ANTAGONISM OF PRO-2-PAM USING CENTRAL AND PERIPHERAL ANTICHOLINERGIC DRUGS

By

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THESIS APPROVAL PAGE

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ABSTRACT

The goal of this study was two-fold. Experiment 1 characterized the intoxicating tremor produced by two known tremor-inducing drugs, oxotremorine and physostigmine in order to characterize the tremoregenic effects of a centrally acting oxime, pro-2-PAM. Pro-2-PAM crosses the blood brain barrier and may have therapeutic value against OP intoxication. Experiment 2 attempted to antagonize the tremor produced by 28.0 mg/kg of pro-2-PAM with a Med Associates System using central and peripheral anticholinergics. The centrally acting anticholinergics were atropine sulfate and scopolamine HBr; the peripherally acting anticholinergics were AMN and SMN. In Experiment 1, the only drugs that showed a tremoregenic effect against their controls were oxotremorine and physostigmine. A high dose of physostigmine (0.4 mg/kg) induced more tremor than 0.1 mg/kg of oxotremorine; only 0.1 mg/kg of oxotremorine induced more tremor than 28.0 mg/kg of pro-2-PAM. In Experiment 2, only the centrally acting anticholinergics reduced the tremoregenic effect of pro-2-PAM.

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CHAPTER 1

INTRODUCTION

Organophosphorus (OP) chemical warfare nerve agents, such as sarin (GB), have been used by terrorists around the world as weapons of mass destruction. In 1995, passengers in the Tokyo subway system were exposed to GB in a terrorist attack causing tonic-clonic convulsions as early as seven minutes post exposure (DeMar et al., 2010). OPs inhibit the enzyme cholinesterase, causing an accumulation of the neurotransmitter acetylcholine (ACh) in the synaptic cleft. Increased amounts of ACh cause problems in the central and peripheral nervous systems. In the central nervous system (CNS), accumulated ACh causes epileptic seizures and neuronal damage. In the peripheral nervous system (PNS), muscle fasciculation and tremor occur along with salivation, lacrimation, and possible paralysis (Gordon et al., 2008).

Treatment during the Tokyo terrorist attack consisted of the current drugs deemed acceptable in the United States for OP poisoning: a combination of atropine sulfate, an antimuscarinic that antagonizes the toxic effects of ACh, pralidoxime chloride (2-PAM), an oxime that reactivates the AChE inhibited by the OP agent, and diazepam, a benzodiazepine anticonvulsant that is used to relieve seizure activity by increasing inhibitory control of nerves and muscles (DeMar et al. 2010; Gordon et al. 2008).

The problem with this combined therapy is the fact that 2-PAM does not enter the brain directly because it does not cross the blood brain barrier (BBB), a mechanism between brain tissues that protects the CNS (Campbell, Reece, & Mitchell, 1999). The BBB allows lipophilic OP agents to enter the brain and block AChE causing the

accumulation of ACh. It also allows atropine sulfate to pass through it, but it blocks 2-PAM from entering the CNS (Kenley, Howd, & Uyeno, 1982). The efficacy of this drug combination increases survival in OP victims, but does not stop seizures from occurring. This status epilepticus (SE), or continuous state of epilepsy, is the result of OP exposure. If OP intoxication is not fatal, one may suffer from decreased cholinesterase levels and long-term personality changes that could be related to anxiety (DeMar et al. 2010).

Because no direct countermeasure to OP poisoning in the CNS exists, there is a need to study new therapeutics (oximes) that will increase survivability and prevent negative long term effects in the CNS (DeMar et al. 2010). Tertiary oximes are of particular interest because they cross the BBB unlike quaternary oximes, such as 2-PAM, which do not enter the CNS (Shih, Skovira, O'Donnell, & McDonough, 2010). 1-Methyl-1,6-dihydropyridine-2-carbaldoxime (pro-2-PAM), the pro-drug formulation of 2-PAM, is lipophilic in nature and capable of entering the CNS. Current research has revealed that once this oxime enters the brain, it oxidizes into its parent compound, 2-PAM, which is able to reactivate the inhibited AChE in the CNS once blocked by the OP. Pro-2-PAM will reactivate more brain AChE than 2-PAM therapy alone, thus further restoring cholinergic function (DeMar et al. 2010).

Although pro-2-PAM may increase survivability and prevent long term brain damage, it is inherently toxic in nature. This toxicity is visible through the tremor that results in guinea pigs after pro-2-PAM administration (Clement, 1979). Past and current research has shown that pro-2-PAM alone cannot be given at very high doses because it will cause small rodents to involuntarily shake; this resulting tremor can lead to death (Clement, 1979; De Mar et al. 2010; Gordon et al. 2008). However, combining pro-2-

PAM with the current treatments deemed acceptable for nerve agent poisoning may provide the FDA with faster working therapeutics for treating OP intoxication.

In order to better understand the therapeutic value of pro-2-PAM, it is necessary to quantify its tremorogenic nature. Therefore, the purpose of this literature review is to first define what tremor is and provide examples of it, followed by an introduction to two known tremor-inducing drugs: oxotremorine and physostigmine. These drugs will provide positive control data and characterization of tremor in part one of the thesis study. Because the part two of the thesis study concentrates on antagonizing the effects of pro-2-PAM, studies utilizing this drug will be addressed, followed by an introduction to both centrally and peripherally acting drugs that will hopefully antagonize the toxic effects of pro-2-PAM. The centrally acting drugs include atropine sulfate and scopolamine hydrobromide; the peripherally acting drugs include atropine methyl nitrate and scopolamine methyl nitrate. Two additional drugs will then be presented that were included in the thesis study, MINA and midazolam. The literature review will conclude by introducing Fast Fourier Transform, which will be used to quantify the tremor data in the proposed thesis experiment. The purpose of the thesis project is two-fold: it will first quantify the tremorogenic effects of pro-2-PAM and then attempt to antagonize the toxicity of this oxime.

CHAPTER 2

LITERATURE REVIEW

Tremor defined

Elble (2009) defined tremor as an approximately rhythmic, involuntary movement that is characterized by a succession of sinusoidal curves showing muscle contraction.

According to Smaga (2003), there are two types of tremor, resting tremor and action tremor. Resting tremor occurs at 4 to 6 Hz when a body part is supported against gravity. This type of tremor occurs when a person has his or her hand resting in the lap. Action tremor is produced by voluntary muscle contraction, such as isometric tremor, which occurs at greater than 5 Hz when a person squeezes an object. Although the mechanism of tremorogenesis is not fully understood, tremor can occur after drug intoxication and is classified as toxic tremor. Toxic tremor may be due to central nervous system dysfunction after drug ingestion (Fowler et al. 2001; Gerhart, Hong, & Tilson, 1983).

According to Elble (2009), different types of tremor may relate to drug-induced tremor experienced by small rodents in animal research. The first type of tremor is essential tremor, which is the most common form of pathologic tremor that affects the head, jaw, hands, and lower limbs. In humans, this type of tremor can begin at any age, but tends to increase with age. Investigators believe that its pathophysiology involves synaptic or neuronal membrane dysfunction, leading to tremorogenic oscillations in the basal ganglia occurring between 4 to 8 hertz (Hz). There is no direct treatment for essential tremor due to a limited understanding of how it works, but deep brain stimulation (DBS) of the thalamus seems to limit essential tremor in patients.

The second type of tremor is orthostatic tremor, which is a postural tremor affecting the cranium, neck, and limbs. Patients exhibiting this kind of tremor lack a sense of balance upon sitting or standing. Orthostatic tremor is best detected with electromyographic (EMG) electrodes, and it usually forms oscillations between 7 to 9 Hz. However, it can occur as high as 13 to 18 Hz. This kind of tremor is not a variant of essential tremor. Its pathophysiology has been studied, but a full understanding has not

been reached. Researchers hypothesize that orthostatic tremor emerges from a central source of oscillation in the body because frequencies as high as 18 Hz are too high to originate in the lower extremities. This central source is thought to formulate the rhythmicity and synchrony that orthostatic tremor produces in the body. Although there is no direct cure for orthostatic tremor, dopaminergic drugs seem to reduce its tremorogenic effect.

The third type of tremor is drug-induced tremor. Neuroleptics (tranquilizers) produce parkinsonian resting tremor, which is often described as pill rolling tremor because patients suffer from tremulous hand movements that resemble the repetitive rolling of a pill. Beta-adrenergic agonists (used to treat cardiovascular disease) and tricyclic antidepressants produce postural tremor, which occurs when rhythmic bursts of contractions occur in opposing muscle groups. Finally, lithium produces kinetic/action tremor, an involuntary trembling that increases during voluntary movement of the muscles. These drug-induced action tremors respond to propanolol, a beta blocker, whereas neuroleptic-induced tremor is treated with an anticholinergic medication (Elble, 2009).

Because pro-2-PAM is a centrally acting drug, its tremoregenic effect can be defined as drug-induced tremor. However, it is possible that pro-2-PAM may produce characteristics of the different types of tremor previously described. The upcoming thesis study will describe the tremoregenic effect of pro-2-PAM in more detail.

Drugs that induce tremor: Oxotremorine and Physostigmine

In order to assess the tremor induced by Pro-2-PAM, it is necessary to address drugs that produce a tremorogenic effect. These drugs will provide positive control data

and characterization of tremor in the proposed thesis study. Two such tremor-inducing drugs include oxotremorine and physostigmine. Whereas oxotremorine is a direct muscarinic acetylcholine receptor agonist that binds to the cholinergic receptor and stimulates it, physostigmine is an indirect agonist. Because it is an indirect agonist, AChE, not physostigmine, binds to the cholinesterase enzyme, inhibiting it and causing an accumulation of ACh. The buildup of ACh stimulates the muscarinic receptors (Bear, Connors & Paradiso, 2001; Philippens, Olivier, & Melchers, 1997; Witkin et al. 1978).

Early studies on oxotremorine by Cox & Potkonjak (1969), reported that oxotremorine's tremorogenic effect may be due to its ability to release acetylcholine (ACh) in the CNS; they hypothesized that tremor may be mediated by ACh. In order to test their theory, these investigators pretreated male Wistar rats with atropine sulfate at different doses (2.5, 5.0, 10.0, and 40.0 mg/kg) followed 30 minutes later by exposure to oxotremorine at a high dose of 2.0 mg/kg. They wanted to assess whether atropine sulfate could antagonize the tremorogenic effects of oxotremorine. All drugs were given as intraperitoneal (IP) injections. The results showed that the highest dose of oxotremorine (2.0 mg/kg) produced a significant increase in the ACh concentration of the brain, even when the rats were pretreated with 5.0 and 10.0 mg/kg of atropine sulfate. Thus, the low doses of atropine sulfate had no antagonizing effect against oxotremorine; the results from this group matched the ACh levels of rats treated with oxotremorine alone. The concentration of ACh after 2.5 mg/kg atropine sulfate and 2.0 mg/kg oxotremorine was significantly higher than the previous groups (5.0 and 10.0 mg/kg atropine sulfate and 2.0 mg/kg oxotremorine). However, a decrease in ACh concentration was seen in the 40.0 mg/kg atropine sulfate group treated with 2.0 mg/kg

oxotremorine. In all of these groups, tremor reached its maximum within 5 minutes of the oxotremorine injection. The results showed that atropine sulfate at 40.0mg/kg prevented the oxotremorine-induced rise in ACh. This study did not show a causal relationship between the increase in ACh and tremor, but the results do not eliminate the possibility that tremor is mediated by ACh.

