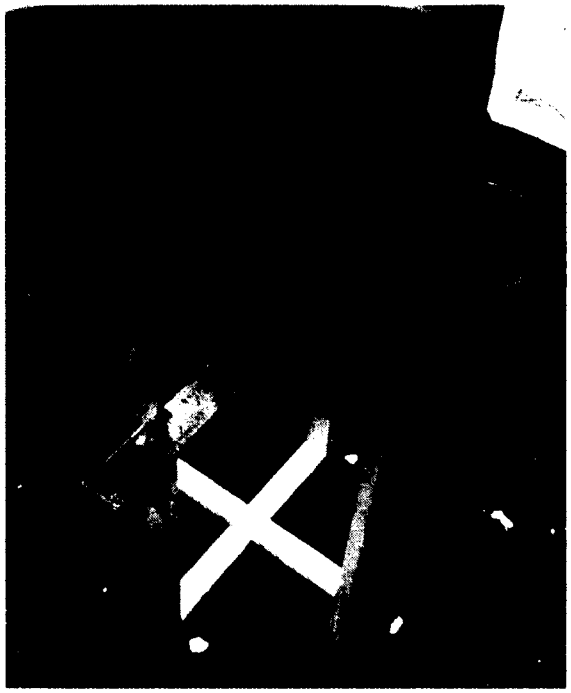

Figure 2-1: Elevated plus maze.

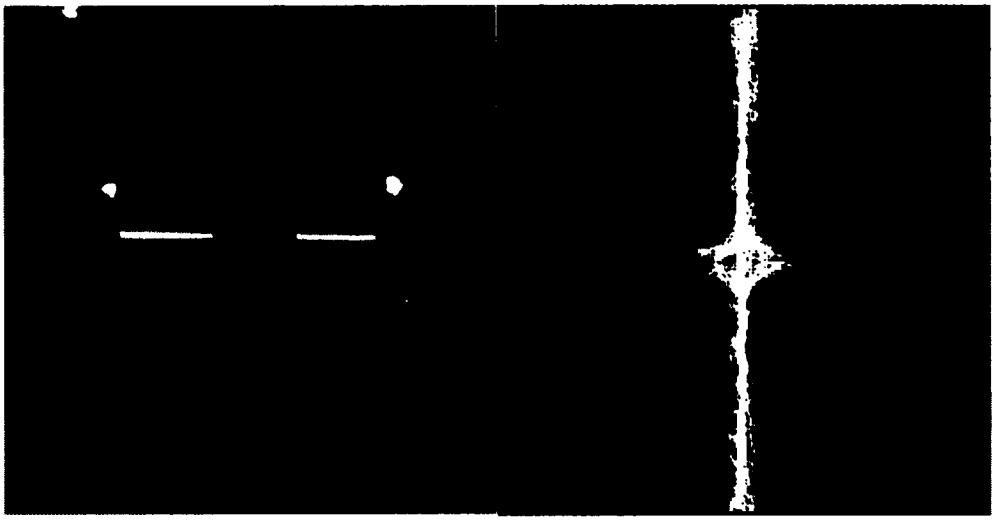

Figure 2-2: Overhead view of the elevated plus maze and the corresponding centroid trace of a typical mouse's path.

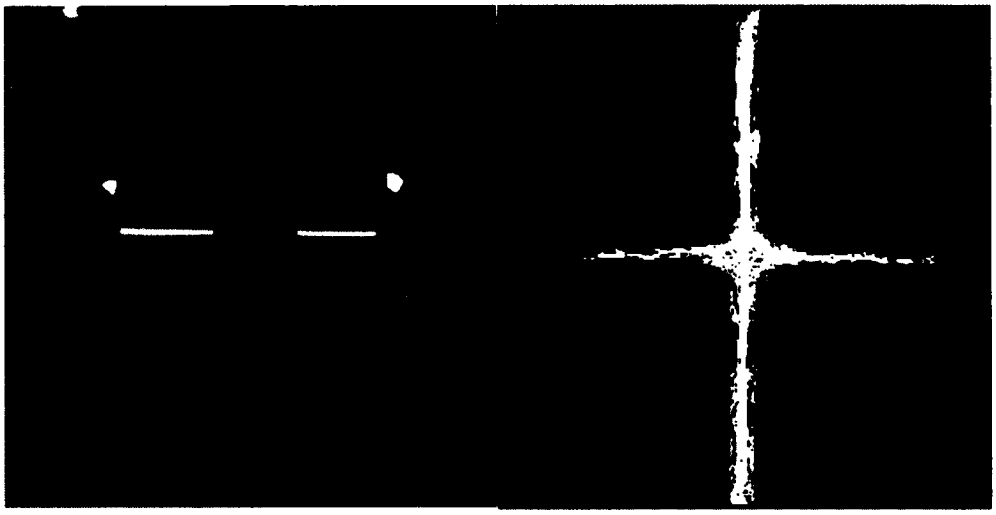

Figure 2-3: Overhead view of the elevated plus maze and the corresponding centroid trace of an adventurous mouse's path.

2.2.3.2 *Light/dark transition test.* For the light/dark transition test, a box comprised of two equally sized rooms (a dark room and a brightly light room) was used to examine the anxiety levels of the mice **Figure 2-4**. Between the rooms, there was an opening with a removable partition that allowed the mouse to move between the rooms. The interior of each chamber was 20 cm by 20 cm with a height of 25 cm. The dark room has a lid covering the top while there is an opening for the light room. The width of the opening was 5 cm wide with a height of 3 cm. The light/dark transition box was composed of medium density fiberboard (MDF), which acts as a sound insulator. The partition door was controlled by a linear actuator that was activated by a limit switch when the lid of the dark chamber closes. A camera was used to record the movement of the mice in the brightly lit room. To begin the test, a mouse was placed inside the dark room and the lid was closed hitting a limit switch **Figure 2-5**. After 3 seconds, the partition wall automatically moves to provide an opening for the mouse to move between the rooms. The movement of the mouse was recorded for 10 minutes. Then the mouse was removed and return to its home cage. The rooms were then cleaned with super hypochlorous water before starting the next trial [44]. Using video analysis, the percentage of time the mouse spends in the light room was measured and compared to the sham mice. Based on the exploratory-anxiety conflict, the more anxious the mouse, the lower the percentage of time the mouse will spend in the light room. Mice ranging in age from 6-9 weeks were used.

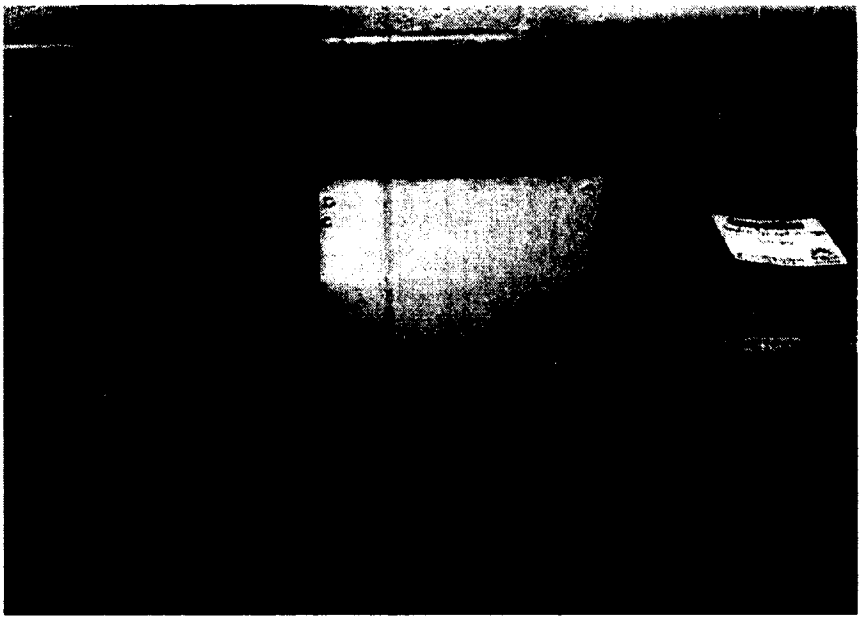

Figure 2-4: Light and dark transition test apparatus.

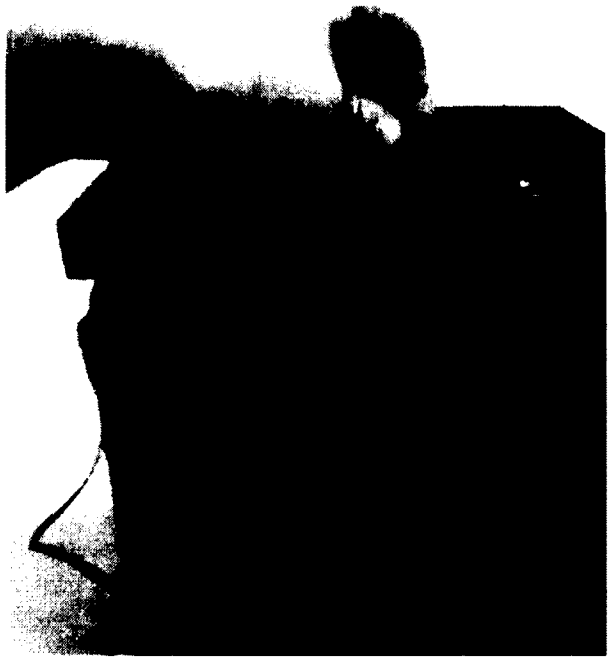

Figure 2-5: The lid and limit switch interaction on the light and dark transition test apparatus.

2.2.3.3 *Open field test.* Four month old mice were used. The weight of the male mice (n=9 control, n = 12 combination, n=9 propranolol, n=13 diphenoxylate) ranged from 23.1-51.1 g. The weight of the female mice (n=11 control, n=8 combination, n=11 propranolol, n=7 diphenoxylate) ranged from 17.6-42.5 g. The volume of administration ranged from 20-50 μL following BSA method of dosage.

The open field test apparatus consisted of an open square wooden container with a 25 cm walls enclosing the perimeter. The walls and floor of the container was spray painted black. On the floor of the container, a white 16 square grid was drawn. A camera mounted above the box and facing perpendicular to floor was used to record the movements of the mice for analysis. To begin the test, a mouse was placed on the peripheral regions within the container. The mouse was allowed to explore the container for 5 minutes before being removed and placed into its home cage. Between trials, super hypochlorous water was used to clean the floor and walls of the container [4]. Using the ImageOF plugin for ImageJ, the percentage of time the mouse spends in each region was measured and compared to the sham mice. Traces of the mouse's movements were also generated by ImageOF (**Figure 2-6**). Based on the exploratory-anxiety conflict, the more anxious the mouse, the lower the percentage of time the mouse will spend in the center region. Two investigators who were blinded to treatment conditions analyzed the videos and scored the frequency of rearing within the 4 squares at center of the chamber (middle 25% of the chamber).

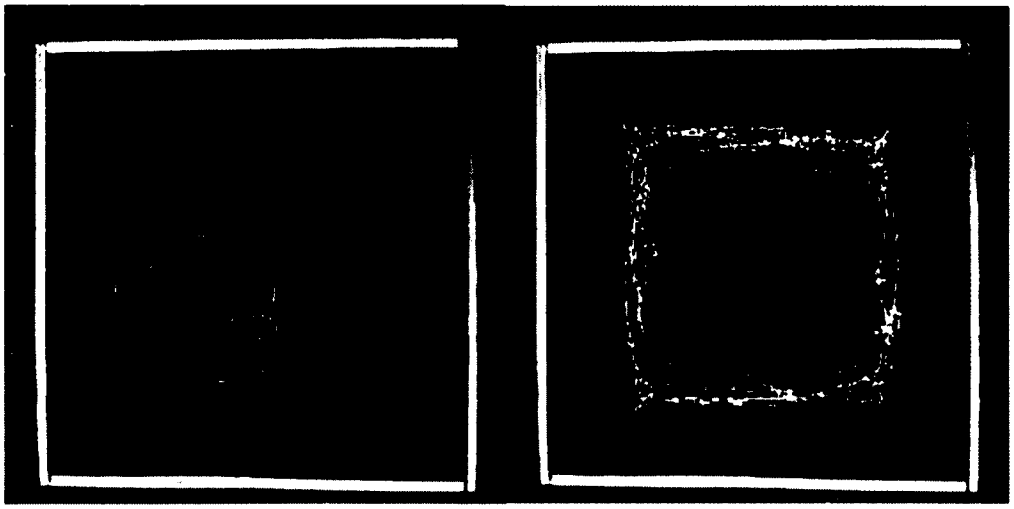

Figure 2-6: Overhead view of the open field apparatus and the corresponding centroid trace of a typical mouse's path.

2.2.3.4 *1-chamber social interaction test.* For the 1-chamber social interaction test, each mouse had a habituation session alone each day in the neutral cage for 10 minutes starting two days prior to the test day [45]. On test day, two mice from different home cages that are unfamiliar with one another were placed in the neutral cage for 10 minutes [45, 46]. Mouse tracking software along with a video camera was used to monitor the behavior of the mice [46, 47]. The time the mice spend in social interaction was measured. If the mice have less social anxiety, the idea is that they will spend more time in social interaction. The percentage of time the mice in the experimental groups spend in social interaction was compared to the sham mice.

2.2.3.5 *3-chamber social approach test*

The apparatus for the social approach test was comprised of three adjacent chambers (**Figure 2-7**). Removal partition doors were placed between the central chamber and the outside chambers. The outside chambers each contained an empty inverted wire cup in the center of the room. A video camera mounted above the apparatus

was used to record the experiment [48-50]. To begin testing, a mouse was placed in the middle chamber with both partition doors to the outside chambers open for a 10 minute habituation period. Then the doors were closed and the mouse was placed back in the central chamber. An unfamiliar mouse was placed in one of the empty inverted wire cups, which were held down by placing 500 g masses on the bases of the inverted cups. The doors were then opened for a 10 minute session. After the session was over, the mouse was placed back in the central room with both partition doors closed. A second unfamiliar mouse was placed in the opposing empty inverted wire cup. The doors were then opened again for an additional 10 minute session. Between trials all chambers was cleaned with super hypochlorous water [48-51].

Figure 2-7: Overhead view of the 3 chamber social interaction test

During both the first and second session the duration in each compartment was measured. The time the subject mouse spent in the chamber with the unfamiliar mouse during the first session was compared to the time spent in the chamber with the empty

cup. For the second session, the time the subject mouse spent in the chamber with the first unfamiliar mouse was compared to second unfamiliar mouse. Wild type mice with normal sociability, motivation, and affiliation will spend more time with the unfamiliar mouse than with the empty cup. The second session was used to estimate social novelty and social memory. Wild type mice will typically spend more time with the second unfamiliar mouse than with the first [49]. The behaviors of the mice in the experimental groups were compared to the sham mice and any significant differences were noted.

2.2.3.6 *Rat exposure test.* The apparatus for the rat exposure test consisted of an exposure chamber ($46 \times 24 \times 21\ cm$) with clear walls and a home chamber ($7 \times 7 \times 12\ cm$) with three opaque walls and one clear wall that were connected together by a clear cylindrical tunnel ($4.4\times 13\ cm$) as shown in **Figure 2-8**. The exposure chamber was divided in half by a wire mesh forming two rooms with equal dimensions ($21 \times 24 \times 21\ cm$). One of the rooms had a tunnel leading to the home chamber, whereas the other was enclosed. To begin the test, a mouse was placed in the room with the tunnel access for 10 minutes a day for 3 days to become habituated. On the fourth day, the mouse was placed in the exposure chamber in the room with tunnel access. The threat stimulus (a rat) was then immediately placed in the enclosed room. The reaction of the mouse was recorded with a video camera. Between trails the apparatus was cleaned with super hypochlorous water and wiped dry with paper towels [52]. Based on the outcomes of the 3-chamber social approach test, a second identical apparatus may be used in another identical room replacing the threat stimulus with a plush toy [52]. The amount of time the mouse spends in the home chamber, tunnel, and exposure chamber was measured. The percentage of time spent in the home chamber and tunnel would be

greater for mice that are afraid of the rat behind the metal mesh. The percentage of time spent in the exposure chamber would be greater for mice that are not afraid. The percentage of time the mice in the experimental groups spend in each compartment was compared to the sham mice and any significant differences were noted.

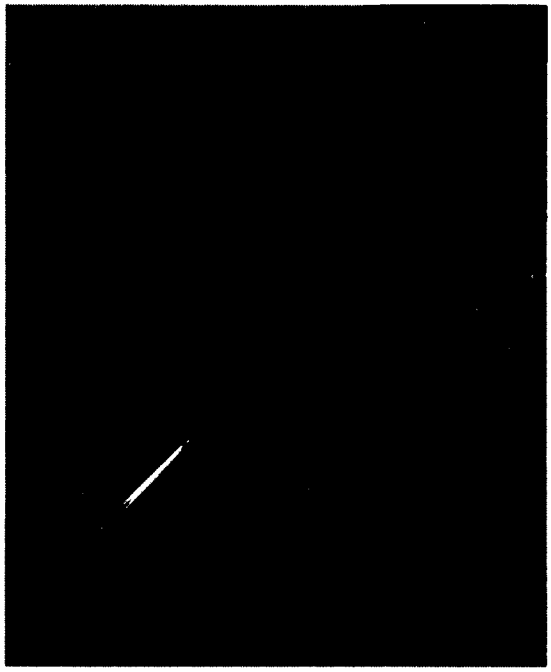

Figure 2-8: Photo of the rat exposure test apparatus.

2.2.3.7 *Cued fear conditioning test.* Mice were first conditioned to associate a cue noise to an electric shock. A mouse was placed in an acrylic rectangular chamber (33 cm x 25 cm x 28 cm) with flooring composed of 0.2 cm diameter steel rods spaced 0.5 cm apart. After 120 seconds a 55 dB auditory cue was played for 30 seconds. During the last 2 seconds of the cue, a 0.5 mA foot shock was delivered to the mouse using a Coulbourn Instruments Precision Regulated Animal Shocker [53]. After a 90 second break, the auditory cue and foot shock was given in the same manner for a second time. This was repeated a third time following another 90 second break after the second shock. After the

third shock, the mouse was left undisturbed in the chamber for 90 seconds and then returned to its home cage [54]. The apparatus was then cleaned with super hypochlorous water, except for the metal grid which was cleaned with 70% ethanol solution.

On the second day, the mice were placed in the fear conditioning chamber without the unconditioned stimulus (electric shock) being presented. However, the condition stimulus (the audible noise) was still presented in 30 second intervals as mentioned above. The mice were recorded with a video camera for 5 minutes and during this duration the amount of time the mice spent freezing was measured. An ImageJ plugin, ImageFZ [54], was utilized to perform video analysis. On the third day, the same procedure was followed as the previous day.

2.2.3.8 *Data analysis.* Using SPSS software, one-way ANOVAs were performed between treatment groups for the behavioral measurements in the OF, EPM, and 3-chamber social interaction test.

2.3 Results

2.3.1 Open Field

2.3.1.1 *Open field total center time.* The time the male and female wild-type mice spent within the center (inner 25% area) of the open field was measured after being given water (n = 9, male; n = 11, female), propranolol (40 mg HED, n = 9, male; n = 11, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 12, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 13, male; n = 8, female) (**Figure 2-9**). There was no significant difference between treatment groups for neither the male or female mice.

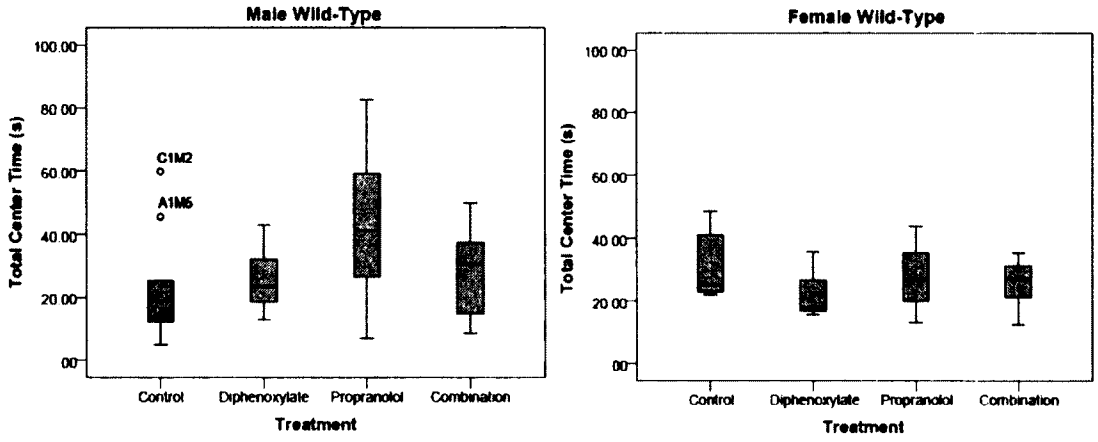

Figure 2-9: The time spent within the center (inner 25% area) of chamber for male and female wild-type mice after being given water (n = 9, male; n = 11, female), propranolol (40 mg HED, n = 9, male; n = 11, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 12, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 13, male; n = 8, female). ($p>0.05$).

The time the male Swiss mice spent within the center (inner 25% area) of the open field was measured after being given water (n = 10), propranolol (40 mg HED, n = 10), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 10), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 11) (**Figure 2-10**). There was no significant difference between treatment groups.

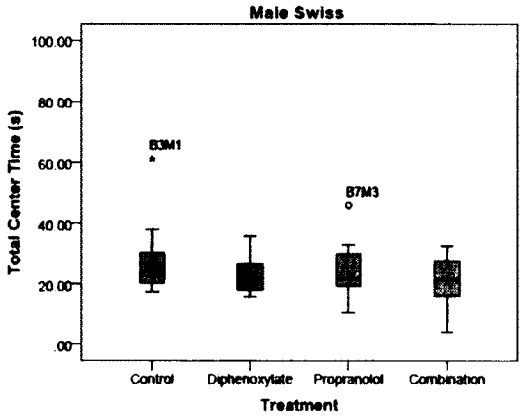

Figure 2-10: The time male Swiss mice spent within the center (inner 25% area) of open field after being given water (n = 10), propranolol (40 mg HED, n = 10), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 10), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 11). ($p>0.05$).

Combining the Swiss male mice with the wild type mice, we obtain **Figure 2-11**. There was no significant difference in total time spent in the center of the open field between treatment groups for both male and female mice ($p>0.05$). In contrast, Propranolol increased entries into in the central region of a circular open field in rats [55]. Stone *et al.* found that L-propranolol inhibited stress-induced increases in open field emergence in mice [56]. Similarly, Benton *et al.* found that naloxone did not alter the time albino mice spent in the center of an open field [57].

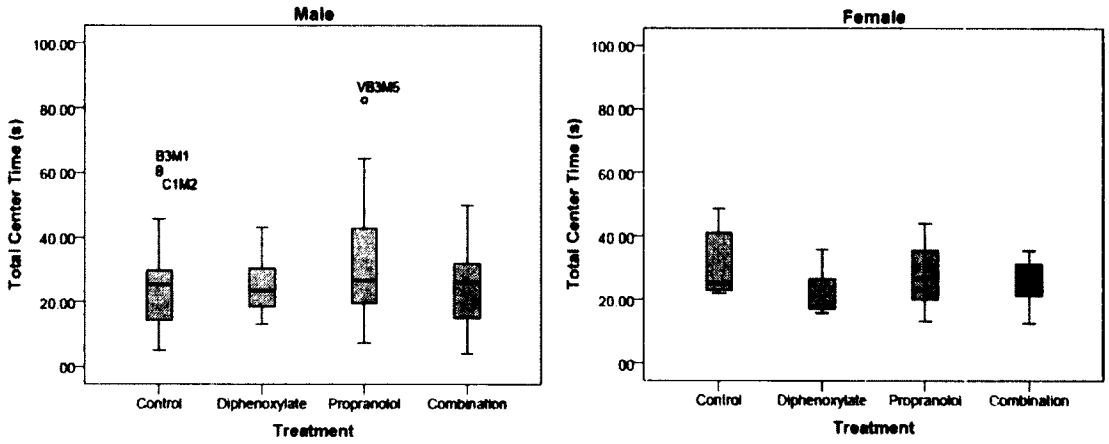

Figure 2-11: The time spent within the center (inner 25% area) of chamber for male and female wild-type mice after being given water (n = 9, male; n = 11, female), propranolol (40 mg HED, n = 9, male; n = 11, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 12, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 13, male; n = 8, female). ($p>0.05$).

2.3.1.2 Open *field rearing frequency.* The rearing frequency of the mice in the center (the 4 inner squares) of the open chamber observed during the same experiment for male and female wild-type mice after being given water, propranolol (40 mg HED), diphenoxylate with atropine (5 mg and 0.05 mg HED), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED) is shown in **Figure 2-12.**

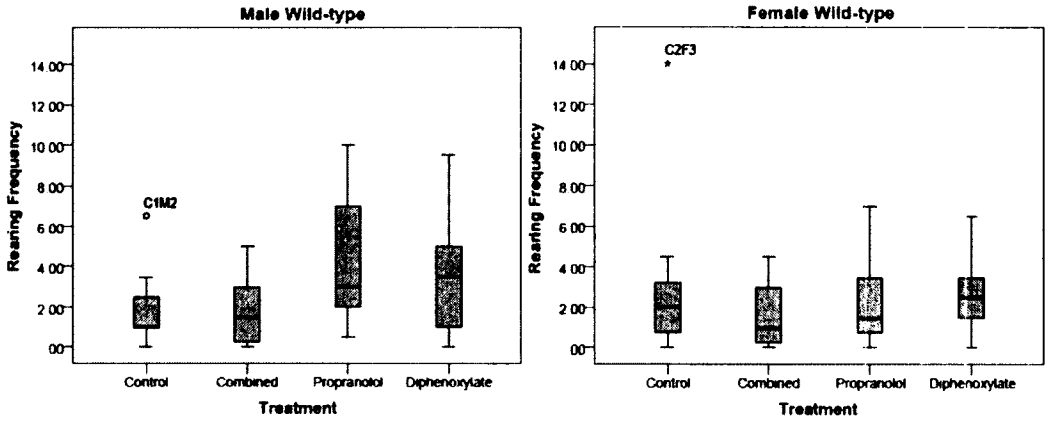

Figure 2-12: The rearing frequency of the mice in the center (the 4 inner squares) of the open chamber observed during the same experiment for male and female wild-type mice after being given water, propranolol (40 mg HED), diphenoxylate with atropine (5 mg and 0.05 mg HED), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED). ($p>0.05$).

A one-way ANOVA was conducted to determine if the rearing frequency of male mice in the open field was different for the different dosage treatments. Participants were classified into four groups: control (n = 9), combined (n = 12), propranolol (n = 9) and diphenoxylate (n = 13). There was one outlier found in the control group (C1M2). This outlier was replaced with the value of the next largest value found within the control group. The data was normally distributed for each group, as assessed by Shapiro-Wilk test ($p > 0.05$); but there was heterogeneity of variances, as assessed by Levene's test of

homogeneity of variances ($p = 0.019$). Data is presented as mean ± SEM. The rearing frequency of the mice was higher for propranolol (4.3 ± 1.2 per 5 minutes) and diphenoxylate (3.4 ± 0.8 per 5 minutes) in comparison to the control (1.8 ± 0.4 per 5 minutes) and combined (1.8 ± 0.5 per 5 minutes). The differences between the groups were not statistically significant, Welch's $F(3, 39) = 2.444$, $p = 0.078$.

For the female mice, a one-way ANOVA was conducted to determine if there were differences in rearing frequency within the open field between the different treatments. Participants were classified into four groups: control ($n = 11$), combined ($n = 8$), propranolol ($n = 11$) and diphenoxylate ($n = 7$). There was one extreme outlier found in the control group (C2F3). This outlier was replaced with the value of the next largest value found within the control group. The data was normally distributed for each group, as assessed by Shapiro-Wilk test ($p > 0.05$); and there was homogeneity of variances, as assessed by Levene's test of homogeneity of variances ($p = 0.746$). Data are presented as mean ± SEM. There was no difference among the control (2.2 ± 0.5 per 5 minutes), combined (1.6 ± 0.6 per 5 minutes), propranolol (2.5 ± 0.7 per 5 minutes), and diphenoxylate (2.7 ± 0.8 per 5 minutes). The differences between the treatment groups was not statistically significant, $F(3, 33) = 0.423$, $p = 0.738$.

2.3.1.3 *Open field total distance.* The distance travelled by the male and female wild type mice in the open field was measured after being given water (n = 9, male; n = 11, female), propranolol (40 mg HED, n = 9, male; n = 11, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 12, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 13, male; n = 8, female) as shown in **Figure 2-13**. There was no significant difference between treatment groups (p>0.05).

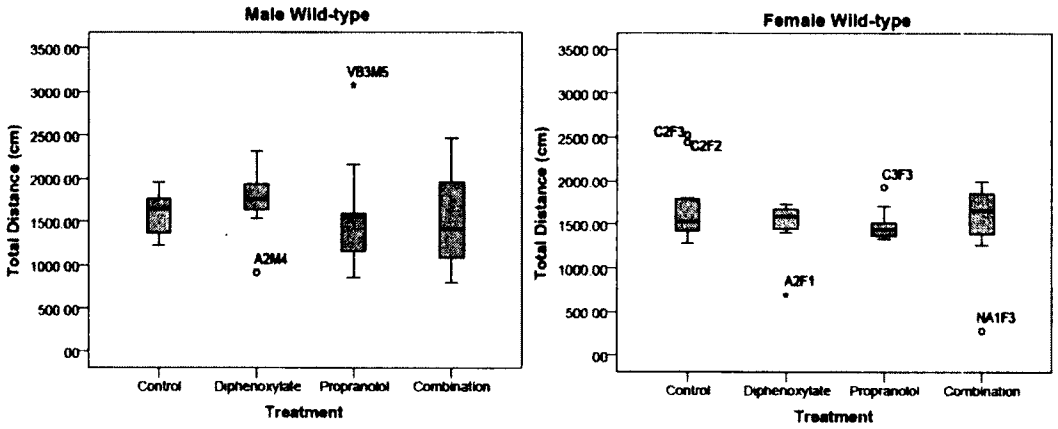

Figure 2-13: The distance travelled by the mice in the open field chamber for (a) male and (b) female wild type mice after being given water (n = 9, male; n = 11, female), propranolol (40 mg HED, n = 9, male; n = 11, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 12, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 13, male; n = 8, female). There was no significant difference between the treatment groups (p>0.05).

Figure 2-14 shows the distance travelled by the male Swiss mice in the open field chamber for after being given water (n = 10), propranolol (40 mg HED, n = 10), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 10), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 11). There was no significant difference between the treatment groups ($p>0.05$).

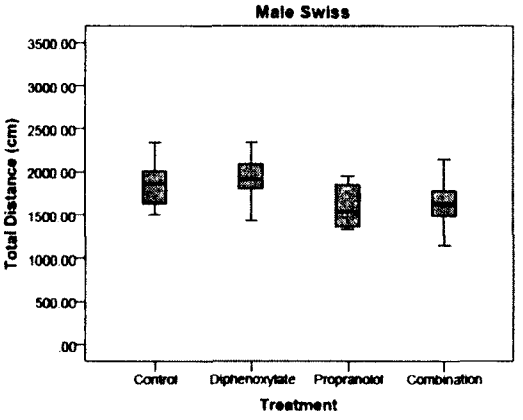

Figure 2-14: The distance travelled by the male Swiss mice in the open field chamber for after being given water (n = 10), propranolol (40 mg HED, n = 10), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 10), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 11). There was no significant difference between the treatment groups ($p>0.05$).

2.3.1.4 *Open field moving speed.* **Figure 2-15** shows the moving speed of the wildtype mice in the open field for male and female mice after being given water (n = 9, male; n = 11, female), propranolol (40 mg HED, n = 9, male; n = 11, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 12, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 13, male; n = 8, female).

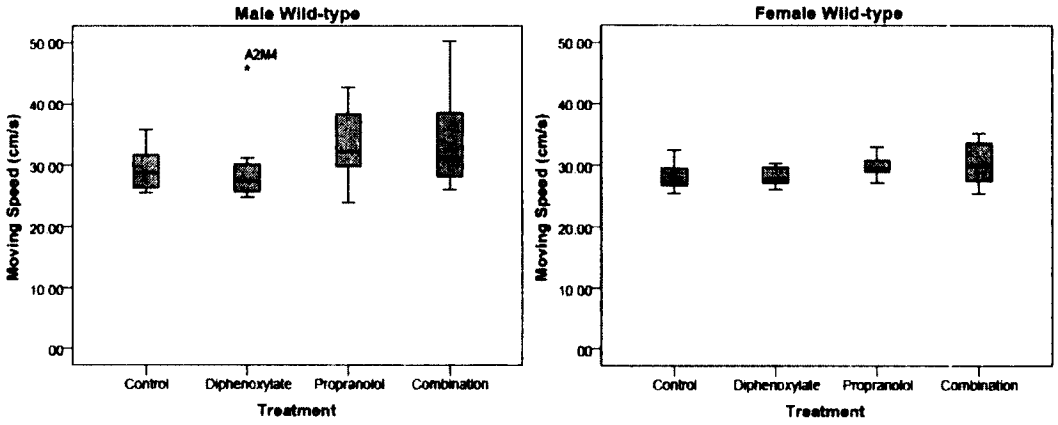

Figure 2-15: The moving speed of the wildtype mice in the open field for male and female mice after being given water (n = 9, male; n = 11, female), propranolol (40 mg HED, n = 9, male; n = 11, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 12, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 13, male; n = 8, female). There was no significant difference between the treatment groups ($p>0.05$).

Figure 2-16 shows the moving speed of the male Swiss mice in the open field after being given water (n = 10), propranolol (40 mg HED, n = 10), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 10), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 11). There was no significant difference between the treatment groups (p>0.05).

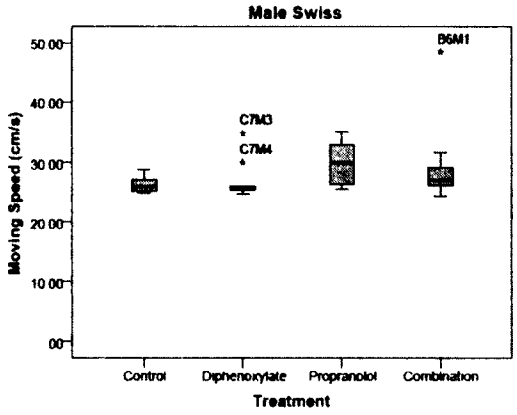

Figure 2-16: The moving speed of the male Swiss mice in the open field after being given water (n = 10), propranolol (40 mg HED, n = 10), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 10), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 11). (p>0.05).

2.3.1.5 *Open* field *stretch-attend posture.* **Figure 2-17** shows the percentage of SAP in the open field for male and female mice after being given water (n = 9, male; n = 11, female), propranolol (40 mg HED, n = 9, male; n = 11, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 12, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 13, male; n = 8, female). There was no significant difference between the treatment groups ($p>0.05$).

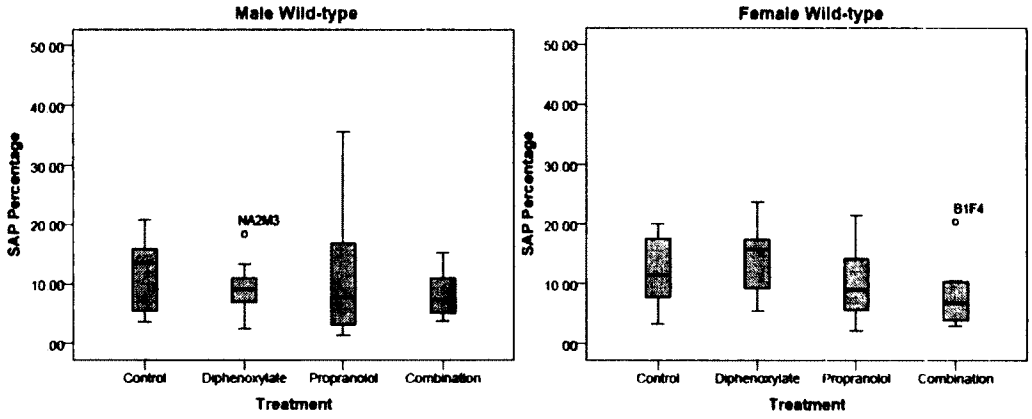

Figure 2-17: The percentage of SAP in the open field for male and female mice after being given water (n = 9, male; n = 11, female), propranolol (40 mg HED, n = 9, male; n = 11, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 12, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 13, male; n = 8, female). ($p>0.05$).

The percentage of SAP expressed by the male Swiss mice in the open field was calculated for after being given water (n = 10), propranolol (40 mg HED, n = 10), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 10), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 11) (**Figure 2-18**). There was no significant difference between the treatment groups ($p>0.05$).

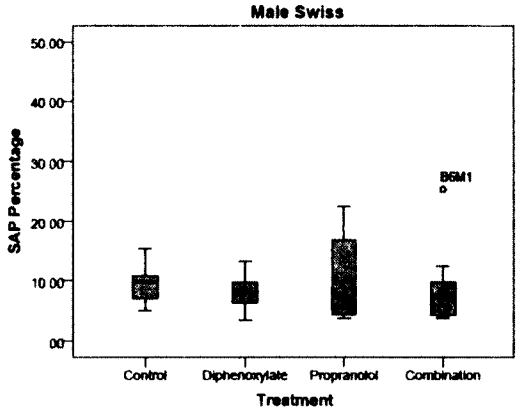

Figure 2-18: The percentage of SAP expressed by the male Swiss mice in the open field chamber for after being given water (n = 10), propranolol (40 mg HED, n = 10), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 10), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 11). ($p>0.05$).

Figure 2-19 shows the frequency of SAP in the open field for male and female mice after being given water (n = 9, male; n = 11, female), propranolol (40 mg HED, n = 9, male; n = 11, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 12, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 13, male; n = 8, female). There was no significant difference between the treatment groups ($p>0.05$).

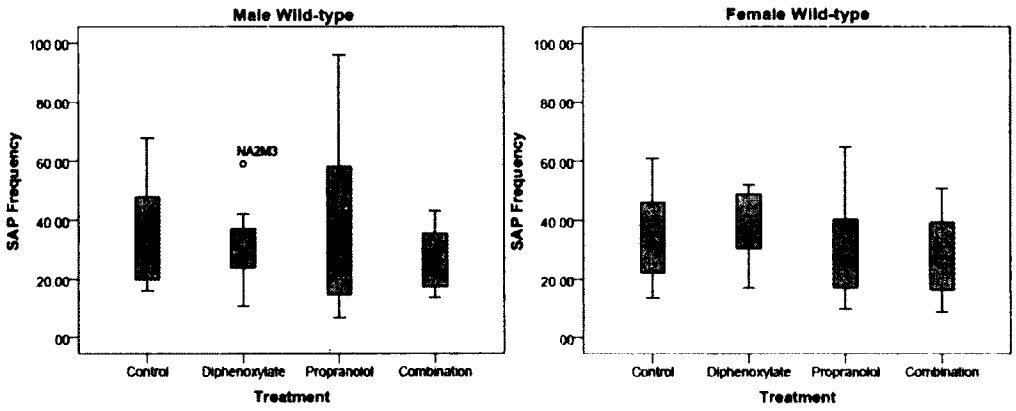

Figure 2-19: The frequency of SAP in the open field for male and female mice after being given water (n = 9, male; n = 11, female), propranolol (40 mg HED, n = 9, male; n = 11, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 12, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 13, male; n = 8, female). ($p>0.05$).

The frequency of SAP expressed by the male Swiss mice in the open field chamber for after being given water (n = 10), propranolol (40 mg HED, n = 10), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 10), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 11). ($p>0.05$) is shown in **Figure 2-20**.

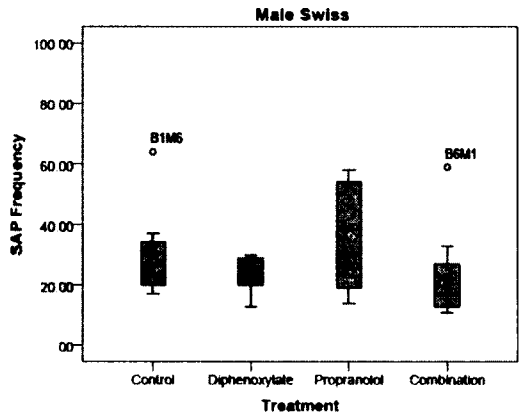

Figure 2-20: The frequency of SAP expressed by the male Swiss mice in the open field chamber for after being given water (n = 10), propranolol (40 mg HED, n = 10), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 10), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 11). ($p>0.05$).

2.3.2 Elevated Plus Maze

2.3.2.1 *Percentage of time in open arms.* As shown in **Figure 2-21**, the percentage of time the male and female wild type mice spent in the open arms was measured after the mice were given water (n = 6, male; n = 7, female), propranolol (40 mg HED, n = 10, male; n = 7, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 8, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 7, male; n = 7, female). There was no statistically significant difference between the treatment groups ($p>0.05$).

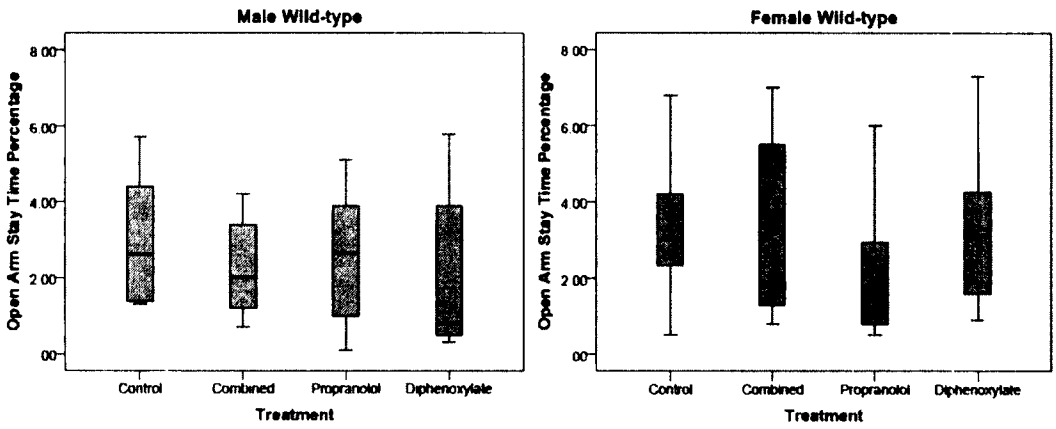

Figure 2-21: The percentage of time spent in the open arms after being given water (n = 6, male; n = 7, female), propranolol (40 mg HED, n = 10, male; n = 7, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 8, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 7, male; n = 7, female) for male and female wild-type mice. There was no statistically significant difference between the treatment groups ($p>0.05$).

2.3.2.2 *Percentage of open arm entries.* **Figure 2-22** shows the percentage of open arm entries the male and female wild-type mice made after being given water, propranolol (40 mg HED), diphenoxylate with atropine (5 mg and 0.05 mg HED), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED). There was no statistically significant difference between the treatment groups (α=0.5).

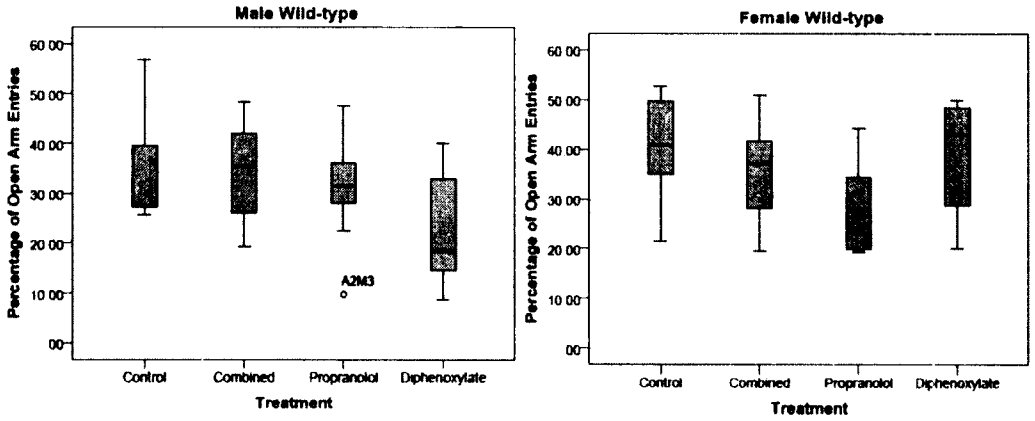

Figure 2-22: The percentage of open arm entries after being given water, propranolol (40 mg HED), diphenoxylate with atropine (5 mg and 0.05 mg HED), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED) for male and female wild-type mice. There was no statistically significant difference between the treatment groups (α=0.5).

2.3.2.3 *Total distance.* The total distance the male and female wild-type mice traveled within the elevated plus maze was calculated after the mice were given water (n = 6, male; n = 7, female), propranolol (40 mg HED, n = 10, male; n = 7, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 8, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 7, male; n = 7, female) as shown in **Figure 2-23**. There was no statistically significant difference between the treatment groups (α=0.5).