A similar study by Witkin et al. (1986) was performed with male Sprague-Dawley rats pre-treated with atropine sulfate (40.0 mg/kg) 40 minutes prior to an IP injection of oxotremorine (5.0 to 30.0 mg/kg). Unlike the previous study, higher doses of oxotremorine were utilized. In comparison to the 5.0 mg/kg dose of oxotremorine, 10.0 mg/kg produced a quicker onset of lacrimation, salivation, and tremor. Also, convulsions from the 10.0 mg/kg dose produced more lethality than the 5.0 mg/kg dose. These high doses of oxotremorine produced clonic convulsions, which are characterized by alternating muscle contraction and relaxation. These clonic convulsions caused an involuntary jerking of the upper torso that resembled seizure activity. The high dose of atropine sulfate (40.0 mg/kg) prevented lacrimation and salivation at the lower dose of oxotremorine (5.0 mg/kg), but could not stop these peripheral responses to oxotremorine in doses greater than 5.0 mg/kg (Witkin et al., 1986).

Another study by Weinstock, Zavadil, Rosin, Chiueh, & Kopin (1978) assessed the action of centrally acting and peripherally acting drugs against tremor induced by oxotremorine in male Sprague-Dawley rats. All drugs were injected subcutaneously (SC). Saline or atropine methyl nitrate (0.5 mg/kg) were given as a pretreatment 30 minutes prior to oxotremorine (0.01, 0.25, 0.5, and 1.5 mg/kg). Although it was not specified when scopolamine HBr (0.5 mg/kg SC) was given, it was used to treat the rats.

Three and five minutes after oxotremorine, tremor intensity was assessed using a rating scale: no tremor (0), occasional muscle twitches or slight tremor (1), moderate intermittent tremor (2), gross tremor with occasional periods of rest (3), gross intense continuous tremor of limbs and body (4). The other assessment made was the amount of salivation produced by oxotremorine. A tissue was held to the mouth and the following scale was used to assess amount of salivation: no wetness (0), up to 0.5 cm diameter (1), 0.5 to 1.0 cm diameter (2), 1.0 to 1.5 cm diameter (3), and greater than 1.5 cm diameter (4). The results showed that oxotremorine (0.25 mg/kg) produced the greatest amount of tremor over the 30 minute period of observations. Atropine methyl nitrate, which does not cross the BBB, prevented salivation and chromodacryorrhea (red tears). Interestingly, animals pretreated with atropine methyl nitrate (0.5 mg/kg) produced less tremor at the higher dose of oxotremorine (1.5 mg/kg) compared to the lower dose (0.25 mg/kg). Scopolamine HBr completely abolished tremor, which is due to its ability to cross the BBB. Thus, the tremor resulting from oxotremorine is due to stimulating the CNS. This is supported by the fact that atropine methyl nitrate could not stop the tremor induced, whereas scopolamine HBr completely abolished it.

The second drug of interest, physostigmine, is also known to produce whole-body tremors in rodents, or hypothermia (Murugaiah & Ukponmwan, 2003; Wang & Fowler, 2001). Physostigmine produces this tremor in rats by increasing cholinergic activity in the brain (Gothoni, Lehtinen, & Fincke, 1983). Stanford & Fowler (1997) hypothesized that if tremor could be detected at lower doses than 0.2 mg/kg, and the tremorogenic effects of physostigmine could be antagonized by an anticholinergic, such as scopolamine HBr, a cholinergic mechanism could be confirmed. Therefore, they conducted a study

using male Sprague-Dawley rats that were trained to press and hold a horizontal disk attached to a force-sensing transducer using water reinforcement. When the rat extended its forelimb to place pressure on a lever, it was also allowed to drink from a dipper, thus stimulating the rat to continually press the lever. The pressure produced allowed tremor to be quantified at certain frequencies. The rat's tongue could reach the dipper as long as the lever was kept above 6.7 grams (g). If the force fell below 6.7 g, the dipper dropped into the water reservoir and could not be reached.

Rats were given SC injections of physostigmine (0.05 and 0.1 mg/kg) or scopolamine HBr (0.1 and 0.2 mg/kg), or both drugs simultaneously. Each drug was diluted in 1.0 ml/kg of saline. Each drug day was preceded by a vehicle injection day (saline), and followed by a day of no injections. Thus, the animals received one of the two doses and were allowed to recover from the drugs administered before being dosed again with the second dose not previously given. Forelimb tremor was quantified by Fourier analysis, which formulates a periodic function of sine and cosine waves that can quantitatively measure drug-induced tremor. Tremor was defined within a frequency of 10 to 25 Hz because previous studies revealed that frequencies below 10 Hz contained power attributable to the licking behavior of the rat, not the tremorogenic effect of the drug. Tremor was characterized through behavioral measures. The first measure was overall task engagement, or time on task, defined as the time it took the rat to apply 1 g of force. The raw data were formulated by a computer program into individual waveforms, which were averaged to calculate the maximum peak force attained by the rat during the first second of pressure, as well as the mean force sustained during the hold force. The hold force was defined as the length of time 20 g of pressure could be applied by the rat

within 3.36 s. Thus, the time domain totaled 4.36 s after 1 g (peak force) and 20 g (hold force) of pressure were quantified.

Results of this study showed that when administered alone, physostigmine increased the peak force, while scopolamine HBr decreased it. At low doses, physostigmine increased power in the 10 to 25 Hz band of the forelimb force power spectrum. This indicated a broad-band elevation in the same frequency band reported for the whole-body tremor produced by the higher dose of physostigmine. Thus, physostigmine increased tremor, whereas scopolamine HBr antagonized the tremorogenic effect of the physostigmine, decreasing tremor. Although more tremor was seen at a higher dose of physostigmine, it may not have been a reflection of increased muscle tone because the hold force was not elevated significantly by physostigmine. Thus, physostigmine produced tremor without necessarily producing an increased force on the transducer. Finally, the tremorogenic effects of physostigmine were antagonized by scopolamine HBr, an anticholinergic. Therefore, AChE inhibition is related to the said tremor and physostigmine is an AChE inhibitor.

As a follow-up, Wang & Fowler (2001) used another tremor-detection instrument, the force plate actometer, to quantitatively describe the frequency characteristics of tremor induced by physostigmine. The previous study presented waveforms that were averaged to provide the peak force and hold force, which were used to quantitatively measure tremor. In this study, the force plate actometer also utilized Fourier analysis to quantify whole-body tremor, but unlike the last study, male Sprague-Dawley rats were exposed to higher doses of physostigmine.

Four groups of rats received daily injections of vehicle (saline), and physostigmine (0, 0.1, 0.25, and 0.5 mg/kg) for four consecutive days. Immediately after injection (route not specified), the rats were placed in the recording chamber for 30 minutes. A Latin square design was used so that each run contained one rat from each of the dosing groups. Data from each session were broken into 45 frames of 40.96 s (2048 samples at a rate of 50 Hz). Fast Fourier transformation resulted in 45 power spectra to be $\log_{10}$ transformed and averaged. The average power in each frequency band (0 to 5 Hz and 5 to 15 Hz) was calculated; the difference between the average power in the 0 to 5 Hz and 5 to 15 Hz bands was labeled tremor index 1, and it specified the peak of the spectrum in the 5 to 15 Hz frequency band. Similarly, the difference between the average power in the 0 to 5 Hz and 15 to 25 Hz bands was labeled tremor index 2, and it specified the peak of the spectrum in the 15 to 25 Hz frequency band. Compared to harmaline, another tremor-inducing drug used in this study, physostigmine treatment produced lower amplitude tremor that was less rhythmic and less intermittent. It also produced brief, high amplitudes that harmaline did not show. Harmaline showed peak amplitudes around 9 to 11 Hz, whereas physostigmine's power was spread over a broader bandwidth.

As previously stated, tremor index 1 showed amplitudes between 5 to 15 Hz, whereas tremor index 2 showed amplitudes between 15 and 25 Hz. Unlike harmaline, physostigmine significantly increased tremor index 1 dose-dependently over the four-day treatments. The animals developed a tolerance for the highest dose of physostigmine, which was seen in a flattening of the dose effect over the four-day period. The results of Wang & Fowler (2001) showed that physostigmine induced myoclonus, which is clonic

muscle spasm, where the muscle tends to rapidly alternate in contracting and relaxing. Thus, whole body tremor was achieved with higher doses of physostigmine.

Overall, oxotremorine and physostigmine are tremor-inducing drugs. The tremor produced can be quantified by Fourier analysis. Because the tremorogenic effect of these drugs is well-established, they can be used as controls to characterize the tremoregenic effects of pro-2-PAM, an oxime of interest for its therapeutic value against OP chemical nerve agents.

Pro-2-PAM: centrally active oxime to be antagonized

As previously mentioned, OP chemical warfare nerve agents inhibit AChE, causing a buildup of ACh, which causes epileptic seizures and neuronal damage. FDA approved treatment consists of a combination of atropine sulfate, which antagonizes the toxic effects of ACh, and 2-PAM, which reactivates the AChE blocked by the OP agent (DeMar et al. 2010; Gordon et al. 2008). 2-PAM is useful in overcoming the effects of nerve agent, but it cannot cross the BBB, and it has poor oral bioavailability. It is also unfavorably distributed because it is mostly found in the plasma, and it has a short half-life (Bodor, 1975). However, the pro-drug of 2-PAM, pro-2-PAM, crosses the BBB and oxidizes into its parent compound, 2-PAM, which then reactivates the inhibited AChE in the CNS once blocked by the OP (DeMar et al. 2010; Gordon et al. 2008). Thus, pro-2-PAM is an oxime of interest in chemical warfare research.

The following studies illustrate the effect of pro-2-PAM in comparison to 2-PAM. The first study was conducted by Clement (1979), whose goal was two-fold. First, a safety study was conducted to investigate the difference in effectiveness between pro-2-PAM and 2-PAM in female guinea pigs. Then, the effectiveness of these drugs was

tested against the toxic effect of OPs (DFP: diisopropyl phosphorofluoridate; sarin: GB; soman: GD). Pro-2-PAM was dissolved in citrate buffer (pH 3.0), which is more stable than an unbuffered aqueous solution, such as saline, or a solution at a higher pH. Preliminary studies showed that in guinea pigs, the intramuscular (IM) LD_{50} of pro-2-PAM was found to be between 40.0 and 80.0 mg/kg. There was 0% fatality in guinea pigs treated at 40.0 mg/kg, versus 100% fatality in guinea pigs treated at 80.0 mg/kg. Thus, pro-2-PAM proved to be toxic via IM injection in the guinea pig.

After completing the preliminary studies, guinea pigs were treated with pro-2-PAM alone, 2-PAM alone, pro-2-PAM plus atropine sulfate, or 2-PAM plus atropine sulfate as a prophylaxis against OP poisoning. Pro-2-PAM was administered as a pre-treatment IM 10 minutes prior to subcutaneous (SC) injection of an LD_{50} dose of DFP, GB, or GD. The results showed that in guinea pigs, pro-2-PAM given alone or combined with atropine sulfate was more effective than 2-PAM against DFP. The animals receiving the combined therapy received pro-2-PAM 10 minutes before the OP, and 17.4 mg/kg of atropine sulfate one minute after OP administration. This ensured that pro-2-PAM was at the site of action before the OP was administered. The combination produced a significant increase in brain AChE post agent exposure. In comparison to guinea pigs treated with 2-PAM and atropine sulfate, guinea pigs receiving pro-2-PAM and atropine sulfate showed more brain AChE activity. Interestingly, the prophylaxis was effective against GD but not GB versus 2-PAM plus atropine sulfate treatment in guinea pigs. Clement (1979) concluded that treating OP victims with pro-2-PAM and atropine sulfate was most effective.

Clement (1979) showed that pro-2-PAM can be effective in reactivating AChE, but it is a toxic substance when given by itself at high doses. Kenley, Howd, & Uyeno (1982) used a conditioned avoidance response (CAR) task to evaluate the behavioral protectiveness of pro-2-PAM. Rats were trained to avoid or escape a foot shock by climbing a 20 cm pole following the onset of a conditioned stimulus, an increase in light intensity, and a 4 kHz tone. Half of the animals learned to climb the pole before getting shocked; 48 rats were trained in this CAR task. Half of the animals were given an IP injection of saline (2.0 mL/kg), while the other half received 2.0 mg/kg of DFP, IP, in 5% ethanol. The rats were then given a 60-trial test for CAR performance. Two hours after the last trial, one third of the rats in each group were injected with saline (2.0 mL/kg, SC), one third of the rats in each group were injected with 2-PAM (50.0 mg/kg in saline, SC), and one third of the rats in each group were injected with pro-2-PAM (50.0 mg/kg in saline, SC). They were then retested in CAR performance again for 60 trials.

The results showed that all DFP rats failed in CAR performance, 2-PAM and pro-2-PAM could not reverse the toxic effect of DFP, and the high dose of pro-2-PAM caused an impairment in CAR performance. Pro-2-PAM was expected to reactivate the inhibited AChE more than 2-PAM because of its ability to cross the BBB. If this were the case, the behavioral effect of DFP may have been reversed. Because the pro-2-PAM dose was so high, its toxic effect may have caused the behavioral deficit. It is unknown whether pro-2-PAM itself or its conversion to 2-PAM in the brain caused the failed CAR response. Like the study by Clement (1979), which showed that high doses of pro-2-PAM injected IP as a pretreatment can cause toxicity, Kenley et al. (1982) also showed toxicity when pro-2-PAM was injected SC as a post treatment.

Although pro-2-PAM is intoxicating at high doses, lower doses of this drug in combination with other therapeutics, like atropine, can increase AChE activity in OP intoxicated victims. Gordon et al. (2008) performed a study with guinea pigs, the model for OP poisoning due to their detoxifying enzymes that more closely resemble the human enzyme complement as compared to mice and rats (DeMar et al. 2010). Gordon et al. (2008) surgically implanted radiotelemetry probes on the back skin of a guinea pig, which were connected to wire leads on the skull. This allowed investigators to record brain activity via electroencephalography (EEG) and heart rate via electrocardiography (ECG). The guinea pigs were pretreated with pyridostigmine bromide (0.026 mg/kg), a reversible inhibitor of AChE that does not cross the BBB. They were then treated with DFP (8.0 mg/kg, SC) followed one minute later by atropine methyl bromide (2.0 mg/kg, IM). Various times later, the animals were injected with equivalent doses of 2-PAM or pro-2-PAM (13.0 mg/kg, IM). This dose approximated the doses given to the military in a Mark 1 antidote kit. Twenty four hours after exposure, the animals were euthanized with 75.0 mg/kg pentobarbital and their forebrains removed for a cholinesterase assay. The results showed that DFP caused prolonged hypothermia and decreased activity during the 24 hours the animals were alive. Unlike 2-PAM, pro-2-PAM given one minute after exposure prevented seizure activity and reduced seizure activity when given 15 minutes after exposure. The cholinesterase assay showed that 2-PAM did not reactivate AChE in the frontal cortex, which was expected because it does not cross the BBB. Pro-2-PAM therapy between 1 and 40 minutes after exposure showed greater than two-fold average increase in frontal cortex AChE activity, inferring it reactivated the CNS AChE.

Unlike Gordon et al. (2008), a more recent pro-2-PAM study by Shih, Guarisco, Myers, Kan & McDonough, (2011) revealed that pro-2-PAM treatment at seizure onset after exposure to VX (nerve agent) is not effective. Guinea pigs were challenged with 2 x LD_{50} of VX (nerve agent) and treated with 0.5 mg/kg of atropine sulfate, IM, one minute after agent exposure. At seizure onset, the animals were treated with 32.0 mg/kg of pro-2-PAM in order to determine if delayed pro-2-PAM treatment would terminate seizure activity produced by VX. The results showed that pro-2-PAM failed to terminate VX-induced seizures if injected at seizure onset. However, in animals treated with pro-2-PAM doses between 17.7 and 35.3 mg/kg one minute after agent exposure, pro-2-PAM successfully reactivated VX-inhibited AChE in more brain tissues. Thus, pro-2-PAM may increase survival in nerve agent victims if given immediately after agent exposure versus being given at seizure onset.

Overall, pro-2-PAM is beneficial because it crosses the BBB, rapidly converts to 2-PAM, and protects the CNS from OP-induced seizures by restoring brain AChE activity. Unlike other centrally active oximes, its rapid conversion to 2-PAM also protects the PNS from intoxication. Within 24 hours, peripheral signs of nerve agent intoxication, such as salivation and lacrimation may be nonexistent with the use of pro-2-PAM, restoring the guinea pig to normal activity (Gordon et al. 2008). Pro-2-PAM may cause toxicity when given alone as a pre- or post-treatment, but when mixed with atropine, it reactivates AChE in OP victims (Clement, 1978; Kenley et al. 1982). There are mixed results as to whether pro-2-PAM is effective if treatment is delayed after nerve agent exposure.

Centrally acting drugs, Atropine Sulfate and Scopolamine HBr, to antagonize Pro-2-PAM

Atropine sulfate is a competitive antagonist for the muscarinic acetylcholine receptor (Dawson & Poretski, 1983). Thus, it has been approved by the FDA to treat nerve agent toxicity because it is an anticholinergic drug that prevents binding at muscarinic receptor sites after nerve agent exposure (Clement, 1979). The FDA has also approved an oxime, 2-PAM, to reactivate the inhibited AChE post exposure (Shih, Rowland, & McDonough, 2006). Recently, the antagonism of physostigmine-induced tremor using the anticholinergic, scopolamine HBr, was addressed (Stanford & Fowler, 1997). Although scopolamine HBr is not approved by the FDA as an anticholinergic to antagonize OP-induced toxicity, studies have been performed to show that it may be beneficial in restoring normal cholinergic function. Like atropine sulfate, scopolamine HBr is a competitive antagonist at the muscarinic acetylcholine receptors, specifically, the M1 receptors (Klinkenberg & Blokland, 2011). It prevents ACh binding at the muscarinic receptor sites after nerve agent exposure. Because previous studies describing oxotremorine and physostigmine in this review have introduced atropine sulfate and scopolamine HBr, the focus here will be on one study encompassing both drugs and their anticholinergic effect on OP intoxication.

Shih et al. (2006) reported that the FDA-approved regimen to treat nerve agent intoxication does not prevent seizure activity. This status epilepticus (SE) can produce irreversible brain damage resulting in cognitive and behavioral deficits. Previously, Shih & McDonough, (2000) and Shih et al. (2003) developed a model (model A) whereby guinea pigs were pretreated with a reversible cholinesterase inhibitor, pyridostigmine

bromide, challenged with 2 x LD_{50} of a nerve agent, and treated one minute later with 2-PAM (25.0mg/kg, IM) and atropine sulfate (2.0 mg/kg, IM). An anticonvulsant, such as diazepam, was given five minutes after seizure onset. They discovered that atropine sulfate could terminate seizures if a high dose of the anticonvulsant was utilized. Therefore, Shih et al. (2006) developed another model (model B), where a lower dose of atropine sulfate (0.1 mg/kg) was given with 25.0 mg/kg of 2-PAM one minute after nerve agent injection, followed by the anticonvulsant given at the time of seizure onset, not five minutes post onset as described in the first model. This model more closely simulated the medical treatment protocol that the military uses when a soldier is exposed to nerve agent. A comparison between models was made emphasizing seizure occurrence and latency to seizure onset, as well as time of seizure termination and 24-hour lethality. The potency of three anticonvulsant drugs, midazolam, diazepam, and scopolamine HBr were tested. The ED_{50} for scopolamine ranged from 0.01 to 0.13 mg/kg for models A and B.

Shih et al. (2006) had guinea pigs undergo EEG surgery one week prior to exposure to nerve agent, and an EEG baseline was established on the day of exposure. Thirty minutes after baseline, the animals were challenged SC with 2 x LD_{50} of various nerve agents: tabun (GA: 240µg/kg), sarin (GB: 84µg/kg), cyclosarin (GF: 114µg/kg), O-ethyl S-(2-(diisopropylamino)ethyl)-methylphosphonothioate (VX: 16µg/kg), or O-isobutyl S-(2-diethylamino)ethyl)-methyl phosphonothioate (VR: 22.6µg/kg). As described in model B, atropine sulfate (0.1 mg/kg) was given with 25.0 mg/kg 2-PAM one minute after nerve agent injection, followed by an anticonvulsant: diazepam, midazolam or scopolamine HBr given IM at seizure onset.

The results showed that a lower atropine sulfate dose allowed seizure occurrence to increase, while time to seizure onset remained constant. Seizure terminations took significantly longer when the atropine sulfate dose was lowered from 2.0 to 0.1 mg/kg in the GF-exposed animals treated with diazepam. However, the latency to seizure termination was not significantly different than model A for the GF guinea pigs treated with midazolam or scopolamine HBr. In general, using the lower dose of atropine sulfate did not change the anticonvulsant effects against all of the nerve agents. Midazolam acted faster than diazepam in terminating seizures from the GA, GD, and VR groups regardless of the atropine sulfate dose. Guinea pigs exhibited more salivation and lacrimation at the lower dose of atropine sulfate. These signs of peripheral intoxication were minimally observed at the higher dose of atropine sulfate used in model A.

To conclude Shih et al. (2006), the lower dose of atropine sulfate contributed most to the observed differences between models A and B. It was the only variable that could influence seizure onset. When 0.1 mg/kg of atropine sulfate was given in model B versus 2.0 mg/kg given in model A, only the ED_{50} for midazolam increased for the nerve agents tested. Thus, scopolamine HBr, worked similarly at both doses of atropine sulfate used in the study. Both atropine sulfate and scopolamine HBr are anticholinergics that can counteract the cholinergic effects of OPs.

Peripherally acting drugs, Atropine Methyl Nitrate and Scopolamine Methyl Nitrate, to antagonize Pro-2-PAM

Unlike the centrally acting drugs that cross the BBB and enter the CNS, atropine methyl nitrate (AMN) and scopolamine methyl nitrate (SMN) are peripherally acting antimuscarinic agents (Dilsaver, Snider, & Alessi, 1987; Hollingsworth & Smith, 1999).

Thus, they do not cross the BBB. Their effect can only be seen in the periphery after a subject is exposed to nerve agent. These drugs play an active role in reducing the toxic signs seen in nerve agent poisoning, such as lacrimation and salivation. The next few studies will address these drugs and their effect on the neurotoxicity produced by OPs.