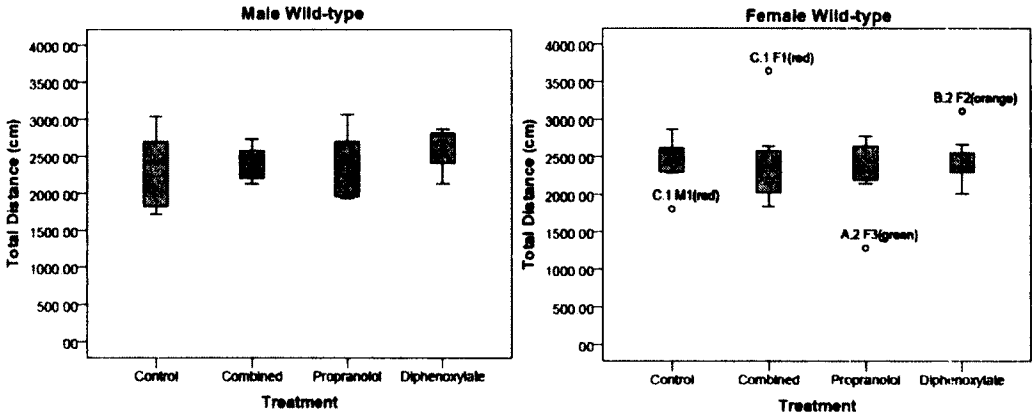

Figure 2-23: Total distance traveled in the elevated plus maze after being given water (n = 6, male; n = 7, female), propranolol (40 mg HED, n = 10, male; n = 7, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 8, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 7, male; n = 7, female) for male and female wild-type mice. There was no statistically significant difference between the treatment groups (α=0.5).

2.3.2.4 *Total entries.* As shown in **Figure 2-24** the total number of entries the male and female wild-type mice took between the arms and center area was measured after being given water (n = 6, male; n = 7, female), propranolol (40 mg HED. n = 10, male; n = 7, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 8, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 7, male; n = 7, female). There was no statistically significant difference between the treatment groups (α=0.5).

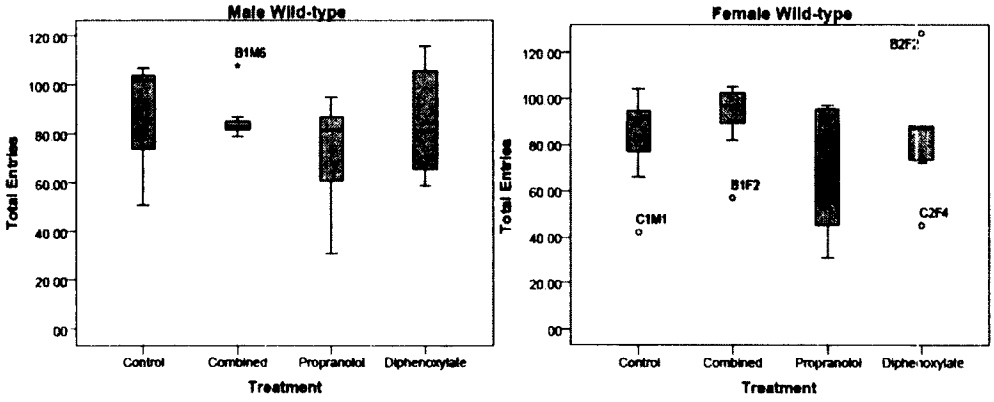

Figure 2-24: Total entries between arms and center area after being given water (n = 6, male; n = 7, female), propranolol (40 mg HED, n = 10, male; n = 7, female), diphenoxylate with atropine (5 mg and 0.05 mg HED, n = 8, male; n = 7, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED, n = 7, male; n = 7, female) for male and female wild-type mice. There was no statistically significant difference between the treatment groups (α=0.5).

2.3.3 3-Chamber Social Interaction

2.3.3.1 *3-chamber social interaction stage 1.* **Figure 2-25** shows the percentage of time the male and female mice spent with the empty chamber during the first session after being given water, propranolol (40 mg HED), diphenoxylate with atropine (5 mg and 0.05 mg HED), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED). There was no significant difference between treatment groups for both male and female mice ($p>0.05$).

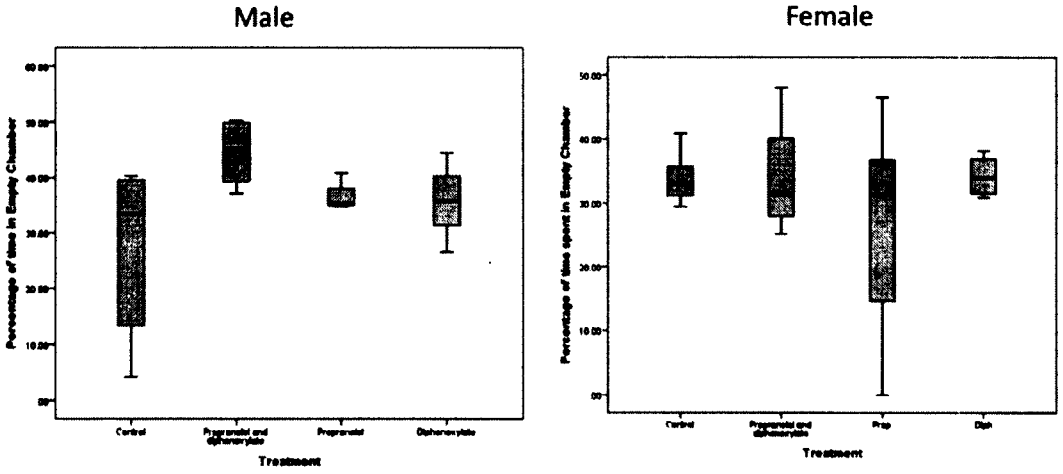

Figure 2-25: The percentage of time the male and female mice spent with the empty chamber during the first session after being given water, propranolol (40 mg HED), diphenoxylate with atropine (5 mg and 0.05 mg HED), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED). There was no significant difference between treatment groups for both male and female mice ($p>0.05$).

Figure 2-26 shows the percentage of time the male and female mice spent with the unfamiliar mouse during the first session after being given water, propranolol (40 mg HED), diphenoxylate with atropine (5 mg and 0.05 mg HED), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED). There was no significant difference between treatment groups for both male and female mice ($p>0.05$).

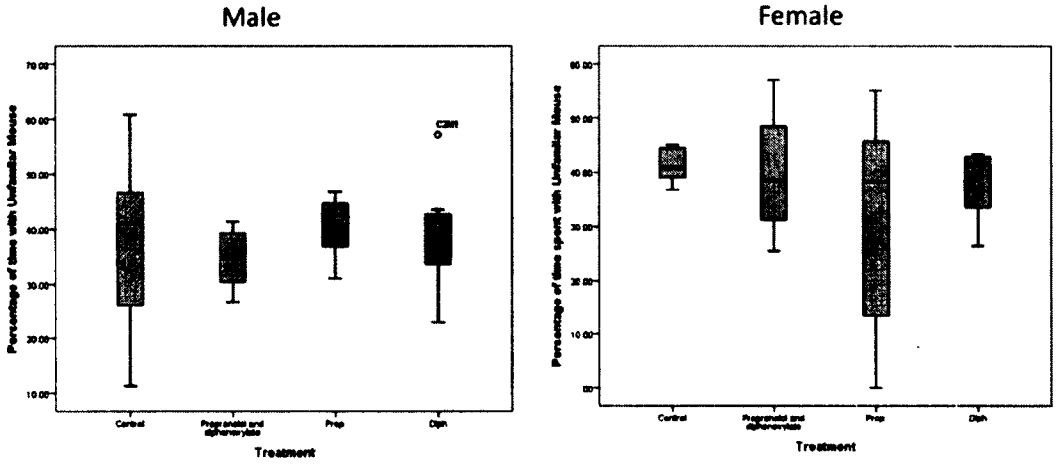

Figure 2-26: The percentage of time the male and female mice spent with the unfamiliar mouse during the first session after being given water, propranolol (40 mg HED), diphenoxylate with atropine (5 mg and 0.05 mg HED), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED). There was no significant difference between treatment groups for both male and female mice ($p>0.05$).

2.3.3.2 *3-chamber social interaction stage 2.* After being given water, propranolol (40 mg HED), diphenoxylate with atropine (5 mg and 0.05 mg HED), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED), the percentage of time the male and female mice spent with the first unfamilar mouse during the second session was measured (**Figure 2-27**). There was no significant difference between treatment groups for both male and female mice ($p>0.05$).

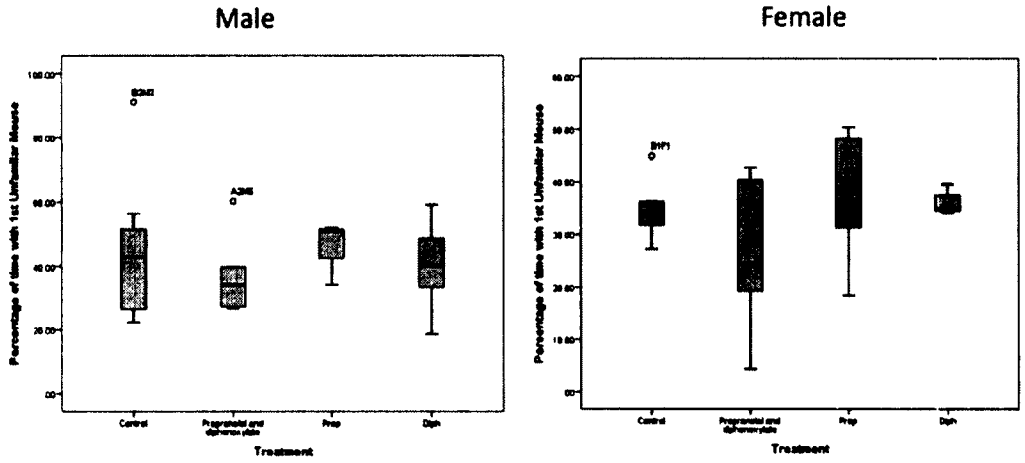

Figure 2-27: The percentage of time the male and female mice spent with the first unfamilar mouse during the second session after being given water, propranolol (40 mg HED), diphenoxylate with atropine (5 mg and 0.05 mg HED), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED). There was no significant difference between treatment groups for both male and female mice ($p>0.05$).

After being given water, propranolol (40 mg HED), diphenoxylate with atropine (5 mg and 0.05 mg HED), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED), the percentage of time the male and female mice spent with the new unfamilar mouse during the second session was measured (**Figure 2-28**). There was no significant difference between treatment groups for both male and female mice ($p>0.05$).

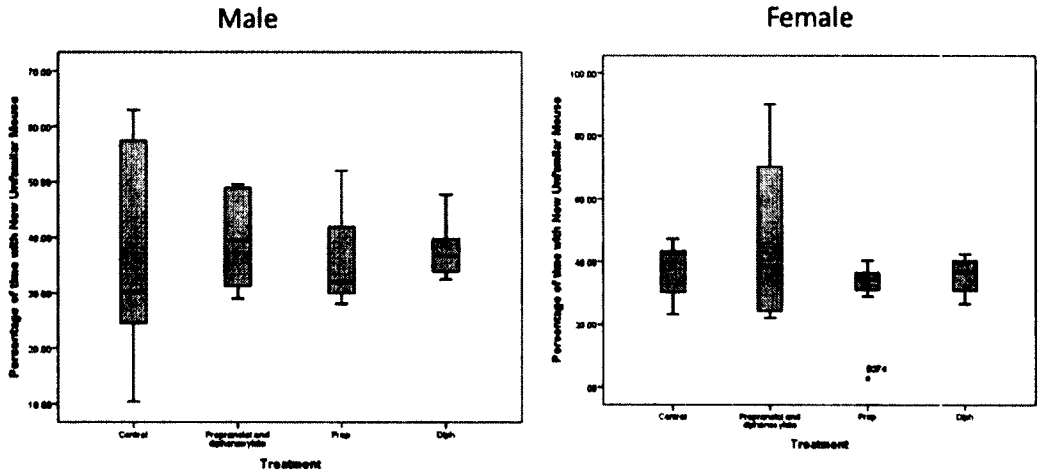

Figure 2-28: The percentage of time the male and female mice spent with the new unfamiliar mouse during the second session after being given water, propranolol (40 mg HED), diphenoxylate with atropine (5 mg and 0.05 mg HED), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED). There was no significant difference between treatment groups for both male and female mice ($p>0.05$).

2.3.1 Light/Dark Transition Test

Figure 2-29 shows the percentage of time the mouse spent in the light chamber of the light and dark transition apparatus after the mouse was given water (n=7, male; n= 7, female), propranolol (40 mg HED; n=9, male; n= 7, female), diphenoxylate with atropine (5 mg and 0.05 mg HED; n=5, male; n= 8, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED; n=7, male; n= 7, female). The average percentage of time spent in the light chamber for the control, propranolol, diphenoxylate, and combination groups were 55.7 ± 3.3%, 51.3 ± 2.4%, 45.3 ± 3.3%, and 43.8 ± 3.6% (SEM), respectively. There was homogenous of variance between the groups based on the Levene's test. A one-way ANOVA test revealed there were no significant differences between the treatment groups (p=0.06).

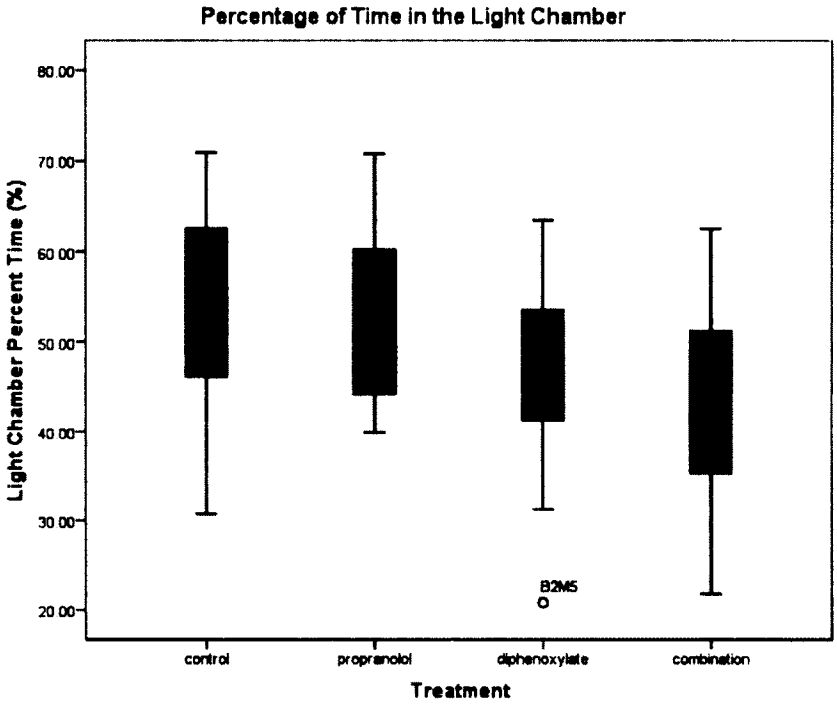

Figure 2-29: The percentage of time the mouse spent in the light after being given water (n=7, male; n= 7, female), propranolol (40 mg HED; n=9, male; n= 7, female), diphenoxylate with atropine (5 mg and 0.05 mg HED; n=5, male; n= 8, female), or the combination of propranolol and diphenoxylate with atropine (40 mg and 5 mg with 0.05 mg HED; n=7, male; n= 7, female). There was no significant difference between the treatment groups ($p>0.05$).

2.3.2 Fear Conditioning Test

The percentage of time the mouse spent freezing on Day 2 of the fear conditioning test after being given water or propranolol (40 mg HED) is shown in **Figure 2-30**. Time was broken down into 16 bins each covering 30 seconds. There was no significant difference between the control (n=8) and propranolol (n=6) treatment groups during the 85 decibel white noise stimulation (bins 5, 9, and 13) ($p>0.05$).

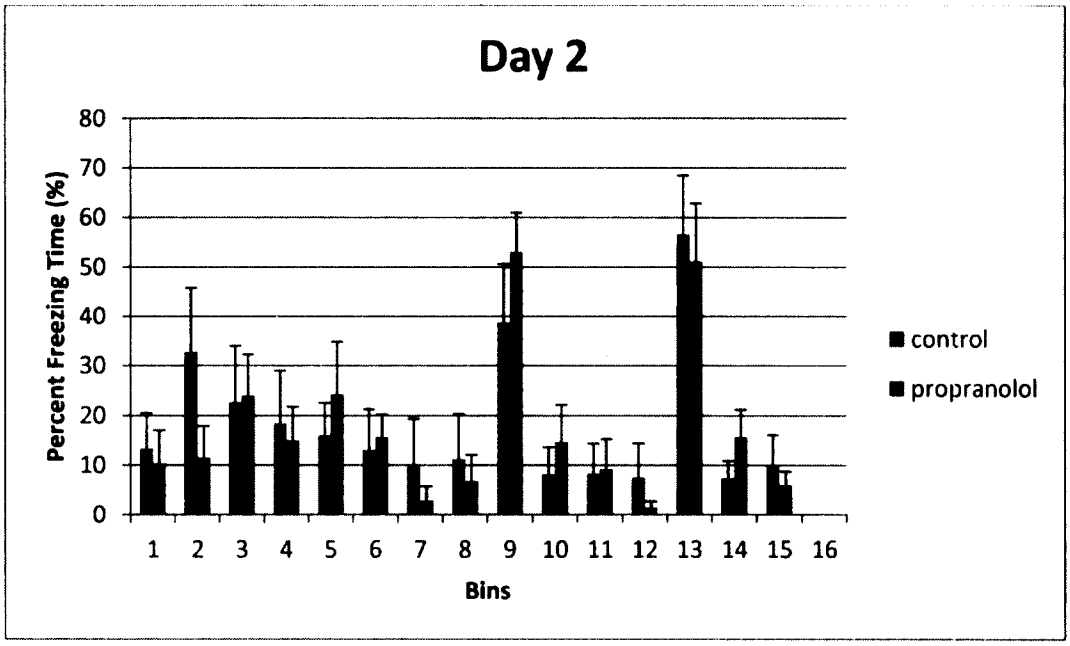

Figure 2-30: The percentage of time the mouse spent freezing in each of the 16 bins (30 seconds) for Day 2 after being given water or propranolol (40 mg HED). Loud speakers exhibited 85 decibel white noise during bins 5, 9, and 13. There was no significant difference between the control (n=8) and propranolol (n=6) treatment groups ($p>0.05$).

The percentage of time the mouse spent freezing on Day 3 of the fear conditioning test after being given water or propranolol (40 mg HED) is shown in **Figure 2-31**. Time was broken down into 16 bins each covering 30 seconds. There was no significant difference between the control (n=8) and propranolol (n=8) treatment groups during the 85 decibel white noise stimulation (bins 5, 9, and 13) ($p>0.05$).

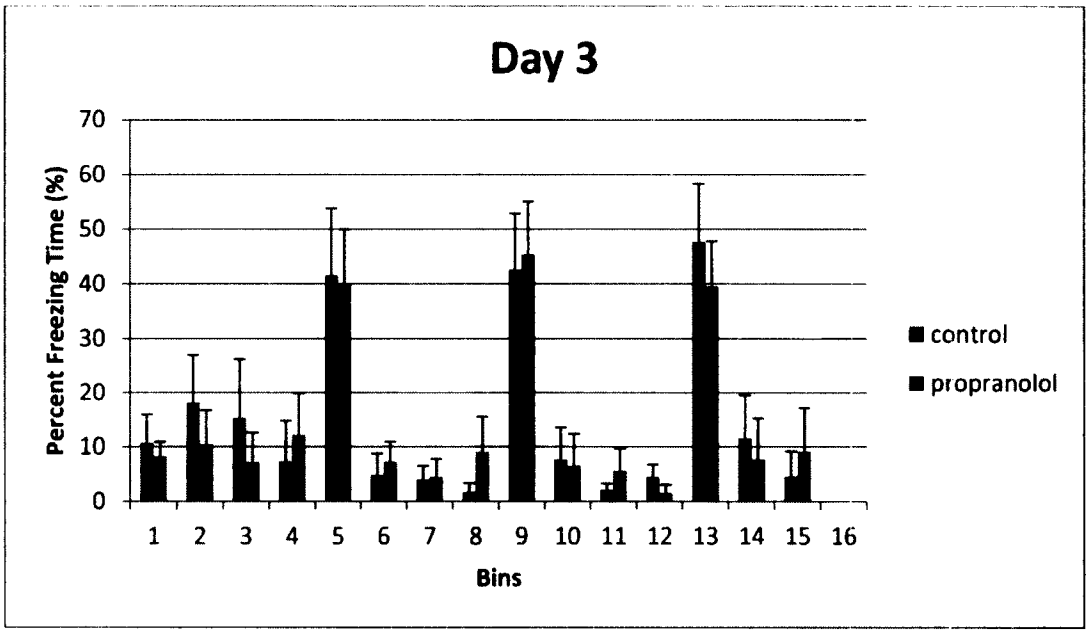

Figure 2-31: The percentage of time the mouse spent freezing in each of the 16 bins (30 seconds) for Day 3 after being given water or propranolol (40 mg HED). Loud speakers exhibited 85 decibel white noise during bins 5, 9, and 13. There was no significant difference between the control (n=8) and propranolol (n=8) treatment groups ($p>0.05$).

2.4 Discussion

2.4.1 Open Field

There was no significant difference in spatiotemporal measures between the experimental groups. In contrast, Stone *et al.* found that L-propranolol inhibited stress-induced increases in open field emergence in mice [56]. This study supports that propranolol has no effect on open field emergence in mice when stress is not induced. Unlike in mice, propranolol increased entries into in the central region of a circular open field in rats [55].

Benton *et al.* found that naloxone did not alter the time albino mice spent in the center of an open field [57]. In our study, diphenoxylate, a μ-opioid antagonist similar to naloxone, did not alter the time in which the mice spent in the center of the open field.

In our study, there was an increase in rearing in the male mice when propranolol was administered, but a nominal difference in female mice. This is contrary to a study with rats where high doses (>10mg/kg) of d,l-propranolol decreased rearing frequencies [58]. However in this rat study, the decrease in rearing could be attributed to sedation effects as locomotion was significantly decreased as well as an increase of immobility duration. This sedation effect could be attributed to the fact that propranolol lowers heart rate and cardiac output [59]. The high doses of propranolol may have lowered the heart rate of the rats so significantly that the rats became easily fatigued.

There was no difference in rearing frequency detected when diphenoxylate plus atropine were administered to the mice. This coincides with Benton *et al.* who found that naloxone, a μ-opioid antagonist, did not alter the rearing frequency in albino mice [57].

However, we also found there was a slight increase in rearing in the male mice when propranolol and diphenoxylate plus atropine was administered, but a nominal difference in female mice. This would suggest a modulatory role of the μ-opioids in inhibiting propranolol.

Schneider *et al.* found that naloxone, a μ-opioid antagonist, decreased freezing times of rats during fear conditioning tests. Propranolol had the same effect, but when combined with naloxone the rats had significantly higher freezing times ($p<0.05$) [60]. In our study, male mice given the combination of diphenoxylate and propranolol had significantly less rearing frequency in the center of an open field than propranolol alone ($p<0.05$).

Propranolol and naloxone both individually inhibit the effects of PCAP 38 had on rearing and locomotion behavior of rats in an open field [61]. Propranolol and naloxone both individually also inhibit CGRP-induced increase in rearing and grooming behavior of rats in an open field [62]. This further suggests that behavior is modulated by the blockage of the beta-adrenergic receptors and μ-opioid receptors. It is possible that μ-opioids modulate the beta-adrenergic receptors [60, 63] as it modulates 5-HT receptors [64].

In passive avoidance paradigms, Kovács *et al.* hypothesized that CGRP, which is mediated by beta-adrenergic, serotonergic, and opiate mechanisms, improves the fear-motivated learning-associated memory formation [62].

2.4.2 Elevated Plus Maze

Based on the exploratory-anxiety conflict, the more anxious the mouse, the lower the percentage of time the mouse will spend in the open arm and lower the amount of

entries the mouse will make in the open arm. When propranolol was injected in the BLA of rats, there was decrease in the percentage of time spent in the open arms, but no difference in the percentage of open arm entries [65]. Naloxone decreased the percentage of time spent in the open arms and the percentage of open arm entries without any effects on locomotion [63]. In our study there was no significant difference in the spatiotemporal or ethological measures between the groups. One possible reason behind these contradictory results could be that rats are neurologically distinct from mice. Another could be the administration through oral gavage is not as potent as intravenous injections. Along the same lines, the high metabolism of mice could require a short timespan from receiving the oral dose to testing. The half-life of propranolol and diphenoxylate could be shorter in this animal model.

2.4.3 3-Chamber Social Interaction Test

There was no significant difference in spatiotemporal measures between the experimental groups. Diphenoxylate did not have any apparent effect on the anxiety levels of them mice during the 3-chamber social interaction test. This coincided with the null effect of naloxone in social interaction test [57].

2.4.4 Fear Conditioning Test

Propranolol has be shown to reduce freezing behaviors in rats during fear conditioning tests [60, 66, 67] but not for mice [68, 69]. However, propranolol was able to reduce freezing behaviors in Ntsr1-KO mice [68]. Our study did not reveal any significance difference in freezing behavior between experimental groups. Chou *et al.* have noted the reactive behavior of Wistar rats in auditory fear conditioning can differ considerably despite being identical in stock, sex, age, and housing conditions [70].

They concluded there is considerable individual differences in the acquisition and expression of conditioned fear even among the same strain [70].

2.4.5 Why Species and Sex Difference?

In order to explain a possible reason for the differences in the effects of propranolol on anxiety related behaviors between species and sex, one needs to understand what happens when the body undergoes stress. Norepinephrine is released from the locus coeruleus. The norepinephrine binds to β2-adrenergic receptors, which in turn causes an increase in production of β2-adrenergic receptors and a decrease in β1-adrenergic receptors. This increases the serotonin levels in the basolateral amygdala (BLA) and the bed of nucleus stria terminalis (BNST), which activates anxiety-related behaviors.

Testosterone is known to have a high affinity for intracellular androgen receptors. Testosterone decreased anxiety-like behaviors in housed male mice. Androgen regimes increase the duration of open arm time in the EPM implying decrease in anxiety levels. Beta-adrenergic receptors in the BLA are known to play a role in anxiety. Upregulation of serotonin in this region is known to cause anxiety and levels are enhanced during stressful experiences. Testosterone upregulates β1-adrenergic receptors, while down regulating β2-adrenergic and β3-adrenergic receptors. β1-adrenergic receptors are known to be coupled with stimulatory Gi protein (Gs). There is an upregulation of β1-adrenergic receptors in the amygdala after fear training and anxiety conditions. The beta blocker, metaprolol, has been shown to inhibit this upregulation in rats after being microinjected into the BLA. Testosterone and betaxolol decreases serotonin concentrations in the BLA. Therefore, it is likely

that the anxiolytic effects of testosterone are due to the reduction in serotonin levels [71].

This could explain why power posture, made famous by Amy Cuddy's Ted Talk presentation, is effective to reducing anxiety and stress prior to interviews [72]. Power postures are known to increase testosterone levels [73].

Propranolol is stress-dependent because the locus coeruleus increases norepinephrine when activated by stress. Propranolol prevents norepinephrine from binding to β2-adrenergic receptors **Figure 2-32**.

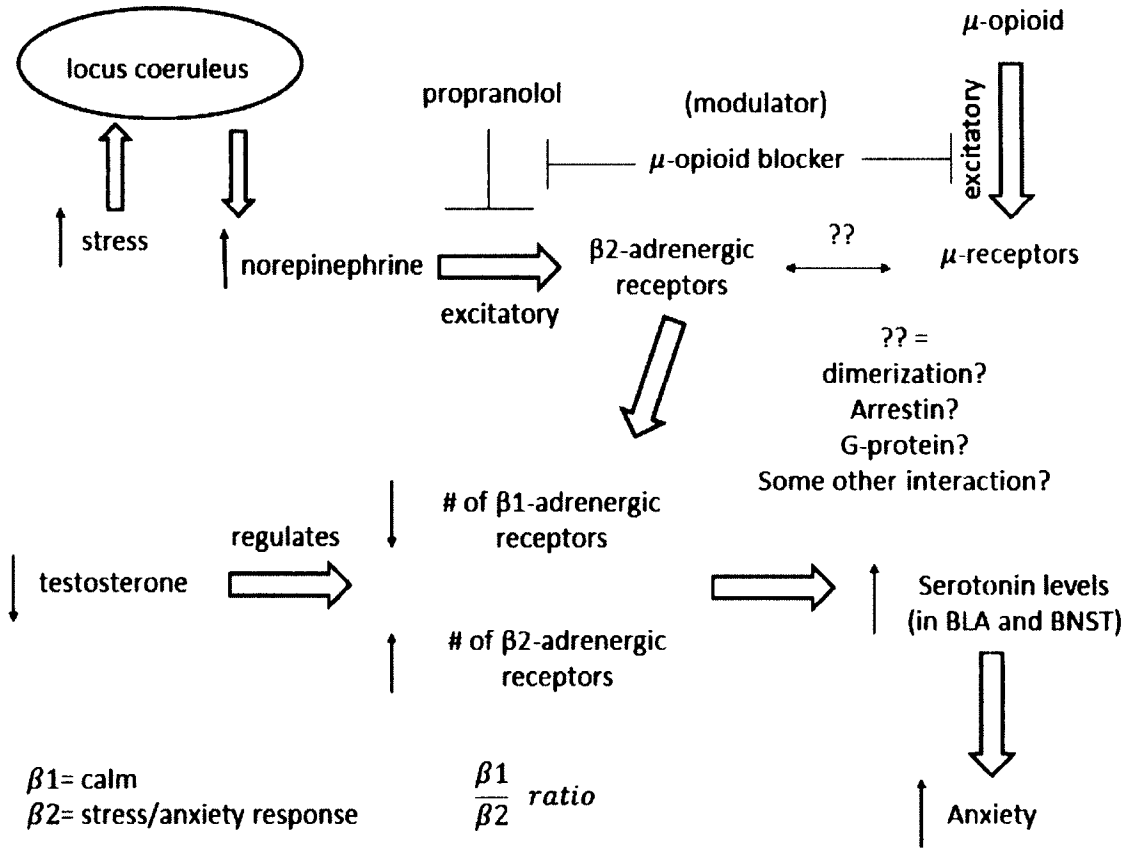

Figure 2-32: Proposed interactions between β_1 and β_2-adrenergic receptors with μ-receptors (Graphic created by Kevin Holly).

The density distribution of β1- and β2-adrenergic receptors in the BNST (an anxiety region) is different between rats and mice. Rats have an even distribution of each receptor subtype, whereas mice have more β1-adrenergic receptors than β2-adrenergic receptors [74]. Knowing the important role that testosterone has on the upregulation of β1-adrenergic receptors, which in turn seems to play a role in decreasing serotonin concentrations levels, one can assume that testosterone would have a greater impact in reducing serotonin levels in the BNST in mice compared to rats. This would result in mice being less anxious than rats with equivalent doses of testosterone.

Also, if the distribution of β1- and β2-adrenergic receptors varies between species, does it vary between male and females? Males have higher testosterone levels than females. How does this affect anxiety levels? We know females are more inclined to experience depression [75], which is an emotion closely related to anxiety. Understanding the effects of testosterone on the serotine levels also may shed light on male and female differences.

Propranolol alone and diphenoxylate alone affect mice rearing in center of open field for male mice, but not female. It was found that the right amygdala is more active than the left amygdala in men during emotional stimuli, whereas the left amygdala is more active in women. The right hemisphere of the brain is biased toward processing more global events while the left hemisphere is more detailed oriented. Cahill and Stegeren found evidence that supports the idea that each amygdala projects predominantly to its ipsilateral hemisphere and that the memory function in the amygdala depends on β-adrenergic activation. They were able to show that propranolol was capable

of impairing big picture memories formed after an emotional arousing story in men without affecting memories pertaining to peripheral story details. In women, propranolol impaired the memories pertaining to peripheral story details without affecting big picture memories [75]. The effects of propranolol has shown to be more pronounced in women than men [76].

2.4.6 The Regulation of Memories and the Modulating Role of Opioids

As mentioned in section 1.3.2, the amygdala regulates the consolidation of emotion memories into the hippocampus. Stress hormones help perpetrate this consolidation by activating the amygdala as mentioned in section 2.4.5. This stress-induced memory strengthening is partially regulated by beta-adrenergic receptors. Propranolol being a β-adrenergic receptor antagonist is known to inhibit consolidation of emotional memories. Interestingly, propranolol seems to stop stress-induced strengthening of learning extinction in rats. Propranolol has been shown to reduce freezing behaviors in rats during fear conditioning tests [60, 66, 67] but not for mice [68, 69]. It was hypothesized in section 2.4.5 that the differences in rat and mouse fear condition results could be due to differences in rat and mouse beta receptors distribution in BNST, an area known for anticipatory anxiety [74]. The mouse BNST had greater densities of beta-1 receptors than beta-2 receptors [74].

Propranolol is known to pass through the brain blood barrier. So, the systematic administration of the drug would lead to the pharmaceutical flooding the whole brain. This would suggest that the distribution of the affected receptors would be of importance. We know that it is a central effect of propranolol that reduces the freezing behavior because the peripheral beta blocker sotalol, which does not pass

through the brain blood barrier, does not reduce the anxiety behaviors. However, the sotalol does inhibit stress-induced memory enhancement, which would suggest that epinephrine's effect on stress enhanced memory consolidation is initiated by peripheral β-adrenoceptors [77]. This implies that the both the peripheral and central β-adrenoceptors are need in the process of stress-induced memory enhancement.

Interestingly, propranolol disrupts the consolidation of emotional, but not neutral memories[78]. This shows that propranolol does not inhibit "normal" memories, but it inhibits the strengthening of stress-induced memories. Recall, this makes sense because propranolol inhibits norepinephrine, which is released during stress. The consolidation of these memories is thought to be performed by long-term potentiation (LTP). Propranolol inhibits LTP of GABA neurons, which in turn inhibit the LTP of thalamic inputs.

So how does β-adrenoceptors affect LTP? When β_1 or β_2-adrenoceptor undergo a conformation change after a ligand binds to the extracellular portion of the receptor, guanine nucleotide-binding regulatory G_s-proteins are activated by binding to the intracellular portion of the beta receptor. The G_s-protein breaks down into subunits and triggers a signal cascade that includes activating adenylate cyclase (AC), which increases intracellular cyclic adenosine monophosphate (cAMP) and activates the protein kinase (PKA) pathway. The phosphorylation effects of PKA on the calcium ion permeability of NMDA receptors affect LTP[79] (see section 1.3.3 for NMDA receptor's role in LTP). In β_2-adrenoceptors, G_i/G_o-proteins mediate the activation of extracellular signal-regulated kinases (ERK), mitogen-activated proein (MAPK), Akt, and tyrosine kinase transactivation. MAPK and ERK are essential to the activation of cAMP response element-binding protein (CREB), which mediates protein transcription. These proteins

that are transcribed support persistent synaptic plasticity, which enables the encoding of long-term memory. The ERK pathway leads to an increase of GluA1 (also known as GluR1) phosphorylation. PKA pathway can increase the surface extrasynaptic pool of GluA1, which is regulated by noradrenaline. Recall from section 1.3.2 that the phosphorylation of the AMPA receptors leads to an increase influx of sodium ions that allows for a more rapid voltage change, which in turns repels magnesium from the NMDA receptors allowing an influx of calcium ions to generate an action potential leading to LTP [17]. In the amygdala, ERK signaling activated by beta adrenergic receptors helps with the formation of new spines , which helps with LTP [80].

Inhibiting beta adrenergic receptors and IL-1 receptors affects the expression of ERK and c-Fos. Social defeated mice showed anxiety-like behaviors and exhibited high levels of ERK and c-Fos. Following social defeat, mice with higher levels of ERK froze more during a fear conditioning test that followed. The presence of ERK reaffirms the ERK pathway's role in LTP of circuits related to fear and anxiety. Social defeat can be used to separate mice that are susceptible to fear conditioning as opposed to resistance mice, since it has been noted that individual mice have a high variation in anxiety/fear expression (future work is needed to find the genetic, environmental, and other factors that cause the individual variation). A future experiment suggested was to see if fear conditioning test can predict whether animals would be susceptible or resistant to sociability following social defeat stress [80, 81].

Kim *et al.* have shown an age-related different in the neurocircuitry of extinction in rats. For adult rats (P24), extinction of conditioned fear involves intracellular activation of the IL and the amygdala. For young rats (P17), only the amygdala was

shown to play a role in the extinction process [82]. This opens up the possibility that propranolol's extinction inhibitory role through injections into the prefrontal cortex could only apply to adult rats [83]. Of course, there are other areas that influence the epinephrine regulation within amygdala during memory consolidation such as the nucleus of the solitary tract (NTS) [77]. The direct administration of propranolol into amygdala posttraining has been shown to block the memory enhancement induced by systemic administration of epinephrine in contrast to norepinephrine infusion which enhance memory retention and attenuated the impairment induced by adrenal demedullation [77]. It is important to note that unlike propranolol, epinephrine can't easily pass through the brain blood barrier [7].

Now that propranolol's role in inhibiting stress-induced enhancement of memories is better established, how does the μ-opioid diphenoxylate come into play? It is hypothesized that there could be cross-talk amongst different GPCRs. Cervantes *et al.* have shown that arrestin orchestrates cross-talk between GPCRs to modulate the spatiotemporal activation of ERK MAPK[84]. The importance of ERK's role in fear, anxiety, and LTP has already been established. Perhaps, cross-talk through arrestin can take place between μ-opioid receptor and beta adrenergic receptor. In this manner, μ-opioids could modulate the effects of beta adrenergic receptors. Note, diphenoxylate similar to propranolol can pass through brain blood barrier. It is also important to note that μ-opioids are the most popular opioid in amygdala, the central modulator of anxiety and fearful memories. Alternatively, cross-talk could occur through dimerization of the μ-opioid receptors with the beta adrenergic receptors. Both receptors could undergo a conformational change after their respective ligand binds

that allows for them to dimerize with one another. This could allow for a unique G-protein signal pathway, which produces a synergic effect in treating performance anxiety. Based on our behavioral studies, this effect does not occur in mice, but it does not eliminate the possibly of it occurring in rats or people as the distribution of the receptors in the brain differ from mice.

It has been proposed that opioid neurotransmission could be used in erasure extinction by preventing and/or reversing the consolidation of memories[85]. To further back this stance, more μ-opioid receptors are found in younger mammals. Opioids being crucial for extinction make sense because younger mammals have more plastic brains. Since they are still developing, they need to be able to weaken all synaptic pathways in order to form new ones. During the early stages of brain development, some cells undergo apoptosis (programed cell death) in order to develop new connections. The weakening of synaptic connections known as LTD is needed in order to develop new memories with LTP. Kim and Richardson proposed that in younger rats, the erasure extinction process is heavily based on the opioid system as opposed to the multiple neurotransmitter systems involved in adult rats [85]. Following Kim and Richardson's thought process, it is hypothesized that the opioid system is still involved in erasure extinction in adult rats, but with a lower effect. The opioid system probably plays more of a modulatory role in the more hard-wired circuitry. Assuming opioid are central to erasure extinction, diphenoxylate should have a greater effect on fear extinction in young mammals and play more of a modulatory role in adults. Kim and Richardson have found that the amygdala is important for extinction the first time, but appears unimportant for re-extinction for P24 and adult rats as opposed to P17 rats [85]. In adult rats, the

amygdala seems to be involved in the initial memory involving in the disassociation of the condition stimulus to the unconditional stimulus. After which, the amygdala seems uninvolved in the extinction process [85].

It seems that propranolol inhibits consolidation where diphenoxylate may have a modulatory role. Based on the idea that opioid propagate the erasure extinction process, diphenoxylate could have an unwanted side effect of inhibiting erasure extinction. Further research is needed using a different animal model than the mouse to test the effects of propranolol and diphenoxylate.

To further validate the involvement of LTP in fear generation, we will examine the use of D-cycloserine as therapeutic treatment. As shown in **Figure 2-33**, D-cycloserine binds to NMDA receptors causing an increase in permeability for calcium ions. This leads to LTP where it plays a role in learning extinction by helping to form new memories. Michael Davis was able to successfully use D-cycloserine for treatment in fear therapy. He had patients with phobias of bridges or elevators wear a virtually reality headset to face virtual heights, bridges, and elevators. After a successful session where the patient faces the phobia and does not have a harmful experience, Davis prescribes them d-cycloserine to strengthen these new memories that do not associate fear and the object of their phobia [13]. This is an example of a success pharmaceutical treatment to fear. Likewise, we desire to develop a treatment for performance anxiety, which could be viewed as a social phobia. Using a terminology emerged in the literature trying define the emotional state that the BNST effect, the drug combination could be used to treat "sustained fear" or "anticipatory anxiety." Understanding the mechanisms behind Davis' work opens up a more methodical procedure in finding novel treatments

for emotional behavioral states. Targeting the LTP and LTD mechanisms in the appropriate brain regions is key to disrupt or enhance the neurocircuitry of a behavior. This requires a holistic understanding of the brain role in behavior from the chemical reactions of the receptors and ligands to the neurocircuitry.

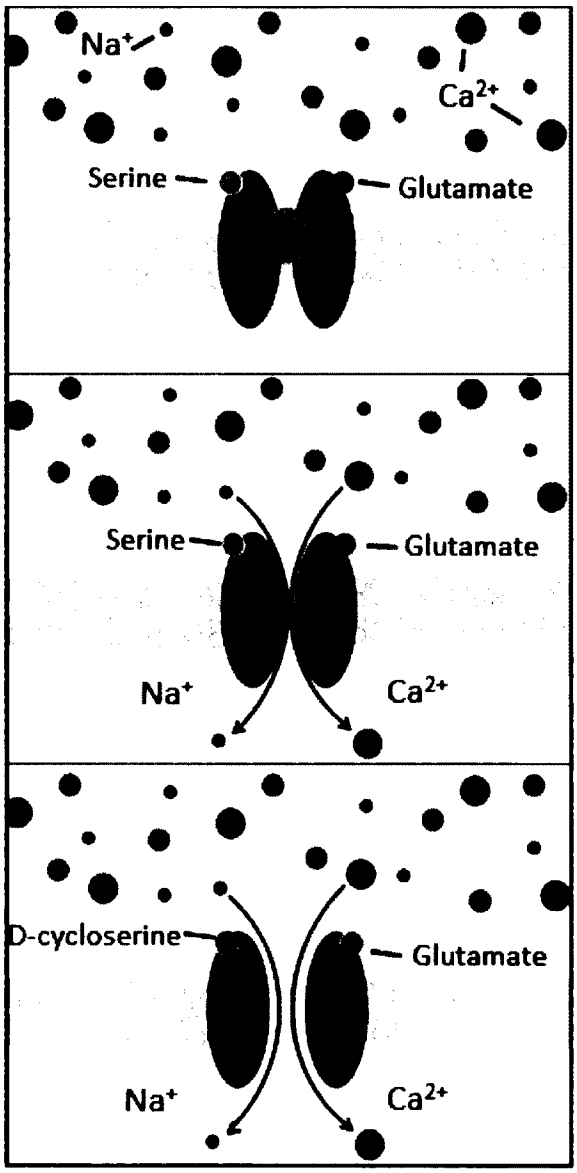

Figure 2-33: D-cycloserine opens the voltage-sensitive NMDA receptors wider than serine or glycine, which allow more calcium ions to flow into the postsynaptic neuron (Graphic created by Kevin Holly).

AMPA potentiator facilitates extinction, but not reconsolidation. It affects the mPFC, but not amygdala. Knowing AMPA receptor allow sodium in the neuron which in turn helps NMDA receptors open up, it seems that NMDA receptors are key to the extinction process.

This makes sense as to why D-cycloserine (a NMDA agonist) helps with the extinction process. However, D-cycloserine is known to affect the basolateral nucleus of the amygdala. Perhaps, the AMPA potentiator used in the study was not enough to open NMDA receptors due to a lack of glycine or serine. It would be interesting to see what would happen if both an AMPA potentiator such as PEPA and D-cycloserine were injected into the basolateral region of the amygdala. D-cycloserine is known is affect reconsolidation as well [68].

2.5 **Conclusion**

This was the first animal study that looked into the combination of propranolol and diphenoxylate to treat anxiety. Research on the role of opioids in anxiety and fear has been lacking, although recently the importance of opioids in panic has been emerging with the understand of their modulatory role toward 5-HT receptors [64]. As we are aware, μ opioid treatment for anxiety has only been tested once systemically with naloxone [60]. Our study has also included female mice, which has been neglected in the past. Based on our study and others, we have noticed that propranolol was only able to reduce fearful behaviors in rats, but not mice. We have uncovered a difference in the distribution of the affected beta adrenergic receptors in the BNST between species. We have hypothesized that in rats propranolol was able to hinder consolidation by inhibiting the LTP process. We also hypothesized that diphenoxylate

may play a role in modulating the beta adrenergic receptors affected by propranolol through a cross-talk between the μ-opioid receptors and beta adrenergic receptors. Possible mechanism could be through the utilization of arrestin or a possible GPCR dimerization between the receptors. We have come to the conclusion after learning of the species differences in mice and rats that looking at receptors alone is not enough. The understanding of neurocircuitry and the mechanism that follow is key to understanding and treating different behavioral or emotional states. Knowing how LTP and LTD affect memory formation is fundamental for a more methodical approach in find new pharmaceutical treatments. Because of the overlap of neurocircuitry between multiple emotional states, a holistic study of fear, anxiety, panic, social defeat, and depression is needed.

CHAPTER 3

MATSAP: AN AUTOMATED ANALYSIS OF STRETCH-ATTEND POSTURE IN RODENT BEHAVIORAL EXPERIMENTS

3.1 Introduction

Rodent behavioral analysis is often used to assess the effects of pharmaceuticals, implanted devices, or surgical procedures in preclinical research. The development of image analysis tools has enabled researchers to quantitatively assess various rodent behaviors quickly and objectively [2, 3]. However, most automated scoring programs track patterns in spatial locomotor exploration and neglect ethological behaviors, such as head dipping and stretch-attend posture (SAP) [2]. Currently, there is no accurate tracking and scoring software that can directly detect SAP [4].

SAP, which is generally associated with anxiety, occurs when the rodent lowers its back, elongates its body, and is either standing still or moving forward very slowly [18]. SAP is a naturally occurring behavior found in rodents, such as hamsters, that can be reliably intensified by certain experimental paradigms, such as placing the rodent in an open-field test [18, 19]. In mice, the SAP behavior occurs when the mouse is undergoing risk-assessment specifically due to an internal exploratory-anxiety conflict. It can also occur under fearful risk-assessment where SAP would be an ambivalent element reflecting an approach-avoidance tendency [18, 20]. When SAP is present during a passive avoidance situation, mice approach or avoid the object at nearly equal rates,

which indicates they are undergoing risk-assessment during an approach-avoidance conflict [19, 20]. SAP is a good identifier for conflict behavior in mice and can be used to evaluate the effects of drugs at reducing these internal conflicts [21]. During exploratory-anxiety conflict situations, SAP can be used as a valid measure of anxiety as anxiolytic drugs have successfully reduced SAP [19, 21-23].

SAP has been evaluated in elevated plus maze (EPM) [6], open field (OF) [28], rat exposure test [29], and canopy stretch attend posture test [22]. Increased SAP behavior of rodents near the entrance of the open arms in EPM and along the border of the canopy in the canopy stretch attend posture test has demonstrated SAP as a risk assessment behavior [22, 30].

In classical anxiety tests, such as EPM and OF, the conventional spatiotemporal measurements may not detect effects of novel anxiolytic medications [6]. SAP has been found to be more sensitive to the effects of classical and atypical anxiolytics than traditional spatiotemporal indices in the murine plus-maze [24, 25]. For example, SAP is especially sensitive to the effects of ligands acting on 5-HT1A receptors [24, 25]. It is hypothesized that SAP can be related more to the cognitively oriented aspects of anxiety [24]. Inclusion of ethological measurements such as SAP in EPM provides a more comprehensive profile on the anxiolytic or anxiogenic effects of a treatment [23, 25, 26]. SAP can also help differentiate between anxiogenesis and sedation effects of drugs [2, 27]. Despite finding that risk assessment measurements are more sensitive to anxiety modulating drugs than traditional indices, Carobrez *et al.* found that only a quarter of studies have adopted them [2].

SAP is usually evaluated using its frequency of occurrence [22, 86, 87], although some have quantified SAP in both duration and frequency [25, 28, 30]. Researchers to date have scored ethological behaviors manually either with the aid of Observer XT [88-90] or without computer-aided assistance [26, 91-93]. Some investigators are even using EthoVision XT for spatiotemporal measurements while using trained observers for manually recording ethological behaviors such as SAP [94-97]. Evaluation with human observers is time-consuming and susceptible to error as people become fatigued and lose concentration during long mental tasks. Human observers may introduce subjectivity into their scoring leading to variable interpretations of observed behaviors between individual observers which decreases inter-observer reliability [98]. Intra-rater variability is also a concern because human observers may have different scores for the same videos when blindly scoring the same set of videos twice. In contrast, computers are consistent and measure objectively. Further, computers do not experience fatigue or require training.

Event-recording programs such as Hindsight and EthoVision require a user to manually press a button when the behavior of interest occurs. These programs facilitate the viewing and counting of the behavior; this process is time consuming and subject to human error. While commercially available software could be used to automatically detect SAP with additional customization, this can be costly for the purchaser. Currently, EthoVision XT can be used to find the speed and elongation of a rodent to detect SAP if the user is given the proper threshold values and if the software is modified to provide the additional output. However, to the best of our knowledge, the use of this program to detect SAP has not appeared in the literature. To encourage a greater use of SAP as a

metric in behavioral tests, we have created an open source MATLAB-based software, MATSAP, to detect SAP.

3.2 Methods

3.2.1 Mice Living Conditions and Institutional Approvals

10 male Swiss mice from Jackson Laboratories were used in this study housed in groups of 4-6 mice per cage to avoid stress and anxiety induced by social isolation [99], although some believe individual housing decreases anxiety in mice [30]. The mice were housed in a 12-hour day/night cycle where food was administered *ad libitum*. Behavioral test procedures were approved by the Louisiana Tech University Institutional Animal Care and Use Committee and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996).

3.2.2 Behavioral Experiments

Four month old male Swiss mice were used in this study (n=10 for OF, n=9 for EPM). The weight of the male mice ranged from 32.5-40.0 g during testing. Behavioral tests were conducted by a male experimenter. For each mouse, there was a minimum of 24 hours between each behavioral test.

3.2.2.1 *Open field test.* The open field (OF) test apparatus consisted of an open square wooden container (30x30cm) with 25-cm high walls enclosing the perimeter. The walls and floor of the container were spray painted black **Figure 2-6**. On the floor of the container, a white 16 square grid was drawn [100]. A camera mounted above the box and facing perpendicular to the floor was used to record the movement of the mice at 29 fps in high-definition MPEG Transport Stream (MTS, .mts) video format. To begin the test, a

mouse was placed in the corner of the container. The mouse was allowed to explore the container for 5 minutes before being removed and placed into its home cage. Between trials, super hypochlorous water was used to clean the floor and walls of the container [4].

3.2.2.2 *Elevated plus maze.* An elevated plus maze (EPM) was built from medium density fiber board using previously established dimensions [3]. Two opposing open arms and two opposing enclosed arms extended 25 cm from a 5 x 5 cm central platform forming a plus shape. Enclosed walls were 25 cm tall and the maze was elevated 50 cm above the floor **Figure 2-1**. To begin the test, a mouse was placed in the central platform facing the south enclosed arm and was allowed to move freely about the maze for 10 minutes. A ceiling-mounted video camera facing perpendicular to the floor with a field of view centered on the central platform recorded mouse movement at 29 fps in MTS video format. The platform of the maze was black to provide color contrast to the white Swiss mice and the testing room was illuminated with standard fluorescent lights. Between trials, the central platform and all four arms were cleaned with super hypochlorous water to remove odors left by the previous mouse [3].

3.2.3 Video Preparations

Videos were converted from MTS files at 29 fps to Audio Video Interleave (AVI, .avi) files at 10 fps without audio and then loaded into ImageJ 1.47t, a public domain image processing program [101]. Videos were converted to grayscale and cropped to the dimensions of the open field box with a 1:1 width to height ratio. Before saving as a multi-TIFF file, an image of the open field without a mouse present was added as the last frame of the video to use for background subtraction in the other frames so that the white rodent is readily distinguished from a black background.

3.2.4 MATSAP Availability

The most recent version of MATSAP can be found at the MathWorks File Exchange (http://www.mathworks.com/matlabcentral/fileexchange/58412-matsap). MATLAB (Version R2012a or greater) along with the Image Processing Toolbox™ is required to run MATSAP. Comments are embedded within the MATLAB code that explains steps, such as analyzing images and optimization steps.

3.2.5 Structural Design of Software

The software allows the users to analyze multi-TIFF video files of rodents from an overhead view. The user will need to convert video files to multi-TIFF files with the last frame being the background. Conversion to TIFF files can be done through the public domain image processing program ImageJ if the video format is AVI [101].

Figure 3-1 is a simplified flowchart of the software program. When the user runs MATSAP, a dialog box will prompt the user to select the folder containing the multi-TIFF files for analysis. The user was asked if the rodents are darker than the background in case the images need to be inverted for analysis. Then the program will give the user the option of opening the MATSAP Threshold Previewer, an interactive preview screen of the videos, to test for an appropriate binary conversion threshold value that was used to convert the images into a highly contrast binary images (**Figure 3-2**).

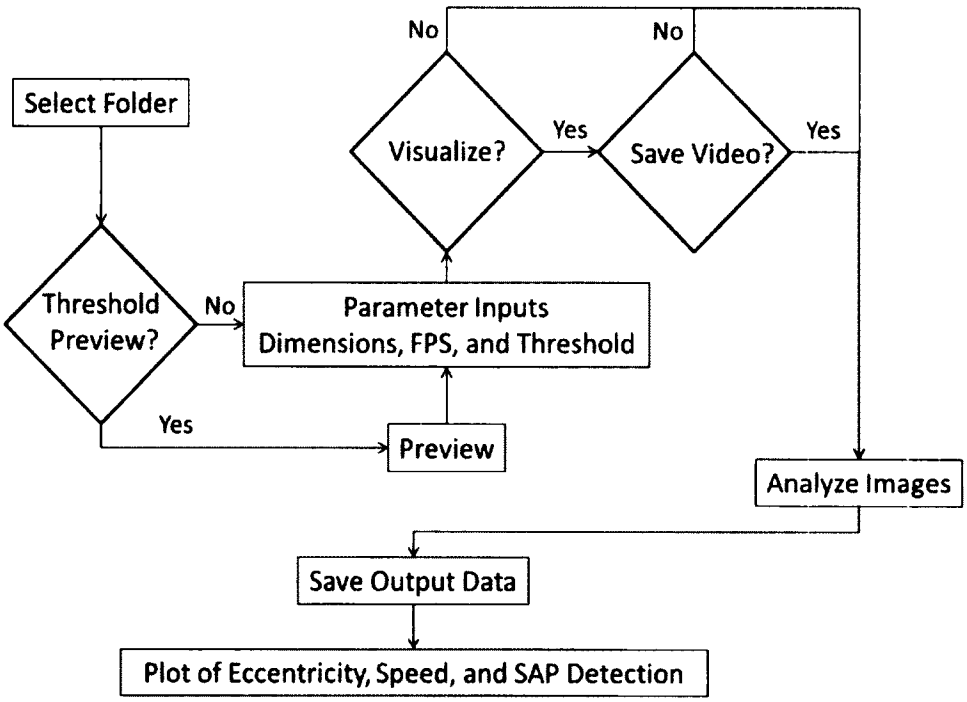

Figure 3-1: A simplified flowchart of MATSAP

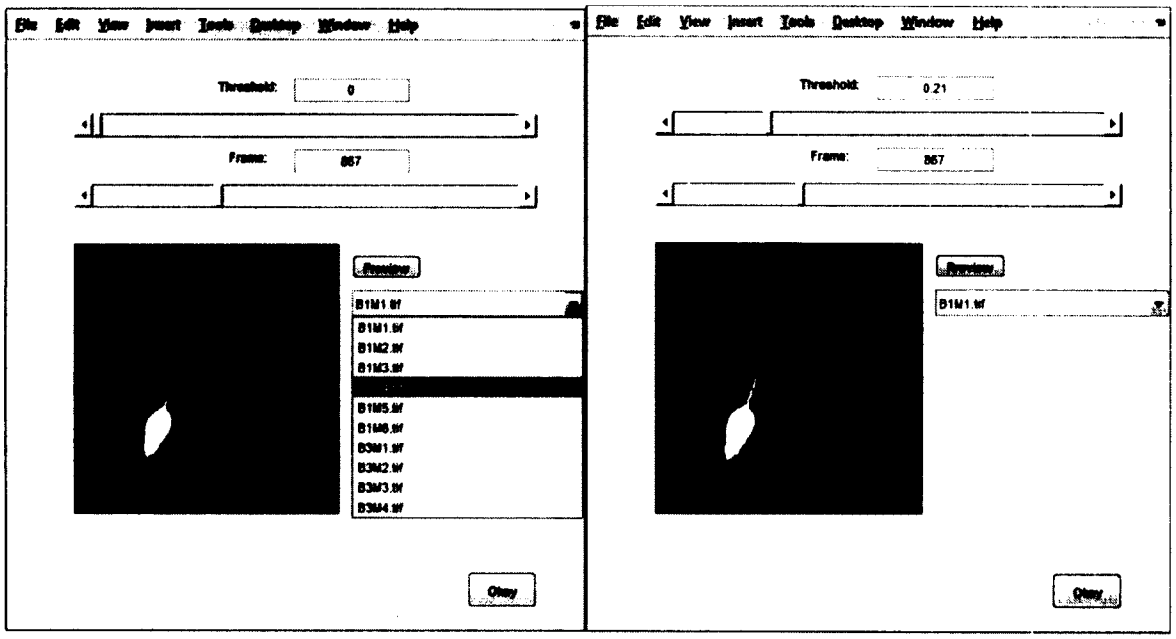

Figure 3-2: The MATSAP Threshold Previewer allows the user to select the appropriate threshold value to create a high contrast binary image.

After viewing the optional threshold preview or selecting "No" in the dialog box, another dialog box appears for the user to input parameters of the video, which includes dimensions of the area covered in the video, the fps, the binary conversion threshold value, and the speed and eccentricity threshold values. After this, the user is given the options "yes" or "no" to have a visualized output of the analysis (**Figure 3-3**). If "yes" is selected, then the option of saving the video is presented to the user. The images are then analyzed and the user is prompted to select the file folder location to save an Excel file containing SAP detection results. Plots of the eccentricity, speed, and SAP detection are displayed and saved in the directory folder (the folder that contained multi-TIFF files for analysis) unless an alternative directory is chosen for output files. The data are also archived in ASCII files in case a user does not have Excel in the default directory, unless the user specifies otherwise. A result summary spreadsheet is also generated containing the total SAP percentage, time, and frequency for each video in the directory folder. If all the input parameters are the same, including the binary conversion threshold value, the dimension, and the fps, the user has the option of skipping subsequent input dialog box prompts for the remainder of the videos in the folder. Furthermore, the user can set MATSAP to run until all of the videos in the folder are analyzed.

Figure 3-3: Output of an open field video (single frame). This image demonstrates that the image analysis successfully formed an ellipse around the body of the rodent.

3.2.6 Image Analysis

After the user enters the required parameters and answers the dialog prompts, MATSAP begins its fully automated image analysis by subtracting the background from each image frame and then converting the frames into black and white, binary images (**Figure 3-4a**). The rodent's tail is removed by eroding the perimeter of the rodent with the built-in imerode MATLAB function. This step creates an ellipse-like form, but it also reduces the overall size of the mouse (**Figure 3-4b**). (In earlier versions of this program, the eccentricity values were distorted by the length and changing position of the tail. This step removes the tail and results in more accurate eccentricity values.) After this automated step, the program restores the binary image of the rodent to its normal size by dilating the perimeter of the rodent with the imdilate function, thus restoring the original girth and length, but without the tail (**Figure 3-4c**) [102]. MATSAP then selects the largest object with the aid of the regionprops MATLAB function and generates an ellipse

around it as described in Steve Eddin's 2010 MathWorks blog post, "Steve on Image Processing." This is done by first finding the longest length of the object and denoting it as the major axis. A perpendicular vector is then created from the centroid of the object and denoted as the minor axis length. With these two lines, the ellipse is generated around the body of the mouse (**Figure 3-4d**). MATSAP provides an option to display the generated ellipse on the image frames so that the user can verify that the program is working properly.

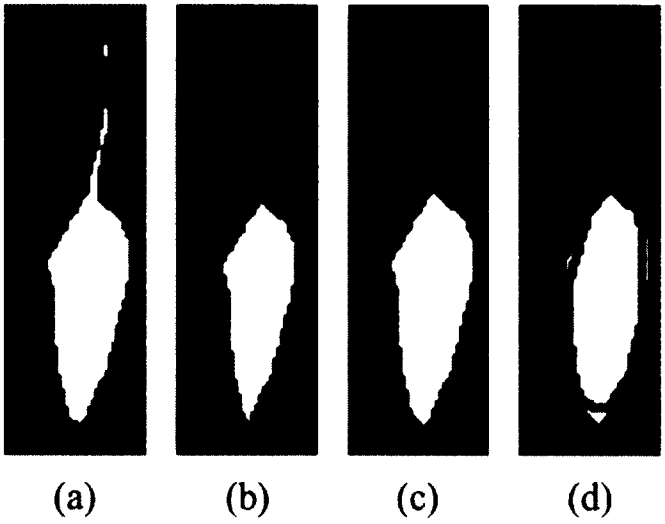

Figure 3-4: The MATSAP software opens a multi-TIFF video of the mouse and then (a) makes a binary image of the rodent, (b) erodes the image which eliminates tail, (c) creates a dilated image that bring size of rodent back to normal, and then (d) places an ellipse around the body of rodent.

The eccentricity value was calculated at each second using **Eq. 3-1**, where the major axis of the ellipse was divided by the distance between the foci points of the ellipse as shown in **Figure 3-5**.

$$\text{Eccentricity} = \frac{\text{Major axis length}}{\text{Distance between Foci}} \qquad \text{Eq. 3-1}$$

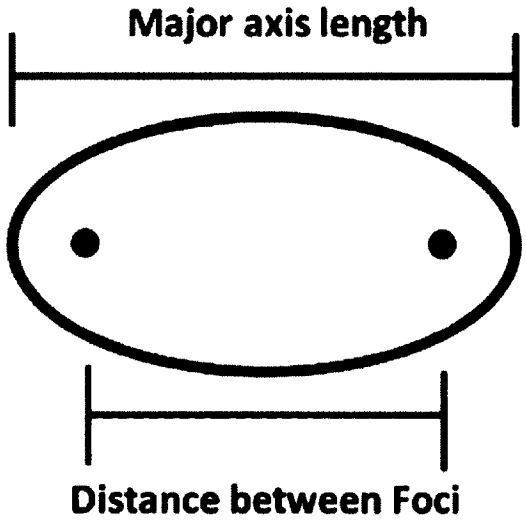

Figure 3-5: Major axis length and distance between foci points measurements depicted on ellipse.

The speed of the rodent is found by using the centroid values of each frame provided by the regionprop command. The pixel distance between the centroids in consecutive frames is calculated. Based on the video's field of view dimensions provided by the user, the actual distance between the centroids is found. The speed of the rodent is then determined using this actual distance multiplying this by the video's fps rate. If the rodent's speed is greater than 12 cm/s (or another specified speed threshold) at an individual time-step, then the SAP detection array is given the value of "0" at the current time-step.

The assumption is made that SAP cannot occur in a time duration less than or equal to 0.5 seconds. So to eliminate false positives, any time there are consecutive 1's of a length less than or equal to half of the fps in the SAP detection array, these ones are changed to zeroes. This eliminates the cases where the mouse is not in a stretch-attend posture but is elongated because it is running.

The program provides plots displaying SAP detection as well as the corresponding speed and eccentricity values (**Figure 3-6**). The plots are saved and the data are archived in Excel and ASCII files. A summary result spreadsheet is also generated providing the duration, percentage, and frequency of SAP for each the multi-TIFF file. If chosen, videos of the image analysis are saved as well.

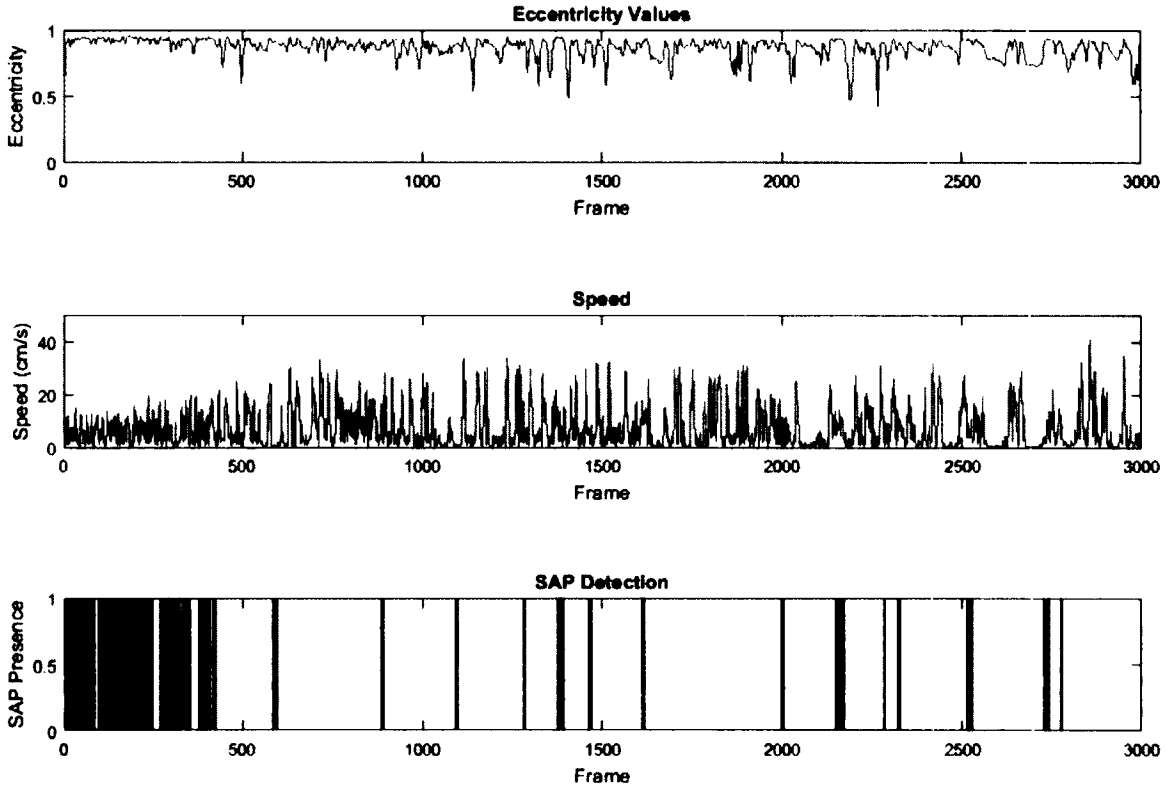

Figure 3-6: MATSAP output plots. The eccentricity values (top panel), speed (middle panel), and the detection of SAP (bottom panel) are shown for each frame in the video.

3.2.7 Evaluation Methods

Ten 5-min videos at 10 frames per second of male Swiss mice in an open field box maze and nine 10-min videos at 10 frames per second of male Swiss mice in an elevated plus maze were evaluated by 5 human observers that were blinded to each other and MATSAP. Both the inside of the OF box and the EPM were painted black for maximizing contrast with the white mouse. For each second of the videos, the scorers determined if SAP was present giving a score of "1" if present and "0" if not. A human consensus score was determined via majority voting of each of the individual scorers at each second. The SAP detection software developed scores of the videos frame by frame. After running MATSAP on MATLAB (Version R2012a), the results were translated from frame-based scoring to a time-based scoring (in s). SAP was considered present in a specific second if SAP was detected in at least one frame MATSAP has a built-in filter removing the durations of SAP less than a half second to minimize false positives. The second-based SAP detection array of 1's and 0's generated by MATSAP was compared to the human consensus SAP detection array; the human consensus was treated as the ground truth.

To determine the runtime of the MATSAP, MATLAB profiler was utilized. A typical laptop was used with an Intel® Core™ i7-3520M core processer at 2.90 GHz and with 6.00 GB of RAM. The runtime of MATSAP for a single video was measured at two different settings, obtaining results (i) with the visualized output being displayed and without saving the video and (ii) without displaying the output visual or saving the video.

3.2.8 Statistical Analysis

Using R software along with the irr package, a two-way agreement average-measure intra-class correlation was used to compute the inter-rater reliability of the 5 human observers that established the ground truth[103]. The accuracy, sensitivity, and specificity of MATSAP compared to the human consensus (ground truth) were determined along with the F-score, MCC, and area under the curve (AUC). Binomial proportion confidence intervals for the accuracy, sensitivity, and specificity were calculated using normal approximation interval (Wald interval) since the sample size (the total seconds of video evaluated) was greater than 30 and the proportions were not close to 0 or 1 [104]. The AUC was approximated in **Eq. 3-2** by the simple trapezoidal method [105, 106].

$$\mathbf{AUC} = \frac{\mathbf{sensitivity} + \mathbf{specificity}}{2} \qquad \textbf{Eq. 3-2}$$

The MCC plots and ROC curves were generated with MATSAP Threshold Optimizer to justify the selection of speed and eccentricity thresholds used to conclude if SAP was present in an image frame. In the ROC curves, which were also generated in Excel, the most optimal threshold would be located in the top left of the graph (Supplementary Fig. S9-S12) as this is where sensitivity and specificity are the highest. For the MCC plots, the speed and eccentricity threshold values that provided the maximum MCC would be the most optimal, albeit also containing a reasonable sensitivity and specificity ratio.

3.3 **Results**

MATSAP detects SAP by generating an ellipse fitted around a rodent in multi-TIFF video files and then it uses the calculated eccentricity value of the ellipse along with

the rodent's calculated speed to discriminate SAP from running. MATSAP provides results in Excel and American Standard Code for Information Interchange (ASCII) files for importing into statistical programs. It also displays plots of the eccentricity, speed, and SAP detection over time. The program also provides optional features, which include a threshold preview screen to aid the user in selecting the appropriate threshold values to convert the multi-TIFF images into binary images for analysis, visualization of the image analysis (**Figure 3-4**), and saving the visualized output (Supplementary Video S1). These features can be used for sample videos in a large batch and then the batch can be run without them or with periodic sampling to reduce run times, if desired. Thus, MATSAP is flexible to allow for the optimization of runtimes.

3.3.1 Open Field

To test the ability of MATSAP to detect SAP, ten 5-minute videos at 10 frames per second (fps) of white mice moving in an open field box with a dark background were first evaluated by 5 blinded human observers (inter-rater reliability 0.83) to reach a "ground truth" consensus score and then the videos were evaluated using MATSAP. An inter-rater reliability above 0.80 is considered an excellent agreement beyond chance according to Fleiss *et al.* and in almost perfect agreement according to Koch and Landis [107, 108]. Based on the human consensus score, SAP was present in 337 seconds (*Positive*) and was not present in 2663 seconds (*Negative*). MATSAP had an accuracy of 90.4% (99% CI: 89.0 – 91.8%) with a sensitivity and specificity of 84.6% (99% CI: 79.5 – 89.6%) and 91.2% (99% CI: 89.8 – 92.6%), respectively, compared to the human consensus score. The average accuracy of the individual observers was 92.7±3.4% (mean ± SD) compared to the consensus score. The F-score was 66.7% and the Matthews

correlation coefficient (MCC) was 0.64. MCC is preferable over the F-score because the *Positive* and *Negative* classes are imbalanced [105]. Since the MCC is closer to 1 than -1, a strong positive relationship is indicated between MATSAP's classification of SAP and the classification by the human consensus [105, 109]. The chosen eccentricity and speed thresholds of 0.90 and 12 cm/s were verified by Receiver Operating Characteristic (ROC) curves (**Figure 3-7** and **Figure 3-8**). The area under the ROC curve (AUC) was 0.8802 (Supplementary Table S1), which is rated "very good" [105].

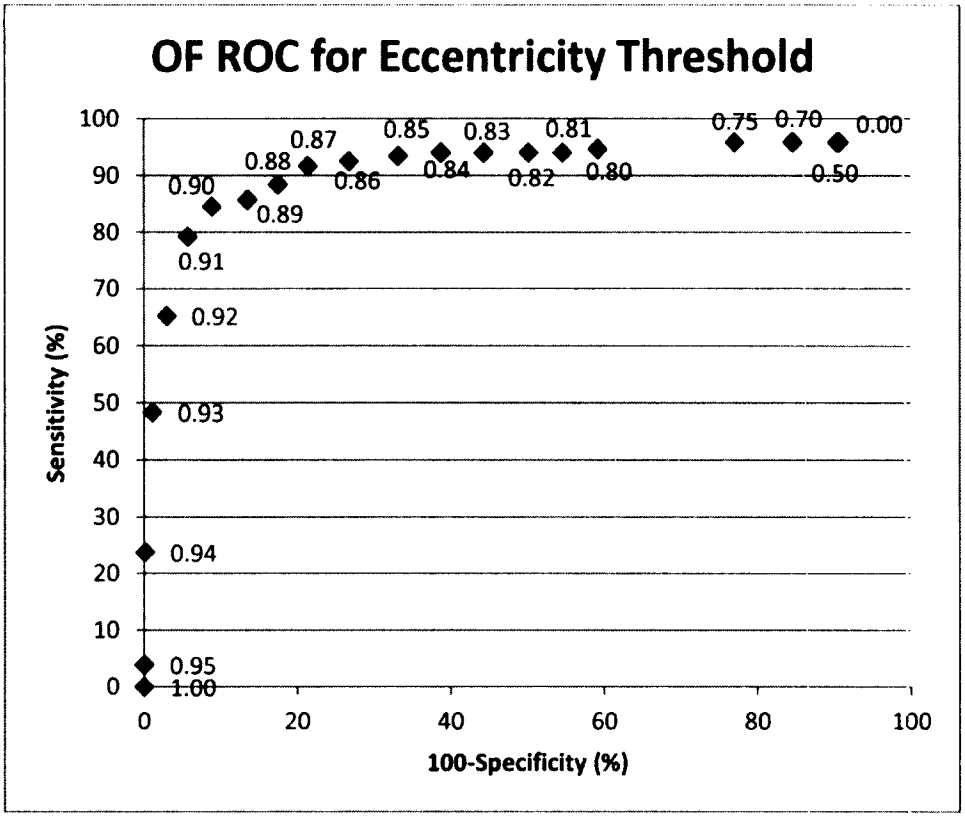

Figure 3-7: ROC curve for eccentricity threshold when speed threshold is set at 12 cm/s for open field. The chosen eccentricity threshold value of 0.90 can be found on the top left of the ROC curve indicating a reasonable value.

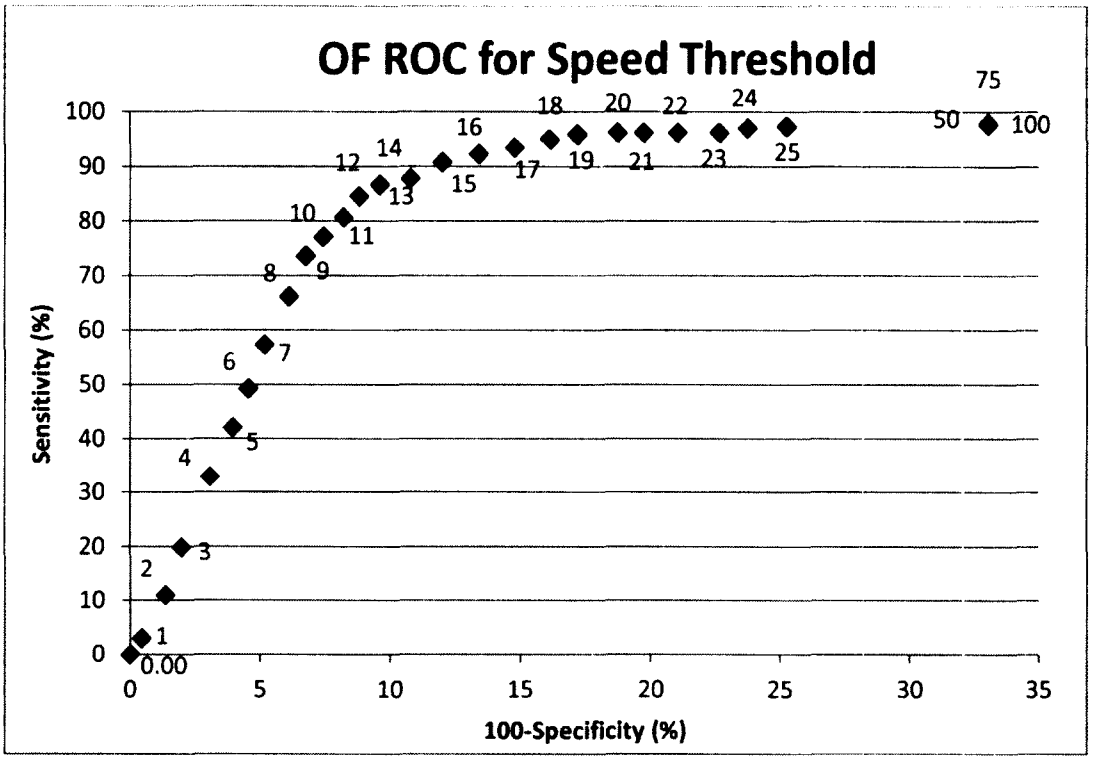

Figure 3-8: ROC curve for speed threshold when eccentricity threshold is set at 0.90 for open field. The chosen speed threshold value of 12 cm/s can be found on the top left of the ROC curve indicating a reasonable value.

3.3.2 Elevated Plus Maze

A similar procedure was employed for an elevated plus maze experiment. Nine 10-minute videos at 10 fps of male Swiss mice in a maze with a dark background were evaluated by 5 blinded human observers (inter-rater reliability 0.86) to reach a "ground truth" consensus score. Based on the human consensus score, SAP was present in 2059 seconds (*Positive*) and was not present in 3341 seconds (*Negative*). In this case, MATSAP had an accuracy of 85.5% (99% CI: 84.3 – 86.7%) with a sensitivity and specificity of 78.3% (99% CI: 74.6 – 82.0%) and 89.9% (99% CI: 88.8 – 91.0%), respectively. The average accuracy of the individual observers was 87.4±6.4 (mean ± SD). The F-score was 80.97% and MCC was 0.69. Again, MCC indicated a strong

positive relationship between MATSAP's classification of SAP to the classification by the human consensus. The chosen eccentricity and speed thresholds of 0.90 and 12 cm/s were verified by ROC curves (**Figure 3-9** and **Figure 3-10**). The AUC was 0.8470 (Supplementary Table S2), which is within the "very good" range [105].

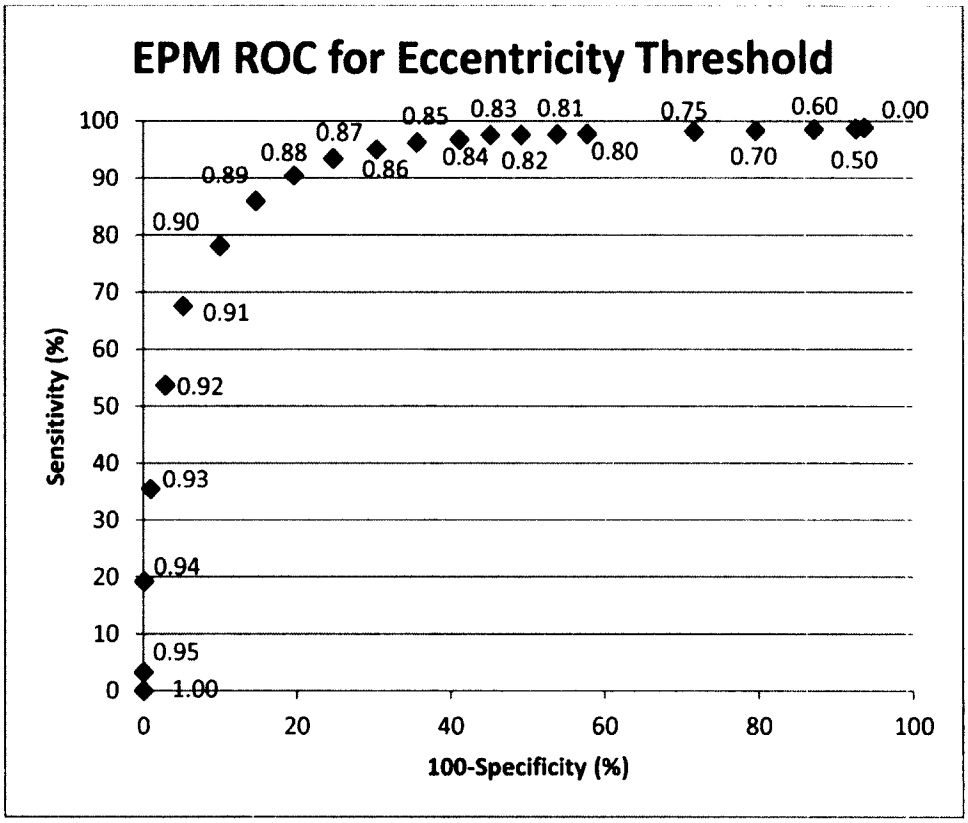

Figure 3-9: ROC curve for eccentricity threshold when speed threshold is set at 8 cm/s for elevated plus maze. The chosen eccentricity threshold value of 0.89 can be found on the top left of the ROC curve indicating a reasonable value.

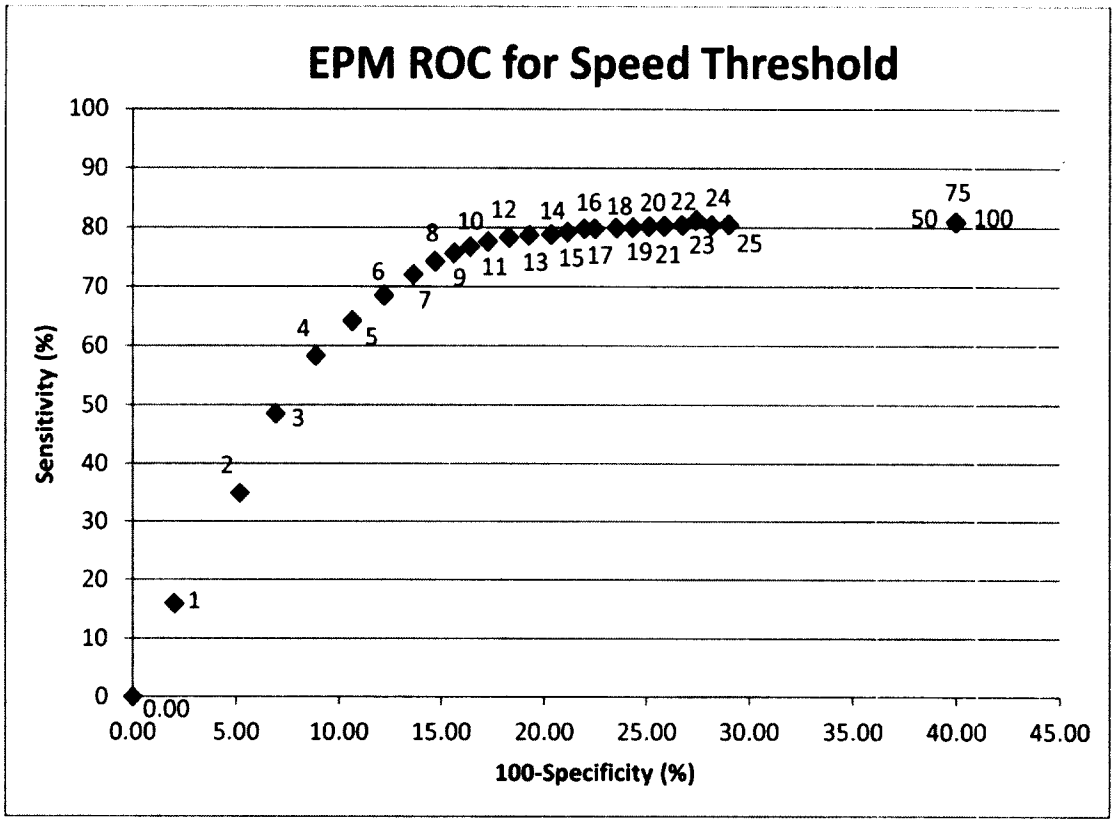

Figure 3-10: ROC curve for speed threshold when eccentricity threshold is set at 0.89 for elevated plus maze. The chosen speed threshold value of 12 cm/s can be found near the top left of the ROC curve indicating an acceptable value.

The speed threshold was lower than the threshold for the open field test as the mice had less space to gain momentum with only 5-cm wide paths. The eccentricity value was lower because the mice would exhibit SAP while bending out toward the open arms. This posture generates a shorter ellipse during SAP. An eccentricity value of 0.90 would fail to detect some of these bent SAP postures, so an eccentricity of 0.89 was used to increase the sensitivity although this change decreased specificity.

3.3.3 Runtimes

The runtime for MATSAP to analyze the videos, as measured by the in-build MATLAB profiler, was markedly shorter than the evaluation time taken by the human observers. On a laptop with an Intel® Core™ i7-3520M core processer at 2.90 GHz and with 6.00 GB of RAM, the runtime for a 5-min video at 10 fps was less than 2 min when the output was visualized at 1 fps without saving the video. Furthermore, the runtime was only about 30 s without the visualized output or saving videos. These runtimes exclude the time spent by the user to answer prompts and to use the threshold preview screen. If the parameters (video dimensions, fps, and threshold) are the same, the user can set the program to run continuously through hundreds of videos. Depending on the human observer's skill level, the evaluation time ranged from 10 to 45 min per 5-min video.

3.3.4 MATSAP Threshold Optimizer

MATSAP is user-friendly and flexible enough to meet different research requirements with respect to adjusting speed and eccentricity parameters to achieve greater sensitivity or specificity. MATSAP Threshold Optimizer can assist users in finding the optimal speed and eccentricity value thresholds based on a sample set of scored videos. These threshold values can be selected based on the nature of the data along with the sensitivity, specificity, accuracy, MCC, F-score, and area under the curve (AUC). For example if an experimenter needs to analyze SAP in 120 videos, ten of the videos can be scored by observers. Then MATSAP Threshold Optimizer can be used to find optimal speed and threshold measurements based on the scored data. Using these calculated thresholds, the 120 videos can be scored.

3.3.4.1 *Optimizing threshold in open field.* Using the MATSAP Threshold Optimizer (Supplementary Software 1), different eccentricity and speed thresholds were explored to optimize SAP detection in the open field. **Table 3-1** provides a summary of this analysis.

Table 3-1: MATSAP Threshold Optimizer output table.

	Speed	Eccentricity	Sensitivity	Specificity	Accuracy	MCC	Fscore	AUC
max MCC	16	92	78.3	94.9	93	0.68	0.72	0.87
max Accuracy	12	92	65.3	97.1	93.5	0.66	0.69	0.81
max F-score	16	92	78.3	94.9	93	0.68	0.72	0.87
max AUC	19	91	93.5	88.1	88.7	0.63	0.65	0.91

The maximum MCC of 0.6803 occurred when the speed and eccentricity values of 16 cm/s and 0.92 are chosen, respectively (**Figure 3-11**). The F-score was also at the maximum of 0.7164 (**Figure 3-12**) and the accuracy was 93.03%. At these thresholds, the sensitivity was 78.34% and the specificity was 94.89%.

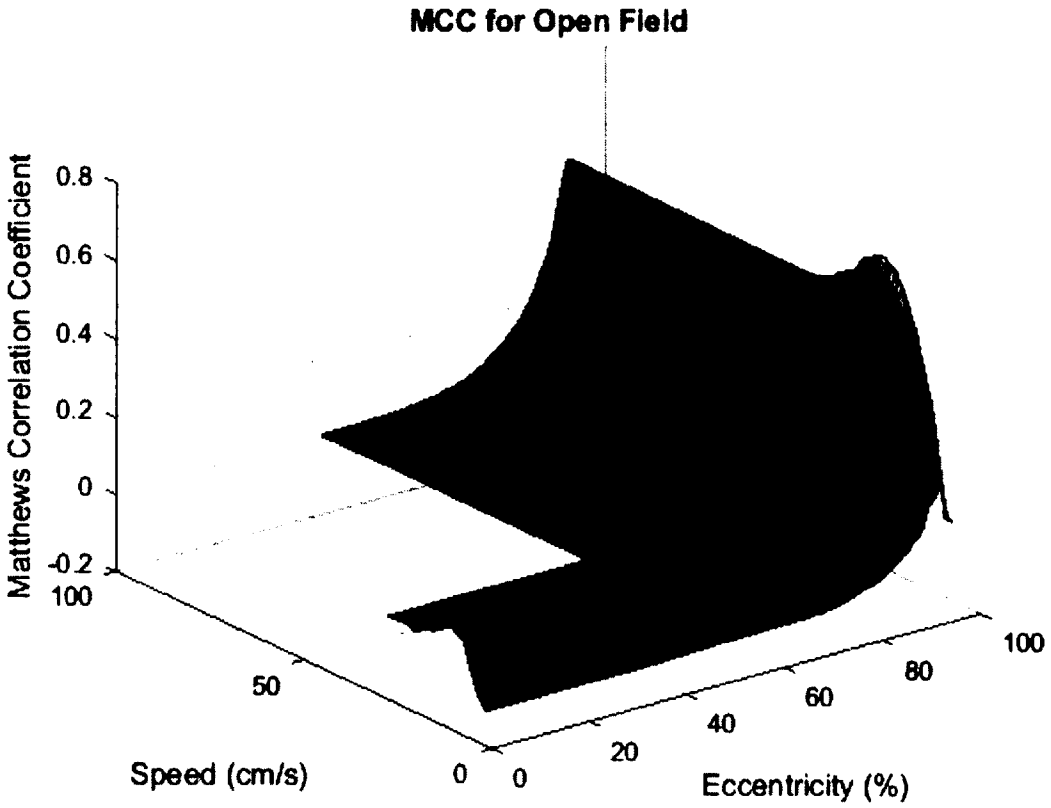

Figure 3-11: MCC of MATSAP analyzing open field videos. Matthews correlation coefficient (MCC) values when analyzing open field videos at different speed and eccentricity thresholds. The maximum MCC of 0.68 occurred with a speed threshold of 16 cm/s and an eccentricity threshold of 92%.