As reported earlier when addressing oxotremorine, Witkin et al. (1986) performed a rat study to analyze the tremorogenic effects of oxotremorine. Rats were pre-treated with atropine sulfate (40.0 mg/kg) 40 minutes prior to oxotremorine (5.0 to 30.0 mg/kg, IP). Oxotremorine (10.0 mg/kg) produced a quicker onset of lacrimation, salivation, and tremor than the 5.0 mg/kg dose. Also, convulsions from the 10.0 mg/kg dose produced more lethality than the 5.0 mg/kg dose. These high doses of oxotremorine produced tremor that resembled seizure activity. In a second experiment, AMN (20.0 or 40.0 mg/kg) was given 15 minutes before oxotremorine (10.0 or 15.0 mg/kg). The said doses prevented lacrimation and salivation, but tremor and convulsions induced by oxotremorine were not inhibited by AMN. Although tremor was not reduced, AMN reduced lethality.

Another study addressed previously, Weinstock et al. (1978), assessed the action of AMN against tremor induced by oxotremorine. The results showed that oxotremorine (0.25 mg/kg) produced the greatest amount of tremor over the 30-minute period of observations. AMN, which does not cross the BBB, prevented salivation and chromodacryorrhea. Interestingly, there was less tremor seen at the higher dose of oxotremorine (1.5 mg/kg) compared to the lower dose of oxotremorine (0.25 mg/kg), where each group was pretreated with AMN (0.5 mg/kg).

The next drug of interest, SMN, is a medical countermeasure being studied in the United Kingdom (U.K.) against nerve agent poisoning. Researchers in this field are striving to develop a therapeutic that will not require a pretreatment (Wetherell, Price, Mumford, Armstrong, & Scott, 2007). This is important because the medical treatment protocol that the military uses when a soldier is exposed to nerve agent does not include a pretreatment to nerve agent poisoning (Shih et al. 2006). A new therapeutic to treat nerve agent poisoning that does not require a pretreatment may also reduce the current FDA requirement of three autoinjectors consisting of 2.0 mg of atropine sulfate and 600.0 mg of 2-PAM (Wetherell et al. 2007; Shih et al. 2006).

Wetherell et al. (2007) performed a guinea pig study to assess the effectiveness of therapy drugs, including SMN, against GD (135µg/kg, equivalent to 5 x LD_{50}, SC). SMN was specifically chosen to assess its ability to prevent intussusception in the gastrointestinal (GI) tract after soman exposure. Intussusception occurs when part of the intestine enfolds upon another section of the intestine, causing a blockage of the GI tract, which could lead to gangrene, internal bleeding, shock, and death. Another drug of interest in this study was HI-6, an oxime used for immediate therapy against nerve agent exposure. In the UK, HI-6 combined with other drugs: physostigmine, scopolamine HBr, avizafone, and atropine sulfate, has provided greater protection against a wide range of nerve agents.

Results of Wetherell et al. (2007) showed that one minute after exposure to GD, the first therapy regimen consisting of SMN (4.0 mg/kg), physostigmine (0.2 mg/kg) and HI-6 (93.6 mg/kg) did not prevent incapacitation and lethality. At 24 hours, 83.3% of the animals were dead; none of these animals had intussusception. Another therapy regimen

consisting of scopolamine HBr (2.0 mg/kg), SMN (2.0 mg/kg), physostigmine (0.2 mg/kg), and HI-6 (93.6 mg/kg) caused 100% survival without evidence of intussusception. There were more survivors at 24 hours after the second therapy regimen, showing that a combination of scopolamine HBr and SMN along with physostigmine and HI-6 improved survival, versus treating with SMN, physostigmine and HI-6.

However, because it is not practicable to deliver 93.6 mg/kg HI-6 in a human autoinjector, Wetherell et al. (2007) performed another study using a lower dose of HI-6 (7.0 mg/kg). Combining the lower dose of HI-6 with scopolamine HBr (2.0 mg/kg or 4.0 mg/kg) and physostigmine (0.2 mg/kg) caused incapacitation; 37.5% of the animals died within 24 hours and had intussusception. However, when the animals were given scopolamine HBr (2.0 mg/kg or 4.0 mg/kg), SMN (2.0 mg/kg), physostigmine (0.2 mg/kg), and HI-6 (7.0 mg/kg), 100% survival at 24 hours occurred and intussusception was less evident. Overall, a high dose of HI-6 showed no evidence of intussusception regardless of using scopolamine HBr or SMN, whereas at the low dose of HI-6, SMN decreased intussusception. Also, because scopolamine HBr was better than SMN at preventing incapacitation and lethality, a combination of these drugs may be beneficial against nerve agent toxicity (Wetherell et al. 2007).

Another realm of pharmacology that utilizes SMN is the study of depression and the symptoms that follow the discontinuation of tricyclic antidepressants (TCA). Dilsaver, Snider & Alessi (1987) suggested that patients who stop taking TCAs suffer from withdrawal-induced cholinergic overdrive. This conclusion arose from the fact that TCA withdrawal symptoms respond to antimuscarinic agents. Investigators hypothesized

that chronic treatment with a TCA (e.g. amitryptiline: AMI) produces supersensitivity in the cholinergic system that can be measured through the hypothermic response induced by oxotremorine.

Male Sprague-Dawley rats underwent surgery to implant thermosensors (for temperature recording) in the peritoneal cavity. After five days of recovery, SMN (1.0 mg/kg, IP) was injected 30 minutes prior to oxotremorine (0, 0.05, 0.10, 0.25, 1.0, and 2.5 mg/kg) to block oxotremorine's peripheral effects. A baseline hypothermic response to oxotremorine was recorded before and after SMN administration. For 16 days, one group of these animals was treated with AMI (10.0 mg/kg, IP twice daily). They were rechallenged with oxotremorine (0.05, 0.1, or 0.25 mg/kg) between days 12 and 16. Another group of these animals were treated with AMI (10.0 mg/kg, IP) for 17 days, after which they received scopolamine HBr (2.0 mg/kg, IP) instead of SMN. This change was used to assess whether a peripheral or central mechanism of action induced hypothermia caused by oxotremorine.

Results of the study indicated that AMI enhanced the hypothermic response to oxotremorine (0.05, 0.1, and 0.25 mg/kg), supporting the hypothesis that TCA withdrawal involves cholinergic overdrive. Scopolamine HBr, but not SMN, blocked the hypothermic response of oxotremorine in the animals. Thus, a central cholinergic mechanism is involved (Dilsaver et al. 1987). SMN, which only works peripherally, could not reverse the effects of oxotremorine that resulted in the hypothermic response.

Overall, AMN and SMN work peripherally by blocking the muscarinic receptors. They may have an effect in the periphery by reducing signs of OP intoxication, such as lacrimation and salivation.

Other Drugs of Interest used in Thesis Study

Two additional drugs of interest, MINA and midazolam, were added to the upcoming thesis study. Like pro-2-PAM, MINA is a tertiary oxime that is toxic in nature. It was a drug of interest as early as the 1950s because of its capability of raising the LD_{50} dose of GB when used alone or in combination with atropine sulfate (Shih, et al. 2010). During this time, both MINA and another tertiary oxime, diacetylmonoxime (DAM), were found to raise the LD_{50} of GB approximately five-fold when given in equal doses as a pretreatment to GB intoxication (Rajapurkar & Koelle, 1958). Like Pro-2-PAM, MINA is highly lipid soluble, allowing it to cross the BBB and reactivate AChE inhibited by the OP. Further research of MINA was discontinued in the 1950s because quaternary oximes, like 2-PAM, were found to reactivate phosphorylated AChE in human red blood cells (Shih et al. 2010). However, MINA's ability to control GB-induced seizures and increase survival has kept it popular in today's research. Because of its toxic nature, it was added to Experiment 1 to quantify its tremorogenic effect compared to pro-2-PAM.

The other drug of interest, midazolam, is a benzodiazepine that shows great anticonvulsant activity against seizure activity (Shih, Duniho, & McDonough, 2003). Because midazolam is a muscle relaxant, it was expected to reduce tremor compared to the tremor-inducing drugs being assessed in the thesis study. According to Olkkola & Ahonen (2008), midazolam is considered to be a GABA modulator; it works on the ionotropic GABA(A) receptors in the CNS. Midazolam does not activate GABA directly but requires GABA to relax the muscles. Compared to other muscle relaxants, midazolam has the shortest recovery profile compared to lorazepam and diazepam.

Because the half lives of the tremor-inducing drugs in the thesis study were less than 120 minutes, midazolam was an ideal choice for comparison.

Use of Fast Fourier Transform to Quantitatively Analyze Tremor

This literature review introduced physostigmine as a drug that characterized tremor; Wang & Fowler (2001) used a tremor-detection instrument, the force plate actometer, to quantitatively describe the frequency characteristics of tremor induced by physostigmine. Thus, it is necessary to first address how tremor is quantified using spectral analytic techniques and Fast Fourier Transform.

Smith (2006) describes digital signal processing as the science of using computers to analyze signals that result from movement. Spectral analytic techniques quantitatively measure tremor by breaking movement down into a set of discrete frequency components that correspond to different rates of movement. Specifically, slow movement corresponds to low frequency changes, whereas fast movement corresponds to high frequency changes. The Fourier transform converts a signal into a sum of sine waves that oscillate at different frequencies. The three parameters of the sine wave consist of magnitude, phase, and frequency, where magnitude and phase are a function of frequency (Newland, 1988). A digital signal is decomposed into components that can be added together and passed through a linear system, characterized by additivity and homogeneity. Additivity means that signals added during the input phase produce signals added in the output phase, and homogeneity means that a change in the input signal's amplitude results in a change in the output signal's amplitude. Furthermore, input and output depends on the type of movement you are studying. For example, in a simple

resistor, the input to the system will be the voltage across the resistor, whereas the output will be the current running through the resistor (Smith, 2006).

In order to further understand input and output signals, it is necessary to define convolution in digital signal processing. According to Smith (2006), convolution is defined in this manner: first, an input signal is decomposed into a set of impulse responses. The output from each impulse is a scaled and shifted version of the impulse response, so that the overall output signal is found by adding the scaled and shifted impulse responses. Thus, the process of decomposing the input signal into impulse responses that are scaled and shifted to form the output signal is convolution. Fast Fourier transform is used to establish convolution, but before it can be defined, Fourier analysis must be addressed in more detail.

According to Smith (2006), Fourier analysis consists of mathematical techniques that decompose signals into sinusoids. Sinusoids are waveforms consisting of sine and cosine waves. Through convolution, the input signal is decomposed into these waves because they have the property of sinusoidal fidelity, which the original signal does not have. This property guarantees that the sinusoidal input will produce a sinusoidal output, the amplitude and the phase of the signal may change, and the frequency and wave shape will never change. Sinusoids are unique due to these properties and are favored over square and triangular waves that do not share these characteristics. Discrete Fourier Transform (DFT) takes the N point input signal and changes it into two N/2 + 1 point output signals, where N is the number of samples in the time domain. Whereas the input signal contains the original, decomposed signal, the output signal contains the amplitudes of the sine and cosine waves. The input signal is in the time domain because the most

common type of signal entering the DFT consists of samples taken at specific intervals of time. Unlike the time domain, the frequency domain describes the amplitudes of the sine and cosine waves, but it has all the features of the time domain in a different form. Knowing one domain will allow the experimenter to calculate the other domain.

Although systems are analyzed in the time domain via convolution, Fourier transform allows a similar analysis to be done in the frequency domain. Through Fourier transform, each input signal is represented by a group of cosine waves having a specific amplitude and phase shift. Therefore, the change in amplitude and phase of cosine waves passing through a linear system is the frequency response. In the frequency domain, the output signal is the result of multiplying the input spectrum by the frequency response. Therefore, convolution occurring in the time domain parallels multiplication occurring in the frequency domain.

Unlike the impulse response, which is a discrete signal, the frequency response is continuous. As stated previously, an N point DFT of the impulse response results in N/2+1 samples of the continuous curve. By making the DFT longer, the resolution will improve and the experimenter will gain a better idea of what the continuous curve looks like. The horizontal axis of the said curve is a fraction of the sampling rate. The horizontal axis values will always be between 0 and 0.5 because discrete data can only contain frequencies between 0 and one-half of the sampling rate. Thus, since the input signal contains any frequency between 0 and 0.5, the system's frequency response must be a continuous curve over the 0 to 0.5 range.

Another way to calculate the DFT in a fast and efficient manner is through Fast Fourier Transform (FFT). To understand FFT, it is necessary to first distinguish between

real DFT and complex DFT. Real DFT is a version of the discrete Fourier transform that uses real numbers to represent the input and output signals, whereas the Complex DFT uses arrays of complex numbers to quantify the signals. FFT is an algorithm for calculating the complex DFT, and real DFT data must be transferred into and out of the complex DFT format. It has already been established that the real DFT transforms the N point time domain signal into two N/2 + 1 point frequency domain signals. These two frequency domain signals are called the real part and the imaginary part. The real part consists of the amplitudes of the cosine waves, whereas the imaginary part consists of the amplitudes of the sine waves. Unlike the real DFT, the complex DFT transforms the two N point time domain signals into two N point frequency domain signals. In this case, the time domain signals, not the frequency domain signals, are called the real and imaginary part. The names real and imaginary stem from the complex DFT; they distinguish real numbers from imaginary numbers. Now that the real DFT and complex DFT have been distinguished, FFT can be described.

FFT operates with N that is a power of two because binary addressing is used in digital data storage, making powers of two a natural signal length. N is usually selected between 32 and 4096, and samples run from 0 to N-1. Three steps are required for a successful FFT. First, the N point time domain signal is decomposed into N time domain signals each consisting of a single point. Second, the N frequency spectra corresponding to the N time domain signals are calculated. Fortunately, this is simple in FFT because the frequency spectrum of a one-point signal is equal to itself. Third, the N spectra are synthesized into a single frequency spectrum. When analyzing data that have undergone FFT, an experimenter may have to calculate the real DFT by means of the complex DFT.

In this case, the experimenter must move the N point signal into the real part of the complex DFT's time domain and set all of the samples in the imaginary part to zero. This results in two signals in the frequency domain, a real and imaginary signal, each composed of N points. The experimenter will then analyze the samples that lie between 0 and N/2, which correspond to the real DFT's spectrum.

In order to analyze the data, an experimenter must understand the physical characteristics of the signal. Notation refers to what the signal looks like, and there are two types of notation that apply to FFT. In the frequency domain, each N/2 + 1 cosine and sine wave has a specific amplitude. The frequency spectra are formulated in rectangular notation by adding the real parts to the real parts and the imaginary parts to the imaginary parts. In other words, each cosine wave (real) is added together, and each sine wave (imaginary) is added together. However, the frequency domain can also be expressed in terms of polar notation. Unlike rectangular notation, the N/2 + 1 cosine waves resulting from decomposition of the N point signal, each have a specified magnitude (amplitude), and a phase shift. Polar notation only uses cosine waves because the sine waves cannot represent the DC component (mean value of the waveform) of the signal. The sine wave of zero frequency is composed of all zeros, so that no mean value can be obtained. Overall, polar notation, not rectangular notation, provides experimenters with a better understanding of the physical characteristics of the resulting signals post FFT.

In order to pull the concepts presented about digital signal processing and Fourier transform together, it is best to work through an example. If you wanted to investigate sounds traveling through the ocean, you would place a microphone in the water and

amplify the electronic signal. A low-pass filter could be used to remove frequencies occurring above 80 hertz so that a signal could be digitized at 160 samples per second. After collecting thousands of samples, you see a noisy waveform whose characteristics cannot be distinguished. Noise is the result of interference with the signal of interest. In this case, if you were only interested in analyzing the sounds of fish moving in the water, but they could also be heard chewing food as they swam, the chewing noise would be an interference. To eliminate the noise, the noisy waveform can be passed through a Hamming window, which is used for smoothing purposes (Smith, 2006). If the signal contains an instantaneous onset or offset, as may occur with drug-induced tremor that turns on and off, the magnitude estimate of tremor at each frequency will leak into other magnitude estimates at adjacent frequencies resulting in contamination (Newland, 1988). Smoothing allows waves that overlap to be distinguished from one another (Smith, 2006). It is performed to reduce the noise produced by the transducers, as well as reduce the effect of high frequency variations in force caused by the animal, such as a footstep (Fowler et al. 2001). Multiplying the waveform by the Hamming window will result in a wave with tails to look like a smooth peak that can be distinguished from other waves (Smith, 2006).

According to Smith (2006), after the Hamming window is applied, DFT and conversion to polar notation will result in a frequency spectrum. Unfortunately, the data still looks noisy. This could be because you do not have enough samples to form a well-behaved curve. Earlier, it was mentioned that using a longer DFT would improve the resolution of the resulting curve. However, in this case, it will not work because a greater number of samples will dilute the information already obtained. To fix this problem, the

original input signal must be broken down into point segments that are multiplied by the Hamming window and undergo DFT to form the frequency spectra. The resulting spectra are then averaged to form a single frequency spectrum for observational purposes. This reduces the noise to a level that will allow the experimenter to analyze distinguishing features of the observed signal. By using point segments and applying a longer DFT, the resolution will also improve.

One other way to reduce spectral noise is to run a low-pass digital filter to smooth the spectrum. However, this reduces the noise at the expense of the resolution. Although this method has the potential for better performance due to manipulation between the noise and resolution, this method is seldom used because applying point segments and using more data from the input signal will reduce the noise and improve the resolution.

Overall, spectral analytical techniques use FFT to quantify signals. In the case of the thesis experiment, FFT will be used to quantify tremor. Based on the research reviewed above, the following thesis study, consisting of two experiments, was formulated to characterize tremor with known tremor-inducing drugs, and to antagonize the toxicity of a tremor-inducing oxime, pro-2-PAM, that crosses the BBB. This study was conducted to further assess the possibility of pro-2-PAM one day gaining FDA approval to treat victims of chemical warfare. For Experiment 1, it was hypothesized that if tremor could be successfully quantified using a Med Associates system, then the tremor could be antagonized through Experiment 2 using centrally and peripherally acting drugs. Furthermore, for Experiment 2, it was hypothesized that atropine sulfate and scopolamine HBr would antagonize the tremor produced by 28.0 mg/kg of pro-2-PAM more than the peripherally acting drugs, AMN and SMN. This is because atropine sulfate and

scopolamine HBr cross the BBB entering the brain, whereas the peripheral drugs do not enter the brain. For both experiments, the independent variable was the dosage of drug used and the dependent variable was the amount of tremor produced.

CHAPTER 3

MATERIALS AND METHODS

Subjects

Male, Hartley guinea pigs (250 g), purchased from Charles River Labs (Kingston, NY, USA), were quarantined for one week to acclimate to the environment. They were housed individually in temperature- and humidity-controlled cages on a 12-hour light-dark schedule. Lights were illuminated at 0600 hours and shut off at 1800 hours. Each guinea pig had access to laboratory chow and water ad libitum while they resided in their home cages.

Materials

All drugs used in this experiment were purchased from commercial sources and prepared in either saline or citrate buffer for IM or SC injections. Oxotremorine, physostigmine, MINA, atropine sulfate, scopolamine HBr, and SMN were purchased from Sigma Chemical Co., St. Louis, MO. AMN was purchased from ChromaDex Inc., Irvine, CA; midazolam was purchased from T.W. Medical Veterinary Supply, Austin, TX; pro-2-PAM was custom synthesized by Southwestern Research Institute, San Antonio, TX. Saline was purchased from APP Pharmaceuticals, Schaumburg, IL. Citrate buffer was made in our laboratory.

Tremor Assessment

Pro-2-PAM is a centrally active oxime that produces a tremorogenic effect in unchallenged (no nerve agent) animals. The literature review showed that the tremor resulting from Pro-2-PAM is a sign of toxicity. The present study produced tremor by giving pro-2-PAM, and attenuated the tremor pharmacologically using different anticholinergic drugs. The study focused on the cholinergic system because of observations that oximes can alter the release of acetylcholine, have interactions with presynaptic and postsynaptic receptors, and their toxicity is modified in the presence of atropine sulfate (Davies & Willey, 1958; Van Helden, Busker, Melchers, &Bruijnzeel, 1996).

In order to assess tremor, a model for its detection and characterization was formulated using an automated system. Successful development of this model for the measurement of tremor allowed more precise and less subjective (bias-free) quantitative measurements of tremor in two experiments. The first experiment characterized the tremor caused by pro-2-PAM by comparing it to known tremor-inducing drugs, including oxotremorine and physostigmine. The second experiment antagonized the toxic effects of pro-2-PAM with anticholinergic drugs. Both experiments determined the safety and efficacy of pro-2-PAM as a potential therapeutic against nerve agent intoxication.

Procedure

Apparatus. Tremor was recorded using a Med-Associates Activity monitor system (St. Albans, VT, 2008), made up of a platform located in an HK sound attenuating chamber. A load cell was used to transduce movement (force) into an electric signal that was stored and analyzed. The Med-Associates system reduced ambulation and provided

a sound- and light-attenuated environment. It was calibrated using a number of weights (1 to 20 g); the gain was adjusted to produce a five-fold amplification. Weights were dropped from a height of one inch and the gain was adjusted to get a reading five times higher than the reading on the display than the actual mass that was dropped.

Experiment 1: Tremor Characterization. Two known tremor-inducing drugs, oxotremorine and physostigmine, provided positive control data and characterization of the tremor-detection system. Pro-2-PAM, the centrally active oxime of interest, was administered at two different doses to characterize tremor and select an effective (tremorogenic) dose. Using a Latin square design, 20 male Hartley guinea pigs (250 g) were randomly assigned to one of five orders of drug presentation. Within each group, the animals received vehicle (saline or citrate buffer), the higher dose of each test drug, and the lower dose of each test drug, until each animal had received all doses throughout the study. Citrate buffer has a low pH and was used as a vehicle for pro-2-PAM and midazolam, while saline was the vehicle used for the rest of the treatments. We used separate vehicles to more clearly determine the effects of each drug dissolved in the vehicle. For oxotremorine, only one low dose was administered due to early fatalities seen at higher doses. No animal received more than one dose on each test day. The study ran for seven weeks, allowing each animal to receive all doses of each drug and recover from the previous dose of drug received. All animals received oxotremorine (0.1 mg/kg, SC), physostigmine (0.1 and 0.4 mg/kg, SC), and pro-2-PAM (24.0 and 28.0 mg/kg, IM), and were immediately placed into the tremor detection system. The animals also received one dose of MINA (200.0 mg/kg, IM) to quantify its tremorogenic effect,

and midazolam (0.5 or 2.0 mg/kg, IM), to show reduced tremor compared to the tremorogenic effect of the other drugs.