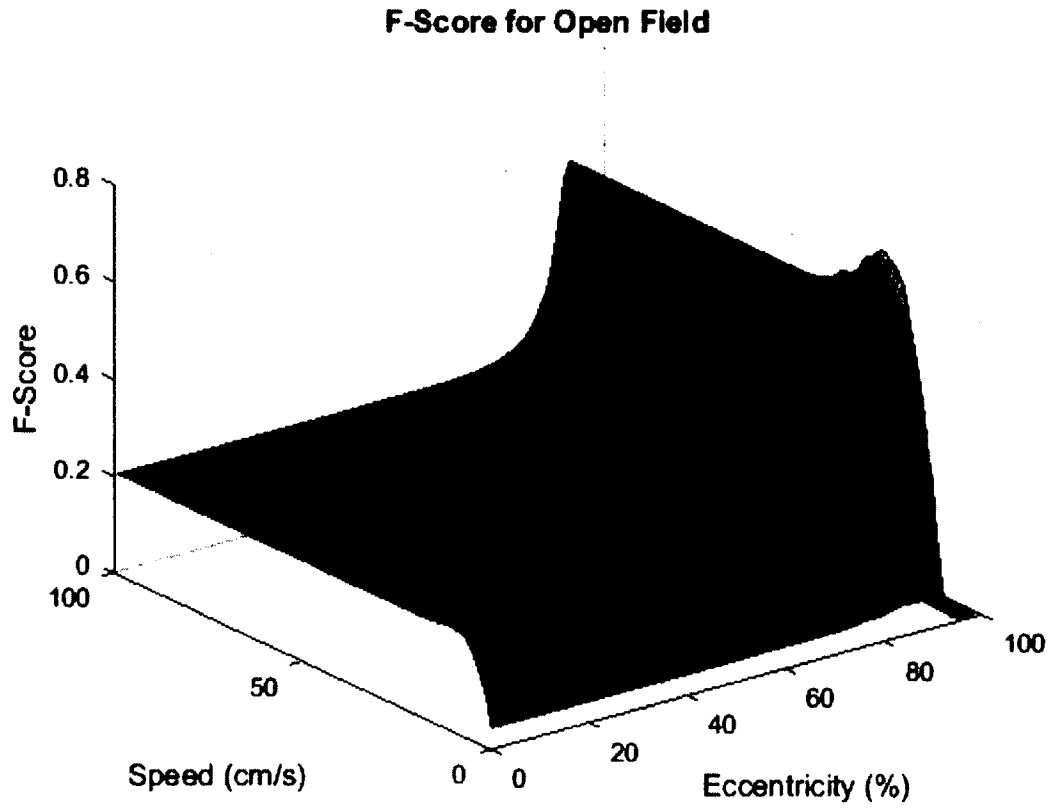

Figure 3-12: F-score of MATSAP analyzing open field videos. The maximum F-score of occurred with a speed threshold of 16 cm/s and an eccentricity threshold of 92%.

Since the negative class (SAP not present) was greater than the positive class (SAP present), the MCC score favored a higher specificity. Since the positive and negative class may be more balanced in other experiments, a relatively balanced sensitivity and specificity was desired in conjunction with a high MCC. This is the rationale we used for selecting 12 cm/s and 0.90 as thresholds values for speed and eccentricity, respectively. The maximum accuracy of 93.50% occurred when the speed and eccentricity values of 12 cm/s and 0.92 were chosen, respectively (**Figure 3-13**). The sensitivity and specificity at these thresholds were 65.28% and 97.07%, respectively. The specificity was favored for accuracy as there were more negative classes present in this

experiment. In our application, we do not desire high specificity at the cost of losing sensitivity.

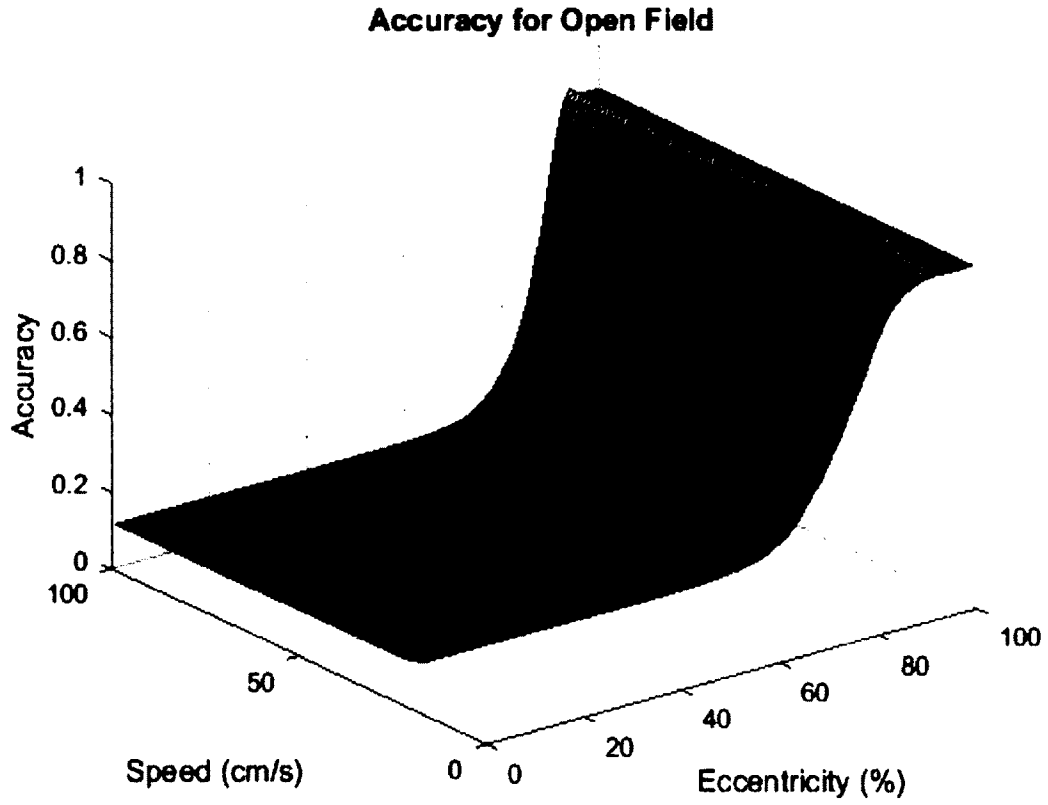

Figure 3-13: Accuracy of MATSAP analyzing open field videos. The maximum accuracy of 93.03% occurred with a speed threshold of 12 cm/s and an eccentricity threshold of 92%.

The maximum AUC of 0.9077 occurred when the speed and eccentricity values of 19 cm/s and 0.91 are chosen, respectively (**Figure 3-14**). At these thresholds, the sensitivity was 92.58% and the specificity was 88.92%. A specificity slightly higher than sensitivity would be preferable since in practice it is more likely that SAP will not be present than present.

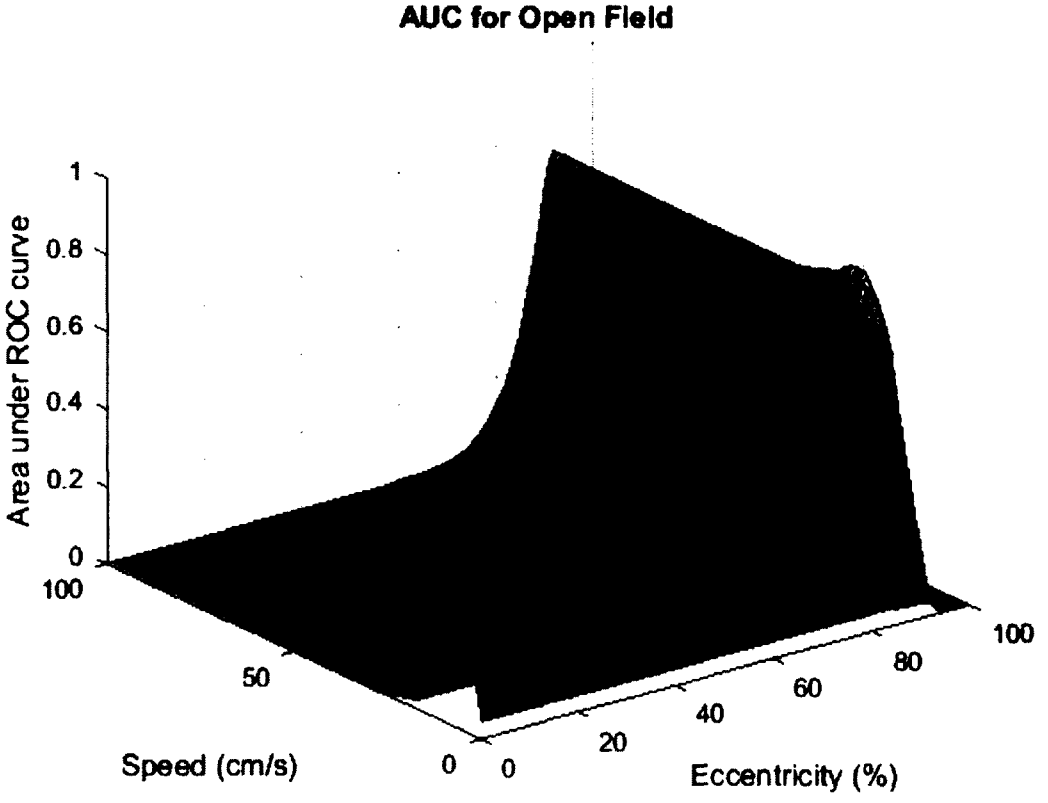

Figure 3-14: Area under the ROC curve for MATSAP analyzing open field videos. The maximum AUC of 0.9077 occurred with a speed threshold of 19 cm/s and an eccentricity threshold of 91%.

3.3.4.2 *Optimizing threshold in elevated plus maze.* MATSAP Threshold Optimizer was also used to explore different eccentricity and speed thresholds in the elevated plus maze. Supplementary Table S2 provides a summary of this analysis. The maximum MCC of 0.7016 occurs when the speed and eccentricity values of 8 cm/s and 0.89 are chosen, respectively (**Figure 3-15**). At these thresholds, the sensitivity was 85.96% and the specificity was 85.30. The accuracy, F-score, and AUC were 85.56%, 0.8194, and 08563, respectively.

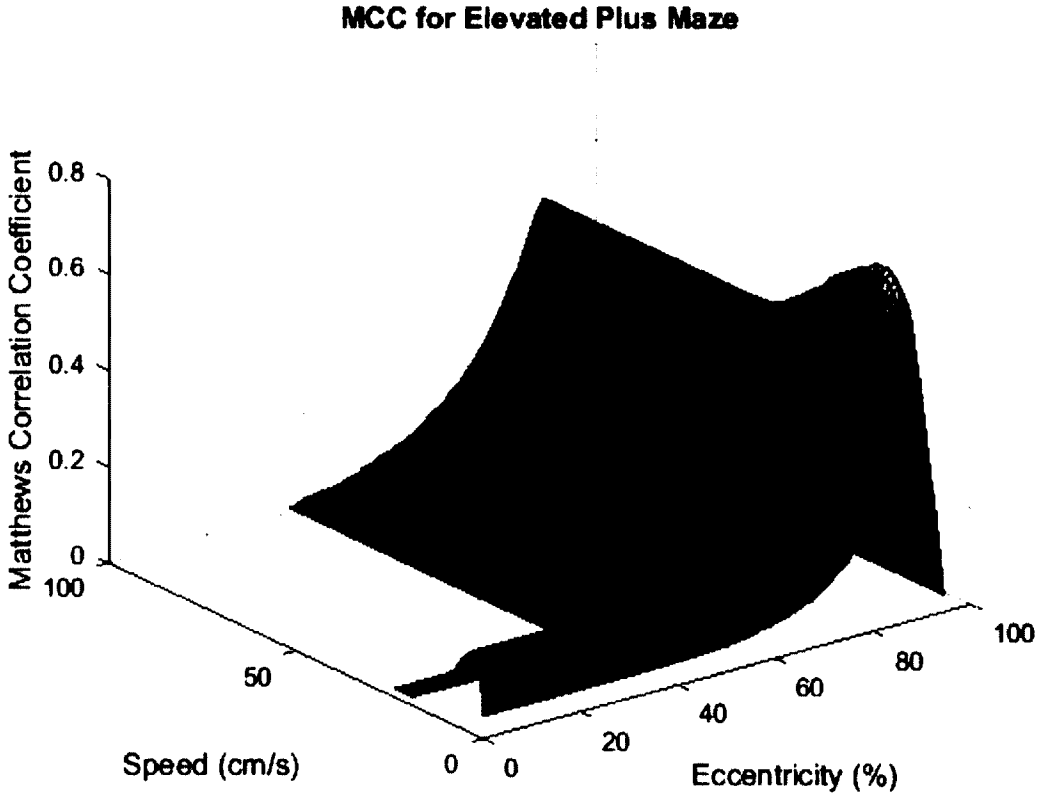

Figure 3-15: MCC of MATSAP analyzing elevated plus maze videos. Matthews correlation coefficient values when analyzing elevated plus maze videos at different speed and eccentricity thresholds. The maximum MCC of 0.70 occurred with a speed threshold of 8 cm/s and an eccentricity threshold of 89%.

The maximum accuracy of 85.63% occurred when the speed and eccentricity values of 8 cm/s and 0.90 were chosen, respectively (**Figure 3-16**). The sensitivity and specificity at these thresholds were 79.41% and 89.46%, respectively.

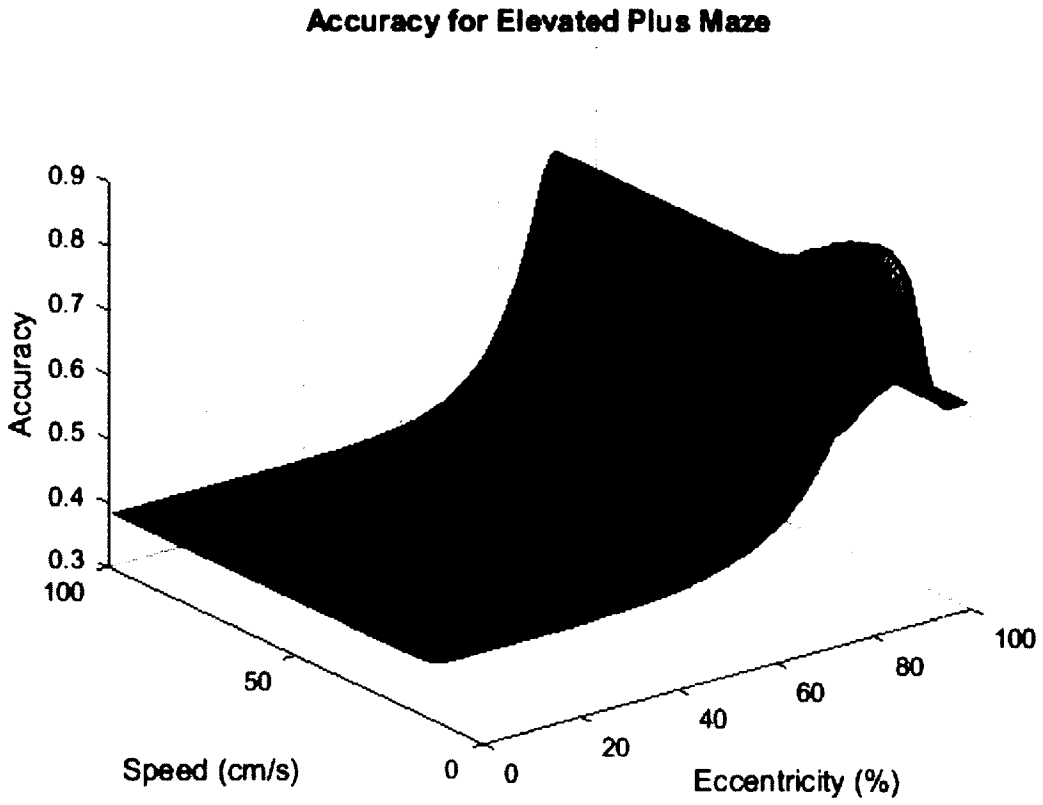

Figure 3-16: Accuracy of MATSAP analyzing elevated plus maze videos. The maximum accuracy of 85.63% occurred with a speed threshold of 8 cm/s and an eccentricity threshold of 90%.

The maximum F-score and maximum AUC both occur when the speed threshold was 9 cm/s and the eccentricity threshold value was 0.89 (**Figure 3-17** and **Figure 3-18**). The sensitivity and specificity values were 87.03% and 84.38%, respectively.

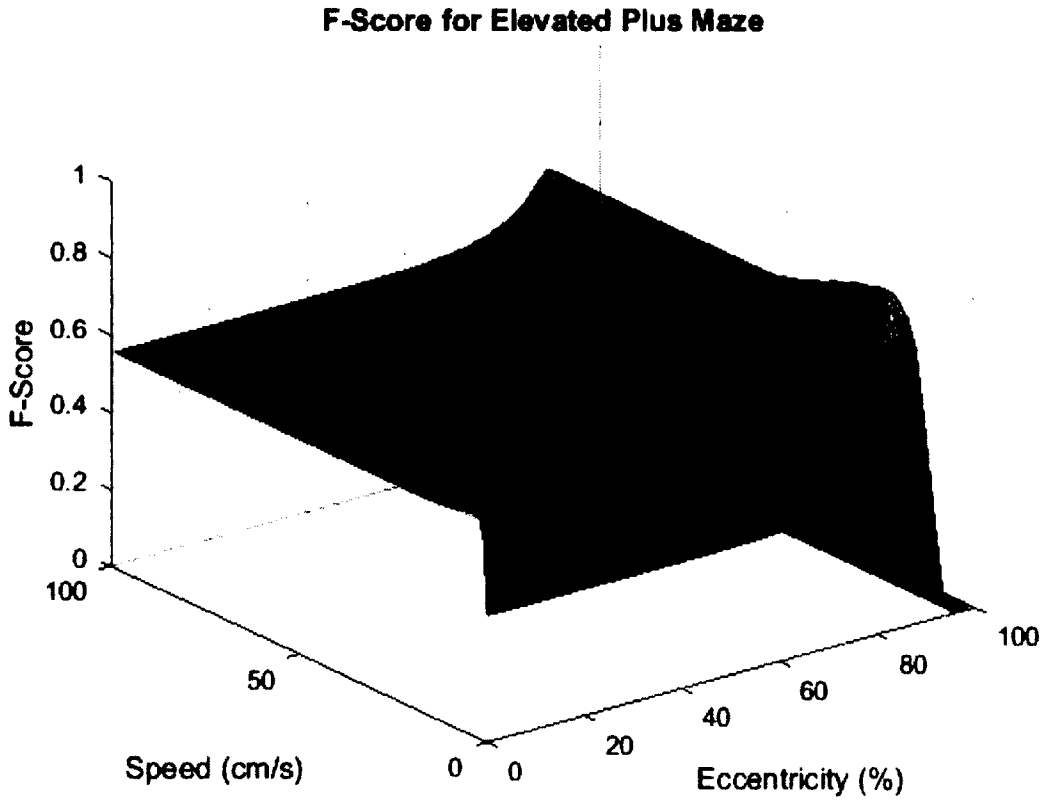

Figure 3-17: F-score of MATSAP analyzing elevated plus maze videos. The maximum F-score of occurred with a speed threshold of 16 cm/s and an eccentricity threshold of 92%.

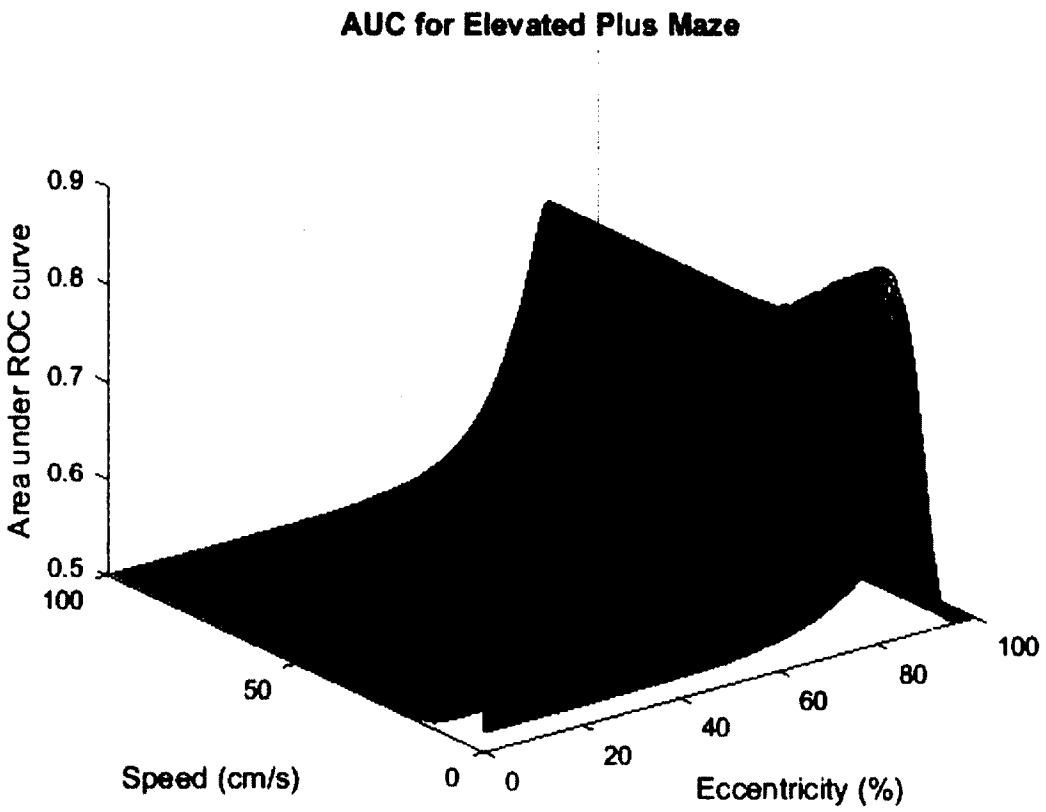

Figure 3-18: Area under the ROC curve for MATSAP analyzing elevated plus maze videos. The maximum AUC of 0.7343 occurred with a speed threshold of 9 cm/s and an eccentricity threshold of 89%.

3.4 Discussion

Currently, there is no commercially available behavioral analysis software that directly detects SAP or any free software that can be readily utilized to detect SAP. Due to limited funding in some research labs, there is a need for inexpensive software that detects SAP in rodents. To meet this need, a freely available, open source software program with a flexible, user-friendly GUI called MATSAP was successfully developed to detect SAP. The program runs in a basic MATLAB installation with the Image Processing Toolbox™. MATSAP allows users to analyze multi-page Tag Image File Format (multi-TIFF, .tif) video files of rodents from an overhead view. MATSAP was a

reliable program for detecting SAP when using male Swiss mice weighing 32.5-40.0 g in both the OF and EPM.

Computers are quick, consistent, and tireless, unlike human observers. The flexibility of the program and the user-friendly interface allows for optimal run times. It takes less than 2 minutes to analyze a 5-minute, 10-fps video using MATSAP. In contrast, it would take a human observer from 10 to 45 minutes depending on the observer's skill level. This 5- to 23-fold decrease in time means that the program is well suited for on-line applications. Furthermore, MATSAP can replace human observers with the exception of an occasional check of the program's output.

Frequency was higher in MATSAP than the human consensus. It is possible that MATSAP could be more accurate than the human consensus, which was assumed to be the "ground truth." The observers may be influenced by the psychological effect known as the law of closure. If there was a small break between SAP behaviors, the human observers may have considered the separate events as one. MATSAP would discriminate these events as separate resulting in a higher frequency, but maintaining roughly the same duration of the behavior as observed in the case of the OF test.

In the EPM, both MATSAP and the individual observers had a lower accuracy, sensitivity, and specificity than in the OF when compared to the human consensus (Table 1). MATSAP detected a lower duration of SAP than the human consensus in EPM. It is possible that MATSAP was not detecting SAP behavior while the mice were bending around a corner peering into an open field arm. The generated ellipse may not have been long enough to pick up all of these instances. The frequency of SAP was slightly higher in EPM than the OF as indicated by both MATSAP and the human consensus results.

There were more transitions between non-SAP and SAP behaviors throughout the EPM test in comparison to the OF test (**Figure 3-6** and **Figure 3-19**). This could have increased the likelihood of discrepancies among the human observers leading to a lower average accuracy of the individual observers and in turn a lower accuracy for MATSAP.

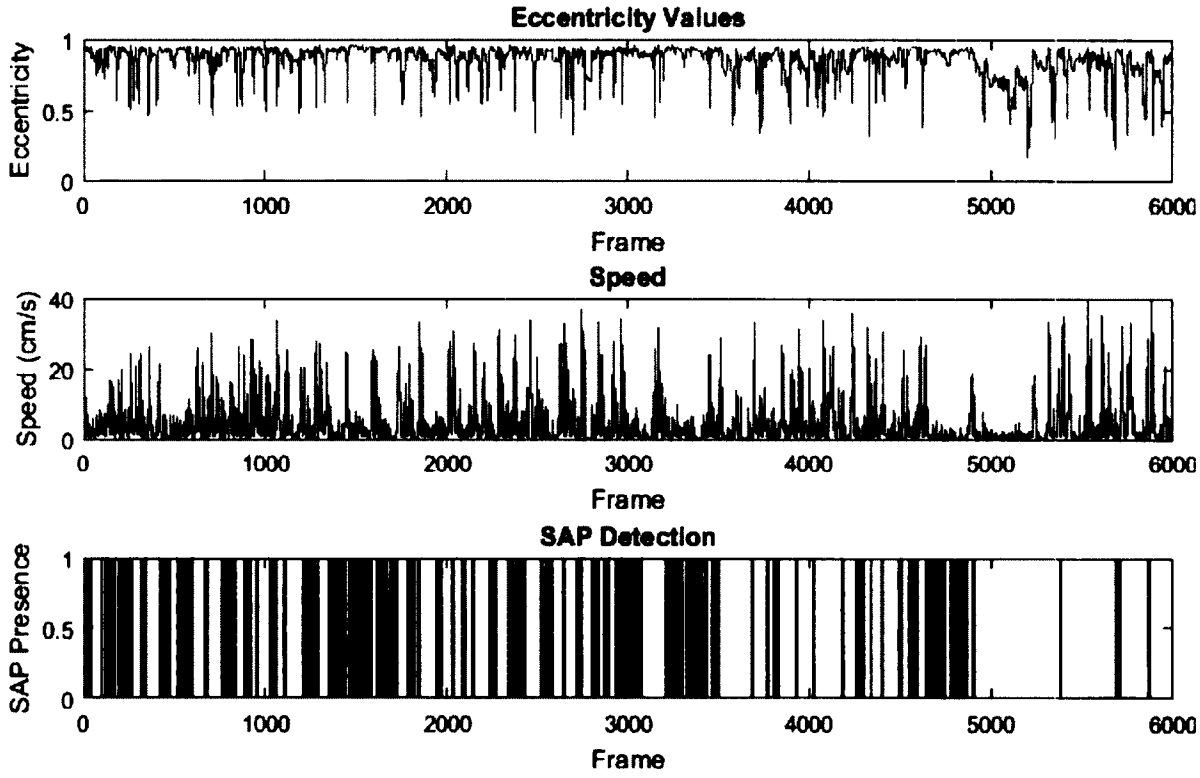

Figure 3-19: Output plots for elevated plus maze video. SAP behavior is more uniform throughout time in the EPM in comparison to the OF.

3.4.1 Flexibility of Software

MATSAP is flexible to meet different research requirements. For example, speed and eccentricity threshold parameters can be adjusted to achieve greater sensitivity or specificity, as needed. In addition to tracking white mice on black surfaces, the software can also track dark mice when the testing apparatus has a white surface. Furthermore, the

program can run on different computer operating systems provided that a MATLAB release is available for the operating system.

Another aim of this program was to have a graphical user interface that makes the program easy to use, especially for optimizing the program runtime for scoring videos. To do this, MATSAP begins with a series of questions that guide the user in selecting the level of visualization that occurs when the program is running. This visualization allows the operator to verify that the program is running properly. To reduce the runtime for all videos, the operator has the option of displaying only one frame of the video per second or not at all. Saving the video output is also optional. Additionally, MATSAP is designed to display the fewest dialog boxes possible between videos. The output files are saved in both Excel and ASCII. If the Excel files fail to save, MATLAB has a built-in function to automatically save them as a Comma Separated Values (CSV, .csv) file. If all of the parameters (thresholds, dimensions, and fps) are the same for a set of videos in a computer file folder, the user can apply them to all videos within that folder, so that the program becomes fully automated without any additional input. This allows the user to analyze hundreds of videos without further input.

3.4.2 Uses and Limitations

MATSAP may also be useful for other behavioral tests such as the canopy test and the rat exposure test. With a slight modification, MATSAP could be used to quantify forward SAP (F-SAP). This would be useful in tracking SAP towards an unfamiliar object, such as in novel object recognition tests, [110] or away from a novel object, such as an electrifiable prod [19]. Combining SAP detection with spatiotemporal measures

could help differentiate between 'protected' (when the rodent is under a covering or in an enclosed area) and 'unprotected' SAP [22, 25, 28, 29, 86, 87].

The eccentricity threshold value of 0.90 can only be confidently used for Swiss male mice with a weight range of 32.5-40.0 g. Different strains of mice or rats may have a different threshold values for speed and eccentricity. Different species, sex and weight of rodents would require the use of different eccentricity and speed threshold values. These can be calculated using the Threshold Optimizer (Supplementary Software 2) that is described in Supplementary Information. Another limitation is that the current version of MATSAP (v1.0) only works in offline mode. This is because video files require preparation, such as cropping videos to a known scale and converting videos to multi-TIFF files before running MATSAP.

3.4.3 Future Work

Our goal is to create a collaborative user group whose participants will provide their optimized eccentricity and speed threshold parameters for other rodents and strains of mice. These would be curated and posted on a user forum. Tables of these threshold values would also contain the species, age, weight and sex associated with the parameter values. Once these tables are established, MATSAP can be modified to include a user-input option to select the rodent type, strain, and weight in order to automatically select the appropriate threshold values. Additionally, we plan to develop real-time analysis capabilities so that users can obtain results while performing an experiment.

MATSAP may also be useful for other behavioral tests such as the canopy test and the rat exposure test and this should be evaluated. With a slight modification, MATSAP could be used to quantify forward SAP (F-SAP). This would be useful in

tracking SAP towards a novel object, such as in novel object recognition tests, [110] or away from a novel object, such as an electrifiable prod [19]. Combining SAP detection with spatiotemporal measures could help differentiate between 'protected' (when the rodent is under a covering or in an enclosed area) and 'unprotected' SAP [22, 25, 28, 29, 86, 87].

There will be continual improvements to MATSAP. We plan to develop online, real-time analysis capabilities where the user can obtain instant results while performing an experiment by using a camera with firewire that can connect directly to a computer running MATSAP. User region selection will be implemented so the user can select a region of known dimensions or a region to crop. This feature can also reduce video preparation time if offline analysis is desired. Saving output videos files without using the MATLAB getframe function is preferred, so the user can save a video of the visualized output without having to display it (thus decreasing the runtime). Another future improvement is to allow the program to read other video formats so the user does not need to convert files into multi-TIFFs.

3.4.4 Conclusion

MATSAP provides a quick, easy, and reliable method to detect the more sensitive rodent anxiety measure, stretch-attend posture, in less time and with less potential subjectivity than the human scorers. The program offers a user-friendly graphical interface and a flexible structure that caters to individual needs and that facilitates the optimization of runtimes. MATSAP enables scoring a large quantity of rodent behavioral videos in a relatively short period of time. This is an advantage when testing a large number of rodents. Future work is needed to establish eccentricity and speed thresholds

tables for different rodent species, strains and sizes, as well as to evaluate other ethological behaviors. This can be accomplished by curating software users' parameters and additional open-source companion programs akin to the user-provided plug-ins for the free image processing software, ImageJ [101].

CHAPTER 4

ETHOSTOCK: AN AUTOMATED ANALYSIS OF ETHOLOGICAL RODENT BEHAVIORS

4.1 Introduction

After the development of MATSAP, EthoStock was developed to detect SAP using elliptic Fourier analysis. This software has the potential to detect a broader array of ethological behaviors such as grooming and rearing. As mentioned in the previous chapter, there is a need for automated software to detect ethological behaviors. The goal of the program is to track an assortment of ethological behaviors of rodents from an overhead view by comparing detected images to a databank. After the development of a successful databank for a particular ethological behavior, the databank will be used to train a neural network to detect the behavior of interest. Using the neural network as opposed to the databank would allow for optimal runtimes.

4.2 Methods

4.2.1 Mice Living Conditions and Institutional Approvals

10 four month old male Swiss mice from Jackson Laboratories were and 30 four month old white wild-type mice from Jackson Laboratories and Mutant Mouse Resource Center (11 female and 19 male) were used in the study. The mice were housed in groups of 4-6 mice per cage to avoid stress and anxiety induced by social isolation [99], although

some believe individual housing decreases anxiety in mice [30]. The mice were housed in a 12-hour day/night cycle where food was administered *ad libitum*. Behavioral test procedures were approved by the Louisiana Tech University Institutional Animal Care and Use Committee and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996).

4.2.2 Behavioral Experiments

10 four month old male Swiss mice were used in open field in order to establish a databank for EthoStock. The weight of the male mice ranged from 32.5-40.0 g during testing. 30 four month old white wild-type mice were used in the open field in order to evaluate EthoStock. Behavioral tests were conducted by a male experimenter. For each mouse, there was a minimum of 24 hours between each behavioral test.

4.2.2.1 *Open field test.* The open field (OF) test apparatus consisted of an open square wooden container (30x30cm) with 25-cm high walls enclosing the perimeter. The walls and floor of the container were spray painted black. On the floor of the container, a white 16 square grid was drawn [100]. A camera mounted above the box and facing perpendicular to the floor was used to record the movement of the mice at 29 fps in high-definition MPEG Transport Stream (MTS, .mts) video format. To begin the test, a mouse was placed in the corner of the container. The mouse was allowed to explore the container for 5 minutes before being removed and placed into its home cage. Between

trials, super hypochlorous water was used to clean the floor and walls of the container [4].

4.2.3 Video Preparations

Videos were converted from MTS files at 29 fps to Audio Video Interleave (AVI, .avi) files at 10 fps without audio and then loaded into ImageJ 1.47t, a public domain image processing program [101]. Videos were converted to grayscale and cropped to the dimensions of the open field box with a 1:1 width to height ratio. Before saving as a multi-TIFF file, an image of the open field without a mouse present was added as the last frame of the video to use for background subtraction in the other frames so that the white rodent is readily distinguished from a black background.

4.2.4 Structural Design of Software

The software allows the users to analyze multi-TIFF video files of rodents from an overhead view. The user will need to convert video files to multi-TIFF files with the last frame being the background. Conversion to TIFF files can be done through the public domain image processing program ImageJ if the video format is AVI [101].

Figure 4-1 is a simplified flowchart of the software program. When the user runs EthoStock, a dialog box will prompt the user to select the folder containing the multi-TIFF files for analysis. The user was asked if the rodents are darker than the background in case the images need to be inverted for analysis. Then the program will give the user the option of opening the EthoStock Threshold Previewer, an interactive preview screen of the videos, to test for an appropriate binary conversion threshold value that was used to convert the images into a highly contrast binary images. After viewing the optional threshold preview or selecting "No" in the dialog box, another dialog box appears for the

user to input parameters of the video, which includes dimensions of the area covered in the video, the fps, the binary conversion threshold value, and the speed and eccentricity threshold values. After this, the user is given the options "yes" or "no" to have a visualized output of the analysis (Supplementary Fig. S2). If "yes" is selected, then the option of saving the video is presented to the user. The images are then analyzed and the user is prompted to select the file folder location to save an Excel file containing SAP detection results. SAP detection plots are displayed and saved in the directory folder (the folder that contained multi-TIFF files for analysis) unless an alternative directory is chosen for output files. The data are also archived in ASCII files in case a user does not have Excel in the default directory, unless the user specifies otherwise. A result summary spreadsheet is also generated containing the total SAP percentage, time, and frequency for each video in the directory folder. If all the input parameters are the same, including the binary conversion threshold value, the dimension, and the fps, the user has the option of skipping subsequent input dialog box prompts for the remainder of the videos in the folder. Furthermore, the user can set EthoStock to run until all of the videos in the folder are analyzed.

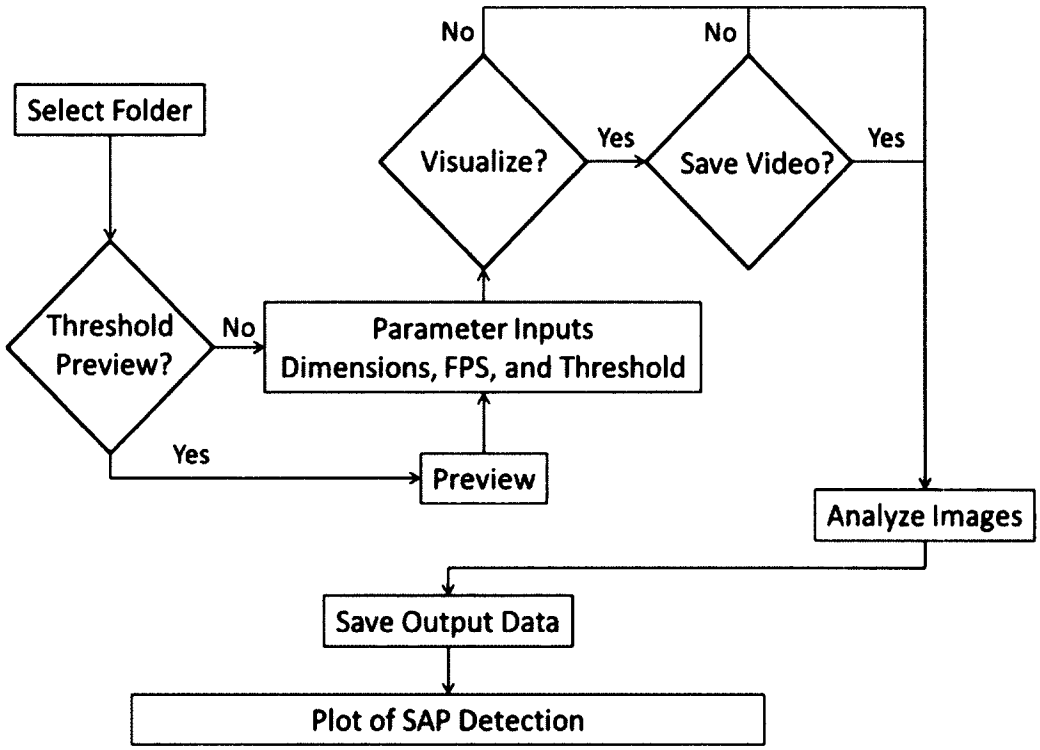

Figure 4-1: A simplified flowchart of EthoStock

4.2.5 Image Analysis

After the user enters the required parameters and answers the dialog prompts, EthoStock begins its fully automated image analysis by subtracting the background from each image frame and then converting the frames into black and white, binary images (**Figure 3-4a**). The rodent's tail is removed by eroding the perimeter of the rodent with the built-in imerode MATLAB function. This step creates an ellipse-like form, but it also reduces the overall size of the mouse (**Figure 3-4b**). After this automated step, the program restores the binary image of the rodent to its normal size by dilating the perimeter of the rodent with the imdilate MATLAB function, thus restoring the original girth and length, but without the tail (**Figure 3-4c**) [102]. EthoStock then selects the largest object (in this scenario, the rodent) with the aid of the regionprops MATLAB

function and crops around the rodent. The silhouette of the rodent is generated with the imperm MATLAB function

After converting videos into binary image stacks of tailless rodents similarly to MATSAP, the image was cropped around the rodent and the silhouette of the rodent was drawn with the imperm MATLAB function (**Figure 4-2**). The perimeter of the rodent was translated into a Freeman chain code so that elliptic Fourier analysis could be performed. EthoStock used elliptic Fourier analysis to form Fourier descriptors that represents the silhouette of the rodent [102, 111]. An ellipse formed around the rodent was molded around the contour of the rodent by two harmonic waves (one in the x-direction and the other in the y-direction) (see **Appendix B** for more details). Each harmonic wave has a real and imaginary coefficient, which are Fourier descriptors. Multiple iterations of these harmonic waves morphing the ellipse was performed until the once ellipse matches the perimeter of the rodent (**Figure 4-3**). Each iteration generated four Fourier descriptors. Ten iterations, which was chosen, generated the desired silhouette of the rodent. This provided 40 Fourier descriptors that represent the silhouette image of the rodent.

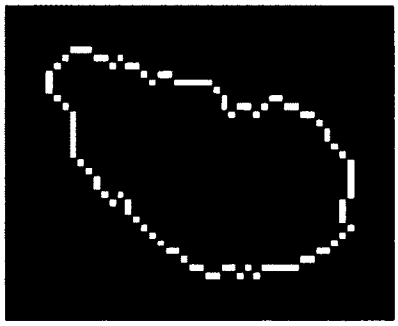

Figure 4-2: Binary image of rodent after implementing the "imperm" MATLAB function.

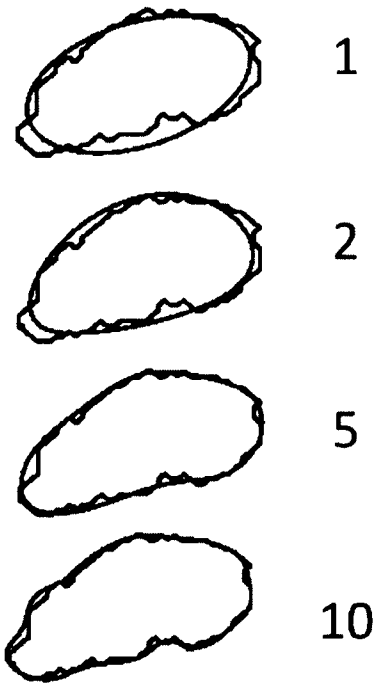

Figure 4-3: The Freeman code (blue) and the deformed ellipse (red) at different iterations.

In order to establish a databank, elliptic Fourier analysis was used to generate Fourier descriptors for each image within a set of training videos and MATSAP was used to determine the presence of SAP for the databank. 30 videos of male Swiss mice and 30 videos of the white wild-type mice in the OF for 5-min videos were used to create databank for EthoStock. The 10 frames per second videos were evaluated by MATSAP for SAP frame by frame.

After generating Fourier descriptors of a new image, EthoStock compared the results to the established databank using K nearest neighbor algorithm. EthosStock finds the three closest matches based on the 40 Fourier descriptors. **Figure 4-4** illustrates the process with only two Fourier descriptors, so it can be visualized in a 2D plane.

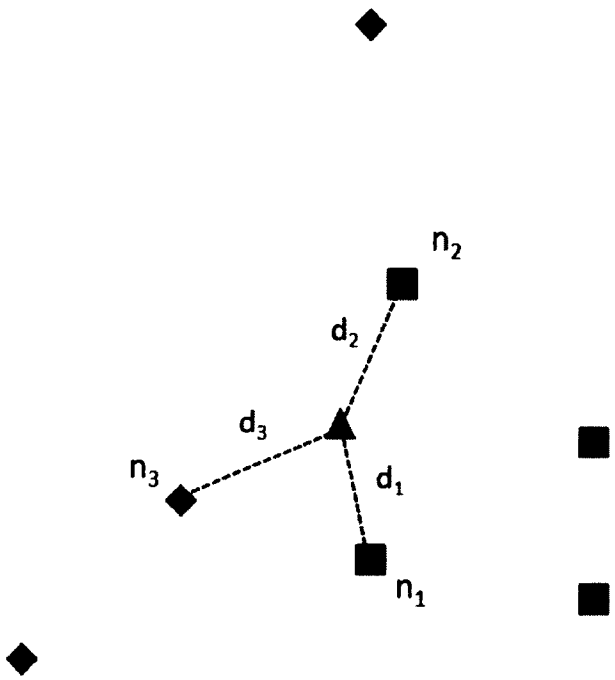

Figure 4-4: Illustration of k nearest neighbor

The three closest neighbors (n1, n2, and n3) are detected along with their distance from the query (d1, d2, and d3). The equation below was used to determine if SAP was present for the point of inquiry. Note the value for each neighbor was either 1 or 0.

$$0.50 \times n_1 + 0.30 \times n_2 + 0.20 \times n_3$$

If the distance to the first neighbor was the same as to the second neighbor, then the equation below was utilized instead.

If $d_1 = d_2$,

$$0.40 \times n_1 + 0.40 \times n_2 + 0.20 \times n_3$$

EthoStock provides an option to display the generated freeman chain code that outlines the silhouette of the rodent with the results of the elliptic Fourier analysis superimposed on it, so the user can verify that the program is working properly.

The assumption is made that SAP cannot occur in a time duration less than or equal to 0.5 seconds. So to eliminate false positives, any time there are consecutive 1's of a length less than or equal to half of the fps in the SAP detection array, these ones are changed to zeroes. This eliminates the cases where the mouse is not in a stretch-attend posture but is elongated because it is running.

4.2.6 Evaluation Methods

Ten 5-min videos at 10 frames per second of male Swiss mice in an open field box maze were evaluated by five human observers that were blinded to each other and by EthoStock. The inside of the OF box was painted black for maximizing contrast with the white mouse. For each second of the videos, the scorers determined if SAP was present giving a score of "1" if present and "0" if not. A human consensus score was determined via majority voting of each of the individual scorers at each second. The SAP detection software developed scores of the videos frame by frame. After running EthoStock on MATLAB (Version R2015b), the results were translated from frame-based scoring to a time-based scoring (in s). The human consensus was treated as the ground truth.

To determine the runtime of the EthoStock, MATLAB profiler was utilized. A typical laptop was used with an Intel® Core™ i7-3520M core processer at 2.90 GHz and with 6.00 GB of RAM. The runtime of EthoStock for a single video was measured at two different settings, obtaining results (i) with the visualized output being displayed and without saving the video and (ii) without displaying the output visual or saving the video.

4.2.7 Statistical Analysis

The accuracy, sensitivity, and specificity of EthoStock compared to the human consensus (ground truth) were determined along with the F-score, MCC, and area under

the curve (AUC). Binomial proportion confidence intervals for the accuracy, sensitivity, and specificity were calculated using normal approximation interval (Wald interval) since the sample size (the total secondss of the videos evaluated) was greater than 30 and the proportions were not close to 0 or 1 [104]. The AUC was approximated as demonstrated earlier in **Eq. 3-2** by the simple trapezoidal method [105, 106].