Four animals were assigned to each of five groups as indicated in *Table 1* of the Appendix. These groups differed only with respect to the order of drug presentation. Previous studies suggest that the half-lives of these drugs are known to be less than 120 minutes (Cox & Potkonjak, 1969; Gordon et al. 2008; Somani & Khalique, 1987). Thus, a twice-weekly dosing schedule provided more than adequate washout of drug effects between doses. Using a Tuesday/Friday dosing schedule, the tremor-inducing drug was administered, and the animals were monitored for 60 minutes. On the next test day, animals initially receiving the drug were then given vehicle, and vice versa. This controlled for order effects and provided a within-subjects comparison, thereby increasing power. Three test days were required for each subject to complete assessment of a given drug (vehicle, low dose, high dose). The entire experiment required 15 test days including the vehicle injections (or approximately seven calendar weeks).

Experiment 2: Antagonism of Pro-2-PAM. We used separate guinea pigs (N=24) to initiate studies designed to antagonize the side effects of 28.0 mg/kg of pro-2-PAM, by targeting its putative mechanism of toxicity. For pro-2-PAM, the presumed mechanism of toxicity is cholinergic, but it is not known whether the peripheral or central nervous system is more responsible for the toxicity produced. Thus, we utilized anticholinergic drugs that entered the brain as well as their methylated analogues that did not enter the brain. Specifically, both atropine sulfate (0, 2.0, 4.0, and 10.0 mg/kg, IM) and atropine methyl nitrate (0, 2.0, 4.0, and 10.0 mg/kg IM), and scopolamine HBr (0,

0.1, 0.3, and 1.0 mg/kg, IM) and SMN (0, 0.1, 0.3, and 1.0 mg/kg, IM) were used. See *Table 2* in the Appendix.

All doses were presented once in a strictly ascending or descending order counterbalanced across subjects in a given group. For atropine sulfate and atropine methyl nitrate, 12 animals were used and the order in which the drugs were presented was counterbalanced. Thus, six animals completed the evaluation of atropine sulfate first, followed by atropine methyl nitrate evaluation. For the remaining six animals, the order of drug presentation was reversed. This method controlled for possible order effects. An analogous study was conducted for scopolamine HBr and SMN utilizing 12 separate animals. All animals were pre-treated with the antagonists 15 minutes before pro-2-PAM administration. Immediately after pro-2-PAM was injected, the animals were placed into the system for tremor analysis.

Data Analysis for Experiments 1 and 2. Power analysis using Piface (http://www.stat.uiowa.edu/~rlenth/Power/) for Experiment 1 indicated a power of 0.8 ($\beta = 0.2$) using 20 subjects to detect a main effect of dose at $\alpha = 0.05$ with a standardized effect size as small as 0.51. For Experiment 2, with 12 subjects per anticholinergic drug and $\alpha=0.05$, Piface indicated a power of 0.8 ($\beta=0.2$) to detect a main effect of anticholinergic compound with a standardized effect size of 0.41, a power of 0.8 ($\beta=0.2$) to detect a main effect of dose with a standardized effect size of 0.4, and a power of 0.8 ($\beta=0.2$) to detect a significant interaction with an effect size as small as 0.57.

The input into the system was the movement (force) transduced into an electric signal, whereas the output (tremor) was the power being analyzed as area under the curve after FFT occurred. Thus, power was defined as a quantitative measure of the tremor

produced. The program to analyze the data was written in Visual Basic for Applications using Microsoft Excel 2007. The program originated from Smith (2006), Table 12-4, which detailed the FFT algorithm used in this experiment. A Hamming window was applied to the data to distinguish the peaks of interest analyzed in this experiment.

The raw data collected for both experiments was acquired at a sampling rate of 100 samples per second, making the frequency 100 Hz. Thus, 6000 samples were produced per minute for a 60 minute session, resulting in 360,000 points per run. We chose a frame size of 4096 for better resolution; according to the Nyquist frequency, the highest frequency that can be estimated is one-half of the sampling frequency (Newland, 1988). Thus, after FFT, we only dealt with 2048 samples at a frequency of 50 Hz. Because FFT transformed the N point time domain signal into two N/2 + 1 point frequency domain signals, the range of frequencies in which tremor was present should have lain between 0 and 50 Hz.

The raw data underwent FFT through the Visual Basic program, decomposing the input signals into additive components that underwent convolution. Although the frame size chosen should have given us better resolution, increased noise resulted. Therefore, the raw data were broken down into segments that underwent FFT and were multiplied by a Hamming Window. The Hamming Window allowed for frequency smoothing, which was necessary because the estimates produced at each frequency leaked into the adjacent frequencies, contaminating the signal. The Hamming Window reduced the artifacts produced by frequency leakage by calculating a three-point moving average, where the center point was more heavily weighted than the adjacent points (Newland, 1988). The output signals produced after the Hamming Window was applied were

converted to polar notation, and the resulting frequency spectra were averaged to calculate area under the curve (amplitude across time), which represented power. Thus, power was a quantitative measure of how much tremor was produced by each drug in Experiment 1, and how much tremor was antagonized with the anticholinergic drugs used in Experiment 2. Bins of 5.45 minutes made up 11 blocks of time that were statistically analyzed for the presence of tremor.

For the following results of Experiments 1 and 2, all figures referred to are in the Appendix.

CHAPTER 4

RESULTS

Experiment 1. In Experiment 1, a repeated measures ANOVA was conducted to ensure that the system could detect a difference in tremor between oxotremorine, physostigmine, pro-2-PAM, midazolam, and MINA, versus their control counterparts. The dosages of each drug of interest were also compared to one another to detect which drug induced more tremor. Pairwise comparisons and Bonferroni post hoc tests were used to assess differences in time course effects over 60 minutes. The physostigmine doses (0.1 and 0.4 mg/kg), and the pro-2-PAM doses (24.0 and 28.0 mg/kg) were compared to one another, as well as the highest dose of physostigmine (0.4 mg/kg) and the highest dose of oxotremorine (0.1 mg/kg). The highest dose of pro-2-PAM (28.0 mg/kg) was then compared to the highest doses of physostigmine (0.4 mg/kg) and oxotremorine (0.1 mg/kg). MINA (200.0 mg/kg) was then compared to both doses of pro-2-PAM (24.0 and 28.0 mg/kg), and the midazolam doses (0.5 and 2.0 mg/kg) were compared to one another. Finally, saline and citrate buffer were compared to one another

to see if a difference in activity occurred between both controls. In Experiment 1, 45% (9/20) of the animals survived all doses of each drug given according to the Latin square design. Therefore, the data of each drug presented have a different N value.

One group of 13 animals was exposed to both oxotremorine (0.1 mg/kg) and saline on different test days. A repeated measures ANOVA comparing oxotremorine to saline indicated a significant effect of drug, $F(1,12) = 34.55$, $p<.05$, partial eta squared = .74, power = 1.00. Because Mauchly's Test of Sphericity was significant for block ($p=.01$), the significance test for block used the Greenhouse-Geisser correction, $F(1,12) = 7.96$, $p<.05$, partial eta squared = .40, power = 1.00. Because Mauchly's Test of Sphericity for the interaction of drug by block was not significant, the test of significance for this effect proceeded with Sphericity Assumed, $F(1,12) = 11.54$, $p<.05$, partial eta squared = .49, power = 1.00. A multivariate test was run to assess whether a mean difference and slope difference between oxotremorine and saline existed. The mean difference was significant, $F(1,12) = 34.55$, $p<.05$, and the slope difference was significant, $F(1,12) = 26.01$, $p<.05$. These results indicated that tremor could be characterized by the system, and that 0.1 mg/kg of oxotremorine induced more tremor in the animals versus saline. See *Figure 1*.

One group of 11 animals was exposed to both physostigmine (0.4 mg/kg) and saline on different test days. A repeated measures ANOVA indicated a significant effect of drug, $F(1,12) = 11.0$, $p<.05$, partial eta squared = .48, power = .86. Because Mauchly's Test of Sphericity was significant for both block ($p=.05$) and the interaction of drug by block ($p=.01$), the significance test for these effects proceeded with the Greenhouse-Geisser Test. For block, $F(1,12) = 7.78$, $p<.05$, partial eta squared = .39,

power = .99, and for the interaction of drug by block, $F(1,12) = 4.88$, $p<.05$, eta squared = .29, power = .92. A multivariate test assessed whether a mean difference and slope difference between 0.4 mg/kg physostigmine and saline existed. The mean difference was significant, $F(1,12) = 11.00$, $p<.05$, and the slope difference was significant, $F(1,12) = 9.57$, $p<.05$. These results indicated that the system could characterize tremor, and that 0.4 mg/kg of physostigmine induced more tremor in the animals versus saline. One group of 10 animals was exposed to both physostigmine (0.1 mg/kg) and saline on different test days. A repeated measures ANOVA indicated a significant effect of drug, $F(1,12) = 8.68$, $p<.05$, partial eta squared = .42, power = .77. Because Mauchly's Test of Sphericity was significant for block ($p=.01$), the significance test for block used the Greenhouse-Geisser correction, $F(1,12) = 3.55$, $p<.05$, partial eta squared = .23, power = .85. Because Mauchly's Test of Sphericity was not significant for the interaction of drug by block the test for this effect proceeded with Sphericity Assumed, $F(1,12) = 2.77$, $p<.05$, eta squared = .19, power = .76. These results indicated that the system could characterize tremor, and that 0.1 mg/kg of physostigmine induced more tremor in the animals versus saline. A multivariate test assessed whether a mean difference and slope difference between 0.1 mg/kg physostigmine and saline existed. Although the slope difference was not significant, the mean difference was significant, $F(1,12) = 8.68$, $p<.05$, suggesting a difference in tremor between 0.1 mg/kg of physostigmine and saline. See *Figure 2.*

One group of 11 animals was exposed to both pro-2-PAM (28.0 mg/kg) and citrate buffer on different test days. A repeated measures ANOVA indicated a significant effect of block, $F(1,9) = 22.88$, $p<.05$, partial eta squared = .72, power = 1.00. There was

no significant effect of drug or the interaction of drug by block. A multivariate test assessed whether a mean difference and slope difference between 28.0 mg/kg of pro-2-PAM and saline existed. The mean difference and slope difference were not significant. These results indicated that 28.0 mg/kg of pro-2-PAM did not induce more tremor in the animals versus citrate buffer. One group of nine animals was exposed to both pro-2-PAM (24.0 mg/kg) and citrate buffer on different test days. A repeated measures ANOVA indicated a significant effect of block, $F(1,8) = 32.78$, $p<.05$, partial eta squared = .80, power = 1.00. There was no significant effect of drug or the interaction of drug by block. A multivariate test assessed whether a mean difference and slope difference between 24.0 mg/kg of pro-2-PAM and saline existed. The mean difference and slope difference were not significant. These results that 24.0 mg/kg of pro-2-PAM did not induce more tremor in the animals versus citrate buffer.