4.3 Results

EthoStock detects SAP by generating Fourier descriptors that describe the posture of the rodent in multi-TIFF video files via elliptic Fourier analysis and then comparing the Fourier descriptors to known values that correlate with SAP using a k nearest neighbor algorithm. EthoStock provides results in Excel and American Standard Code for Information Interchange (ASCII) files for importing into statistical programs. It also displays SAP detection plots over time. The program also provides optional features, which include a threshold preview screen to aid the user in selecting the appropriate threshold values to convert the multi-TIFF images into binary images for analysis, visualization of the image analysis (**Figure 3-4**), and saving the visualized output. These features can be used for sample videos in a large batch and then the batch can be run without them or with periodic sampling to reduce run times, if desired. Thus, EthoStock is flexible to allow for the optimization of runtimes.

4.3.1 Open Field

To test the ability of EthoStock to detect SAP, ten 5-minute videos at 10 frames per second (fps) of male Swiss mice moving in an open field box with a dark background were first evaluated by five observers to reach a "ground truth" score and then the videos were evaluated using EthoStock. Based on the human consensus score, SAP was present

in 337 seconds (*Positive*) and was not present in 2663 seconds (*Negative*). EthoStock had an accuracy of 87.9% (99% CI: 86.3 – 89.4%) with a sensitivity and specificity of 57.0% (99% CI: 50.0 – 64.0%) and 91.8% (99% CI: 90.4 – 93.2%), respectively, compared to the human consensus score. The F-score was 51.4% and the Matthews correlation coefficient (MCC) was 0.45. MCC is preferable over the F-score because the *Positive* and *Negative* classes are imbalanced [105]. Since the MCC is closer to 1 than -1, a strong positive relationship is indicated between EthoStock's classification of SAP and the classification by MATSAP [105, 109].

4.3.2 Runtimes

The runtime for EthoStock to analyze the videos, as measured by the in-build MATLAB profiler, was markedly shorter than the evaluation time taken by the human observers. On a laptop with an Intel® Core™ i7-3520M core processer at 2.90 GHz and with 6.00 GB of RAM, the runtime for a 5-min video at 10 fps was less than 10 min when the output was visualized at 1 fps. Furthermore, the runtime was only about 5 min without the visualized output. These runtimes exclude the time spent by the user to answer prompts and to use the threshold preview screen. If the parameters (video dimensions, fps, and threshold) are the same, the user can set the program to run continuously through hundreds of videos. Depending on the human observer's skill level, the evaluation time ranged from 10 to 45 min per 5-min video.

4.4 Discussion

The current version of EthoStock serves as a proof of concept for analyzing ethological behaviors using Fourier elliptic analysis in conjunction with a database. Although this technique has been examined in the past to analyze rodents, no software

has been developed to be used in the field by psychologist or biologists. EthoStock will be the first program specifically designed for the implementation in other labs. To the best of our knowledge, there is no other available program that uses this technique. EthoStock has a user-friendly interface and uses a contemporary programming language, MATLAB.

With the current databank, EthoStock had a reasonable accuracy and specificity. However, the sensitivity was too low for implementation in practice. A lower sensitivity is more preferable than a lower specificity in this scenario because it suggests that a larger databank that included more SAP postures could lead to an increase in sensitivity without hindering the specificity. If the specificity was low, there would have been a concern because it would suggest that the Fourier descriptors could not discriminate between SAP present and SAP no present postures. The hope is that if missing SAP postures are added to the databank, the sensitivity will increase.

4.4.1 Flexibility of Software

EthoStock was designed to be able to address ethological behaviors.

As long as there is a visual contrast between the rodent and the background, MATSAP can detect SAP. This includes a white rodent on black surfaces, a dark rodent on white surfaces, and an infrared video of a rodent on a cool surface. Furthermore, the program can run on different computer operating systems provided that a MATLAB release is available for the operating system.

EthoStock has a graphical user interface (GUI) that models after MATSAP. The GUI makes the program easy to use, especially for optimizing the program runtime for scoring videos. EthoStock begins with a series of questions that guide the user in

selecting the level of visualization that occurs when the program is running. This visualization allows the operator to verify that the program is running properly. To reduce the runtime for all videos, the operator has the option of displaying only one frame of the video per second or not at all. Additionally, EthoStock is designed to display the fewest dialog boxes possible between videos. The output files are saved in both Excel and ASCII. If the Excel files fail to save, MATLAB has a built-in function to automatically save them as a Comma Separated Values (CSV, .csv) file. If all of the parameters (thresholds, dimensions, and fps) are the same for a set of videos in a computer file folder, the user can apply them to all videos within that folder, so that the program becomes fully automated without any additional input. This allows the user to analyze hundreds of videos without further input.

4.4.2 Uses and Limitations

EthoStock has the potential to address an array of ethological behaviors. At its current state, a larger databank is needed to detect these behaviors. The detection of SAP as a proof of concept showed that EthoStock has potential, but is not ready to be implemented as a SAP detection software.

4.4.3 Future Work

A larger databank of SAP postures is needed to increase sensitivity, which in turn will increase the accuracy of EthoStock. Once a databank is established with suitable measures, the databank will be used to train a neural network through machine learning. This would provide a more optimal runtime as the larger the databank, the slower the software will become. The end goal of the program is to track an assortment of ethological behaviors of rodents from an overhead view. After establishing a decent

databank for SAP and trained neural network, other ethological behaviors will be explored such as grooming and rearing, which there is a greater need for in the field. First a databank will be established for each of these behaviors and then a neural network would be trained with said databank. Further databanks can be used to continually train and approve the detection of each ethological behavior.

EthoStock can also be trained and tested with other apparatuses such as the elevated plus maze, 3-chamber social interaction test, or novel object recognition test.

4.4.4 Conclusion

EthoStock provides a quick and objective method to detect the underutilized rodent anxiety measure, SAP. The program offers a user-friendly graphical interface and a flexible structure that caters to individual needs and that facilitates the optimization of runtimes. EthoStock enables scoring a large quantity of rodent behavioral videos in a relatively short period of time. Future work is needed to increase the accuracy, sensitivity, and specificity of the software. This can be done through extensive work by building a larger databank of SAP postures. This larger databank will provide better results at the cost of slowing down the program. The slower runtime can be circumvented by training a neural network with larger databank. Once the neural network is refined through machine learning, this neural network can be used to determine if the Fourier descriptors of a particular image indicates SAP without having to refer to a large databank.

CHAPTER 5

TBI SAP

5.1 Introduction

With the ease of detecting SAP available due to MATSAP, the application of SAP detection was explored. We decided to evaluate the underutilized SAP measure in rodent traumatic brain injury (TBI) anxiety studies. TBI patients are known to experience anxiety for extended periods of time after experiencing the injury. Anxiety hinders the patient's quality of life and leads to difficulties during treatment. Post-TBI anxiety has been studied in rodent models using classical anxiety paradigms such as the elevated plus maze and the open field. However, these studies neglect the examination of the ethological behaviors such as SAP, which could provide more insight into the anxiety state of the rodents. Multiple studies have shown that TBI can lead to hyperactivity in animal models [112-114]. Hyperactivity will cause increased exploration in the classical paradigms, which calculate spatiotemporal measures to detect anxiety. Traditionally, locomotive measurements are taken in this apparatuses to make sure the anxiety measurements are not influenced by increased motor activity. Looking at ethological behavior such as SAP could help validate the spatiotemporal measurements are contributed to anxiety rather than locomotive behavioral changes.

TBI can also hinder cognitive functions such as spatial mapping or working memory, which can cause increase exploration in rodents [113]. Spatiotemporal anxiety

measures in the EPM have been indirectly correlated with the severity of TBI (Schwarz), while cognitive deficits have been directly correlated with TBI severity [112, 115, 116]. Additionally, mice treated with minocycline after receiving a severe TBI did not exhibit cognitive deficits and showed a decrease in the total distance explored in OF [116].

In order to better understand the anxious behavior that follows in some cases of TBI, a more refined measure of anxiety is required. Stretch-attend-posture (SAP) is a non-social measure of anxiety that is quantified by the speed and elongation of a rodent's body. SAP is a risk-assessment behavior, which is generally associated with anxiety, that occurs when the rodent lowers its back, elongates its body, and is either standing still or moving forward very slowly [18]. SAP has been found to be more sensitive to the effects of classical and atypical anxiolytics than traditional spatiotemporal indices in the murine plus-maze [24, 25]. For example, SAP is especially sensitive to the effects of ligands acting on 5-HT1A receptors [24, 25]. It is hypothesized that SAP can be related more to the cognitively oriented aspects of anxiety [24]. Inclusion of ethological measurements such as SAP in EPM provides a more comprehensive profile on the anxiolytic or anxiogenic effects of a treatment [23, 25, 26]. SAP can also help differentiate between anxiogenesis and sedation effects of drugs [2, 27]. Despite finding that risk assessment measurements are more sensitive to anxiety modulating drugs than traditional indices, Carobrez *et al.* found that only a quarter of studies have adopted them [2]. SAP frequency and duration is often used by ethologists studying anxiety [25, 28, 30], however we have found no evidence that the measure has been applied to TBI models.

In the past, SAP frequency measurement has been very personnel intensive. Recordings would have to be watched and scored by multiple researchers to gain a

reliable result. Meanwhile, multiple tracking programs exist to measure spatiotemporal coordinates during OF and EPM trials. However, with the development of MATSAP, there is now an open-source and publicly available program that can measure SAP frequency in an automated manner. MATSAP allows the user to measure SAP frequency throughout an EPM or OF trial based on the same recordings used to determine spatiotemporal coordinates.

The goal of this study is to show SAP frequency is a reliable measure of post-TBI anxiety in mice that have received a moderate injury that provides further insight into the behavior. This study will also show that MATSAP, which has previously been used to detect similar behaviors in white mice, is flexible enough to detect SAP frequency in black mice.

5.2 **Methods**

5.2.1 Mice Living Conditions and Institutional Approvals

29 wild-type C57BL6 male mice aged 9 weeks old were ordered from Envigo and quarantined for 1 week after arrival. The mice were housed in a 12-hour day/night cycle where food was administered ad libitum. Mice were housed individually after quarantine was lifted to prevent discrepancies between control mice and those undergoing surgery, which would need to be individually housed after the procedure to prevent aggravation of the surgical site. It also served to prevent discrepancies in the anxiety levels in the mice during behavioral tests [30]. Behavioral test procedures were approved by the Louisiana Tech University Institutional Care and Use Committee and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996).

5.2.2 Behavioral Experiments

At 10 weeks, mice began initial behavioral tests. The following week, mice were given a moderate TBI or sham surgery. Ten days after surgery, mice resumed behavioral testing, which continued for an additional 3 weeks. Behavioral tests were conducted by female experimenters. For each mouse, there was a minimum of 24 hours between each behavioral test. Mice were recorded in both the EPM and OF to measure anxiety based on traditional spatiotemporal location. SAP frequency was measured in these recordings. Mice were also given a novel object recognition (NOR) test to detect and compare any cognitive decline that occurred over the course of the study.

5.2.3 Test Schedule

Mice were divided into groups of six and tests were staggered between groups to ensure surgery occurred exactly one week after the initial tests were performed on each mouse. On day one, mice were placed in the EPM for 10 minutes. On day two, mice were placed in the OF for 5 minutes. Immediately after the OF test concluded, the NOR test began in the same apparatus. This allowed the OF test to also act as the pre-NOR test acclimation period, necessary because anxiety can have negative effects on the results of cognitive tests. During the NOR test, two novel objects were presented for 5 minutes, after which a new object replaced one of the originals. The position of the new object was alternated between mice to prevent bias. Each test was repeated once a week over the three weeks following the post-op rest period. We also tested retrograde memory loss by pairing the non-novel object from week 1 of the behavioral tests with a completely novel object during post-TBI NOR tests.

5.2.4 Elevated Plus Maze

An EPM was built from medium density fiber board using previously established dimensions [3]. Two opposing open arms and two opposing enclosed arms extended 25 cm from a 5 x 5 cm central platform forming a plus shape. Enclosed walls were 25 cm tall and the maze was elevated 50 cm above the floor. To begin the test, a mouse was placed in the central platform facing the south enclosed arm and was allowed to move freely about the maze for 10 minutes. A ceiling-mounted video camera facing perpendicular to the floor with a field of view centered on the central platform recorded mouse movement at 29 fps in MTS video format. The platform of the maze was white to provide color contrast to the black C57BL6 mice and the testing room was illuminated with standard fluorescent lights. Between trials, the central platform and all four arms were cleaned with 70% ethanol water to remove odors left by the previous mouse [3].

The public domain ImageJ program developed by Wayne Rasband at the National Institute of Mental Health along with plugin called ImageEP developed by Tsuyoshi Miyakawa was utilized to perform video analysis. The distance traveled, the number of entries into each arm, the time spent in each arm, and the percent of entries into the open arms are calculated by the ImageEP program as well as the generation of traces of the mouse's movement (**Figure 2-2:** Overhead view of the elevated plus maze and the corresponding centroid trace of a typical mouse's path. and **Figure 2-3**). The distance traveled measurement served to detect if locomotion was reduced due to the medication, thus skewing results. The percentage of time spent in the open arm and the percent of entries into the open arms was used to determine the anxiety levels of the mice. The

results of the experimental groups were compared to the sham mouse and any significant differences were noted. Based on the exploratory-anxiety conflict, the more anxious the mouse, the lower the percentage of time the mouse will spend in the open arm and lower the amount of entries the mouse will make in the open arm. SAP was measure using MATSAP. MATSAP Threshold Optimizer was used to establish speed and eccentricity thresholds for the C57BL6 male mice in the EPM.

5.2.5 Open Field Test

The OF test apparatus consisted of an open square wooden container (30x30cm) with 25-cm high walls enclosing the perimeter. The walls and floor of the container were spray painted white. On the floor of the container, a black 16 square grid was drawn [100]. A camera mounted above the box and facing perpendicular to the floor was used to record the movement of the mice at 29 fps in high-definition MPEG Transport Stream (MTS, .mts) video format. To begin the test, a mouse was placed in the corner of the container. The mouse was allowed to explore the container for 5 minutes before being removed and placed into its home cage. Between trials, 70% ethanol was used to clean the floor and walls of the container [4].

Using the ImageOF plugin for ImageJ, the percentage of time the mouse spends in each region was measured and compared to the sham mice. Traces of the mouse's movements were also generated by ImageOF (**Figure 2-6**). Based on the exploratory-anxiety conflict, the more anxious the mouse, the lower the percentage of time the mouse will spend in the center region. SAP was measure using MATSAP. MATSAP Threshold Optimizer was used to establish speed and eccentricity thresholds for the C57BL6 male mice in the OF.

5.2.6 Novel Object Recognition Test

For the NOR test, the OF apparatus was used. The OF test that preceded the NOR allowed the mice to acclimate with the environment. Between the OF and NOR test, the mouse was removed from the container and placed temporarily in its home cage. After the open container was clean with 70% ethanol solution and was dry, two identical novel wooden objects was placed inside the open container near a corner (the two corners were adjacent to each other and the novel objects were 5 cm from each wall forming the corner). The mouse was placed inside the open container near the center of the wall opposing the novel objects and was allowed to explore for 5 minutes. Then the mouse was removed from the container again and placed temporarily in its home cage. After the open container was cleaned with 70% ethanol solution and was dry, one object identical to the previously placed novel objects and a new novel object were placed inside the open container near a corner. The mouse was placed inside the open container near the center of the wall opposing the novel objects and was allowed to explore for 5 minutes. The sessions was videoed and later analyzed using a custom MATLAB code to determine the recognition index and the exploration time to aid in evaluating cognitive and locomotive abilities, respectively [116, 117].

The recognition index was calculated by

$$\textbf{Recognition index} = \frac{\textbf{time spent with novel object} \times 100}{\textbf{time spent with novel object} + \textbf{time spent with identical object}}$$

5.2.7 Behavioral Video Preparations

Videos were converted from MTS files at 29 fps to Audio Video Interleave (AVI, .avi) files at 10 fps without audio and then loaded into ImageJ 1.47t, a public domain image processing program [101]. Videos were converted to grayscale and

cropped to the dimensions of the apparatus (i.e. OF or EPM) with a 1:1 width to height ratio in pixels. Before saving as a multi-TIFF file, an image of the apparatus without a mouse present was added as the last frame of the video to use for background subtraction in the other frames so that the black rodent is readily distinguished from a white background.

5.2.8 Surgery

After the initial week of behavioral test, an injury hub was fitted on the mice as previously described [118]. The mice were first anesthetize with an intraperitoneal injection of ketamine/xylazine cocktail (10mg/mL ketamine, 0.1mL/10g) before the surgical site was cleared of fur with scissors. Then, the mice were positioned in a Cunningham Mouse Adapter (Stoelting) with a nose cone attachment on a stereotaxic frame. Using Kent SomnoSuite (Kent Scientific), 1.0% isoflurane gas was delivered for anesthesia for the duration of the surgery. A 3 mm outer diameter trephine was used to drill during a craniectomy. The injury hub, which will later receive the pressure pulse, was composed of a 20 gauge hypodermic needle female end Luer-lock that was previously cut and sterilized in 91% isopropyl alcohol. Using forceps, the hub was placed over the craniectomy and sealed with a thin layer of cyanoacrylate (Loctite Ultra-gel) followed by a layer of dental cement (RelyX Aplicap). After the operation, the mice remain under 1.0% isoflurane for 20 minutes while the dental cement dried. Then, they were placed in an empty cage that sat on a microwaveable heating pad (Braintree Scientific). Once the mice were conscious, they were returned to their home cage where food and water was available *ad libitum*.

5.2.9 Injury

A fluid percussion device (Custom Design & Fabrication) was used to injury the mice on the same day as surgery. Prior to injury, the mice were allowed to acclimate to the room holding the fluid percussion device before being anesthetized in a 4.5% isoflurane filled induction chamber. The lack of righting response and depth of respiration was evaluated to determine the level of anesthesia. While the mice were being anesthetized the fluid percussion device was primed and the pressure transducer output was recorded. Once the mice were confirmed to be anesthetized, sterile saline was injected into the injury hub and a male Luer-lock on the end of medical tubing attached to the percussion device was locked onto the hub. The mouse was then positioned on its side and the pendulum was dropped when the rodent's breathing returned back to normal. The device delivered an impact of (AA) atm. Righting time was used to evaluate the severity of the injury, where moderate concussion ranged between 200 and 540 seconds [115]. The mice were again anesthetized with 4.5% isoflurane after righting and the injury hub was removed. The isoflurane was reduced to 2.0% after the removal of the hub. The mice were inspected at the site of the craniectomy to ensure the brain was not herniated through the opening. If the brain was herniated, the mice were immediately euthanized. If the craniectomy was clear, a thin layer of agarose gel was applied to seal the opening in order to protect the brain. Then a round cover glass was placed onto top followed by a later of cyanoacrylate (Locitite). This procedure was able to prevent infections and also served as a proof of concept for future studies in which brain imaging will be performed through this sealed window post-TBI. Sham mice followed the same injury procedure,

except no impact was delivered to the rodent. Once the rodents recovered, they were returned to their home cage.

5.2.10 MATSAP Evaluation Method

Ten 5-min videos at 10 frames per second of male C57BL6 mice in an open field box maze and ten 10-min videos at 10 frames per second of male C57BL6 mice in an elevated plus maze were evaluated by 5 human observers that were blinded to each other and MATSAP. Both the inside of the OF box and the EPM were painted with for maximizing contrast with the black mouse. For each second of the videos, the scorers determined if SAP was present giving a score of "1" if present and "0" if not. A human consensus score was determined via majority voting of each of the individual scorers at each second. The SAP detection software developed scores of the videos frame by frame. After running MATSAP on MATLAB (Version R2012a), the results were translated from frame-based scoring to a time-based scoring (in s). The second-based SAP detection array of 1's and 0's generated by MATSAP was compared to the human consensus SAP detection array; the human consensus was treated as the ground truth.

MATSAP Threshold Optimizer was utilized to obtain the optimal eccentricity and speed thresholds used to detect SAP in the C57BL6 male mice.

5.2.11 Statistical Analysis

Using SPSS software, one-way ANOVAs were performed between the TBI treatment groups for the behavioral measurements in the OF and EPM. Levene's test of homogenous variance was used to ensure there was no significant difference in variance between the treatment groups. A post-hoc Tukey test was performed if there was no

significant variance between the groups. Welch's test was utilized in the case of unequal variance and Games-Howell post-hoc was conducted.

Using R software along with the irr package, a two-way agreement average-measure intra-class correlation was used to compute the inter-rater reliability of the 5 human observers that established the ground truth [103]. The accuracy, sensitivity, and specificity of MATSAP compared to the human consensus (ground truth) were determined along with the F-score, MCC, and area under the curve (AUC). Binomial proportion confidence intervals for the accuracy, sensitivity, and specificity were calculated using normal approximation interval (Wald interval) since the sample size (the total seconds of video evaluated) was greater than 30 and the proportions were not close to 0 or 1 [104]. The AUC was approximated in **Eq. 3-2** by the simple trapezoidal method [105, 106].

The MCC plots and ROC curves were generated with MATSAP Threshold Optimizer to justify the selection of speed and eccentricity thresholds used to conclude if SAP was present in an image frame. In the ROC curves, which were also generated in Excel, the most optimal threshold would be located in the top left of the graph as this is where sensitivity and specificity are the highest.

5.3 Results

5.3.1 MATSAP Validation

5.3.1.1 *Optimizing threshold in open field.* To test the ability of MATSAP to detect SAP, ten 5-minute videos at 10 frames per second (fps) of the dark C57BL/6 mice moving in an open field box with a white background were first evaluated by 5 blinded human observers (inter-rater reliability = 0.35) to reach a "ground truth" consensus score and then the videos were evaluated using MATSAP Threshold Optimizer. Removal of one of the human observers that scored liberally provided an inter-rater reliability of 0.55 between the remaining observers.

Using the MATSAP Threshold Optimizer, different eccentricity and speed thresholds were explored to optimize SAP detection in the open field. **Table 5-1** provides a summary of this analysis.

Table 5-1: MATSAP Threshold Optimizer output table

	Speed	Eccentricity	Sensitivity	Specificity	Accuracy	MCC	Fscore	AUC
max MCC	15	93	54.1	96.6	95	0.42	0.44	0.75
max Accuracy	3	94	3.7	99.9	96.4	0.15	0.07	0.52
max F-score	15	93	54.1	96.6	95	0.42	0.44	0.75
max AUC	15	91	80.7	80.6	80.6	0.28	0.23	0.81

The maximum MCC of 0.42 and the maximum F-score of 0.44 occurred when the speed and eccentricity values of 15 cm/s and 0.93 are chosen, respectively (**Figure 5-1** and **Figure 5-2**). At these thresholds, the sensitivity was 54.1%, the specificity was 96.6%, and the accuracy was 95.0%.

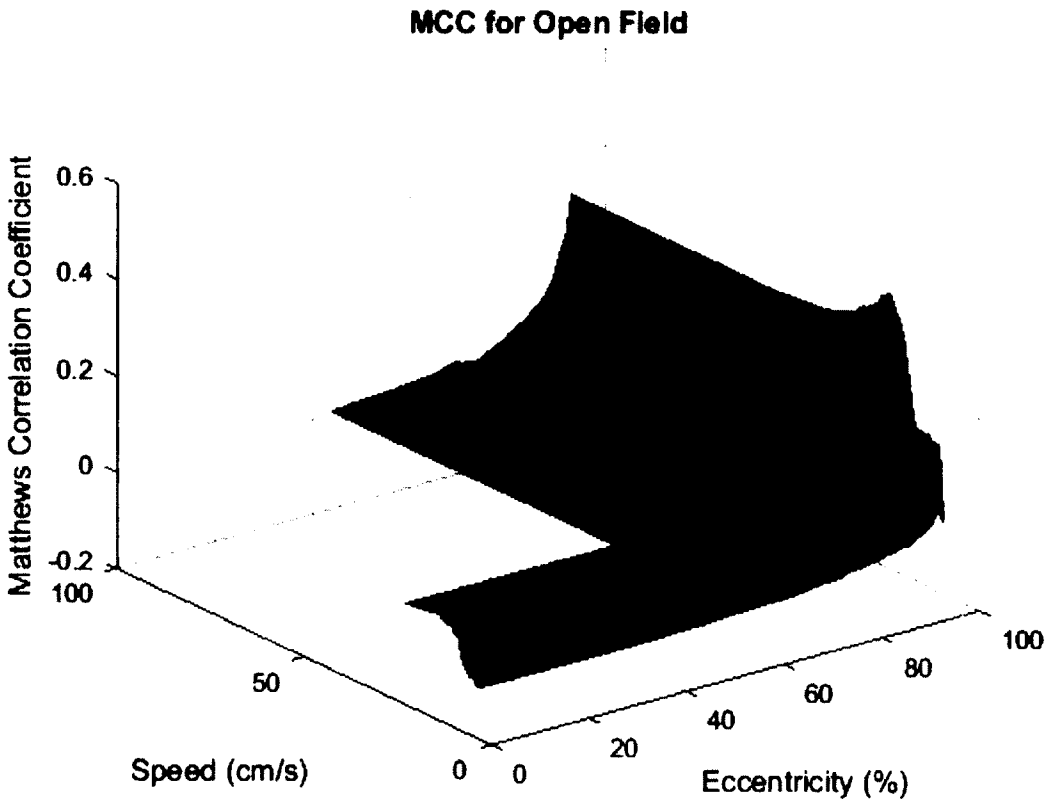

Figure 5-1: MCC of MATSAP analyzing open field videos. Matthews correlation coefficient (MCC) values when analyzing open field videos at different speed and eccentricity thresholds. The maximum MCC of 0.42 occurred with a speed threshold of 15 cm/s and an eccentricity threshold of 93%.

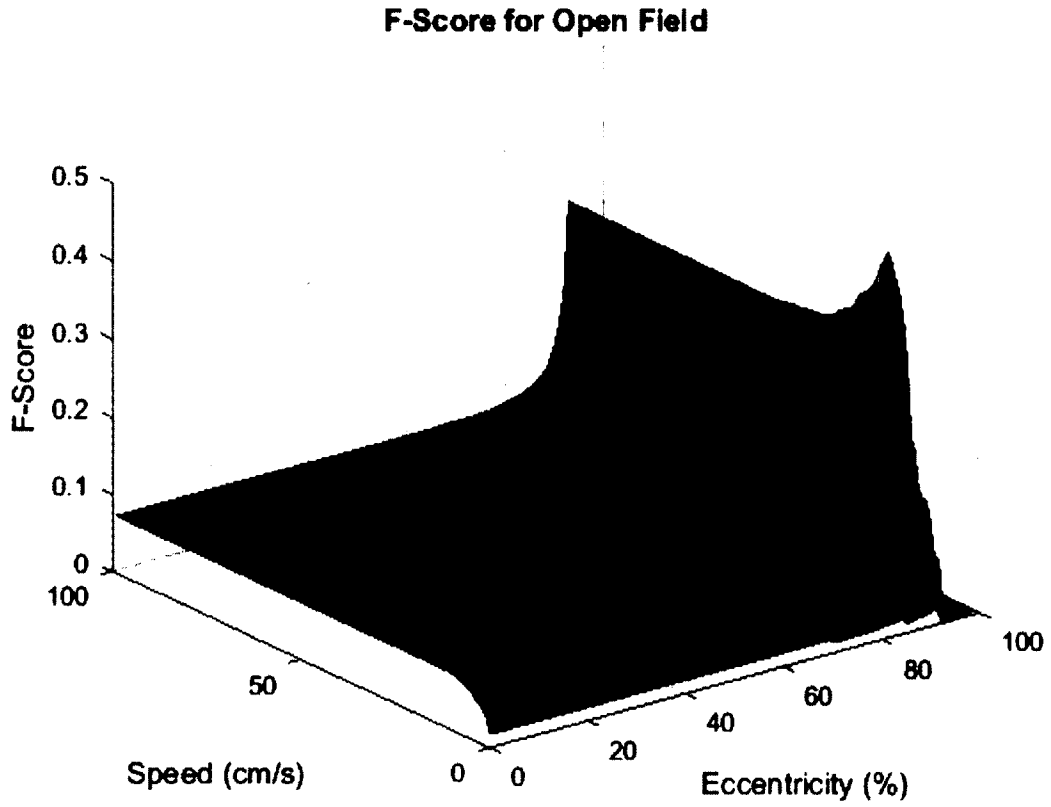

Figure 5-2: F-score of MATSAP analyzing open field videos. The maximum F-score of 0.44 occurred with a speed threshold of 15 cm/s and an eccentricity threshold of 93%.

Since the positive and negative class may be more balanced in other experiments, a relatively balanced sensitivity and specificity was desired. This is the rationale we used for selecting 15 cm/s and 0.91 as thresholds values for speed and eccentricity, respectively. The maximum accuracy of 96.4% occurred when the speed and eccentricity values of 3 cm/s and 0.94 were chosen, respectively (**Figure 5-3**). The sensitivity and specificity at these thresholds were 3.7% and 99.9%, respectively. The specificity was favored for accuracy as there were more negative classes present in this experiment. In our application, we do not desire high specificity at the cost of losing sensitivity.

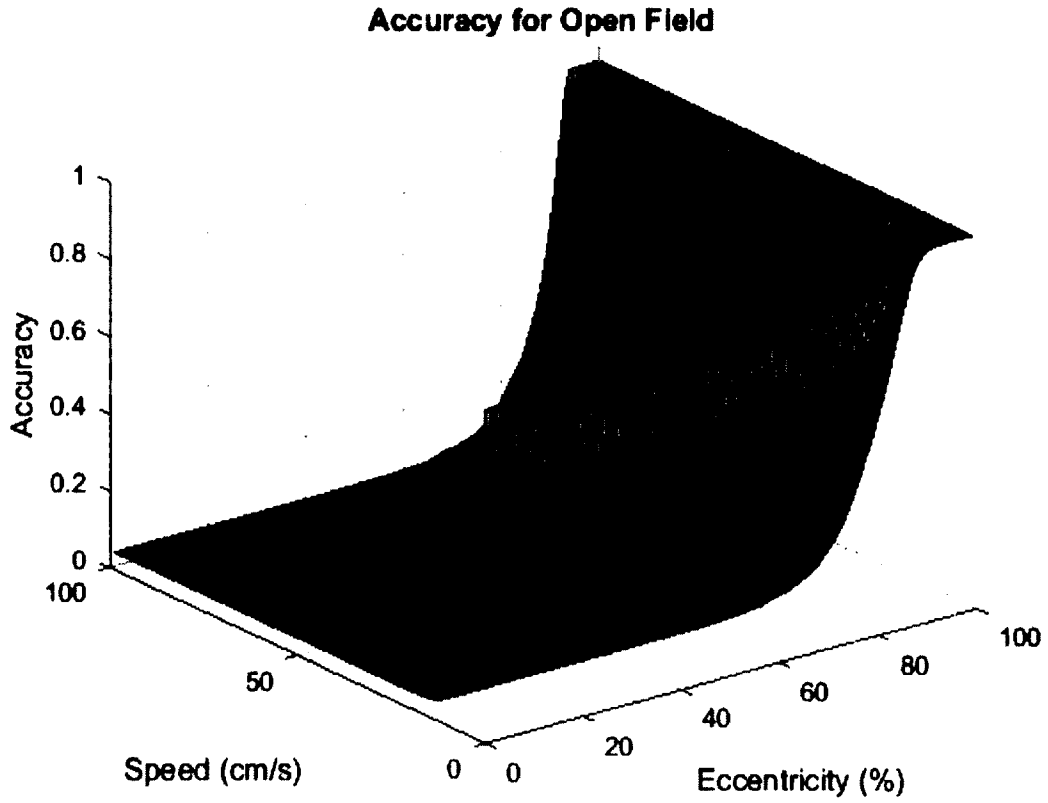

Figure 5-3: Accuracy of MATSAP analyzing open field videos. The maximum accuracy of 96.4% occurred with a speed threshold of 3 cm/s and an eccentricity threshold of 94%.

The maximum AUC of 0.81 occurred when the speed and eccentricity values of 15 cm/s and 0.91 are chosen, respectively (**Figure 5-4**). At these thresholds, the sensitivity was 92.58% and the specificity was 88.92%. A specificity slightly higher than sensitivity would be preferable since in practice it is more likely that SAP will not be present than present.

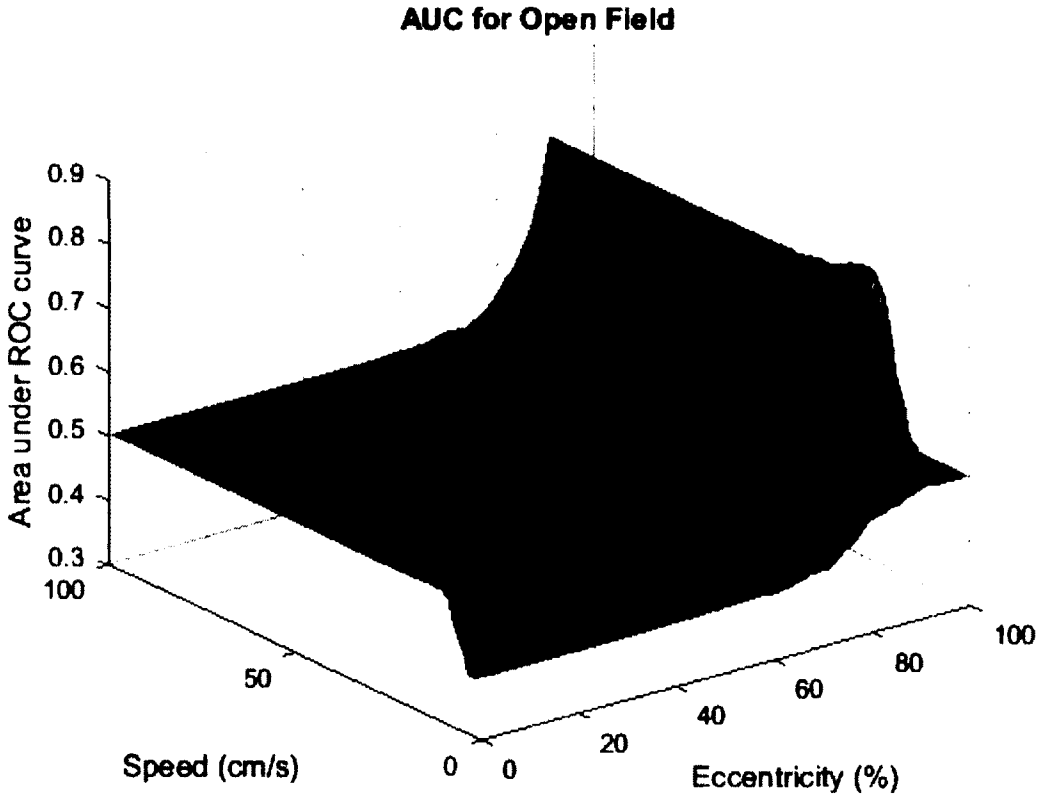

Figure 5-4: Area under the ROC curve for MATSAP analyzing open field videos. The maximum AUC of 0.81 occurred with a speed threshold of 15 cm/s and an eccentricity threshold of 91%.

5.3.1.2 *Optimizing threshold in elevated plus maze.* To test the ability of MATSAP to detect SAP, ten 10-minute videos at 10 frames per second (fps) of C57BL/6 mice moving in an open field box with a white background were first evaluated by 5 blinded human observers (inter-rater reliability 0.70) to reach a "ground truth" consensus score and then the videos were evaluated using MATSAP Threshold Optimizer. Removal of one of the human observers that scored liberally provided an inter-rater reliability of 0.81 between the remaining observers.

MATSAP Threshold Optimizer was used to explore different eccentricity and speed thresholds in the elevated plus maze. **Table 5-2** provides a summary of this analysis.

Table 5-2: MATSAP Threshold Optimizer output table

	Speed	Eccentricity	Sensitivity	Specificity	Accuracy	MCC	Fscore	AUC
max MCC	8	92	64.5	89.5	85.4	0.51	0.59	0.77
max Accuracy	9	93	47.8	95	87.3	0.49	0.55	0.71
max F-score	8	92	64.5	89.5	85.4	0.51	0.59	0.77
max AUC	8	90	83.4	76.4	77.6	0.47	0.55	0.8

The maximum MCC of 0.51 and the maximum F-score of 0.59 occur when the speed and eccentricity values of 8 cm/s and 0.92 are chosen, respectively (**Figure 5-5** and **Figure 5-6**). At these thresholds, the sensitivity was 64.5% and the specificity was 89.5%. The accuracy and AUC were 85.4%, 0.51, and 0.59, respectively.

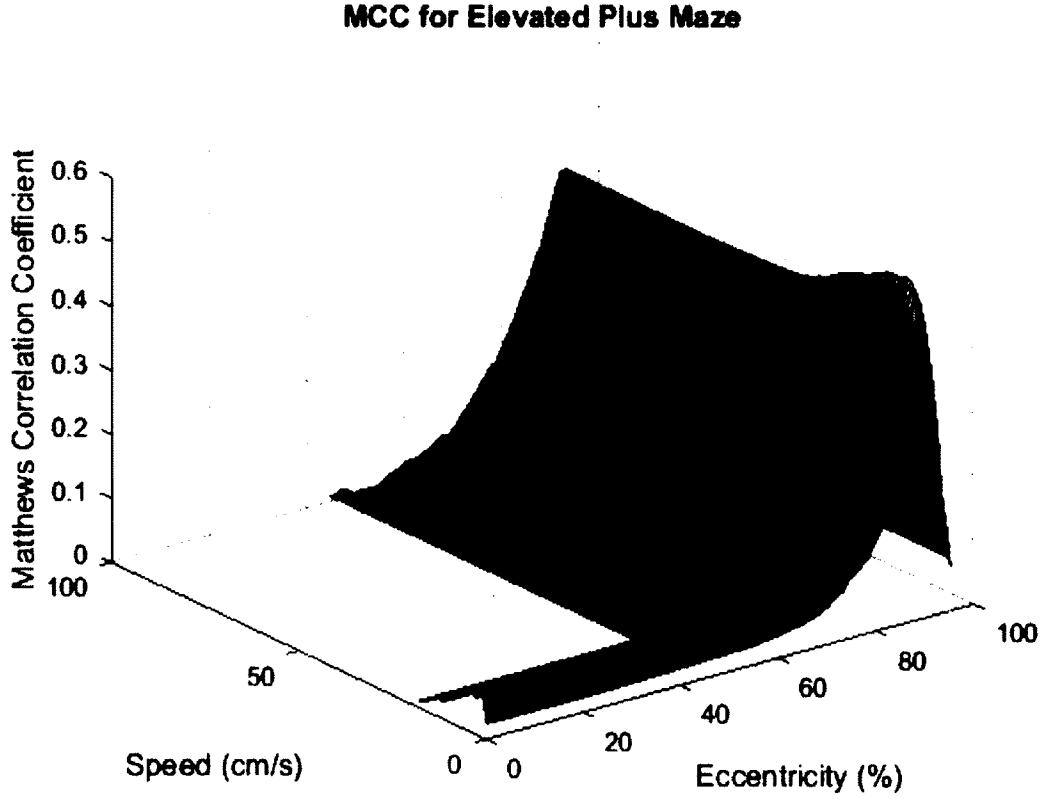

Figure 5-5: MCC of MATSAP analyzing elevated plus maze videos. Matthews correlation coefficient values when analyzing elevated plus maze videos at different speed and eccentricity thresholds. The maximum MCC of 0.51 occurred with a speed threshold of 8 cm/s and an eccentricity threshold of 92%.

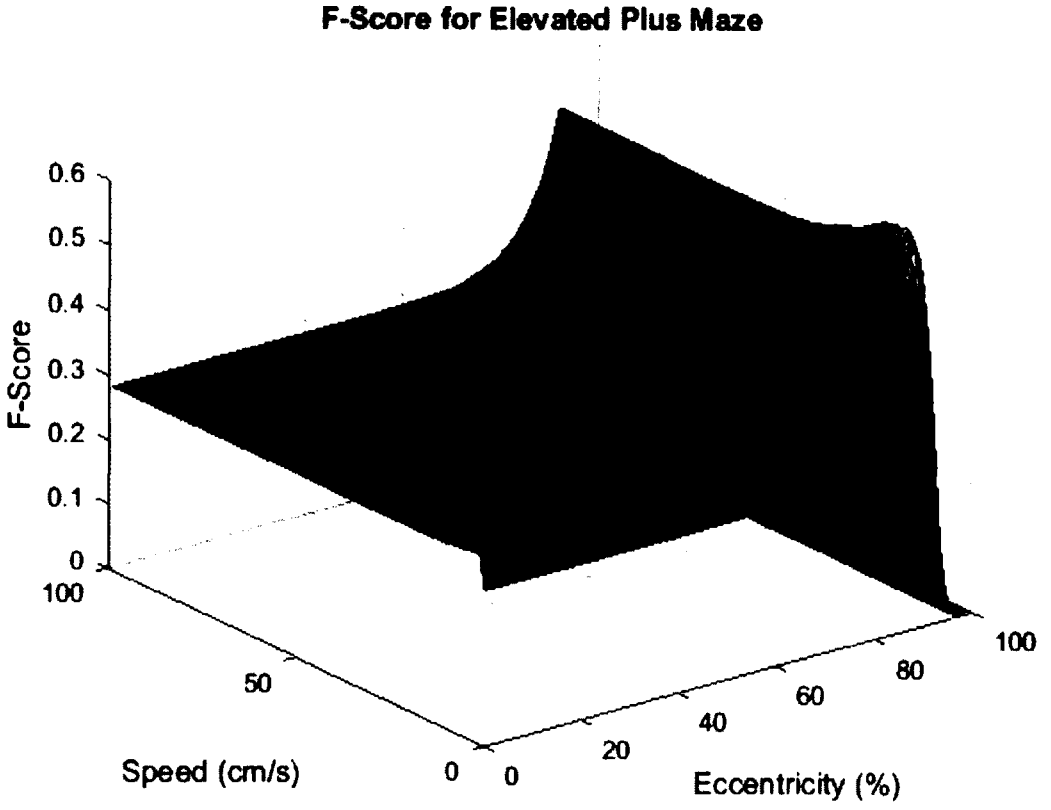

Figure 5-6: F-score of MATSAP analyzing elevated plus maze videos. The maximum F-score of 0.59 occurred with a speed threshold of 8 cm/s and an eccentricity threshold of 92%.

The maximum accuracy of 87.3% occurred when the speed and eccentricity values of 9 cm/s and 0.93 were chosen, respectively (**Figure 5-7**). The sensitivity and specificity at these thresholds were 47.8% and 95.0%, respectively.

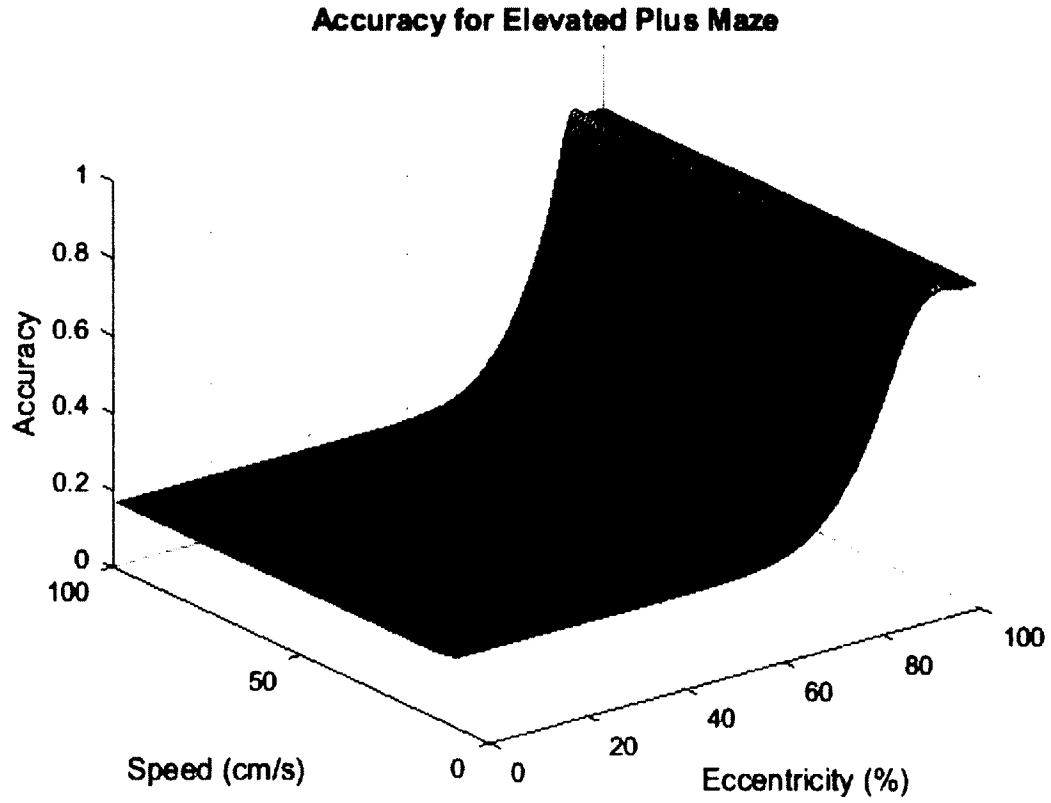

Figure 5-7: Accuracy of MATSAP analyzing elevated plus maze videos. The maximum accuracy of 87.3% occurred with a speed threshold of 8 cm/s and an eccentricity threshold of 93%.

The maximum AUC of 0.80 occurs when the speed threshold was 8 cm/s and the eccentricity threshold value was 0.90 (**Figure 5-8**). The sensitivity and specificity values were 83.4% and 76.4%, respectively.