One group of 10 animals was exposed to both midazolam (2.0 mg/kg) and citrate buffer on different test days. A repeated measures ANOVA indicated a significant effect of block, $F(1,9) = 9.80$, $p<.05$, partial eta squared = .52, power = 1.00. There was also a significant interaction of drug by block, $F(1,9) = 4.15$, $p<.05$, partial eta squared = .32, power = 1.00. A multivariate test assessed whether a mean difference and slope difference between 2.0 mg/kg of midazolam and saline existed. Although the slope difference was significant, $F(1,9) = 9.00$, $p<.05$, the mean difference was not significant. These results indicated that there was no difference in tremor produced by 2.0 mg/kg of midazolam and citrate buffer. One group of 10 animals was exposed to both midazolam (0.5 mg/kg) and citrate buffer on different test days. A repeated measures ANOVA indicated a significant effect of block, $F(1,9) = 15.46$, $p<.05$, partial eta squared = .63,

power = 1.00. There was also a significant interaction of drug by block, $F(1,9) = 3.04$, $p<.05$, partial eta squared = .25, power = .97. A multivariate test assessed whether a mean difference and slope difference between 0.5 mg/kg of midazolam and saline existed. Neither the mean difference nor the slope difference were significant. These results indicated that the tremor induced by 0.5 mg/kg of midazolam was not different than the tremor induced by the citrate buffer injection.

One group of 13 animals was exposed to both MINA (200.0 mg/kg) and saline on different test days. A repeated measures ANOVA indicated no significant effect of drug. Because Mauchly's Test of Sphericity was not significant for block, the test of significance for this effect proceeded with Sphericity Assumed, $F(1,12) = 35.40$, $p<.05$, partial eta squared = .75, power = 1.00. Because Mauchly's Test of Sphericity was significant for the interaction of drug by block, the significance test for the interaction used the Greenhouse-Geisser correction, $F(1,12) = 6.80$, $p<.05$, eta squared = .36, power = .99. A multivariate test assessed whether a mean difference and slope difference between 200.0 mg/kg of MINA and saline existed. Although the slope difference was significant, the mean difference was not significant, indicating that there was no difference in tremor between 200.0 mg/kg of MINA and citrate buffer.

A repeated measures ANOVA was also used to compare the two doses of physostigmine (0.1 and 0.4 mg/kg). No significant effect of drug was found. Because Mauchly's Test of Sphericity was significant for block and the interaction of drug by block ($p=.01$), the significance test for block used the Greenhouse-Geisser correction. For block, $F(1,12) = 5.84$, $p<.05$, partial eta squared = .33, power = 1.00, and for the interaction of drug by block, $F(1,12) = 3.44$, $p<.05$, partial eta squared = .22, power = .80.

A multivariate test assessed whether a mean difference and slope difference between 0.1 and 0.4 mg/kg of physostigmine existed. Although the slope difference was significant, $F(1,12) = 5.95$, $p<.05$, the mean difference was not significant, indicating that there was no difference in tremor between 0.1 and 0.4 mg/kg of physostigmine.

Using the same method, both doses of pro-2-PAM (24.0 and 28.0 mg/kg) were compared to one another. No significant effect of drug was found. However, a significant effect of block occurred, $F(1,10) = 25.43$, $p<.05$, partial eta squared = .76, power = 1.00. A multivariate test assessed whether a mean difference and slope difference occurred between 24.0 and 28.0 mg/kg of pro-2-PAM. The slope difference and mean difference were not significant, indicating that no difference in tremor was found between 24.0 and 28.0 mg/kg of pro-2-PAM.

A repeated measures ANOVA was also used to compare the highest dose of physostigmine (0.4 mg/kg) to oxotremorine (0.1 mg/kg). Because Mauchly's Test of Sphericity was not significant for drug or the interaction of drug by block, the test for these effects proceeded with Sphericity Assumed. A significant effect was found for drug, $F(1,12) = 16.35$, $p<.05$, eta squared = .58, power = .96. There was also a significant effect for block, $F(1,12) = 7.77$, $p<.05$, eta squared = .39, power = 1.00, and a significant effect for the interaction of drug by block, $F(1,12) = 6.52$, $p<.05$, eta squared = .35, power = 1.00. A multivariate test assessed whether a mean difference and slope difference occurred between 0.4 mg/kg of physostigmine and 0.1 mg/kg of oxotremorine. The mean difference was significant, $F(1,12) = 16.35$, $p<.05$, and the slope difference was significant, $F(1,12) = 8.24$, $p<.05$, indicating that 0.4 mg/kg of physostigmine induced more tremor than 0.1 mg/kg of oxotremorine. See *Figure 3.*

The highest dose of physostigmine (0.4 mg/kg) and oxotremorine (0.1 mg/kg) were then compared to the highest dose of pro-2-PAM (28.0 mg/kg) using a repeated measures ANOVA. A significant effect of block and an interaction of drug by block were found between 0.4 mg/kg of physostigmine and 28.0 mg/kg of pro-2-PAM. For block, $F(1,9) = 12.02$, $p<.05$, partial eta squared = .57, power = 1.00, and for the interaction of drug by block, $F(1,9) = 4.44$, $p<.05$, partial eta squared = .33, power = 1.00. A multivariate test assessed whether a mean difference and slope difference occurred between 0.4 mg/kg of physostigmine and 28.0 mg/kg of pro-2-PAM. Although the slope difference was not significant, the mean difference was significant, $F(1,9) = 27.22$, $p<.05$, indicating that a difference in tremor was found between 0.4 mg/kg of physostigmine and 28.0 mg/kg of pro-2-PAM. According to *Figure 4*, however, it is unknown which drug produced more tremor because there was no significant effect of drug.

The next comparison was made between 0.1 mg/kg of oxotremorine and 28.0 mg/kg of pro-2-PAM. A significant effect of drug, block and an interaction of drug by block occurred. For drug, $F(1,9) = 16.28$, $p<.05$, partial eta squared = .64, power = .95. For block, $F(1,9) = 19.63$, $p<.05$, partial eta squared = .69, power = 1.00. For the interaction of drug by block, $F(1,9) = 4.54$, $p<.05$, partial eta squared = .34, power = 1.00. A multivariate test assessed whether a mean difference and slope difference occurred between 0.1 mg/kg of oxotremorine and 28.0 mg/kg of pro-2-PAM. Although the slope difference was not significant, the mean difference was significant, $F(1,9) = 16.28$, $p<.05$, indicating a difference in tremor between 0.1 mg/kg of oxotremorine and 28.0 mg/kg of pro-2-PAM. According to *Figure 5,* 0.1 mg/kg of oxotremorine induced more tremor than 28.0 mg/kg of pro-2-PAM.

A repeated measures ANOVA was used to compare 200.0 mg/kg of MINA to both doses of pro-2-PAM (24.0 and 28.0 mg/kg). A multivariate test assessed whether a mean difference and slope difference occurred between 200.0 mg/kg of MINA and 24.0 mg/kg of pro-2-PAM, and no significant effect was found. Thus, there was no significant difference in the tremor produced by 200.0 mg/kg of MINA and 24.0 mg/kg of pro-2-PAM. The same test was run comparing 200.0 mg/kg of MINA to 28.0 mg/kg of pro-2-PAM. Once again, no significant difference was found, indicating no difference in the tremor produced by 200.0 mg/kg of MINA and 28.0 mg/kg of pro-2-PAM. Finally, the midazolam doses (0.5 and 2.0 mg/kg) were compared to one another using a repeated measures ANOVA to indicate whether there was a significant difference in the resting activity between both doses. A multivariate test did not show any significant difference between either dose of midazolam, meaning there was no difference in resting activity for either dose of midazolam.

Finally, citrate buffer and saline were compared with a repeated measures ANOVA to indicate whether a difference in activity occurred between the controls. There was a significant effect of drug, $F(1,10) = 6.20$, $p<.05$, partial eta squared = .41, power = .60. There was also a significant effect of block, $F(1,10) = 16.06$, $p<.05$, partial eta squared = .64, power = 1.00, and a significant interaction of drug by block, $F(1,10) = 9.34$, $p<.05$, partial eta squared = .51, power = 1.00. A multivariate test was run to assess whether a mean difference and slope difference between the controls existed. The mean difference was significant, $F(1,10) = 6.20$, $p<.05$, and the slope difference was significant, $F(1,10) = 26.26$, $p<.05$. These results indicated that citrate buffer showed more activity on the system than saline. See *Figure 6.*

Peak amplitude for Experiment 1. The Appendix contains figures that show the peak amplitudes of tremor reached after exposure to the drugs of interest in Experiment 1. *Figure 7* shows that on average, oxotremorine had the highest peak amplitude over the 60 minute period versus both doses of physostigmine (0.1 and 0.4 mg/kg) and saline. For the tremoregenic drugs, tremor was prominent at approximately 0.5 Hz, fell at approximately 1.5 Hz, and was non-existent at 3 Hz. *Figure 8* shows that on average, citrate buffer induced more tremor than both doses of pro-2-PAM (24.0 and 28.0 mg/kg), and saline. There was no significant difference between the tremor produced by both doses of pro-2-PAM (24.0 and 28.0 mg/kg), even though the figure shows that 28.0 mg/kg produced more tremor than 24.0 mg/kg of pro-2-PAM. It also looks like both doses of pro-2-PAM produced more tremor than 200.0 mg/kg MINA, but this cannot be concluded because there was no significant difference between MINA and both doses of pro-2-PAM. On average, saline produced the least tremor. For the oximes, tremor was prominent at approximately 0.5 Hz, fell at approximately 1.5 Hz, and was non-existent at 3 Hz.

Experiment 2. After characterizing tremor in Experiment 1, Experiment 2 was performed to antagonize the tremor produced by the highest dose of pro-2-PAM (28.0 mg/kg). Because some fatalities occurred before all treatment doses could be given, regression analyses were used to account for missing data points. The centrally acting drugs (atropine sulfate and scopolamine HBr) were compared to one another because they crossed the BBB, entering the brain, whereas the peripherally acting drugs (AMN and SMN) were compared because they did not enter the brain. Finally, atropine sulfate and AMN were compared, as well as scopolamine HBr and SMN because each

comparison has atropine or scopolamine in common. In Experiment 2, 46% (11/24) of the animals survived all doses of each drug administered in the Latin square design. Therefore, the data of each drug comparison presented have a different N value. Before analyzing these data, a percent difference score was calculated for each animal that measured how well each dose of drug antagonized the tremor produced by pro-2-PAM. The percent difference scores were used to apply an imputed regression analysis to generate missing data due to lethality, a 1 between groups and 1 within treatment ANOVA yielded the results that follow. For the following sets of data, "low," "medium," and "high" doses referred to the lowest dose, the next highest dose, and the highest dose of each drug used to antagonize pro-2-PAM, respectively.

The first comparison was between atropine sulfate (N=8) and scopolamine HBr (N=9). Comparing atropine sulfate (low: 2.0 mg/kg, medium: 4.0 mg/kg, and high: 10.0 mg/kg) to scopolamine HBr (0.1 mg/kg, 0.3 mg/kg, and 1.0 mg/kg), the missing values were determined with 78% accuracy (R squared = .78). There was a significant effect of dose, $F(1,15) = 6.81$, $p<.05$, partial eta squared = .31, power = .89, and a significant interaction of drug by dose, $F(1,15) = 3.64$, $p<.05$, partial eta squared = .20, power = .63. There was a significant between subjects effect, $F(1,15) = 32.53$, $p<.05$, partial eta squared = .68, power = 1.00. All doses of Scopolamine HBr antagonized the tremor produced by pro-2-PAM more than all doses of atropine sulfate. There was no significant difference between the atropine doses (low: 2.0 mg/kg; medium: 4.0 mg/kg; high: 10. mg/kg), but there was a significant difference between the high dose of scopolamine HBr (1.0 mg/kg) and the medium (0.3 mg/kg) and low (0.1 mg/kg) doses. Overall, the high dose of scopolamine HBr antagonized the tremor produced by 28.0

mg/kg of pro-2-PAM more than the low and medium doses of scopolamine HBr. See *Figure 9*. *Figure 10* shows that the doses of atropine sulfate used (2.0, 4.0, and 10.0 mg/kg) were not significantly different from one another in antagonizing the tremor produced by 28.0 mg/kg of pro-2-PAM. However, the figure also shows that for the scopolamine HBr doses (0.1, 0.3, and 1.0), the highest dose was significantly different than both the low and medium doses in antagonizing the tremor produced by 28.0 mg/kg of pro-2-PAM.