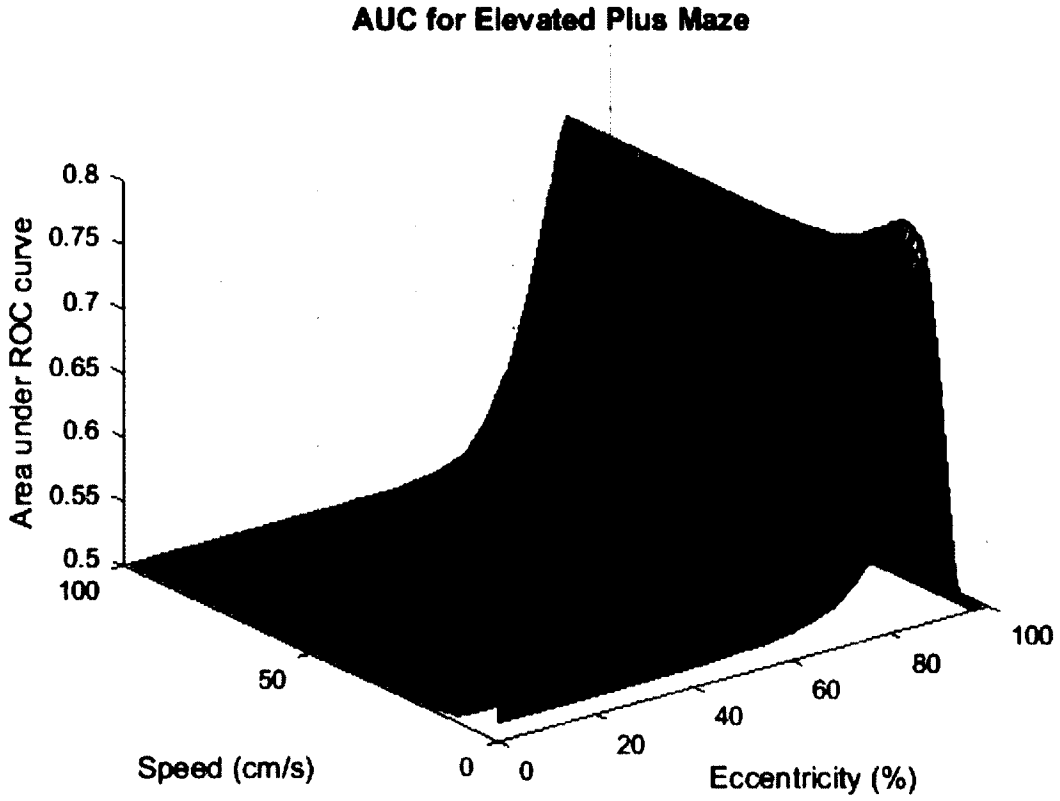

Figure 5-8: Area under the ROC curve for MATSAP analyzing elevated plus maze videos. The maximum AUC of 0.80 occurred with a speed threshold of 8 cm/s and an eccentricity threshold of 90%.

5.3.1 Elevated Plus Maze

The C57BL6 mice were smaller and more hyperactivity than Swiss mice utilized in Chapter 2. The C57BL6 were more willing to explore the open arms. Due to this exploration, the mice fell off the elevated plus maze in greater portion than white Swiss that fell during the propranolol and diphenoxylate studies, which was 1 out of 120. As the trials increased, the more mice fell as they were more willing to explore the open arms. The typical process of falling off the elevated plus mace starts when a mouse walks out onto the 5 cm open arm and looks over the edge facing perpendicular to the extending closed arms. Then after looking downwards, the mouse backs up in a startled response when it's hind legs land past the opposing edge. This is when the mouse falls. These mice whom fell off the edge were excluded from the study.

5.3.1.1 *Spatiotemporal measures.*

5.3.1.1.1 Normalized total distance traveled. The total distance traveled within the elevated plus maze was used as a gauge for hyperactivity. The greater the distance the rodent traveled, the more hyperactive the rodent. Of the six different treatment groups, the rodents with mild TBI expressed the most hyperactivity. As shown in **Figure 5-9**, the mild TBI mice (n = 2) travelled a normalized distance of 1.64 ± 0.68 (SEM) more 10 days after injury than they did preinjury. The control (n = 9), sham (n = 3), mild/moderate (n = 2), moderate (n = 4), and severe (n = 3) groups travelled a normalized distance of 0.88 ± 0.09, 0.80 ± 0.15, 0.94 ± 0.07, 1.02 ± 0.05, and 0.88 ± 0.05 (SEM), respectively. The mild TBI travelled significantly greater distance than the control ($p<0.05$).

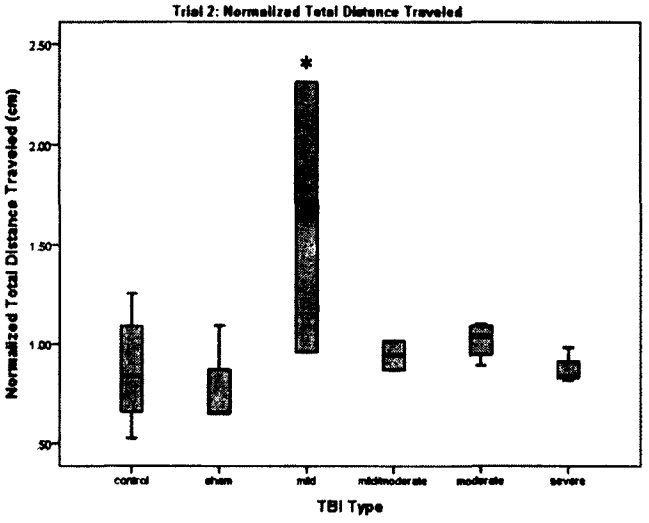

Figure 5-9: The normalized total distance traveled in the elevated plus maze during trial 2. The control (n = 9), sham (n = 3), mild (n = 2), mild/moderate (n = 2), moderate (n = 4), and severe (n = 3) groups travelled a normalized distance of 0.88 ± 0.09, 0.80 ± 0.15, 1.64 ± 0.68, 0.94 ± 0.07, 1.02 ± 0.05, and 0.88 ± 0.05 (SEM), respectively. (*$p<0.05$).

The trend of the mild group having the greatest hyperactivity continued during trials 3 and 4. During trial 3, The control (n = 10), sham (n = 3), mild (n = 2), mild/moderate (n = 2), moderate (n = 4), and severe (n = 3) groups travelled a normalized distance of 0.69 ± 0.06, 0.81 ± 0.15, 1.21 ± 0.10, 1.02 ± 0.03, 0.96 ± 0.11, and 0.69 ± 0.07 (SEM), respectively (**Figure 5-10**). The mild TBI travelled significantly greater distance than the control ($p<0.05$).

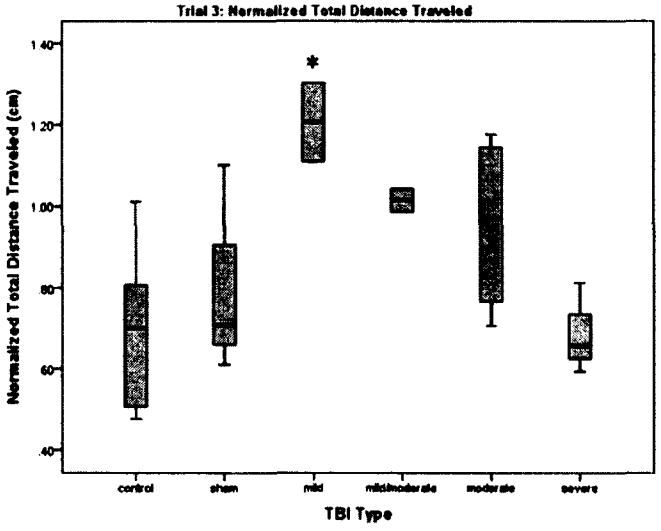

Figure 5-10: The normalized total distance traveled in the elevated plus maze during trial 3. The control (n = 10), sham (n = 3), mild (n = 2), mild/moderate (n = 2), moderate (n = 4), and severe (n = 3) groups travelled a normalized distance of 0.69 ± 0.06, 0.81 ± 0.15, 1.21 ± 0.10, 1.02 ± 0.03, 0.96 ± 0.11, and 0.69 ± 0.07 (SEM), respectively. (*$p<0.05$).

During trial 4, The control (n = 8), sham (n = 3), mild (n = 2), mild/moderate (n = 2), moderate (n = 3), and severe (n = 1) groups travelled a normalized distance of 0.82 ± 0.08, 0.77 ± 0.10, 1.47 ± 0.60, 0.80 ± 0.15, 0.88 ± 0.04, and 0.80 ± 0.00 (SEM), respectively (**Figure 5-11**). The mild TBI travelled significantly greater distance than the control ($p<0.05$).

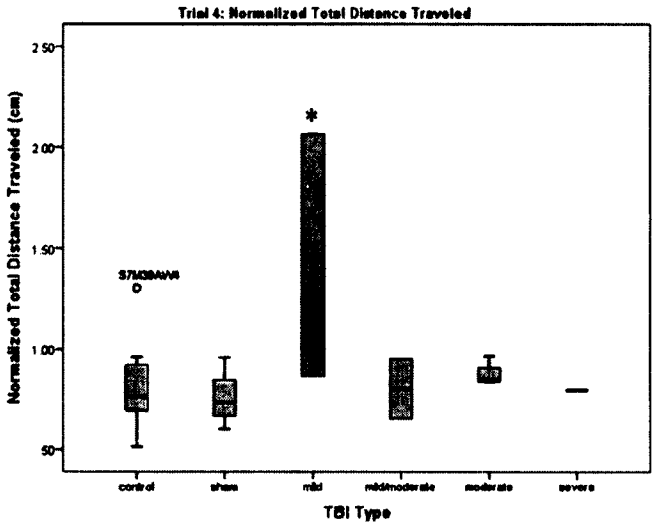

Figure 5-11: The normalized total distance traveled in the elevated plus maze during trial 4. The control (n = 8), sham (n = 3), mild (n = 2), mild/moderate (n = 2), moderate (n = 3), and severe (n = 1) groups travelled a normalized distance of 0.82 ± 0.08, 0.77 ± 0.10, 1.47 ± 0.60, 0.80 ± 0.15, 0.88 ± 0.04, and 0.80 ± 0.00 (SEM), respectively. (*$p<0.05$).

5.3.1.1.2 Open arm time difference. The time the mice spent in the open arms was used as a gauge to determine the anxiety levels of the mice. There was no significant difference in the percent difference of time spent in the open arms between the TBI groups in any of the post-injury trials (trial 2, 3, and 4) to the pre-injury trial, trial 1 ($p>0.05$). In trial 2, the control (n = 9), sham (n = 3), mild (n = 2), mild/moderate (n = 2), moderate (n = 4), and severe (n = 3) groups had a percent difference of -1.52 ± 2.77%, 2.83 ± 2.19%, 0.05 ± 0.35%, 2.15 ± 2.45%, -0.78 ± 0.49%, and -0.50 ± 0.49% (SEM), respectively (**Figure 5-12**).

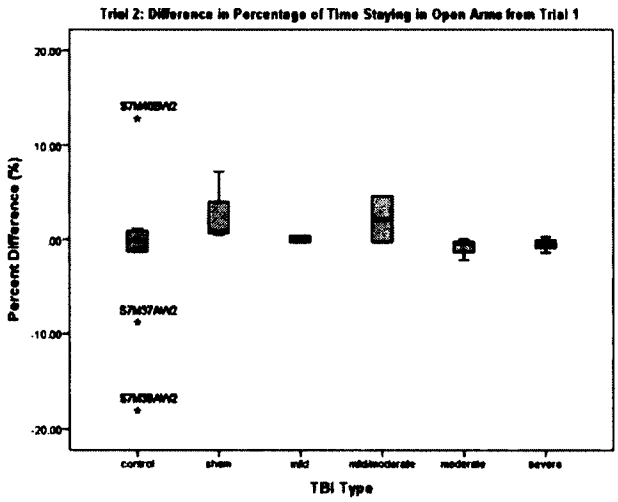

Figure 5-12: The percent difference of time spent in the open arms in the elevated plus maze during trial 2 compared to trial 1. The control (n = 9), sham (n = 3), mild (n = 2), mild/moderate (n = 2), moderate (n = 4), and severe (n = 3) groups had a percent difference of -1.52 ± 2.77%, 2.83 ± 2.19%, 0.05 ± 0.35%, 2.15 ± 2.45%, -0.78 ± 0.49%, and -0.50 ± 0.49% (SEM), respectively

In trial 3, the control (n = 10), sham (n = 3), mild (n = 2), mild/moderate (n = 2), moderate (n = 4), and severe (n = 3) groups had a percent difference of -3.04 ± 2.99%, 2.40 ± 3.10%, -1.40 ± 1.60%, 5.00 ± 0.40%, -0.95 ± 0.33%, and -0.27 ± 0.22% (SEM), respectively (**Figure 5-13**).

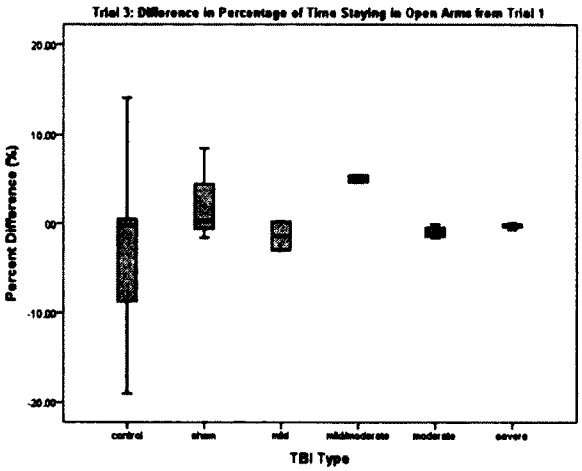

Figure 5-13: The percent difference of time spent in the open arms in the elevated plus maze during trial 3 compared to trial 1. The control (n = 10), sham (n = 3), mild (n = 2), mild/moderate (n = 2), moderate (n = 4), and severe (n = 3) groups had a percent difference of -3.04 ± 2.99%, 2.40 ± 3.10%, -1.40 ± 1.60%, 5.00 ± 0.40%, -0.95 ± 0.33%, and -0.27 ± 0.22% (SEM), respectively.

In trial 4, the control (n = 8), sham (n = 3), mild (n = 2), mild/moderate (n = 2), moderate (n = 3), and severe (n = 1) groups had a percent difference of -1.98 ± 2.66%, 6.00 ± 6.72%, -1.75 ± 1.35%, -0.70 ± 0.00%, -0.73 ± 0.52%, and 0.10 ± 0.00% (SEM), respectively (**Figure 5-14**).

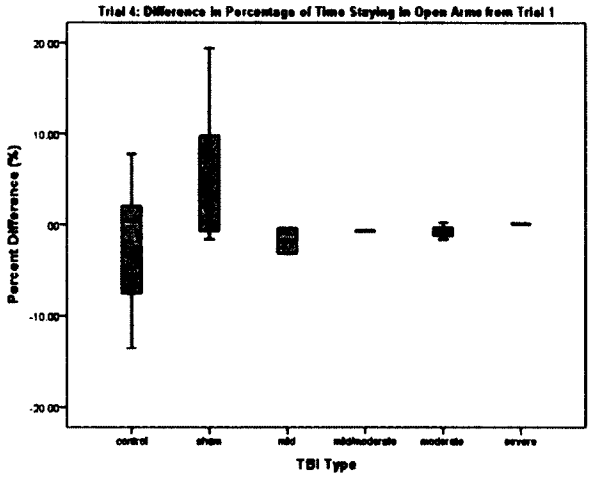

Figure 5-14: The percent difference of time spent in the open arms in the elevated plus maze during trial 4 compared to trial 1. The control (n = 8), sham (n = 3), mild (n = 2), mild/moderate (n = 2), moderate (n = 3), and severe (n = 1) groups had a percent difference of -1.98 ± 2.66%, 6.00 ± 6.72%, -1.75 ± 1.35%, -0.70 ± 0.00%, -0.73 ± 0.52%, and 0.10 ± 0.00% (SEM), respectively.

5.3.1.2 *SAP measures.*

5.3.1.2.1 Normalized SAP duration in elevated plus maze. The SAP duration in the EPM was calculated with MATSAP in trials 1, 2, 3, and 4. The SAP measurements from trials 2, 3, and 4 were normalized by SAP measurements taken in trial 1. During trial 2, the control (n = 9), sham (n = 3), mild (n = 1), mild/moderate (n = 2), moderate (n = 2), and severe (n = 3) groups had a normalized SAP duration of 0.83 ± 0.06, 0.57 ± 0.07, 0.36 ± 0.00, 0.76 ± 0.20, 0.55 ± 0.01, and 0.57 ± 0.08 (SEM), respectively (**Figure 5-15**). There was no significant difference in SAP duration between the groups ($p>0.05$).

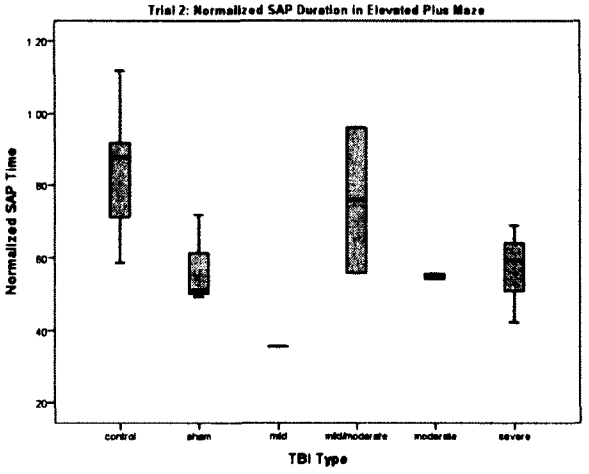

Figure 5-15: The normalized SAP duration in the elevated plus maze during trial 2. The control (n = 9), sham (n = 3), mild (n = 1), mild/moderate (n = 2), moderate (n = 2), and severe (n = 3) groups had a normalized SAP duration of 0.83 ± 0.06, 0.57 ± 0.07, 0.36 ± 0.00, 0.76 ± 0.20, 0.55 ± 0.01, and 0.57 ± 0.08 (SEM), respectively. There was no significant difference in SAP duration between the groups ($p>0.05$).

During trial 3, the control (n = 10), sham (n = 3), mild (n = 1), mild/moderate (n = 2), moderate (n = 4), and severe (n = 2) groups had a normalized SAP duration of 0.85 ± 0.09, 0.67 ± 0.13, 0.41 ± 0.00, 0.44 ± 0.09, 0.62 ± 0.11, and 0.54 ± 0.15 (SEM), respectively (**Figure 5-16**). There was no significant difference in SAP duration between the groups ($p>0.05$).

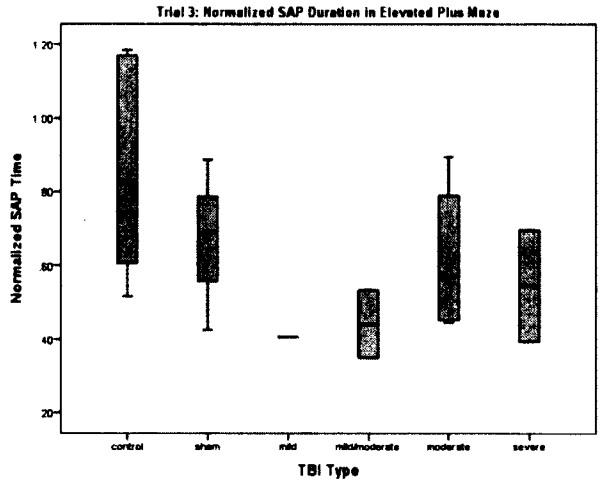

Figure 5-16: The normalized SAP duration in the elevated plus maze during trial 3. The control (n = 10), sham (n = 3), mild (n = 1), mild/moderate (n = 2), moderate (n = 4), and severe (n = 2) groups had a normalized SAP duration of 0.85 ± 0.09, 0.67 ± 0.13, 0.41 ± 0.00, 0.44 ± 0.09, 0.62 ± 0.11, and 0.54 ± 0.15 (SEM), respectively. There was no significant difference in SAP duration between the groups ($p>0.05$).

During trial 4, the control (n = 8), sham (n = 3), mild (n = 1), mild/moderate (n = 1), moderate (n = 3), and severe (n = 1) groups had a normalized SAP duration of 0.69 ± 0.06, 0.58 ± 0.04, 0.40 ± 0.00, 0.73 ± 0.00, 0.64 ± 0.06, and 0.44 ± 0.00 (SEM), respectively (**Figure 5-17**). There was no significant difference in SAP duration between the groups ($p>0.05$).

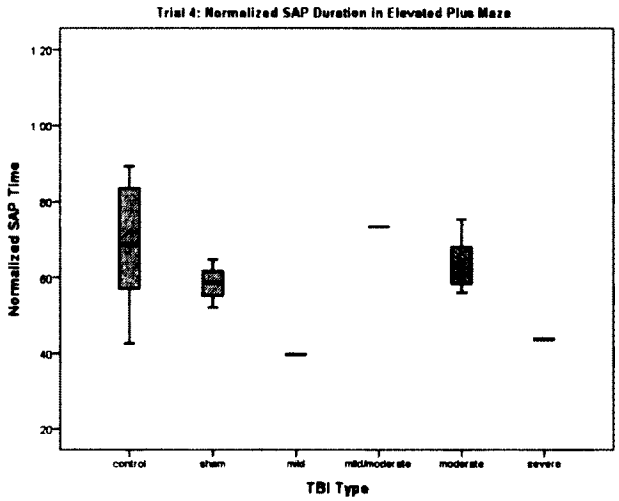

Figure 5-17: The normalized SAP duration in the elevated plus maze during trial 4. The control (n = 8), sham (n = 3), mild (n = 1), mild/moderate (n = 1), moderate (n = 3), and severe (n = 1) groups had a normalized SAP duration of 0.69 ± 0.06, 0.58 ± 0.04, 0.40 ± 0.00, 0.73 ± 0.00, 0.64 ± 0.06, and 0.44 ± 0.00 (SEM), respectively. There was no significant difference in SAP duration between the groups ($p>0.05$).

5.3.1.2.2 Normalized SAP frequency in elevated plus maze. The SAP frequency in the EPM was calculated with MATSAP in trials 1, 2, 3, and 4. The SAP measurements from trials 2, 3, and 4 were normalized by SAP measurements taken in trial 1. During trial 2, the control (n = 9), sham (n = 3), mild (n = 1), mild/moderate (n = 2), moderate (n = 2), and severe (n = 3) groups had a percent difference of 0.76 ± 0.07, 0.57 ± 0.09, 0.53 ± 0.00, 0.71 ± 0.08, 0.66 ± 0.06, and 0.70 ± 0.12 (SEM), respectively (**Figure 5-18**). There was no significant difference in SAP duration between the groups ($p>0.05$).

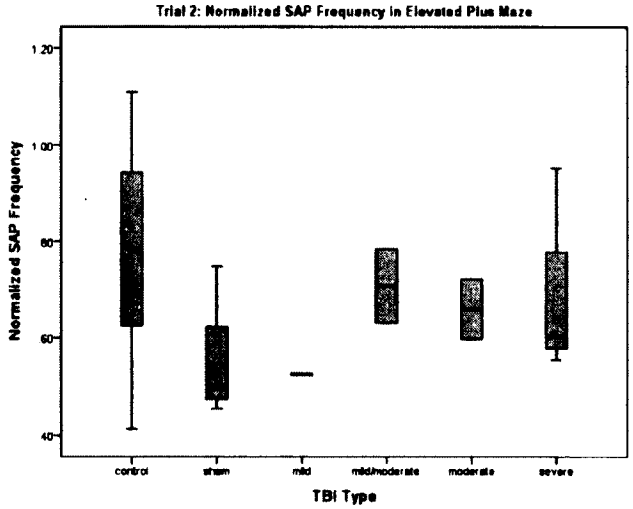

Figure 5-18: The normalized SAP frequency in the elevated plus maze during trial 2. The control (n = 9), sham (n = 3), mild (n = 1), mild/moderate (n = 2), moderate (n = 2), and severe (n = 3) groups had a percent difference of 0.76 ± 0.07, 0.57 ± 0.09, 0.53 ± 0.00, 0.71 ± 0.08, 0.66 ± 0.06, and 0.70 ± 0.12 (SEM), respectively. There was no significant difference in SAP duration between the groups ($p>0.05$).

During trial 3, the control (n = 10), sham (n = 3), mild (n = 1), mild/moderate (n = 2), moderate (n = 4), and severe (n = 2) groups had a percent difference of 0.66 ± 0.08, 0.58 ± 0.06, 0.52 ± 0.00, 0.58 ± 0.12, 0.60 ± 0.08, and 0.56 ± 0.09 (SEM), respectively (**Figure 5-19**). There was no significant difference in SAP duration between the groups ($p>0.05$).

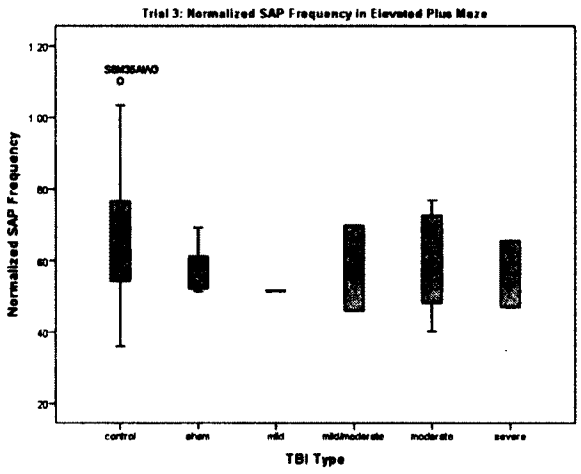

Figure 5-19: The normalized SAP frequency in the elevated plus maze during trial 3. The control (n = 10), sham (n = 3), mild (n = 1), mild/moderate (n = 2), moderate (n = 4), and severe (n = 2) groups had a percent difference of 0.66 ± 0.08, 0.58 ± 0.06, 0.52 ± 0.00, 0.58 ± 0.12, 0.60 ± 0.08, and 0.56 ± 0.09 (SEM), respectively. There was no significant difference in SAP duration between the groups ($p>0.05$).

During trial 4, the control (n = 8), sham (n = 3), mild (n = 1), mild/moderate (n = 1), moderate (n = 3), and severe (n = 1) groups had a percent difference of 0.58 ± 0.08, 0.47 ± 0.08, 0.57 ± 0.00, 0.69 ± 0.00, 0.64 ± 0.08, and 0.48 ± 0.00 (SEM), respectively (**Figure 5-20**). There was no significant difference in SAP duration between the groups ($p>0.05$).

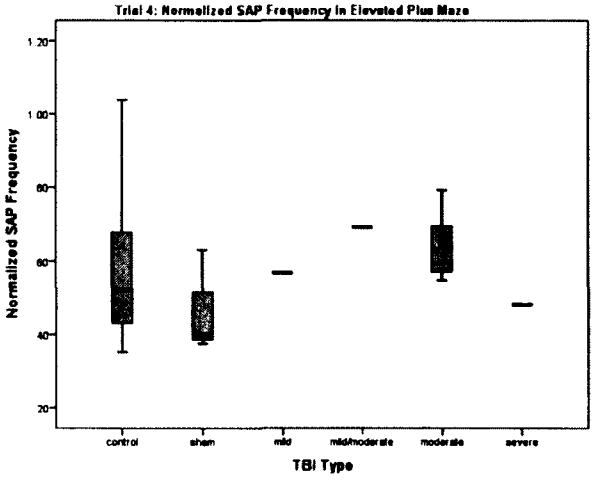

Figure 5-20: The normalized SAP frequency in the elevated plus maze during trial 4. The control (n = 8), sham (n = 3), mild (n = 1), mild/moderate (n = 1), moderate (n = 3), and severe (n = 1) groups had a percent difference of 0.58 ± 0.08, 0.47 ± 0.08, 0.57 ± 0.00, 0.69 ± 0.00, 0.64 ± 0.08, and 0.48 ± 0.00 (SEM), respectively. There was no significant difference in SAP duration between the groups ($p>0.05$).

5.3.2 Open Field

5.3.2.1 *Spatiotemporal measures*

5.3.2.1.1 Normalized total distance traveled. The total distance traveled within the open field was used to assess hyperactivity. The greater the distance the rodent traveled, the more hyperactive the rodent. Of the 6 different treatment groups, the rodents with mild/moderate TBI expressed the most hyperactivity. As shown in **Figure 5-21**, the mild/moderate TBI mice (n = 3) travelled a normalized distance of 1.40 ± 0.06 (SEM) more 10 days after injury than they did preinjury. The control (n = 10), sham (n = 4), mild (n = 2), moderate (n = 5), and severe (n = 3) groups travelled a normalized distance of 0.67 ± 0.04, 0.97 ± 0.16, 1.15 ± 0.08, 1.06 ± 0.10, and 1.09 ± 0.12 (SEM), respectively. The mild/moderate TBI group travelled significantly greater normalized distance than the control ($p<0.01$).

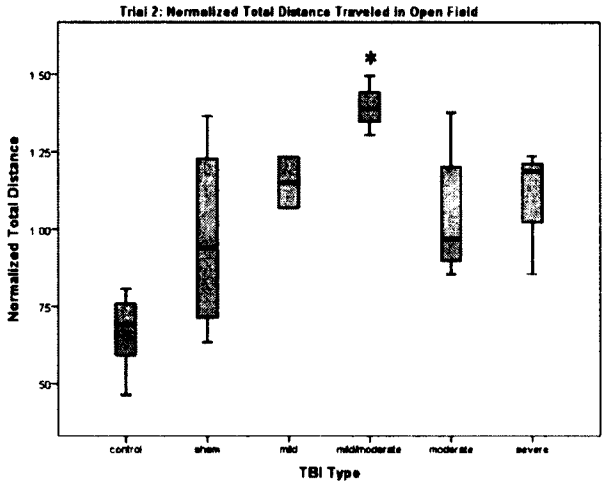

Figure 5-21: The total normalized distance travelled in the open field during trial 2. The control (n = 10), sham (n = 4), mild (n = 2), moderate (n = 5), and severe (n = 3) groups travelled a normalized distance of 0.67 ± 0.04, 0.97 ± 0.16, 1.15 ± 0.08, 1.06 ± 0.10, and 1.09 ± 0.12 (SEM), respectively. The mild/moderate groups has a significantly greater normalized difference from the control (*$p<0.01$).

The trend of the mild/moderate group having the greatest hyperactivity continued during trials 3 and 4. During trial 3, the control (n = 8), sham (n = 4), mild (n = 2), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups travelled a normalized distance of 0.70 ± 0.07, 0.79 ± 0.18, 1.02 ± 0.17, 1.19 ± 0.05, 1.03 ± 0.07, and 0.90 ± 0.10 (SEM), respectively (**Figure 5-22**). The mild TBI travelled significantly greater normalized distance than the control ($p<0.05$).

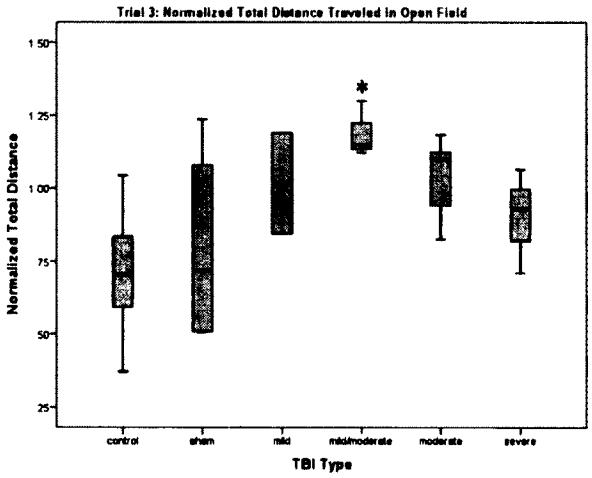

Figure 5-22: The total normalized distance travelled in the open field during trial 3. The control (n = 8), sham (n = 4), mild (n = 2), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups travelled a normalized distance of 0.70 ± 0.07, 0.79 ± 0.18, 1.02 ± 0.17, 1.19 ± 0.05, 1.03 ± 0.07, and 0.90 ± 0.10 (SEM), respectively. The mild/moderate groups had a significantly greater normalized difference from the control (*$p<0.05$).

During trial 4, the control (n = 10), sham (n = 4), mild (n = 2), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups travelled a normalized distance of 0.63 ± 0.03, 0.95 ± 0.16, 0.89 ± 0.05, 1.08 ± 0.12, 0.93 ± 0.08, and 0.79 ± 0.48 (SEM). respectively (**Figure 5-23**). The mild/moderate TBI group travelled significantly greater normalized distance than the control ($p<0.05$).

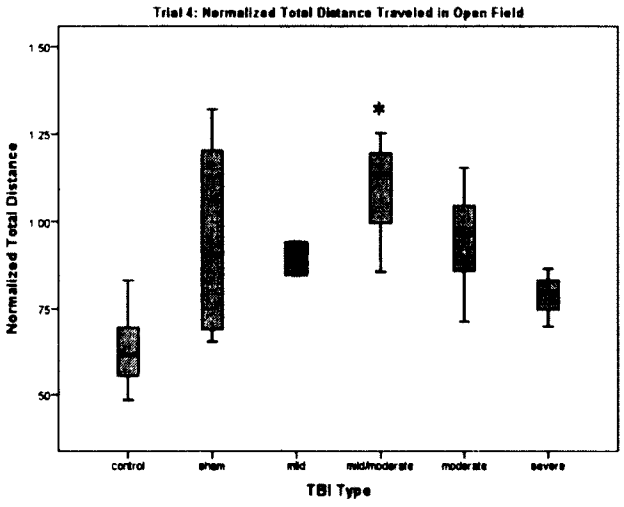

Figure 5-23: The total normalized distance travelled in the open field during trial 4. The control (n = 10), sham (n = 4), mild (n = 2), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups travelled a normalized distance of 0.63 ± 0.03, 0.95 ± 0.16, 0.89 ± 0.48, 1.08 ± 0.12, 0.93 ± 0.08, and 0.79 ± 0.48 (SEM), respectively. (*$p<0.05$).

5.3.2.1.2 Average speed in open field (normalized). The average speed of the rodent travelling in the open field can be used to evaluate the hyperactivity levels of the mice. As shown in **Figure 5-24**, the mild/moderate TBI mice (n = 3) travelled with an average normalized speed of 1.40 ± 0.06 (SEM) more 10 days after injury than they did preinjury. The control (n = 10), sham (n = 4), mild (n = 2), moderate (n = 5), and severe (n = 3) groups travelled with an average normalized speed of 0.67 ± 0.04, 0.96 ± 0.16, 1.18 ± 0.08, 1.05 ± 0.10, and 1.09 ± 0.11 (SEM), respectively. The mild/moderate TBI group travelled with a significantly greater average normalized speed than the control (p=0.001).

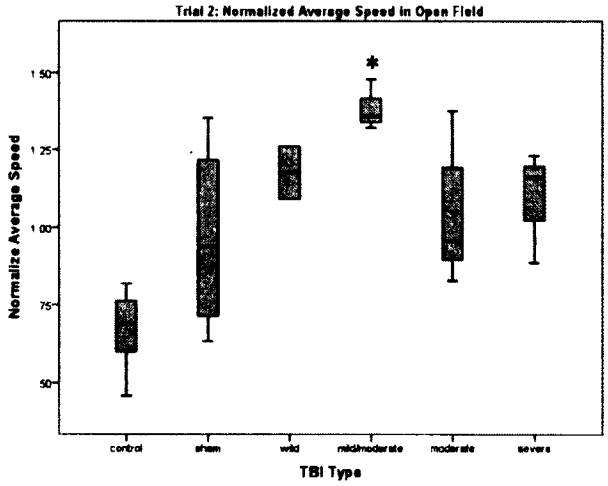

Figure 5-24: The normalized average speed of the mice in open field during trial 2. The control (n = 10), sham (n = 4), mild (n = 2), moderate (n = 5), and severe (n = 3) groups travelled with an average normalized speed of 0.67 ± 0.04, 0.96 ± 0.16, 1.18 ± 0.08, 1.05 ± 0.10, and 1.09 ± 0.11 (SEM), respectively. The mild/moderate TBI group travelled with a significantly greater average normalized speed than the control (*p=0.001).

The trend of the mild/moderate group having the greatest hyperactivity continued during trials 3 and 4. During trial 3, the control (n = 8), sham (n = 4), mild (n = 2), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups travelled with an average normalized speed of 0.71 ± 0.07, 0.79 ± 0.17, 1.03 ± 0.16, 1.19 ± 0.07, 1.03 ± 0.06, and 0.91 ± 0.10 (SEM), respectively (**Figure 5-25**). The mild TBI travelled with a significantly greater normalized average speed than the control ($p<0.05$).

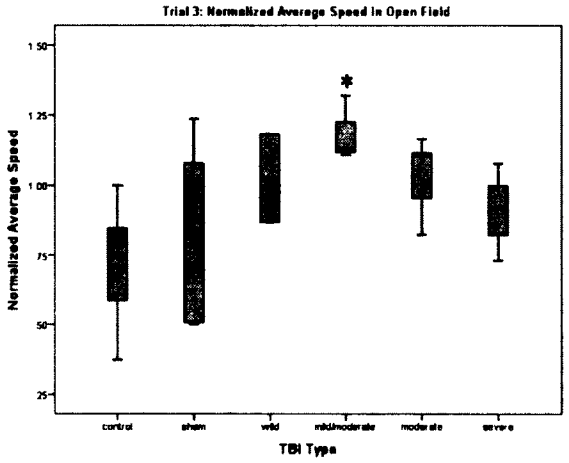

Figure 5-25: The normalized average speed of the mice in open field during trial 3. The control (n = 8), sham (n = 4), mild (n = 2), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups travelled with an average normalized speed of 0.71 ± 0.07, 0.79 ± 0.17, 1.03 ± 0.16, 1.19 ± 0.07, 1.03 ± 0.06, and 0.91 ± 0.10 (SEM), respectively. (*$p<0.05$).

During trial 4, the control (n = 10), sham (n = 4), mild (n = 2), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups travelled with an average normalized average speed of 0.63 ± 0.03, 0.96 ± 0.16, 0.91 ± 0.46, 1.07 ± 0.13, 0.93 ± 0.07, and 0.79 ± 0.06 (SEM), respectively (**Figure 5-26**). The mild/moderate TBI group travelled with a significantly greater normalized average speed than the control ($p<0.05$).

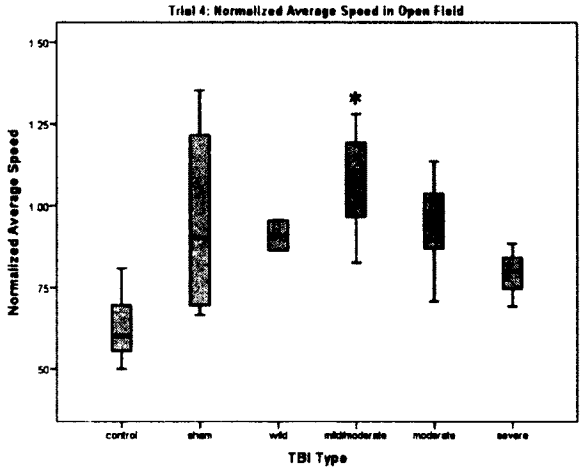

Figure 5-26: The normalized average speed of the mice in open field during trial 4. The control (n = 10), sham (n = 4), mild (n = 2), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups travelled with an average normalized average speed of 0.63 ± 0.03, 0.96 ± 0.16, 0.91 ± 0.46, 1.07 ± 0.13, 0.93 ± 0.07, and 0.79 ± 0.06 (SEM), respectively. (*$p<0.05$).

5.3.2.1.3 Total center time (normalized). The time the rodent spends in the center of the open field is an indication of its anxiety level as the exploratory creatures do not like open areas. As shown in **Figure 5-27**, the control (n = 10), sham (n = 4), mild (n = 2), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups had a total normalized center time of 0.34 ± 0.08, 0.16 ± 0.06, 0.09 ± 0.01, 0.39 ± 0.20, and 0.13 ± 0.07 (SEM), respectively. There was no significant difference among the experimental groups ($p>0.05$).

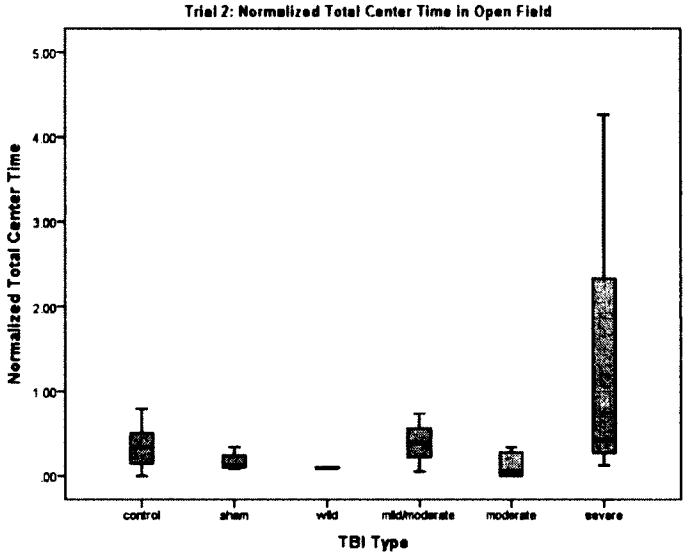

Figure 5-27: The total normalized time spent in the center of the open field during trial 2. The control (n = 10), sham (n = 4), mild (n = 2), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups had a total normalized center time of 0.34 ± 0.08, 0.16 ± 0.06, 0.09 ± 0.01, 0.39 ± 0.20, and 0.13 ± 0.07 (SEM), respectively.

During trial 3, the control (n = 8), sham (n = 4), mild (n = 2), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups spent a total normalized time of 1.04 ± 0.30, 1.15 ± 0.59, 1.25 ± 0.91, 1.45 ± 0.96, 0.32 ± 0.19, and 0.99 ± 0.56 (SEM) in the center of the open field, respectively (**Figure 5-28**). There was no significant difference between treatment groups for the normalized center time ($p>0.05$).

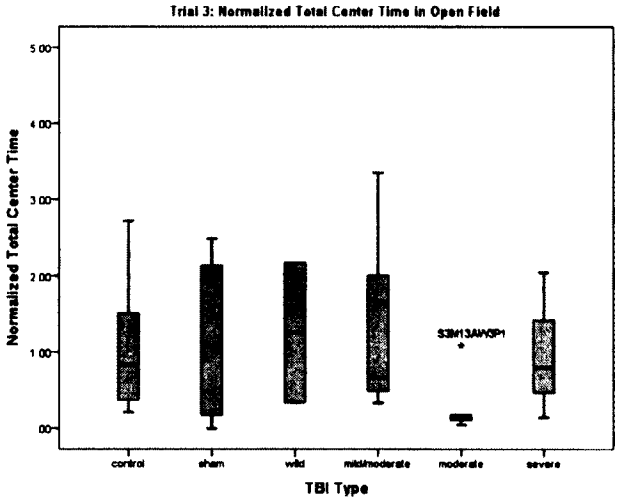

Figure 5-28: The total normalized time spent in the center of the open field trial 3. The control (n = 8), sham (n = 4), mild (n = 2), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups spent a total normalized time of 1.04 ± 0.30, 1.15 ± 0.59, 1.25 ± 0.91, 1.45 ± 0.96, 0.32 ± 0.19, and 0.99 ± 0.56 (SEM) in the center of the open field, respectively. There was no significant difference between treatment groups for the normalized center time ($p>0.05$).

During trial 4, the control (n = 10), sham (n = 4), mild (n = 2), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups had a total normalize center time of 0.39 ± 0.12, 0.67 ± 0.25, 0.23 ± 0.11, 0.90 ± 0.49, 0.29 ± 0.08, and 1.16 ± 0.93 (SEM), respectively (**Figure 5-29**). There was no significant difference between treatment groups for the normalized center time ($p>0.05$).

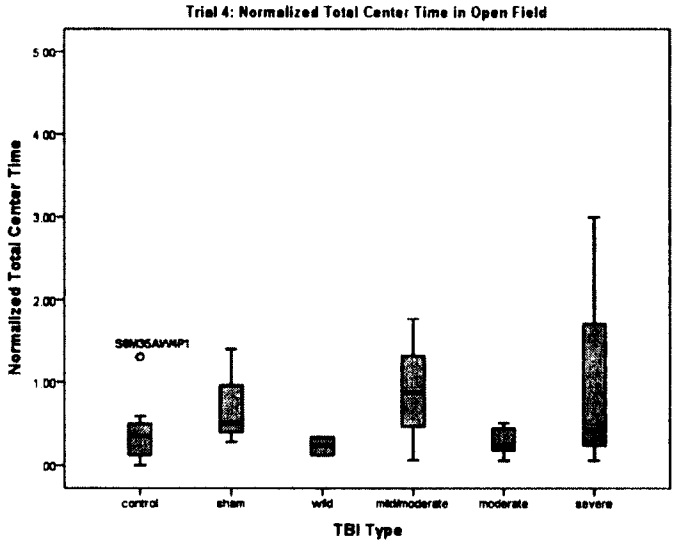

Figure 5-29: The total normalized time spent in the center of the open field during trial 4. The control (n = 10), sham (n = 4), mild (n = 2), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups had a total normalize center time of 0.39 ± 0.12, 0.67 ± 0.25, 0.23 ± 0.11, 0.90 ± 0.49, 0.29 ± 0.08, and 1.16 ± 0.93 (SEM), respectively.

5.3.2.2 *SAP measures.*

5.3.2.2.1 Normalized SAP duration in open field. The SAP duration in the OF was calculated with MATSAP in trials 1, 2, 3, and 4. The SAP measurements from trials 2, 3, and 4 were normalized by SAP measurements taken in trial 1. During trial 2, the control (n = 10), sham (n = 3), mild (n = 1), mild/moderate (n = 3), moderate (n = 5), and severe (n = 2) groups had a normalized SAP duration of 0.51 ± 0.11, 0.16 ± 0.04, 0.13 ± 0.00, 0.31 ± 0.11, 0.28 ± 0.08, and 0.28 ± 0.03 (SEM), respectively (**Figure 5-30**). There was no significant difference between treatment groups for the normalized center time ($p>0.05$).

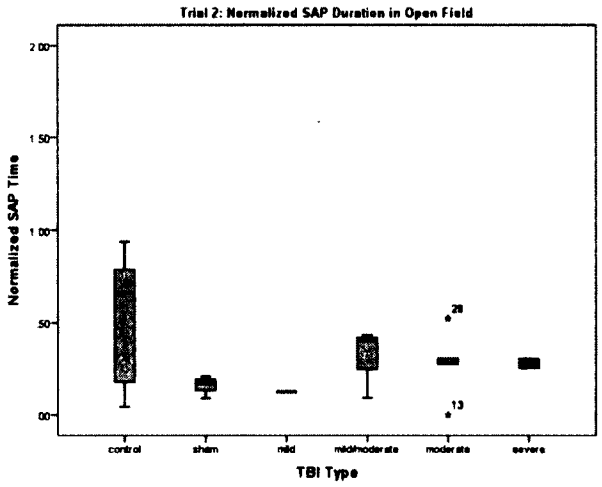

Figure 5-30: The normalized SAP duration in the elevated plus maze during trial 2. The control (n = 10), sham (n = 3), mild (n = 1), mild/moderate (n = 3), moderate (n = 5), and severe (n = 2) groups had a normalized SAP duration of 0.51 ± 0.11, 0.16 ± 0.04, 0.13 ± 0.00, 0.31 ± 0.11, 0.28 ± 0.08, and 0.28 ± 0.03 (SEM), respectively. There was no significant difference between treatment groups for the normalized center time ($p>0.05$).

During trial 3, the control (n = 8), sham (n = 3), mild (n = 1), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups had a normalized SAP duration of 0.44 ± 0.06, 0.33 ± 0.04, 0.20 ± 0.00, 0.65 ± 0.44, 0.19 ± 0.05, and 0.19 ± 0.08 (SEM), respectively (**Figure 5-31**). There was no significant difference between treatment groups for the normalized center time ($p>0.05$). There was no significant difference between treatment groups for the normalized center time ($p>0.05$).

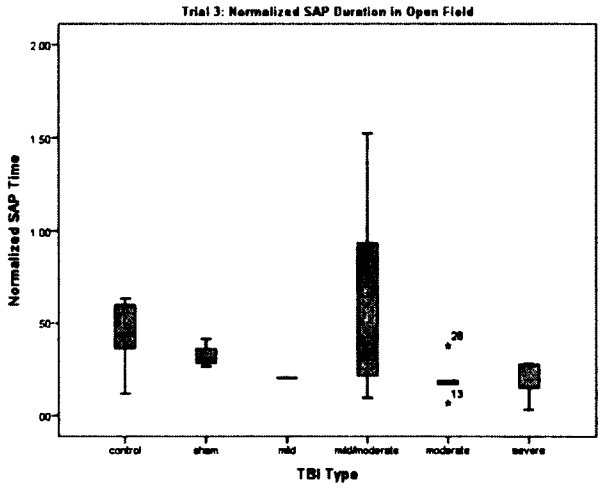

Figure 5-31: The normalized SAP duration in the elevated plus maze during trial 3. The control (n = 8), sham (n = 3), mild (n = 1), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups had a normalized SAP duration of 0.44 ± 0.06, 0.33 ± 0.04, 0.20 ± 0.00, 0.65 ± 0.44, 0.19 ± 0.05, and 0.19 ± 0.08 (SEM), respectively. There was no significant difference between treatment groups for the normalized center time ($p>0.05$). There was no significant difference between treatment groups for the normalized center time ($p>0.05$).

During trial 4, the control (n = 9), sham (n = 4), mild (n = 1), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups had a normalized SAP duration of 0.25 ± 0.03, 0.11 ± 0.03, 0.29 ± 0.00, 0.27 ± 0.15, 0.09 ± 0.04, and 0.14 ± 0.02 (SEM), respectively (**Figure 5-32**). There was no significant difference between treatment groups for the normalized center time ($p>0.05$).

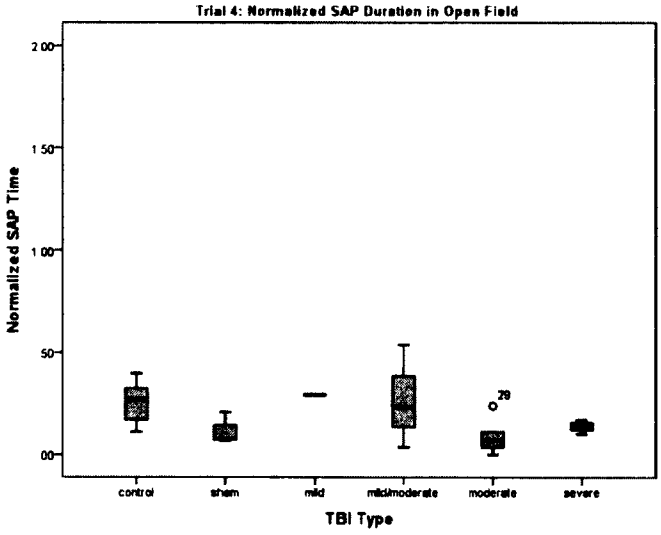

Figure 5-32: The normalized SAP duration in the elevated plus maze during trial 4. The control (n = 9), sham (n = 4), mild (n = 1), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups had a normalized SAP duration of 0.25 ± 0.03, 0.11 ± 0.03, 0.29 ± 0.00, 0.27 ± 0.15, 0.09 ± 0.04, and 0.14 ± 0.02 (SEM), respectively. There was no significant difference between treatment groups for the normalized center time ($p>0.05$).

5.3.2.2.2 Normalized SAP frequency in open field. The SAP frequency in the OF was calculated with MATSAP in trials 1, 2, 3, and 4. The SAP measurements from trials 2, 3, and 4 were normalized by SAP measurements taken in trial 1. During trial 2, the control (n = 10), sham (n = 3), mild (n = 1), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups had a normalized SAP frequency of 0.47 ± 0.10, 0.19 ± 0.05, 0.15 ± 0.00, 0.40 ± 0.15, 0.36 ± 0.13, and 0.36 ± 0.02 (SEM), respectively (**Figure 5-33**). There was no significant difference between treatment groups for the normalized center time ($p>0.05$).

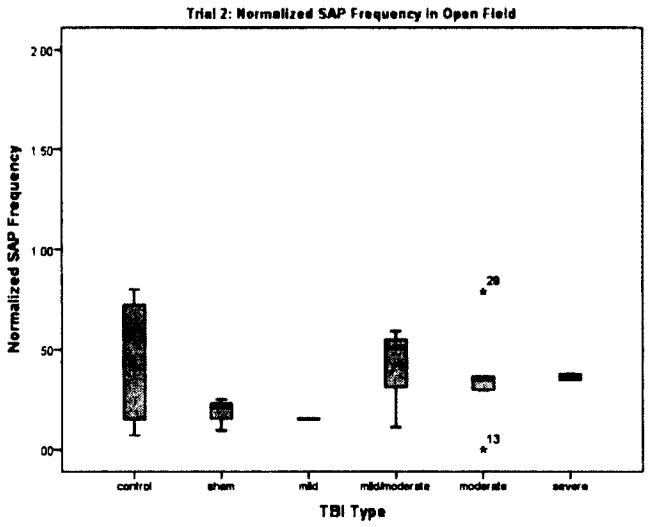

Figure 5-33: The normalized SAP frequency in the elevated plus maze during trial 2. The control (n = 10), sham (n = 3), mild (n = 1), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups had a normalized SAP frequency of 0.47 ± 0.10, 0.19 ± 0.05, 0.15 ± 0.00, 0.40 ± 0.15, 0.36 ± 0.13, and 0.36 ± 0.02 (SEM), respectively. There was no significant difference between treatment groups for the normalized center time ($p>0.05$).

During trial 3, the control (n = 8), sham (n = 3), mild (n = 1), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups had a normalized SAP frequency of 0.44 ± 0.06, 0.40 ± 0.05, 0.25 ± 0.00, 0.83 ± 0.56, 0.26 ± 0.10, and 0.18 ± 0.07 (SEM), respectively (**Figure 5-34**). There was no significant difference between treatment groups for the normalized center time ($p>0.05$).

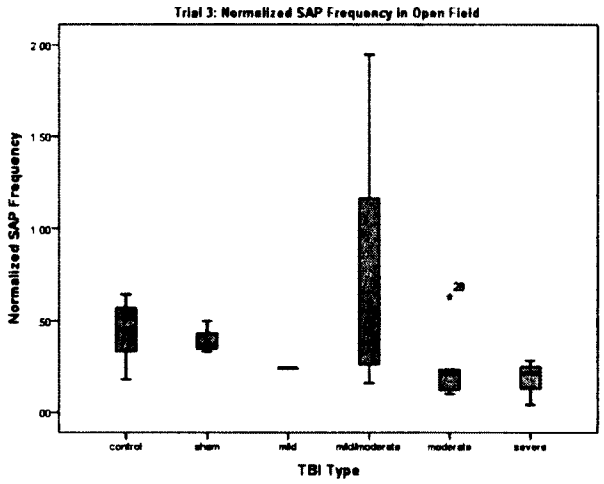

Figure 5-34: The normalized SAP frequency in the elevated plus maze during trial 3. The control (n = 8), sham (n = 3), mild (n = 1), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups had a normalized SAP frequency of 0.44 ± 0.06, 0.40 ± 0.05, 0.25 ± 0.00, 0.83 ± 0.56, 0.26 ± 0.10, and 0.18 ± 0.07 (SEM), respectively. There was no significant difference between treatment groups for the normalized center time ($p>0.05$).

During trial 4, the control (n = 9), sham (n = 4), mild (n = 1), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups had normalized SAP frequency of 0.23 ± 0.03, 0.16 ± 0.04, 0.31 ± 0.00, 0.29 ± 0.15, 0.12 ± 0.05, and 0.15 ± 0.02 (SEM), respectively (**Figure 5-35**). There was no significant difference between treatment groups for the normalized center time ($p>0.05$).

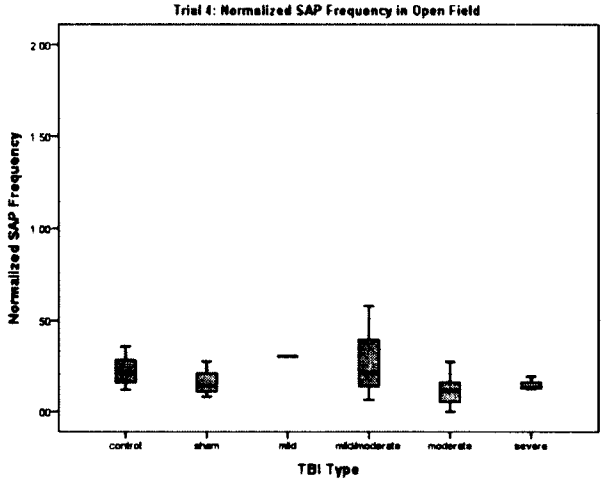

Figure 5-35: The normalized SAP frequency in the elevated plus maze during trial 4. The control (n = 9), sham (n = 4), mild (n = 1), mild/moderate (n = 3), moderate (n = 5), and severe (n = 3) groups had normalized SAP frequency of 0.23 ± 0.03, 0.16 ± 0.04, 0.31 ± 0.00, 0.29 ± 0.15, 0.12 ± 0.05, and 0.15 ± 0.02 (SEM), respectively. There was no significant difference between treatment groups for the normalized center time ($p>0.05$).

5.4 Discussion

5.4.1 MATSAP Validation

MATSAP Threshold Optimizer was used to obtain the optimal eccentricity and speed values of 91 and 15 cm/s for the OF, respectively. MATSAP Threshold Optimizer was used to obtain the optimal eccentricity and speed values of 90 and 8 cm/s for the EPM, respectively. The MATSAP results using the optimal thresholds were validated by a human consensus score developed by 5 blinded observers. After MATSAP was shown to be a reliable method to detect C57BL6 male mice, MATSAP was used to detect SAP for the remaining videos.

5.4.2 Elevated Plus Maze and Open Field Spatiotemporal Inferences

The spatiotemporal measurements have shed light on the both the hyperactivity and anxiety levels of the rodents. The normalized total distance and the normalized average speed in the OF revealed that the mild/moderate group were more hyperactivity than the other TBI groups throughout the post-injury trails in comparison to the pre-injury trial. Despite traveling faster and longer distances, these mice were on par with the other treatment groups in respect to the normalized time spent in the center of the open field during in the post-injury trials. This suggests that the mildly injured mice exhibit hyperactivity without inducing anxiety.

When looking at the outcomes of the EPM, we see a similar trend, but this time with the mild/moderate group. This group exhibits a greater increase of hyperactivity in the trials after being subjected to injury than the other treatment groups. However, the mild/moderate group displays an average change of anxiety levels during the post-injury

trials when compared to the other treatment groups. This suggests that the hyperactivity can be induced in mild and mild/moderate mice without hindering anxiety levels.

5.4.3 Elevated Plus Maze and Open Field Ethological Confirmation

To be sure that the anxiety-related spatiotemporal measurements of EPM and OF properly reflect the anxiety levels of the mice, the risk assessment behavior, SAP, was measured using MATSAP. For both the EPM and OF, there was no significant difference in the normalized display of SAP between the control, sham, and TBI groups in neither duration nor frequency. However, the number of rodents per treatment group could have been higher for more certainty. The corroborative evidence from the spatiotemporal measurements leads to the conclusion that the TBI does not affect the anxiety level of the mice.

5.5 Conclusion

The study has demonstrated the successful use of MATSAP in detecting SAP in C57BL6 mice. The utilization of the ethological behavior SAP in TBI anxiety rodent studies provides corroborative evidence in conjunction with the spatiotemporal measurements to ensure the correct appraisal of the mouse's anxiety state. There is evidence to suggest that TBI may attribute to hyperactivity in the mild and/or mild/moderate groups without increase anxiety levels.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

The anxiolytic effects of propranolol and diphenoxylate were investigated in the mouse model through a series of tests. There was no significant difference between the treatment groups. Based on the literature, propranolol was known to affect rats, but not mice in fear conditioning tests. Further investigation into the literature revealed a difference in the distribution of beta-adrenergic receptors in the BNST. With the knowledge that propranolol floods the entire brain when administered orally, we hypothesized that this could explain why propranolol was effective in the rats, but not the mice. The neurocircuitry of fear and anxiety were examined and possible explanations were speculated based on known mechanism of LTP and LTD. We also hypothesized possible ways diphenoxylate modulated the beta-adrenergic system.

During the process of evaluating the behavioral experiments, it was discovered that there was no known freely available automated program to detect SAP. Based on this need, MATSAP, an open source MATLAB based software, was successfully developed, which provided a quick and objective analysis of SAP from an overhead view for male Swiss mice weighing 32.5-40.0 g in both the OF and EPM.

Another approach to detect SAP was also taken using of elliptic Fourier analysis. This lead to the creation of the MATLAB based software called EthoStock. EthoStock

not only has the potential to detect SAP, but also other ethological behaviors such as rearing and grooming.

After the development of MATSAP, the use of underutilized ethological behavior, SAP, was explored. We decided to use MATSAP to evaluate SAP in a mouse TBI study in order to examine how TBI affects anxiety levels. In this study, C57BL6 mice were utilized instead of male Swiss mice so the threshold parameters (speed and eccentricity) used in MATSAP had to be validated. The utilization of MATSAP to detect the SAP behavior provided further validation in the study that the change in anxiety levels did not differ between the experimental groups by corroborating with the anxiety-related spatiotemporal measurements. The MATSAP Threshold Optimizer detection of optimal speed and threshold parameter made the translation from detecting Swiss mice to C57BL6 a smooth process. For both the EPM and OF, only 10 sample videos of each paradigm was needed to determine the speed and threshold values needed to effectively detect SAP. After which, 100 videos were able to be analyzed in batch taking only 30 to 60 seconds a piece depending on video length. Of course, this was after videos were prepped into multi-TIFF files, which had to be done regardless in order to obtain the spatiotemporal measurement with the ImageJ plugins, ImageOF and ImageEP.

6.2 **Future Work**

Further studies of the anxiolytic effects of propranolol and diphenoxylate will need to be examined in other animal models besides the mouse. The motivation behind this is due to the anecdotal evidence that the combination of propranolol and diphenoxylate has a synergic effect on humans to reduce performance anxiety. This would be greatly useful for treating public speaking phobias, which is the most highly

ranked phobia in the world. The review of the literature has shown a clear discrepancy in the effects of propranolol during fear conditioning test between rats and mice. Since there is such a stark difference between rats and mice, re-evaluation of antiphobic or anxiolytic pharmaceuticals that have only been tested on one of the species in the past is needed. The hypotheses of the mechanism of action of propranolol and diphenoxylate will need to be verified, if it holds true that they are effective in other animal models. A clinical trial of the effects of propranolol and diphenoxylate will be of most usefulness given the desired application.

For MATSAP, it is desired to develop tables of speed and eccentricity threshold values for different species, ages, weights, and sex. The user forum at MATLAB File Exchange, which hosts the most recent version of MATSAP, will be utilized to curate and post the finding of a collaborative user group whose participants will provide their optimized eccentricity and speed threshold parameters for other rodents and strains of mice. Once these tables are established, MATSAP can be modified to include a user-input option to select the rodent type, strain, and weight in order to automatically select the appropriate threshold values. Additionally, we plan to develop real-time analysis capabilities so that users can obtain results while performing an experiment.

MATSAP may also be useful for other behavioral tests such as the canopy test and the rat exposure test and this should be evaluated. With a slight modification, MATSAP could be used to quantify forward SAP (F-SAP). This would be useful in tracking SAP towards a novel object, such as in novel object recognition tests, [110] or away from a novel object, such as an electrifiable prod [19]. Combining SAP detection with spatiotemporal measures could help differentiate between 'protected' (when the

rodent is under a covering or in an enclosed area) and 'unprotected' SAP [22, 25, 28, 29, 86, 87].

There will be continual improvements to MATSAP. We plan to develop online, real-time analysis capabilities where the user can obtain instant results while performing an experiment by using a camera with firewire that can connect directly to a computer running MATSAP. User region selection will be implemented so the user can select a region of known dimensions or a region to crop. This feature can also reduce video preparation time if offline analysis is desired. Saving output videos files without using the MATLAB getframe function is preferred, so the user can save a video of the visualized output without having to display it (thus decreasing the runtime). Another future improvement is to allow the program to read other video formats so the user does not need to convert files into multi-TIFFs.

For EthoStock, a larger databank of SAP postures is needed to increase the accuracy, sensitivity and specificity of the program. Once a suitable databank is established, the databank will be used to train a neural network through machine learning. The establishment of a trained neural network would provide optimal runtimes by circumventing the issue of have slower software due to a large databank. The end goal of the program is to track several different ethological behaviors of rodents from an overhead view. After establishing a decent databank for SAP and trained neural network, other ethological behaviors will be explored such as grooming and rearing, which there is a greater need for in the field. First a databank will be established for each of these behaviors and then a neural network would be trained with said databank. Further

databanks can be used to continually train and approve the detection of each ethological behavior.

APPENDIX A

MATSAP SUPPLEMENTARY INFORMATION

A.1 MATSAP Threshold Optimization Guide

This guide will assist users in optimizing the eccentricity and speed threshold values for a particular experiment.

1. Manual Scoring: Evaluate the presence of SAP in sample videos

    a. Classify the presence of SAP in videos on a second by second basis

        i. When SAP is present, give the classification is "1". When SAP is not present, give the classification "0".

        ii. Record classifications for each second of video. List the scores (0 or 1) in consecutive order by time.

        iii. Compile consensus classifications for all sample videos into a single spreadsheet column. Place the classifications in order of the sample videos arranged in alphabetical/numerical order.

    b. Use 2 expert raters (inter-rater reliability >0.90) or 4 – 5 trained, but non-expert raters (inter-rater reliability >0.75) to assess sample videos.

    c. Calculate the consensus classification at each second via majority voting between raters in step b.

    d. In our study, we evaluated ten 5 minute open field videos at 10 fps. We had 5 observers classify if SAP was present at each second, totaling 3000 seconds.

e. Save the single column spreadsheet containing the binary classification of SAP for all sample videos as a .csv file named "Consensus.csv".

2. Video Preparation

a. Convert videos into multi-TIFF files

i. Convert video files into AVI files (ImageJ can read AVI videos) and adjust fps if needed. This can be done with Any Video Converter, AVS Video Converter, or other video converting program.

ii. Use ImageJ to convert files into multi-TIFF. If needed, crop image to known dimensions and create a background image (see Appendix B.2 for detailed instructions).

b. Place multi-TIFF files that will be used to optimize the threshold into a folder without any other TIFF files present.

3. Run MATSAP Threshold Optimizer

a. Follow instructions as prompted

b. If the program is running slowly or you receive a memory error, consider reducing the sample size. Try using multi-TIFF files with lower fps. Alternatively, try using fewer or shorter sample videos (this would require adjusting the consensus data, so lowering the fps may be preferable).

c. Once the videos are analyzed, you should obtain 8 figures and a summary table similar to **Table A-1**.

Table A-1: MATSAP Threshold Optimizer summary table

	Speed	Eccentricity	Sensitivity	Specificity	Accuracy	MCC	Fscore	AUC
max MCC	16	92	78.3	94.9	93	0.68	0.72	0.87
max Accuracy	12	92	65.3	97.1	93.5	0.66	0.69	0.81
max F-score	16	92	78.3	94.9	93	0.68	0.72	0.87
max AUC	19	91	93.5	88.1	88.7	0.63	0.65	0.91

d. Enter the commands below in the command window of MATLAB to explore additional speed and eccentricity threshold values that are not provided by the table.

Commands (case-sensitive):

MCC(S,E)

Accuracy(S,E)

Sensitivity(S,E)

Specificity(S,E)

Fscore(S,E)

AUC(S,E)

When entering the above commands in the MATLAB command window, enter numerical values for S and E (S= Speed threshold value (cm/s), E= Eccentricity Threshold value (%)).

Example:

Let's say we want to investigate the MCC when the eccentricity threshold is 90% and the speed threshold is 12 cm/s. The command below would be written in the MATLAB command window.

MCC(12,90)

The MCC value will then be given and the MATLAB command window would be ready to receive more commands from the user.

A.2 **Video Preparation Protocol with ImageJ**

1. File>Open (Ctrl + O)
    i. Select AVI video you wish to convert to multi-TIFF
    ii. Select "OK" when "AVI Reader" dialog prompt appears.
2. If you need to crop image,
    i. Select the region you wish to crop
    ii. Image>Crop (Ctrl + Shift + X)
3. Create a background frame,
    i. Move to the last frame of video (scroll slide bar to the right)
    ii. Image>Stacks>Add Slice
    iii. Move to the previous frame
    iv. Edit>Selection>Select All (Ctrl+A)
    v. Edit>Copy (Ctrl + C)

vi. Move to the last frame

vii. Edit>Paste (Ctrl + V)

viii. Take note of the location of the rodent and move to a previous frame where rodent is not present in that particular location.

ix. Select the empty region where rodent was present in the last frame.

x. Edit>Copy (Ctrl + C)

xi. Move to the last frame without deselecting region

xii. Edit>Paste (Ctrl + V)

APPENDIX B

ELLIPTIC FOURIER ANALYSIS

The Elliptic Fourier Analysis technique used in EthoStock was developed by Kuhl and Giardina [119]. After obtaining the Freeman chain code of the object of interest, the Fourier series expansion defined in Eq. B-4 was used to describe the complete contour in the x projection based on the chain code.

Equation B-3

$$x(t) = A_0 + \sum_{n=1}^{\infty} a_n cos\left(\frac{2n\pi t}{T}\right) + b_n sin\left(\frac{2n\pi t}{T}\right),$$

where

$$A_0 = \frac{1}{T}\int_0^T x(t)\,dt,$$

$$a_n = \frac{2}{T}\int_0^T x(t)\cos\left(\frac{2n\pi t}{T}\right)dt,$$

$$b_n = \frac{2}{T}\int_0^T x(t)\sin\left(\frac{2n\pi t}{T}\right)dt.$$

The time derivative of the Fourier series described in Eq. B-4 can be represented as a Fourier series as shown in Eq. B-5.

Equation B-4

$$\dot{x}(t) = \sum_{n=1}^{\infty} \alpha_n cos\left(\frac{2n\pi t}{T}\right) + \beta_n sin\left(\frac{2n\pi t}{T}\right),$$

where

$$\alpha_n = \frac{2}{T}\int_0^T \dot{x}(t)\cos\left(\frac{2n\pi t}{T}\right)dt,$$

$$\beta_n = \frac{2}{T}\int_0^T \dot{x}(t)\sin\left(\frac{2n\pi t}{T}\right)dt.$$

Then

$$a_n = \frac{T}{2n^2\pi^2}\sum_{p=1}^{K}\frac{\Delta x_p}{\Delta t_p}\left[cos\left(\frac{2n\pi t_p}{T}\right) - cos\left(\frac{2n\pi t_{p-1}}{T}\right)\right]$$

and

$$b_n = \frac{T}{2n^2\pi^2}\sum_{p=1}^{K}\frac{\Delta x_p}{\Delta t_p}\left[sin\left(\frac{2n\pi t_p}{T}\right) - sin\left(\frac{2n\pi t_{p-1}}{T}\right)\right] .$$

The y projection of the chain code can be represented by the Fourier series

$$y(t) = C_0 + \sum_{n=1}^{\infty} c_n cos\left(\frac{2n\pi t}{T}\right) + d_n sin\left(\frac{2n\pi t}{T}\right),$$

where

$$c_n = \frac{T}{2n^2\pi^2}\sum_{p=1}^{K}\frac{\Delta x_p}{\Delta t_p}\left[cos\left(\frac{2n\pi t_p}{T}\right) - cos\left(\frac{2n\pi t_{p-1}}{T}\right)\right],$$

$$d_n = \frac{T}{2n^2\pi^2}\sum_{p=1}^{K}\frac{\Delta x_p}{\Delta t_p}\left[sin\left(\frac{2n\pi t_p}{T}\right) - sin\left(\frac{2n\pi t_{p-1}}{T}\right)\right] .$$

APPENDIX C

MATSAP SOURCE CODE

C.1 MATSAP Source Code

```
%MATSAP v1.0 developed by Kevin Holly
%
%Image Processing Toolbox required
%
%Version v1.0 - 27 Jul 2016
%
%Required m-files:
%
%MATSAP.m
%MATSAP_Threshold_Previewer.m
%pp_call.m
%preview_pushbutton1_Callback.m
%preview_pushbutton2_Callback.m
%SliderCallback.m
%

%clear

Contpreview = 'Yes';
allsame = 'No.';
visall = 'No.';
AFall = 'No.';
SFall = 'No.';

waitfor(msgbox({'Welcome to MATSAP!';' ';'The SAP Dectection
program.';' ';'Please select the folder containing the multi-TIFF video
files you wish to analyze for SAP Detection.'},'MATSAP v1.0'))

DefaultDir=uigetdir('C:\');
files=dir(fullfile(DefaultDir,'*.tif'));
curr_folder=pwd;
cd(DefaultDir);

%preallocating for optimal runtime
```

```
M = zeros(length(files),1);
N = zeros(length(files),1);
O = zeros(length(files),1);
Q = zeros(length(files),1);

global invert
invert = questdlg('Are the rodents darker than the background?',
'Invert',...
            'Yes','No.','No.');

for i=1:length(files)

    info = imfinfo(files(i).name);
    num_images = numel(info);%Find how many slices (or frames)are in
multi-TIFF file
    J = imread(files(i).name,num_images, 'Info', info);%Creates an
array based on background image's pixel values
    dimJ = size(J); % output = (xpixelength, ypixelength, #ofChannels)

    xpixelength = dimJ(1);
    ypixelength = dimJ(2);


    if Contpreview == 'Yes'
        preview = questdlg('Do you want to preview threshold effects?',
'Preview',...
            'Yes','No.','No.');

        if preview == 'Yes'
            global preview2
            preview2 = 1;
            MATSAP_Threshold_Previewer
            uiwait(gcf);
            cd(DefaultDir);
        end
        if i == 1
            Contpreview = questdlg('Would you like to view the
threshold preview screen before each video?', 'Preview',...
                'Yes','No.','No.');
        end
    end


    if allsame == 'No.'
        prompt = {'Enter binary conversion threshold value:','Enter fps
of video:','Enter x-length of video (cm):','Enter eccentricity
threshold value:','Enter speed threshold (cm/s):'};
        dlg_title = 'Input video and analysis parameters';
        num_lines = 1;
        def = {'0.20','10','30','0.90','12'};
        answer = inputdlg(prompt,dlg_title,num_lines,def);
        threshold = answer{1};
        threshold = str2num(threshold);
        fps = answer{2};
        fps = str2num(fps);
```

```
        xdistance = answer{3};
        xdistance = str2num(xdistance);
        Eccenthreshold = answer{4};
        Eccenthreshold = str2num(Eccenthreshold);
        Speedthreshold = answer{5};
        Speedthreshold = str2num(Speedthreshold);
        if i == 1
            allsame = questdlg('Do these parameters apply to all videos
in folder?', 'Parameters',...
                'Yes','No.','No.');
        end
    end

    if visall == 'No.'
        VT=questdlg('Do you want to visualize the image analysis?',
'Options',...
            'Yes','No.','No.');
        if i == 1
            visall = questdlg('Does your answer apply to all videos in
folder?', 'Visualization',...
                'Yes','No.','No.');
        end
    end

    if VT == 'Yes'

        if AFall == 'No.'
            AF = questdlg('Display all frames?', 'Options',...
                'Yes','No.','No.');
            if i == 1
                AFall = questdlg('Does your answer apply to all videos
in folder?', 'Video Display',...
                    'Yes','No.','No.');
            end
        end
    else
        AF = VT;
    end

    if VT == 'Yes'

        if SFall == 'No.'
            SF = questdlg('Do you want to save the visualized image
analysis as an AVI video file?', 'Options',...
                'Yes','No.','No.');
            if i == 1
                SFall = questdlg('Does your answer apply to all videos
in folder?', 'Video Display',...
                    'Yes','No.','No.');
            else
                if SF == 'Yes'
                    viddir = uigetdir('C:\');
                    mkdir([viddir,filesep,'Videos'])
                end
            end
        end
```

```
    else
        SF = VT;
    end

    if SF == 'Yes'
        if i == 1
            viddir = uigetdir('C:\');
            mkdir([viddir,filesep,'Videos'])
        end
        writerObj =
VideoWriter([viddir,filesep,'Videos',filesep,strrep(files(i).name,
'.tif','') '.avi']);
        if  AF == 'Yes'
            writerObj.FrameRate = fps;
        else
            writerObj.FrameRate = 1;
        end
        open(writerObj);
    end

    if i == 1
        nostop = questdlg('Automatically close output plots after
autosaving?', 'Preview',...
            'Yes','No.','No.');
        defoutdir = questdlg('Save output files in default directory?',
'Default Directory?',...
            'Yes','No.','No.');
        if defoutdir == 'Yes'
            mkdir([DefaultDir,filesep,'Figures'])
            mkdir([DefaultDir,filesep,'Spreadsheet Outputs'])
            mkdir([DefaultDir,filesep,'Notepad Outputs'])
        else
            waitfor(msgbox('Please select the directory to save the
outputs'))
            Outputdir = uigetdir('C:\');
            mkdir([Outputdir,filesep,'Figures'])
            mkdir([Outputdir,filesep,'Spreadsheet Outputs'])
            mkdir([Outputdir,filesep,'Notepad Outputs'])
        end
    end

    %preallocating variabls for runtime optimization
    Eccentricity = zeros((num_images-1),1);
    SAPDetection = zeros((num_images-1),1);
    xbars = zeros((num_images-1),1);
    ybars = zeros((num_images-1),1);
    Distance = zeros((num_images-1),1);
    Speed = zeros((num_images-1),1);

    for k = 1:1:(num_images-1)%For loop from first frame to the second
last frame of video (The last frame is of the background)

        A = imread(files(i).name, k, 'Info', info); %Creates an array
based on for image k's pixel values
        if invert == 'Yes'
            J=255-J;
```

```
            A=255-A;
        end
        C = A-J; %Subtracts background
        bw4=im2bw(C,threshold); %Makes black and white with user's
threshold input
        %bw4 = imfill(bw4, 'holes'); %If needed, this can fill in the
body of rodent, but it will increase the runtime of software.

        %%
        %Detecting ellipses (Note: it will find multiple for each
image)
        se = strel('disk',2);
        bw4=imerode(bw4,se); %Erodes the perimeter of the rodent to
remove the tail
        bw4=imdilate(bw4,se); %Dilates the perimeter of the rodent to
bring back to normal size

        s = regionprops(bw4,'Orientation', 'MajorAxisLength', ...
            'MinorAxisLength', 'Eccentricity', 'Centroid');

        if VT == 'Yes'
            if AF == 'Yes'
                figure(1)
                imshow(bw4) %display background for plot
                hold on
            else
                if ~mod(k,fps)
                    figure(1)
                    imshow(bw4) %display background for plot
                    hold on
                end
            end
        end
        phi = linspace(0,2*pi,50);
        cosphi = cos(phi);
        sinphi = sin(phi);

        ellipsenum = length(s); %Number of ellipses detected in frame

        if ellipsenum < 2,
            input = 1;
        elseif ellipsenum > 1,
            for l = 1:1:length(s);
                [sizesofellipses(l)] =
s(l).MajorAxisLength*s(l).MinorAxisLength; %Finds the rectangular area
of ellipse
            end
            [biggest, input] = max(sizesofellipses);%Determine input
that creates largest ellipse based on rectangular area
            clear 'sizesofellipses'; %Clears array of rectangular areas
b/c next iteriation may have less ellipes, so this ensures it doesn't
pick a value from a previous iteriation.
        end

        %%
        %Display largest ellipse values
```

```
        s(input);

        %Display plot of largest ellipse (Used code from Steve Eddins'
blog article,"Visualizing regionprops ellipse measurements" % _Steve
Eddins_% _Copyright 2010 The MathWorks, Inc)
        if VT == 'Yes'
            if AF == 'Yes'
                for v = input
                    xbar = s(v).Centroid(1);
                    ybar = s(v).Centroid(2);

                    a = s(v).MajorAxisLength/2;
                    b = s(v).MinorAxisLength/2;

                    theta = pi*s(v).Orientation/180;
                    R = [ cos(theta)   sin(theta)
                        -sin(theta)   cos(theta)];

                    xy = [a*cosphi; b*sinphi];
                    xy = R*xy;

                    x = xy(1,:) + xbar;
                    y = xy(2,:) + ybar;


                    plot(x,y,'r','LineWidth',2);
                end
                if SF == 'Yes'
                    frame = getframe(gcf);
                    writeVideo(writerObj,frame);
                end
            else
                if ~mod(k,fps)
                    for v = input
                        xbar = s(v).Centroid(1);
                        ybar = s(v).Centroid(2);

                        a = s(v).MajorAxisLength/2;
                        b = s(v).MinorAxisLength/2;

                        theta = pi*s(v).Orientation/180;
                        R = [ cos(theta)   sin(theta)
                            -sin(theta)   cos(theta)];

                        xy = [a*cosphi; b*sinphi];
                        xy = R*xy;

                        x = xy(1,:) + xbar;
                        y = xy(2,:) + ybar;


                        plot(x,y,'r','LineWidth',2);
                    end
                    if SF == 'Yes'
```

```
                    frame = getframe(gcf);
                    writeVideo(writerObj,frame);
                end
            end
        end
    end

    if VT == 'Yes'
        if SF == 'No.'
            pause(.0001)%This ensures the video will have time to
be displayed on screen
        end
    end
    %%
    Eccentricity(k) = s(input).Eccentricity; %Creates array of
Eccentricity values from the first to second last frame (as the last
frame is the background).
    xbars(k) = s(input).Centroid(1);
    ybars(k) = s(input).Centroid(2);
    if k > 1
        Distance(k)= sqrt((xbars(k-1)-xbars(k))^2+(ybars(k-1)-
ybars(k))^2);
        Speed(k)=Distance(k)*(xdistance/xpixelength)*(fps);
    end

    if Eccentricity(k) <= Eccenthreshold
        SAPDetection(k)=0;
    else SAPDetection(k)=1;
    end
    if k > 1
        if Speed(k) >Speedthreshold,
            SAPDetection(k)=0;
        end
    end
    frame=k   %Displays current frame under analysis in MATLAB
command window
end

%Counter is utilized to remove SAP detection that lasted only up to
a half of a second and measure the SAP frequency.
count = 0;  %Counter to keep track of how many 1's are in a row.
Threshold = 0.5*fps;  %How many 1's are allowed in a row
freqSAP = 0; %frequency of SAP detected

for k = 1:(num_images-1)   %Goes through 1-D array
    if SAPDetection(k,1) == 1
        count = count + 1;  %Keeps track of how many 1's are in a
row
    else
        if count >= Threshold   %If True, restart counter
            count = 0;
            freqSAP = freqSAP + 1;
        else                    %Else, turn all 1's to zeroes
            while count >0
                SAPDetection(k-count,1) = 0;
                count = count - 1;
```

```
            end
        end
    end
end

if SF == 'Yes'
    close(writerObj);
end

figure(2)
subplot(311)
plot(Eccentricity)
title('Eccentricity Values')
xlabel('Frame') % x-axis label
ylabel('Eccentricity') % y-axis label
axis([0 (num_images-1) 0 1]); %Sets axis limits

subplot(312)
plot(Speed)
title('Speed')
xlabel('Frame') % x-axis label
ylabel('Speed (cm/s)') % y-axis label
axis([0 (num_images-1) ylim]); %Sets axis limits

subplot(313)
bar(SAPDetection)
title('SAP Detection')
xlabel('Frame') % x-axis label
ylabel('SAP Presence') % y-axis label
axis([0 (num_images-1) 0 1]); %Sets axis limits


set(gcf,'color','w');

%Save plots
fnam=[strrep(files(i).name, '.tif','') '.fig'];
if defoutdir == 'Yes'
    saveas(gcf,[DefaultDir,filesep,'Figures',filesep,fnam],'fig');
else
    saveas(gcf,[Outputdir,filesep,'Figures',filesep,fnam],'fig');
end

if nostop == 'Yes'
    close(gcf)
end

%Vectors to save into files
A = {'Frame','Eccentricity','Speed (cm/s)','SAP Detected',' ', 'SAP
time (s)', 'Total time (s)','SAP percentage'};
B = 1:1:(num_images-1);
C = Eccentricity;
D = Speed;
E = SAPDetection;
F = sum(E)/fps;
G = (num_images-1)/fps;
```

```
    H = (F/G)*100;

    %Creates Excel spreadsheet with Eccentricity, Speed, and SAP
deteciotn Data
    if defoutdir == 'Yes'
        sheet = 1;
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','') '.xls'],A,sheet,'A1')
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],B(:),sheet,'A2')
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],C(:),sheet,'B2')
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],D(:),sheet,'C2')
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],E(:),sheet,'D2')
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],F(:),sheet,'F2')
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],G(:),sheet,'G2')
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],H(:),sheet,'H2')
    else
        sheet = 1;
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','') '.xls'],A,sheet,'A1')
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],B(:),sheet,'A2')
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],C(:),sheet,'B2')
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],D(:),sheet,'C2')
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],E(:),sheet,'D2')
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],F(:),sheet,'F2')
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],G(:),sheet,'G2')
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],H(:),sheet,'H2')    % end
    end
```

```
    %Creates Notpad files to archive data
    if defoutdir == 'Yes'

        header1 = 'Frame';
        header2 = 'Eccentricity';
        header3 = 'Speed (cm/s)';
        header4 = 'SAP Detection';
        fid=fopen([DefaultDir,filesep,'Notepad
Outputs',filesep,strrep(files(i).name, '.tif','')
'_Eccentricity.txt'],'w');
        fprintf(fid, [ header1 ' ' header2 '\n']);
        fprintf(fid, '%f %f \n', [B(:) C(:)]');
        fclose(fid);
        fid=fopen([DefaultDir,filesep,'Notepad
Outputs',filesep,strrep(files(i).name, '.tif','') '_Speed.txt'],'w');
        fprintf(fid, [ header1 ' ' header3 '\n']);
        fprintf(fid, '%f %f \n', [B(:) D(:)]');
        fclose(fid);
        fid=fopen([DefaultDir,filesep,'Notepad
Outputs',filesep,strrep(files(i).name, '.tif','')
'_SAPDetection.txt'],'w');
        fprintf(fid, [ header1 ' ' header3 '\n']);
        fprintf(fid, '%f %f \n', [B(:) E(:)]');
        fclose(fid);

        header5 = 'SAP time (s)';
        header6 = 'Total time (s)';
        header7 = 'SAP percentage';
        header8 = 'SAP Frequency';
        fid=fopen([DefaultDir,filesep,'Notepad
Outputs',filesep,strrep(files(i).name, '.tif','') '_Results.txt'],'w');
        fprintf(fid, [ header5 ' ' header6 ' ' header7 ' ' header8
'\n']);
        fprintf(fid, '%f %f %f %f \n', [F(:) G(:) H(:) freqSAP(:)]');
        fclose(fid);
    else

        header1 = 'Frame';
        header2 = 'Eccentricity';
        header3 = 'Speed (cm/s)';
        header4 = 'SAP Detection';
        fid=fopen([Outputdir,filesep,'Notepad
Outputs',filesep,strrep(files(i).name, '.tif','')
'_Eccentricity.txt'],'w');
        fprintf(fid, [ header1 ' ' header2 '\n']);
        fprintf(fid, '%f %f \n', [B(:) C(:)]');
        fclose(fid);
        fid=fopen([Outputdir,filesep,'Notepad
Outputs',filesep,strrep(files(i).name, '.tif','') '_Speed.txt'],'w');
        fprintf(fid, [ header1 ' ' header3 '\n']);
        fprintf(fid, '%f %f \n', [B(:) D(:)]');
        fclose(fid);
        fid=fopen([Outputdir,filesep,'Notepad
Outputs',filesep,strrep(files(i).name, '.tif','')
'_SAPDetection.txt'],'w');
        fprintf(fid, [ header1 ' ' header3 '\n']);
```

```
        fprintf(fid, '%f %f \n', [B(:) E(:)]');
        fclose(fid);

        header5 = 'SAP time (s)';
        header6 = 'Total time (s)';
        header7 = 'SAP percentage';
        header8 = 'SAP Frequency';
        fid=fopen([Outputdir,filesep,'Notepad
Outputs',filesep,strrep(files(i).name, '.tif','') '_Results.txt'],'w');
        fprintf(fid, [ header5 ' ' header6 ' ' header7 ' ' header8
'\n']);
        fprintf(fid, '%f %f %f %f \n', [F(:) G(:) H(:) freqSAP(:)]');
        fclose(fid);
    end

    if nostop == 'No.'
        uiwait(gcf);
    end

    L=strrep(files(i).name, '.tif','');
    if defoutdir == 'Yes'
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],{L},sheet,['A',num2str(i+1)])
    else
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],{L},sheet,['A',num2str(i+1)])
    end
    M(i)=F;
    N(i)=G;
    O(i)=H;
    Q(i)=freqSAP;
end

%Saves all results in one Excel sheet

J={'File Name','SAP time (s)', 'Total time (s)','SAP percentage','SAP
Frequency'};

if defoutdir == 'Yes'
    sheet = 1;
    xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],J,sheet,'A1')
    xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],M(:),sheet,'B2')
    xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],N(:),sheet,'C2')
    xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],O(:),sheet,'D2')
    xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],Q(:),sheet,'E2')
else
    sheet = 1;
    xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],J,sheet,'A1')
    xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],M(:),sheet,'B2')
```

```
    xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],N(:),sheet,'C2')
    xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],O(:),sheet,'D2')
    xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],Q(:),sheet,'E2')
end
display('Analysis Complete!')
cd(curr_folder)
clear global
```

C.2 MATSAP Threshold Previewer

```
function MATSAP_Threshold_Previewer
global preview2

if preview2 == 1
    waitfor(msgbox({'Welcome to MATSAP Threshold Preview Screen!';' ';'
';'Please select the folder containing the multi-TIFF video files you
wish to preview for threshold selection.'},'MATSAP v1.0 Threshold
Preview Screen'))
    str='C:\';
    preview_folder_name=uigetdir(str);
    files=dir(fullfile(preview_folder_name,'*.tif'));
    curr_folder=pwd;
    cd(preview_folder_name);

else
    global invert
    waitfor(msgbox({'Welcome to MATSAP Threshold Preview Screen!';' ';'
';'Please answering the following question and then select the folder
containing the multi-TIFF video files you wish to preview for threshold
selection.'},'MATSAP v1.0 Threshold Preview Screen'))

    invert = questdlg('Are the rodents darker than the background?',
'Invert',...
        'Yes','No.','No.');

    str='C:\';
    preview_folder_name=uigetdir(str);
    files=dir(fullfile(preview_folder_name,'*.tif'));
    curr_folder=pwd;
    cd(preview_folder_name);
end



filelist = files(1).name;
info = imfinfo(files(1).name);
num_images = numel(info);%Find how many slices (or frames)are in multi-
TIFF file
for i=2:length(files)
```

```
    info = imfinfo(files(i).name);
    filelist = char(filelist,files(i).name);
    num_images = numel(info);%Find how many slices (or frames)are in
multi-TIFF file
end


%// Create GUI controls
handles.figure = figure('Position',[100 100 500 500],'Units','Pixels');

handles.axes1 = axes('Units','Pixels','Position',[60,100,229,229]);

A= imread(files(1).name, 'tif');
imshow(A,[])

handles.Slider1 = uicontrol('Style','slider','Position',[60 420 400
20],'Min',0,'Max',1,'SliderStep',[1/100
1/100],'Callback',@SliderCallback);
handles.Slider2 = uicontrol('Style','slider','Position',[60 360 400
20],'Min',0,'Max',(num_images-1),'SliderStep',[1/(num_images-1)
1/(num_images-1)],'Callback',@SliderCallback);

handles.Edit1 = uicontrol('Style','Edit','Position',[250 450 100
20],'String','Update Me');
handles.Text1 = uicontrol('Style','Text','Position',[180 450 60
20],'String','Threshold:');
handles.Edit2 = uicontrol('Style','Edit','Position',[250 390 100
20],'String','Update Me');
handles.Text2 = uicontrol('Style','Text','Position',[180 390 60
20],'String','Frame:');

handles.popup1 = uicontrol('style','pop',...
                  'units','pixels',...
                  'position',[300 250 200 40],...
                  'string',{filelist});
set(handles.popup1,'callback',{@pp_call,handles});  % Set the callback.

pb = uicontrol('Style','pushbutton','String','Preview',...
                'Position',[300 300 60 20],...
                'Callback',@preview_pushbutton1_Callback);

pb2 = uicontrol('Style','pushbutton','String','Okay',...
                'Position',[400 20 60 30],...
                'Callback',@preview_pushbutton2_Callback);

handles.xrange = 0:0.1:50; %// Use to generate dummy data to plot.

guidata(handles.figure,handles); %// Update the handles structure.
```

C.2.1 Slider Callback

```
function SliderCallback(~,~) %// This is the slider callback, executed
when you release the it or press the arrows at each extremity.
```

```
handles = guidata(gcf);

threshold = get(handles.Slider1,'Value');
threshold = round(100*threshold)/100;
set(handles.Edit1,'String',num2str(threshold));

k = get(handles.Slider2,'Value');
k = round(k);
set(handles.Edit2,'String',num2str(k));
end
```

C.2.2 Preview pushbutton 1 Callback

```
function preview_pushbutton1_Callback(hObject, eventdata, handles)
%preview_folder_name= 'C:\';
files=dir(fullfile(pwd,'*.tif'));
curr_folder=pwd;
%cd(preview_folder_name);

info = imfinfo(files(1).name);
num_images = numel(info);%Find how many slices in tiff file
J = imread(files(1).name,num_images, 'Info', info);%create an array
based on background image's pixel values
dimJ = size(J); % output = (xpixelength, ypixelength, #ofChannels)

xpixelength = dimJ(1);
ypixelength = dimJ(2);

handles = guidata(gcf);

threshold = get(handles.Slider1,'Value');
threshold = round(100*threshold)/100;
set(handles.Edit1,'String',num2str(threshold));

k = get(handles.Slider2,'Value');
k = round(k);
if k <= 0
    k = 1;
end
set(handles.Edit2,'String',num2str(k));

P = get(handles.popup1,{'string','val'});
info = imfinfo(files(P{2}).name);
A= imread(files(P{2}).name,k, 'Info', info);
num_images = numel(info);
J= imread(files(P{2}).name,num_images, 'Info', info);
global invert
if invert == 'Yes'
    J=255-J;
    A=255-A;
end


if threshold > 0
```

```
    C = A-J; %subtract background
    bw4=im2bw(C,threshold); %Make black and white with 0.35 threshold
    bw4 = imfill(bw4, 'holes');
    imshow(bw4,[])
else
    imshow(A)
end
end
```

C.2.3 Preview pushbutton 2 Callback

```
function preview_pushbutton2_Callback(hObject, eventdata, handles)
close(gcf);
end
```

C.2.4 PP Callback

```
function [] = pp_call(varargin)
% Callback for the popup.
handles = varargin{3};  % Get the structure.
P = get(handles.popup1,{'string','val'});  % Get the user's choice.
```

APPENDIX D

MATSAP THRESHOLD OPTIMIZER SOURCE CODE

```
%MATSAP Threshold Optimizer v1.0 developed by Kevin Holly
%
%Image Processing Toolbox required
%
%Version v1.0 - 27 Jul 2016
%
%Required m-files:
%
%MATSAP_Threshold_Optimizer.m
%MATSAP_Threshold_Previewer.m
%pp_call.m
%preview_pushbutton1_Callback.m
%preview_pushbutton2_Callback.m
%SliderCallback.m

clear

waitfor(msgbox({'Welcome to MATSAP Threshold Optimizer!';' ';'The
MATSAP threshold optimization program.';' ';'Please Select the folder
containing the multi-TIFF video files you wish to analyze for SAP
Detection.'},'MATSAP v1.0 Threshold Optimizer'))

folder_name=uigetdir('C:\');
files=dir(fullfile(folder_name,'*.tif'));
curr_folder=pwd;

global invert
invert = questdlg('Are the rodents darker than the background?',
'Invert',...
            'Yes','No.','No.');

preview = questdlg('Do you want to preview threshold effects?',
'Preview',...
            'Yes','No.','No.');

if preview == 'Yes'
    global preview2
    preview2 = 1;
    MATSAP_Threshold_Preview_Screen
    uiwait(gcf);
```

```
    cd(curr_folder);
end
prompt = {'Enter binary conversion threshold value:','Enter fps of
video:','Enter x-length of video (cm):',};
        dlg_title = 'Input video and analysis parameters';
        num_lines = 1;
        def = {'0.20','10','30'};
        answer = inputdlg(prompt,dlg_title,num_lines,def);
        threshold = answer{1};
        threshold = str2num(threshold);
        fps = answer{2};
        fps = str2num(fps);
        xdistance = answer{3};
        xdistance = str2num(xdistance);
%% Gather Raw Eccentricity and Speed Data
cd(folder_name);

for i=1:length(files)

    info = imfinfo(files(i).name);
    num_images = numel(info);%Find how many slices (or frames)are in
multi-TIFF file
    J = imread(files(i).name,num_images, 'Info', info);%Creates an
array based on background image's pixel values
    dimJ = size(J); % output = (xpixelength, ypixelength, #ofChannels)

    xpixelength = dimJ(1);
    ypixelength = dimJ(2);

    for k = 1:1:(num_images-1)%For loop from first frame to the second
last frame of video (The last frame is of the background)

        A = imread(files(i).name, k, 'Info', info); %Creates an array
based on for image k's pixel values
        if invert == 'Yes'
            J=255-J;
            A=255-A;
        end
        C = A-J; %Subtracts background
        bw4=im2bw(C,threshold); %Makes black and white with user's
threshold input
        %bw4 = imfill(bw4, 'holes'); %If needed, this can fill in the
body of rodent, but it will increase the runtime of software.

        %%
        %Detecting ellipses (Note: it will find multiple for each
image)
        se = strel('disk',2);
        bw4=imerode(bw4,se); %Erodes the perimeter of the rodent to
remove the tail
        bw4=imdilate(bw4,se); %Dilates the perimeter of the rodent to
bring back to normal size

        s = regionprops(bw4,'Orientation', 'MajorAxisLength', ...
            'MinorAxisLength', 'Eccentricity', 'Centroid');
```

```
        ellipsenum = length(s); %Number of ellipses detected in frame

        if ellipsenum < 2,
            input = 1;
        elseif ellipsenum > 1,
            for l = 1:1:length(s);
                [sizesofellipses(l)] =
s(l).MajorAxisLength*s(l).MinorAxisLength; %Finds the rectangular area
of ellipse
            end
            [biggest input] = max(sizesofellipses);%Determine input
that creates largest ellipse based on rectangular area
            clear 'sizesofellipses'; %Clears array of rectangular areas
b/c next iteriation may have less ellipes, so this ensures it doesn't
pick a value from a previous iteriation.
        end

        %%
        Eccentricity(k) = s(input).Eccentricity; %Creates array of
Eccentricity values from the first to second last frame (as the last
frame is the background).
        xbars(k) = s(input).Centroid(1);
        ybars(k) = s(input).Centroid(2);
        if k > 1
            Distance(k)= sqrt((xbars(k-1)-xbars(k))^2+(ybars(k-1)-
ybars(k))^2);
            Speed(k)=Distance(k)*(xdistance/xpixelength)*(fps);
        end

        frame=k   %Displays current frame under analysis in MATLAB
command window
    end


    %Vectors to save into files
    C = Eccentricity;
    D = Speed;

    %Creates Output file contaiining timebased SAP Detection
    if i==1
        mkdir([folder_name,filesep,'Spreadsheet Outputs'])
        csvwrite([folder_name,filesep,'Spreadsheet
Outputs',filesep,'OutputEccentricity.csv'],C',0)
        csvwrite([folder_name,filesep,'Spreadsheet
Outputs',filesep,'OutputSpeed.csv'],D',0)
    else
        dlmwrite ([folder_name,filesep,'Spreadsheet
Outputs',filesep,'OutputEccentricity.csv'], C', '-append');
        dlmwrite ([folder_name,filesep,'Spreadsheet
Outputs',filesep,'OutputSpeed.csv'], D', '-append');
    end

end
display('Raw Data Collected!')
```

```
%% Process and Generate Plots
cd([folder_name,filesep,'Spreadsheet Outputs'])

Eccentricity = dlmread([folder_name,filesep,'Spreadsheet
Outputs',filesep,'OutputEccentricity.csv']);
display('Eccentricity Data Loaded')

Speed = dlmread([folder_name,filesep,'Spreadsheet
Outputs',filesep,'OutputSpeed.csv']);
display('Speed Data Loaded')

if size(Eccentricity,1) == size(Speed,1)
display('Data is of equal dimensions')
else
waitfor(msgbox({'Data selected is not of equal dimensions'},'ERROR'))
end

waitfor(msgbox({'Please select the folder containing the file
Consensus.csv'},'Selecting folder containing Consensus.csv'))
Consensus_folder_name=uigetdir('C:\');
Consensus = dlmread([Consensus_folder_name,filesep,'Consensus.csv']);
display('Consensus Data Loaded')

SAPDetection = zeros(size(Speed,1),100,100);

for k = 1:1:size(Speed,1)
    for l = 1:1:100
        for m = 1:1:100
            if Eccentricity(k) <= m/100
                SAPDetection(k,l,m) = 0;
            else
                SAPDetection(k,l,m) = 1;
            end
            if Speed(k) > l
                SAPDetection(k,l,m) = 0;
            end
        end
    end
k
end
display('Threshold done')
Threshold = fps/2;

for l = 1:1:100
    for m = 1:1:100
        count=0;
        for k = 2:1:size(Speed,1)
            if SAPDetection(k,l,m) == 1
                count = count + 1;  %Keeps track of how many 1's are in
a row
            else
                if count >= Threshold   %If True, restart counter
                    count = 0;
                else                    %Else, turn all 1's to zeroes
                    while count >0
```

```
                    SAPDetection(k-count,l,m) = 0;
                    count = count - 1;
                end
            end
        end
    end
l
end
timebasedSAPDetection = zeros(size(Speed,1)/fps,100,100);
timebasedSAPDetectionFreq = zeros(size(Speed,1)/fps,100,100);
for u = 1:size(Speed,1)/fps
    for l = 1:1:100
        for m = 1:1:100
            for ii=1:1:fps
                timebasedSAPDetectionFreq(u,l,m)=
timebasedSAPDetectionFreq(u,l,m)+SAPDetection(ii+fps*(u-1),l,m);
            end
            if timebasedSAPDetectionFreq(u,l,m) > 0
                timebasedSAPDetection(u,l,m)= 1;
            else timebasedSAPDetection(u,l,m)= 0;
            end
        end
    end
u
end
display('Filter done')
Sensitivity = zeros(100,100);
Specificity = zeros(100,100);
FNR = zeros(100,100);
FPR = zeros(100,100);
MCC = zeros(100,100);
Fscore = zeros(100,100);
Accuracy = zeros(100,100);
for l = 1:1:100
    for m = 1:1:100
        for u = 1:1:size(Speed,1)/fps
            if [timebasedSAPDetection(u,l,m)+Consensus(u)] == 2
                TP(u)=1;
            else
                TP(u)=0;
            end
            if timebasedSAPDetection(u,l,m)>Consensus(u)
                FP(u)=1;
            else
                FP(u)=0;
            end
            if timebasedSAPDetection(u,l,m)<Consensus(u)
                FN(u)=1;
            else
                FN(u)=0;
            end
            if [timebasedSAPDetection(u,l,m)+Consensus(u)] == 0
                TN(u)=1;
            else
                TN(u)=0;
            end
```

```
        end
        Sensitivity(l,m) = sum(TP)/(sum(TP)+sum(FN));
        Specificity(l,m) = sum(TN)/(sum(TN)+sum(FP));
        FNR(l,m) = 1-Sensitivity(l,m);
        FPR(l,m) = 1-Specificity(l,m);
        MCC(l,m) = (sum(TP)*sum(TN)-
sum(FP)*sum(FN))/sqrt((sum(TP)+sum(FP))*(sum(TP)+sum(FN))*(sum(TN)+sum(
FP))*(sum(TN)+sum(FN)));
        Fscore(l,m) = (2*(sum(TP)))/(2*(sum(TP))+ sum(FP)+ sum(FN));
        Accuracy(l,m) =
(sum(TP)+sum(TN))/(sum(TP)+sum(FP)+sum(FN)+sum(TN));

    end
l
end
display('Analysis Complete!')

x=Specificity(:);
y=Sensitivity(:);
figure(1)
for q=1:1:100
plot(1-x([(q-1)*100+1]:q*100),y([(q-1)*100+1]:q*100))
hold on
end
title('Speed ROC curves at various Eccentricity threshold values')
xlabel('1-Specificity')
ylabel('Sensitivity')
hold off

Specificity2=Specificity';
Sensitivity2=Sensitivity';
x2=Specificity2(:);
y2=Sensitivity2(:);
figure(2)
for q=1:1:100
plot(1-x2([(q-1)*100+1]:q*100),y2([(q-1)*100+1]:q*100))
hold on
end
title('Eccentrcity ROC curves at various Speed threshold values')
xlabel('1-Specificity')
ylabel('Sensitivity')
hold off

figure(3)
surf(MCC)
title('MCC')
xlabel('Eccentricity (%)')
ylabel('Speed (cm/s)')
zlabel('Matthews Correlation Coefficient')

figure(4)
surf(Sensitivity)
title('Sensitivity')
xlabel('Eccentricity (%)')
ylabel('Speed (cm/s)')
zlabel('Sensitivity')
```

```
figure(5)
surf(FPR)
title('1-Specificity')
xlabel('Eccentricity (%)')
ylabel('Speed (cm/s)')
zlabel('1-Specificity')

figure(6)
surf(Accuracy)
title('Accuracy')
xlabel('Eccentricity (%)')
ylabel('Speed (cm/s)')
zlabel('Accuracy')

figure(7)
surf(Fscore)
title('F-Score')
xlabel('Eccentricity (%)')
ylabel('Speed (cm/s)')
zlabel('F-Score')

AUC=(Sensitivity+Specificity)/2;
figure(8)
surf(AUC)
title('AUC')
xlabel('Eccentricity (%)')
ylabel('Speed (cm/s)')
zlabel('Area under ROC curve')

maxnames = {'max MCC'; 'max Accuracy'; 'max F-score'; 'max AUC'};

[maxAUC, Eccen4maxAUC] = max(max(AUC));
[maxAUC, Speed4maxAUC]=max(AUC(:,Eccen4maxAUC));

Sensitivity4maxAUC=Sensitivity(Speed4maxAUC, Eccen4maxAUC);
Specificity4maxAUC=Specificity(Speed4maxAUC, Eccen4maxAUC);
Accuracy4maxAUC=Accuracy(Speed4maxAUC, Eccen4maxAUC);
MCC4maxAUC=MCC(Speed4maxAUC, Eccen4maxAUC);
Fscore4maxAUC=Fscore(Speed4maxAUC, Eccen4maxAUC);

[maxMCC, Eccen4maxMCC] = max(max(MCC));
[maxMCC, Speed4maxMCC]=max(MCC(:,Eccen4maxMCC));

Sensitivity4maxMCC=Sensitivity(Speed4maxMCC, Eccen4maxMCC);
Specificity4maxMCC=Specificity(Speed4maxMCC, Eccen4maxMCC);
Accuracy4maxMCC=Accuracy(Speed4maxMCC, Eccen4maxMCC);
Fscore4maxMCC=Fscore(Speed4maxMCC, Eccen4maxMCC);
AUC4maxMCC=AUC(Speed4maxMCC, Eccen4maxMCC);

[maxFscore, Eccen4maxFscore] = max(max(Fscore));
[maxFscore, Speed4maxFscore]=max(Fscore(:,Eccen4maxFscore));

Sensitivity4maxFscore=Sensitivity(Speed4maxFscore, Eccen4maxFscore);
Specificity4maxFscore=Specificity(Speed4maxFscore, Eccen4maxFscore);
```

```
Accuracy4maxFscore=Accuracy(Speed4maxFscore, Eccen4maxFscore);
MCC4maxFscore=MCC(Speed4maxFscore, Eccen4maxFscore);
AUC4maxFscore=AUC(Speed4maxFscore, Eccen4maxFscore);

[maxAccuracy, Eccen4maxAccuracy] = max(max(Accuracy));
[maxAccuracy, Speed4maxAccuracy]=max(Accuracy(:,Eccen4maxAccuracy));

Sensitivity4maxAccuracy=Sensitivity(Speed4maxAccuracy,
Eccen4maxAccuracy);
Specificity4maxAccuracy=Specificity(Speed4maxAccuracy,
Eccen4maxAccuracy);
AUC4maxAccuracy=AUC(Speed4maxAccuracy, Eccen4maxAccuracy);
MCC4maxAccuracy=MCC(Speed4maxAccuracy, Eccen4maxAccuracy);
Fscore4maxAccuracy=Fscore(Speed4maxAccuracy, Eccen4maxAccuracy);

Speedcolumn = [Speed4maxMCC Speed4maxAccuracy Speed4maxFscore
Speed4maxAUC];
Eccencolumn = [Eccen4maxMCC Eccen4maxAccuracy Eccen4maxFscore
Eccen4maxAUC];
Sensitivitycolumn = [Sensitivity4maxMCC Sensitivity4maxAccuracy
Sensitivity4maxFscore Sensitivity4maxAUC];
Specificitycolumn = [Specificity4maxMCC Specificity4maxAccuracy
Specificity4maxFscore Specificity4maxAUC];
Accuracycolumn = [Accuracy4maxMCC maxAccuracy Accuracy4maxFscore
Accuracy4maxAUC];
MCCcolumn = [maxMCC MCC4maxAccuracy MCC4maxFscore MCC4maxAUC];
Fscorecolumn = [Fscore4maxMCC Fscore4maxAccuracy maxFscore
Fscore4maxAUC];
AUCcolumn = [AUC4maxMCC AUC4maxAccuracy AUC4maxFscore maxAUC];

Sensitivitycolumn=round(Sensitivitycolumn,3);
Specificitycolumn=round(Specificitycolumn,3);
Accuracycolumn=round(Accuracycolumn,3);
MCCcolumn=round(MCCcolumn,2);
Fscorecolumn=round(Fscorecolumn,2);
AUCcolumn=round(AUCcolumn,2);

Optimization_table =
table(Speedcolumn',Eccencolumn',Sensitivitycolumn'*100,Specificitycolum
n'*100,Accuracycolumn'*100,MCCcolumn',Fscorecolumn',AUCcolumn','RowName
s',maxnames,'VariableNames',{'Speed' 'Eccentricity' 'Sensitivity'
'Specificity' 'Accuracy' 'MCC' 'Fscore' 'AUC'})

clear global
```

APPENDIX E

ETHOSTOCK SOURCE CODE

```
%EthoStock v0.6 developed by Kevin Holly
%
%Image Processing Toolbox required
%
%EthoStock version 0.6   4 April 2016
%
%Required m-files:
%
%EthoStockv06.m
%chaincode.m
%fefourier.m
%plot_chain_code.m
%plot_fourier_approx.m
%MATSAP_Threshold_Previewer.m
%pp_call.m
%preview_pushbutton1_Callback.m
%preview_pushbutton2_Callback.m
%SliderCallback.m
%
%clear

Contpreview = 'Yes';
allsame = 'No.';
visall = 'No.';
AFall = 'No.';
SFall = 'No.';

waitfor(msgbox({'Welcome to EthoStock SAP Detector!';' ';'The SAP
Dectection program based on a stock of ethological data.';' ';'Please
select the folder containing the multi-TIFF video files you wish to
analyze for SAP Detection.'},'EthoStock SAP Detector v0.6'))

DefaultDir=uigetdir('C:\');
files=dir(fullfile(DefaultDir,'*.tif'));
curr_folder=pwd;
cd(DefaultDir);

%preallocating for optimal runtime
M = zeros(length(files),1);
N = zeros(length(files),1);
```

```
O = zeros(length(files),1);
Q = zeros(length(files),1);

global invert
invert = questdlg('Are the rodents darker than the background?',
'Invert',...
    'Yes','No.','No.');

for i=1%:length(files)

    cd(DefaultDir);
    info = imfinfo(files(i).name);
    num_images = numel(info);%Find how many slices (or frames)are in
multi-TIFF file
    J = imread(files(i).name,num_images, 'Info', info);%Creates an
array based on background image's pixel values
    dimJ = size(J); % output = (xpixelength, ypixelength, #ofChannels)

    xpixelength = dimJ(1);
    ypixelength = dimJ(2);


    if Contpreview == 'Yes'
        preview = questdlg('Do you want to preview threshold effects?',
'Preview',...
            'Yes','No.','No.');

        if preview == 'Yes'
            global preview2
            preview2 = 1;
            MATSAP_Threshold_Previewer
            uiwait(gcf);
            cd(DefaultDir);
        end
        if i == 1
            Contpreview = questdlg('Would you like to view the
threshold preview screen before each video?', 'Preview',...
                'Yes','No.','No.');
        end
    end


    if allsame == 'No.'
        prompt = {'Enter binary conversion threshold value:','Enter fps
of video:','Enter x-length of video (cm):'};
        dlg_title = 'Input video and analysis parameters';
        num_lines = 1;
        def = {'0.20','10','30'};
        answer = inputdlg(prompt,dlg_title,num_lines,def);
        threshold = answer{1};
        threshold = str2num(threshold);
        fps = answer{2};
        fps = str2num(fps);
        xdistance = answer{3};
        xdistance = str2num(xdistance);
```

```
        if i == 1
            allsame = questdlg('Do these parameters apply to all videos
in folder?', 'Parameters',...
                'Yes','No.','No.');
        end
    end

    if visall == 'No.'
        VT=questdlg('Do you want to visualize the image analysis?',
'Options',...
            'Yes','No.','No.');
        if i == 1
            visall = questdlg('Does your answer apply to all videos in
folder?', 'Visualization',...
                'Yes','No.','No.');
        end
    end

    if VT == 'Yes'

        if AFall == 'No.'
            AF = questdlg('Display all frames?', 'Options',...
                'Yes','No.','No.');
            if i == 1
                AFall = questdlg('Does your answer apply to all videos
in folder?', 'Video Display',...
                    'Yes','No.','No.');
            end
        end
    else
        AF = VT;
    end

    if VT == 'Yes'

        if SFall == 'No.'
            SF = questdlg('Do you want to save the visualized image
analysis as an AVI video file?', 'Options',...
                'Yes','No.','No.');
            if i == 1
                SFall = questdlg('Does your answer apply to all videos
in folder?', 'Video Display',...
                    'Yes','No.','No.');
            else
                if SF == 'Yes'
                    viddir = uigetdir('C:\');
                    mkdir([viddir,filesep,'Videos'])
                end
            end
        end
    else
        SF = VT;
    end

    if SF == 'Yes'
        if i == 1
```

```
            viddir = uigetdir('C:\');
            mkdir([viddir,filesep,'Videos'])
        end
        writerObj =
VideoWriter([viddir,filesep,'Videos',filesep,strrep(files(i).name,
'.tif','') '.avi']);
        if  AF == 'Yes'
            writerObj.FrameRate = fps;
        else
            writerObj.FrameRate = 1;
        end
        open(writerObj);
    end

    if i == 1
        nostop = questdlg('Automatically close output plots after
autosaving?', 'Preview',...
            'Yes','No.','No.');
        defoutdir = questdlg('Save output files in default directory?',
'Default Directory?',...
            'Yes','No.','No.');
        if defoutdir == 'Yes'
            mkdir([DefaultDir,filesep,'Figures'])
            mkdir([DefaultDir,filesep,'Spreadsheet Outputs'])
            mkdir([DefaultDir,filesep,'Notepad Outputs'])
        else
            waitfor(msgbox('Please select the directory to save the
outputs'))
            Outputdir = uigetdir('C:\');
            mkdir([Outputdir,filesep,'Figures'])
            mkdir([Outputdir,filesep,'Spreadsheet Outputs'])
            mkdir([Outputdir,filesep,'Notepad Outputs'])
        end
    end

    %preallocating variabls for runtime optimization
    Eccentricity = zeros((num_images-1),1);
    SAPDetection = zeros((num_images-1),1);
    xbars = zeros((num_images-1),1);
    ybars = zeros((num_images-1),1);
    Distance = zeros((num_images-1),1);
    Speed = zeros((num_images-1),1);
    mat=zeros(40,num_images-1);


    for k = 1:1:(num_images-1)%For loop from first frame to the second
last frame of video (The last frame is of the background)

        cd(DefaultDir);
        A = imread(files(i).name, k, 'Info', info); %Creates an array
based on for image k's pixel values
        if invert == 'Yes'
            J=255-J;
            A=255-A;
        end
        C = A-J; %Subtracts background
```

```
        bw4=im2bw(C,threshold); %Makes black and white with user's
threshold input
        %bw4 = imfill(bw4, 'holes'); %If needed, this can fill in the
body of rodent, but it will increase the runtime of software.

        %%
        %Detecting ellipses (Note: it will find multiple for each
image)
        se = strel('disk',2);
        bw4=imerode(bw4,se); %Erodes the perimeter of the rodent to
remove the tail
        bw4=imdilate(bw4,se); %Dilates the perimeter of the rodent to
bring back to normal size

        s = regionprops(bw4,'Orientation', 'MajorAxisLength', ...
            'MinorAxisLength', 'Eccentricity', 'Centroid', 'Area');

        %%
        if VT == 'Yes'
            if AF == 'Yes'
                figure(1)
                imshow(bw4) %display background for plot
                hold on
            else
                if ~mod(k,fps)
                    figure(1)
                    imshow(bw4) %display background for plot
                    hold on
                end
            end
        end
        phi = linspace(0,2*pi,50);
        cosphi = cos(phi);
        sinphi = sin(phi);

        ellipsenum = length(s); %Number of ellipses detected in frame

        if ellipsenum < 2,
            input = 1;
        elseif ellipsenum > 1,
            for l = 1:1:length(s);
                [sizesofellipses(l)] =
s(l).MajorAxisLength*s(l).MinorAxisLength; %Finds the rectangular area
of ellipse
            end
            [biggest, input] = max(sizesofellipses);%Determine input
that creates largest ellipse based on rectangular area
            clear 'sizesofellipses'; %Clears array of rectangular areas
b/c next iteriation may have less ellipes, so this ensures it doesn't
pick a value from a previous iteriation.
        end

        %%
        %Display largest ellipse values
        s(input);
```

```
        Area=[s(input).Area];
        maxarea=max(Area);
        idxBig= find(maxarea == Area);
        it2=ismember(bw4,idxBig);
        [r c]=find(it2);
        maxc=max(c)+5;
        minc=min(c)-5;
        maxr=max(r)+5;
        minr=min(r)-5;
        % This is the updated bbox for trackbg to know where to look
        bbox=[minc minr maxc-minc maxr-minr];
        boxsize=50;

        % ------------------------------------------------------------
---------
        % Writing down the first bounding box used to speed up
trackgo.m
        nextbboxref(1,:)=[bbox(1)-boxsize  bbox(2)-boxsize
bbox(3)+2*boxsize  bbox(4)+2*boxsize];

        bboxref(i,:)=bbox;
        % Now box in the arena reference system (with respect to the
entire frame)
        absolutebbox = [bboxref(i,1)+nextbboxref(i,1)
bboxref(i,2)+nextbboxref(i,2) bboxref(i,3) bboxref(i,4)];
        % A reference to crop the next picture:
        nextbbox=[absolutebbox(1)-boxsize absolutebbox(2)-boxsize
absolutebbox(3)+2*boxsize absolutebbox(4)+2*boxsize];
        if nextbbox(1)<0
            nextbbox(1)=0;
        end
        if nextbbox(2)<0
            nextbbox(2)=0;
        end
        %
        % Finally, just use the current final good finalbbox
        nextbboxref((i+1),:)=absolutebbox;
        %nextbboxref((i+1),:)=nextbbox;


        % ---
        % Relevant data to save
        %
        % (A) The position of the bounding box in the absolute
reference frame
        finalbbox(i,:)=absolutebbox;
        %
        % (B) Output images:
        % The raw cropped image
        %smallImage0=imcrop(myframe,absolutebbox);
        % The processed and cropped image
        smallimage=imcrop(it2,bbox);
        %imshow(smallimage)
        perm=bwperim(smallimage);
```

```
        if VT == 'Yes'
            if AF == 'Yes'
                figure(2);imshow(perm) %display background for plot
                %hold on
            else
                if ~mod(k,fps)
                    figure(2);imshow(perm) %display background for plot
                    %hold on
                end
            end
        end

        %f = getframe(gcf);
        %writeVideo(writerObjbbox,f);

        %f = getframe(gcf);
        %imwrite(f.cdata, fullfile(savelocation,
strcat('BBox_',files(i).name)) ,'tif', 'Compression', 'none',
'WriteMode', 'append')

        %get first connected component
        [start_r,start_c] = find(perm,1,'first');
        [max_r,max_c] = size(perm);

        %as bwtraceboundary needs an intial direction, choose the
        %first one that works
        if start_c < max_c
            if start_c <= 1
                if start_r < max_r
                    if perm(start_r+1,start_c+1) == 1
                        direction = 'SE';
                    elseif perm(start_r,start_c+1) == 1
                        direction = 'E';
                    elseif perm(start_r-1,start_c+1) == 1
                        direction = 'NE';
                    elseif perm(start_r-1,start_c) == 1
                        direction = 'N';
                    elseif perm(start_r+1,start_c) == 1
                        direction = 'S';
                    else
                        assert(0);
                    end
                else
                    if perm(start_r,start_c+1) == 1
                        direction = 'E';
                    elseif perm(start_r-1,start_c+1) == 1
                        direction = 'NE';
                    elseif perm(start_r-1,start_c) == 1
                        direction = 'N';
                    else
                        assert(0);
                    end
                end
            else
                if start_r < max_r
```

```
            if perm(start_r+1,start_c+1) == 1
                direction = 'SE';
            elseif perm(start_r,start_c+1) == 1
                direction = 'E';
            elseif perm(start_r-1,start_c+1) == 1
                direction = 'NE';
            elseif perm(start_r-1,start_c) == 1
                direction = 'N';
            elseif perm(start_r-1,start_c-1) == 1
                direction = 'NW';
            elseif perm(start_r,start_c-1) == 1
                direction = 'W';
            elseif perm(start_r+1,start_c-1) == 1
                direction = 'SW';
            elseif perm(start_r+1,start_c) == 1
                direction = 'S';
            else
                assert(0);
            end
        else
            if perm(start_r,start_c+1) == 1
                direction = 'E';
            elseif perm(start_r-1,start_c+1) == 1
                direction = 'NE';
            elseif perm(start_r-1,start_c) == 1
                direction = 'N';
            elseif perm(start_r-1,start_c-1) == 1
                direction = 'NW';
            elseif perm(start_r,start_c-1) == 1
                direction = 'W';
            else
                assert(0);
            end
        end
    end
else
    if start_r < max_r
        if perm(start_r+1,start_c-1) == 1
            direction = 'SW';
        elseif perm(start_r+1,start_c) == 1
            direction = 'S';
        elseif perm(start_r-1,start_c) == 1
            direction = 'N';
        elseif perm(start_r-1,start_c-1) == 1
            direction = 'NW';
        elseif perm(start_r,start_c-1) == 1
            direction = 'W';
        else
            assert(0);
        end
    else
        if perm(start_r,start_c+1) == 1
            direction = 'E';
        elseif perm(start_r-1,start_c) == 1
            direction = 'N';
        elseif perm(start_r-1,start_c-1) == 1
            direction = 'NW';
```

```
                elseif perm(start_r,start_c-1) == 1
                    direction = 'W';
                else
                    assert(0);
                end
            end
        end
        chain = bwtraceboundary(perm,[start_r,start_c],direction);

        cd(curr_folder)
        [cc]=chaincode(chain);
        c = cc.code;
        if VT == 'Yes'
            if AF == 'Yes'
                figure(3);plot_chain_code(c');
                axis([-50 50 -50 50]);
                hold on
                figure(3);plot_fourier_approx(c', 10, 100, 0, 'r');
                %axis([-50 50 -50 50]);
                hold off
            else
                if ~mod(k,fps)
                    figure(3);plot_chain_code(c');
                    axis([-50 50 -50 50]);
                    hold on
                    figure(3);plot_fourier_approx(c', 10, 100, 0, 'r');
                    %axis([-50 50 -50 50]);
                    hold off
                end
            end
        end

        FSD=fEfourier(chain,10,1,1);
        Hmm =
[FSD(1);FSD(2);FSD(3);FSD(4);FSD(5);FSD(6);FSD(7);FSD(8);FSD(9);FSD(10)
;FSD(11);FSD(12);FSD(13);FSD(14);FSD(15);FSD(16);FSD(17);FSD(18);FSD(19
);FSD(20);FSD(21);FSD(22);FSD(23);FSD(24);FSD(25);FSD(26);FSD(27);FSD(2
8);FSD(29);FSD(30);FSD(31);FSD(32);FSD(33);FSD(34);FSD(35);FSD(36);FSD(
37);FSD(38);FSD(39);FSD(40)];
        mat(1+40*(k-1):40*k) = Hmm;

        %f = getframe(gcf);
        %writeVideo(writerObj,f);

        %imwrite(f.cdata, fullfile(savelocation,
strcat('FourierDescript_',files(i).name)) ,'tif', 'Compression',
'none', 'WriteMode', 'append')
        % Efficient array format to save the images
        %imraw{i}=smallImage0;
        impro{i}=smallimage;

        if k > 1
            xbars(k) = s(input).Centroid(1);
            ybars(k) = s(input).Centroid(2);
            Distance(k)= sqrt((xbars(k-1)-xbars(k))^2+(ybars(k-1)-
ybars(k))^2);
```

```
            Speed(k)=Distance(k)*(xdistance/xpixelength)*(fps);
        end
        frame = k    %Displays current frame under analysis in MATLAB
command window
    end

    %%
    %SAP Analysis

    %hierarchical clustering with dendrogram
    %test = pdist(mat');
    %tree = linkage(test,'centroid');
    %leafOrder = optimalleaforder(tree,test);
    %[H,T,outperm] = dendrogram(tree,'reorder',leafOrder)

    %neural network
    vec=myNeuralNetworkFunction(mat');
    %vec=nnf36(mat');
    cluster_index = vec2ind(vec');
    ci=vec2ind(vec');

    for j = 1:1:3000
        if ci(j)==2|| ci(j)==7 || ci(j)==9 || ci(j)==10 ...
                || ci(j)==11 || ci(j)==13 || ci(j)==15 || ci(j)==16 ...
                || ci(j)==18 || ci(j)==19 || ci(j)==20 || ci(j)==26 ...
                || ci(j)==28 || ci(j)==30 || ci(j)==32 || ci(j)==33 ...
                || ci(j)==35

            SAPDetection(j)=1;
        else
            SAPDetection(j)=0;
        end
        j
    end

    %Counter is utilized to remove SAP detection that lasted only up to
a half of a second and measure the SAP frequency.
    count = 0;  %Counter to keep track of how many 1's are in a row.
    Threshold = 0.5*fps;  %How many 1's are allowed in a row
    freqSAP = 0; %frequency of SAP detected

    for k = 1:(num_images-1)   %Goes through 1-D array
        if SAPDetection(k,1) == 1
            count = count + 1;  %Keeps track of how many 1's are in a
row
        else
            if count >= Threshold   %If True, restart counter
                count = 0;
                freqSAP = freqSAP + 1;
            else                    %Else, turn all 1's to zeroes
                while count >0
                    SAPDetection(k-count,1) = 0;
                    count = count - 1;
                end
            end
```

```
        end
    end

    if SF == 'Yes'
        close(writerObj);
    end

    for u = 1:((num_images-1)/fps)
        time_based(u)=SAPDetection(1+10*(u-1))+SAPDetection(2+10*(u-
1))+SAPDetection(3+10*(u-1))+SAPDetection(4+10*(u-
1))+SAPDetection(5+10*(u-1))+SAPDetection(6+10*(u-
1))+SAPDetection(7+10*(u-1))+SAPDetection(8+10*(u-
1))+SAPDetection(9+10*(u-1))+SAPDetection(10+10*(u-1));
    end


    %Vectors to save into files
    A = {'Frame','Class','Speed (cm/s)','SAP Detected','SAP Timebased',
'SAP time (s)', 'Total time (s)','SAP percentage'};
    B = 1:1:(num_images-1);
    C = ci;
    D = Speed;
    E = SAPDetection;
    F = sum(E)/fps;
    G = (num_images-1)/fps;
    H = (F/G)*100;
    P = time_based;

    %Creates Excel spreadsheet with Eccentricity, Speed, and SAP
deteciotn Data
    if defoutdir == 'Yes'
        sheet = 1;
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','') '.xls'],A,sheet,'A1')
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],B(:),sheet,'A2')
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],C(:),sheet,'B2')
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],D(:),sheet,'C2')
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],E(:),sheet,'D2')
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],F(:),sheet,'F2')
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],G(:),sheet,'G2')
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],H(:),sheet,'H2')
```

```
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],P(:),sheet,'E2')
    else
        sheet = 1;
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','') '.xls'],A,sheet,'A1')
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],B(:),sheet,'A2')
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],C(:),sheet,'B2')
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],D(:),sheet,'C2')
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],E(:),sheet,'D2')
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],F(:),sheet,'F2')
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],G(:),sheet,'G2')
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],H(:),sheet,'H2')
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,strrep(files(i).name, '.tif','')
'.xls'],P(:),sheet,'E2')% end
    end

    %Creates Notpad files to archive data
    if defoutdir == 'Yes'

        header1 = 'Frame';
        header2 = 'Class';
        header3 = 'Speed (cm/s)';
        header4 = 'SAP Detection';
        fid=fopen([DefaultDir,filesep,'Notepad
Outputs',filesep,strrep(files(i).name, '.tif','')
'_Eccentricity.txt'],'w');
        fprintf(fid, [ header1 ' ' header2 '\n']);
        fprintf(fid, '%f %f \n', [B(:) C(:)]');
        fclose(fid);
        fid=fopen([DefaultDir,filesep,'Notepad
Outputs',filesep,strrep(files(i).name, '.tif','') '_Speed.txt'],'w');
        fprintf(fid, [ header1 ' ' header3 '\n']);
        fprintf(fid, '%f %f \n', [B(:) D(:)]');
        fclose(fid);
        fid=fopen([DefaultDir,filesep,'Notepad
Outputs',filesep,strrep(files(i).name, '.tif','')
'_SAPDetection.txt'],'w');
        fprintf(fid, [ header1 ' ' header3 '\n']);
        fprintf(fid, '%f %f \n', [B(:) E(:)]');
```

```
        fclose(fid);

        header5 = 'SAP time (s)';
        header6 = 'Total time (s)';
        header7 = 'SAP percentage';
        header8 = 'SAP Frequency';
        fid=fopen([DefaultDir,filesep,'Notepad
Outputs',filesep,strrep(files(i).name, '.tif','') '_Results.txt'],'w');
        fprintf(fid, [ header5 ' ' header6 ' ' header7 ' ' header8
'\n']);
        fprintf(fid, '%f %f %f %f \n', [F(:) G(:) H(:) freqSAP(:)]');
        fclose(fid);
    else

        header1 = 'Frame';
        header2 = 'Class';
        header3 = 'Speed (cm/s)';
        header4 = 'SAP Detection';
        fid=fopen([Outputdir,filesep,'Notepad
Outputs',filesep,strrep(files(i).name, '.tif','')
'_Eccentricity.txt'],'w');
        fprintf(fid, [ header1 ' ' header2 '\n']);
        fprintf(fid, '%f %f \n', [B(:) C(:)]');
        fclose(fid);
        fid=fopen([Outputdir,filesep,'Notepad
Outputs',filesep,strrep(files(i).name, '.tif','') '_Speed.txt'],'w');
        fprintf(fid, [ header1 ' ' header3 '\n']);
        fprintf(fid, '%f %f \n', [B(:) D(:)]');
        fclose(fid);
        fid=fopen([Outputdir,filesep,'Notepad
Outputs',filesep,strrep(files(i).name, '.tif','')
'_SAPDetection.txt'],'w');
        fprintf(fid, [ header1 ' ' header3 '\n']);
        fprintf(fid, '%f %f \n', [B(:) E(:)]');
        fclose(fid);

        header5 = 'SAP time (s)';
        header6 = 'Total time (s)';
        header7 = 'SAP percentage';
        header8 = 'SAP Frequency';
        fid=fopen([Outputdir,filesep,'Notepad
Outputs',filesep,strrep(files(i).name, '.tif','') '_Results.txt'],'w');
        fprintf(fid, [ header5 ' ' header6 ' ' header7 ' ' header8
'\n']);
        fprintf(fid, '%f %f %f %f \n', [F(:) G(:) H(:) freqSAP(:)]');
        fclose(fid);
    end

    if nostop == 'No.'
        uiwait(gcf);
    end

    L=strrep(files(i).name, '.tif','');
    if defoutdir == 'Yes'
        xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],{L},sheet,['A',num2str(i+1)])
```

```
    else
        xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],{L},sheet,['A',num2str(i+1)])
    end
    M(i)=F;
    N(i)=G;
    O(i)=H;
    Q(i)=freqSAP;
end

%Saves all results in one Excel sheet

J={'File Name','SAP time (s)', 'Total time (s)','SAP percentage','SAP
Frequency'};

if defoutdir == 'Yes'
    sheet = 1;
    xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],J,sheet,'A1')
    xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],M(:),sheet,'B2')
    xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],N(:),sheet,'C2')
    xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],O(:),sheet,'D2')
    xlswrite([DefaultDir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],Q(:),sheet,'E2')
else
    sheet = 1;
    xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],J,sheet,'A1')
    xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],M(:),sheet,'B2')
    xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],N(:),sheet,'C2')
    xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],O(:),sheet,'D2')
    xlswrite([Outputdir,filesep,'Spreadsheet
Outputs',filesep,'Results.xls'],Q(:),sheet,'E2')
end
display('Analysis Complete!')
cd(curr_folder)
clear global
```

APPENDIX F

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL LETTERS

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
Louisiana Tech University

29 July 2013

Teresa Murray, Ph.D.
Kevin Holly B.S.
Biomedical Engineering
Louisiana Tech University
Campus Box # 58

Dear Dr. Murray:

The Louisiana Tech University's Institutional Animal Care and Use Committee (IACUC) examined amendment 2012-1A2 to your protocol 2012-1 and via the designated review process approved the amendment to the protocol entitled:

In Vivo Imaging and Neural Electrophysiological Recording in Rodent Brain for Biomedical Research

The amendment was to study the effects of propranol HCL and diphenoxylated HCL with atropine sulfate on the anxiety levels of mice. The protocol calls for the animals to be tested for anxiety using a behavioral maze.

You have provided evidence that you and your student are trained for the procedures associated with the protocol and adequate rationale to support the study.

I estimate that your charges will be about $500 per year for this protocol. This includes the cost housing them for the estimated duration of the work. We will determine the charges on a semiannual basis and provide you with a bill.

Please remember that you are required to keep adequate and accurate records of all procedures, results, and the number of animals used in this protocol for three years after termination of the project. These records must be available for review by the IACUC or state and federal animal use agencies. Each year in October you will be required to complete a summary of animals used for the United States Agricultural Agency (USDA). Note that failure to follow this protocol as approved may result in the termination of research.

If you have any questions concerning the animal part of your research please contact me via e-mail at jgspauld@latech.edu.

Sincerely,

James G. Spaulding, Chair
Louisiana Tech University IACUC

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
Louisiana Tech University

January 29, 2016

Teresa Murray, Ph.D.
Biomedical Engineering
Louisiana Tech University
Campus Box #58

Dear Dr. Murray:

The Louisiana Tech University's Institutional Animal Care and Use Committee (IACUC) examined your protocol and via the committee review process approved your protocol entitled:

In Vivo Imaging and Neural Electrophysiological Recording in Rodent Brain for Biomedical Research

Your protocol has been assigned the following number: **2016-01**. All changes and procedures have been noted. If changes to your research are necessary, please know you will need prior approval from the IACUC. This protocol will expire **January 29, 2019.**

Please remember that you are required to keep adequate and accurate records of all procedures, results, and the number of animals used in this protocol for three years after termination of the project. These records must be available for review by the IACUC or state and federal animal use agencies. Each year in October you will be required to complete a summary of animals used for the United States Agricultural Agency (USDA). Note, that failure to follow this protocol as approved may result in the termination of research.

If you have any questions concerning the animal part of your research please contact me via e-mail at eborn@latech.edu.

Sincerely,

Emily Born, IACUC Chair
Louisiana Tech University

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