The next comparison was between AMN (N=8) and SMN (N=9). Comparing AMN (low: 2.0 mg/kg, medium: 4.0 mg/kg, and high: 10.0 mg/kg) to SMN (0.1 mg/kg, 0.3 mg/kg, and 1.0 mg/kg), the missing values were determined with 61% accuracy (R squared = 0.61). There was no significant effect of dose or the interaction of dose by drug. There was a significant between subjects effect, $F(1,15) = 36.12$, $p<.05$, partial eta squared = .71, power = 1.00. SMN appeared to antagonize the tremor produced by pro-2-PAM more than AMN, but because no significant effect of drug occurred, this cannot be concluded. See *Figure 11*. *Figure 12* shows that there was no significant effect between all doses of AMN and SMN against 28.0 mg/kg of pro-2-PAM. Thus, it cannot be concluded that either drug antagonized the tremoregenic effect of pro-2-PAM.

The next two comparisons were made between atropine sulfate and AMN, and scopolamine HBr and SMN. Although each drug in these comparisons works differently, where atropine sulfate and scopolamine HBr cross the BBB, and AMN and SMN do not, it was necessary to compare each centrally acting drug to its analog because they come from the same family of drugs, having atropine or scopolamine in common. Whereas the last two comparisons were made between different animals exposed to each antagonist,

the following analyses are within subject comparisons because the same animals were exposed to the drugs being compared.

The first comparison was between atropine sulfate (N=8) and AMN (N=8). Comparing atropine sulfate (low: 2.0 mg/kg, medium: 4.0 mg/kg, and high: 10.0 mg/kg) to AMN (low: 2.0 mg/kg, medium: 4.0 mg/kg, and high: 10.0 mg/kg), there was no significant effect of drug, dose, or the interaction of drug by dose. See *Figure 13.*

The final comparison was between scopolamine HBr (N=9) and SMN (N=9). The missing values for scopolamine HBr were determined with 82% accuracy (R squared = .82), and the missing values for SMN were determined with 60% accuracy (R squared = .602). Comparing scopolamine HBr (low: 0.1 mg/kg, medium: 0.3 mg/kg, and high: 1.0 mg/kg) to SMN (low: 0.1 mg/kg, medium: 0.3 mg/kg, and high: 1.0 mg/kg), there was no significant effect of drug, dose, or the interaction of drug by dose. See *Figure 14.*

Peak amplitude for Experiment 2. In Experiment 2, each central and peripheral antagonist was compared to its respective saline control in antagonizing the tremor produced by pro-2-PAM. Thus, each zero dose represented the animal receiving a saline pre-treatment followed by 28.0 mg/kg of pro-2-PAM. Graph A of *Figure 15* compares each dose of atropine sulfate in antagonizing 28.0 mg/kg of pro-2-PAM. The statistics showed that none of the atropine sulfate doses were significantly different from one another as seen by the overlapping amplitudes in Graph A. The most tremor occurred between 0 and 1 Hz, fell at 2 Hz, and was non-existent at 3 Hz. The statistics also indicated that 1.0 mg/kg of scopolamine HBr antagonized the tremor produced by 28.0 mg/kg of pro-2-PAM more than the other doses of scopolamine HBr (0.1 and 0.3 mg/kg),

as seen in Graph B of *Figure 15*. Tremor appeared to occur between 0 and 1.5 Hz, fell at 1.5 Hz and was non-existent at 3 Hz.

There was no significant difference between the AMN and SMN doses tested in this study. Graph A of *Figure 16* shows that for 28.0 mg/kg of pro-2-PAM, tremor occurred between 0 and 1.5 Hz, fell at 1.5 Hz, and was non-existent at 3 Hz. Similarly, Graph B of *Figure 16* shows that for 28.0 mg/kg of pro-2-PAM and the SMN doses working against pro-2-PAM, tremor occurred between 0 and 1.5 Hz, fell at 1.5 Hz, and was non-existent at 3 Hz.

CHAPTER 5

DISCUSSION

The goal of this study was two-fold. Experiment 1 characterized the intoxicating tremor produced by known tremoregenic drugs, oxotremorine and physostigmine, two centrally acting oximes, pro-2-PAM and MINA, and a muscle relaxant, midazolam, using a Med Associates System. Experiment 2 attempted to antagonize the tremor produced by 28.0 mg/kg of pro-2-PAM using central and peripheral anticholinergics. In Experiment 1, the only drugs that showed a tremoregenic effect against their controls were oxotremorine and physostigmine. A high dose of physostigmine (0.4 mg/kg) induced more tremor than 0.1 mg/kg of oxotremorine; only 0.1 mg/kg of oxotremorine induced more tremor than 28.0 mg/kg of pro-2-PAM. MINA did not show a significant tremoregenic effect, and midazolam did not show resting tremor. Successful characterization of tremor using the automated system justified attempting to antagonize the tremor produced by 28.0 mg/kg of pro-2-PAM in Experiment 2, which was performed in order to support future studies using pro-2-PAM as a therapeutic against OP

intoxication. Only the centrally acting anticholinergics reduced the tremoregenic effect of pro-2-PAM.

The problem with both of these experiments is that tremoregenic activity was only shown between 0 and 2 Hz. We expected higher frequencies of tremor in Experiment 1, especially from physostigmine, which was shown by Wang & Fowler (2001) to show tremor between 5 and 25 Hz. We also expected lower frequencies of tremor in Experiment 2 after attempting to antagonize the tremoregenic effect of pro-2-PAM. According to Elble (2009) and Smaga (2003), tremor is not defined by frequencies of 0 to 2 Hz, and according to Wang & Fowler (2001), frequencies below 10 Hz were attributable to the licking behavior of a rat versus the tremoregenic effect of physostigmine. Therefore, it is necessary to first discuss why the Med Associates System failed to detect tremor at higher frequencies in both studies by first addressing how the force plate actometer and load cell transducer have detected tremor in past research.

Unlike the Med Associates System, Fowler et al. (2001) used a force plate actometer to detect tremor in rats. The force plate employs three or more transducers that convert the force of the animal into quantitative measures that can be statistically analyzed after FFT occurs. Transducers convert the mechanical energy of tremor to electrical energy that is digitized (Newland, 1988). The force sensing plate used in Fowler et al. (2001), quantified the animal's movements as it stood, walked, or laid on the plate. If a force is applied to the force plate that is equidistant from the four support points, then the forces sensed at those points will equal the applied force in the center of the plate. The animal is the load on the force plate representing the center of force. The center of force varies with the applied force so that the position of the center of force (the

moving animal), will vary with time every instant the animal moves. This is why the force plate can detect the tremor of an animal as it moves on the plate.

Unlike the force plate having four transducers, the Med Associates System in our study utilized a load cell (one transducer) to measure tremor. According to Newland (1988), load cells and strain gauges are types of force transducers. They measure movement based on the principle that displacement of a spring is proportional to the applied force, so that deflection will provide a measure of force that can be detected by the spring. Force measures are applied using load cells by placing a platform containing a rat and a load cell and analyzing the spectral content of the entire system. Load cells can successfully measure the tremorogenic effect of drugs, as demonstrated by Gerhart, Hong, Uphouse, & Tilson (1982) who studied the tremorogenic effect of chlordecone on rats. Chlordecone is an insecticide that causes neurotoxicity. Gerhart et al. (1982) exposed male Fischer rats to various doses of chlordecone (0, 10.0, 50.0 or 100.0 mg/kg) to measure tremor. The animals were placed onto a platform (12.7 x 12.7 cm) sitting on a plexiglass chamber (15.9 x 16.5 x 30.5 cm); the platform was freely moving and attached to a 0.23 kg load cell transducer. The analog output generated by the transducer passed through a spectral analyzer that quantified tremor through FFT. Power was quantified by successive 2.5 Hz bands from 2.5 to 20 Hz over a 2.5 minute period.

The question, then, is if a load cell can successfully measure tremor, why did the Med Associates System fail to measure tremor at high frequencies? The Med Associates System differed from the load cell used in Gerhart et al. (1982) in two ways. First, a heavier 6 kg load cell was utilized by the system, versus the 0.23 kg load cell used in Gerhart et al. (1982). It is possible that the mass of the platform was too much to

quantify the tremorogenic effect of the drugs in this study. A less massive plate may have produced a better signal. Second, the rats used in Gerhart et al. (1982) were allowed to walk within a plexiglass chamber, whereas our guinea pigs were confined to stand in a plastic container (11.5 cm x 11.5 cm) with a suspended restraint chamber over their bodies that only allowed for postural movements. None of the cited research restrained the animals to measure tremor on a force plate actometer or a load cell (Gerhart et al. (1982);Wang & Fowler, (2001). By not allowing the animal to move freely, we may have prevented the transducer from properly reading the movement of the animal on the platform. Before beginning the study, we reasoned that if the guinea pig remained stationary over the center of the plate, the tremor would be fully quantified by the transducer. The fact that we did get a response means that the system picked up some activity, but whether restraining the animals interrupted the signal to the transducer is a question that can only be answered if the study is replicated with the animal being able to freely move on the plate.

Now that the Med Associates System has been discussed in great detail, it is necessary to discuss how the species of animals chosen for this experiment may have limited our ability to detect tremor and antagonize it. Unlike previous tremorogenic research, this experiment utilized guinea pigs, not rats to study tremor. Wang & Fowler (2001) showed tremor between 5 and 25 Hz in the physostigmine-exposed rats, and Gerhart et al. (1982) found peak amplitudes at 12 Hz after exposing rats to chlordecone. To our knowledge, this study was the first of its kind to measure tremor in the guinea pig using a load cell and spectral analysis. According to DeMar et al. (2010), the detoxifying enzyme levels of guinea pigs make them more useful than rats and mice for studying OP

intoxication. Like humans, their low carboxylesterase levels and relative levels of AChE make them advantageous in studying recovery from OP intoxication (Maxwell, Lieske, & Brecht, 1993). Perhaps this advantage does not conform to studying the tremorogenic effect of intoxicating substances. It may have been more advantageous for us to use rats on the Med Associates System to ensure that tremor could be detected before testing guinea pigs. Had we received a tremorogenic response from the rats and not the guinea pigs, we would have been able to conclude that guinea pigs do not display tremor in the same manner as rats and mice.

The next issue of discussion is the tremoregenic effect that 28.0 mg/kg of pro-2-PAM produced throughout these studies and our attempts to antagonize it. Unlike oxotremorine and physostigmine, the tremor produced by pro-2-PAM in Experiment 1 was not found to be significantly different from its citrate buffer control. Also, oxotremorine produced more tremor than pro-2-PAM in this study. From these results we concluded that pro-2-PAM's tremoregenic effect may not be as profound as that of oxotremorine, which is an established tremoregenic drug. Interestingly, citrate buffer produced just as much activity on the system as pro-2-PAM, making it look like a tremorogenic substance. In reality, however, citrate buffer and pro-2-PAM were highly acidic at an approximate pH of 3. The acidity of pro-2-PAM and citrate buffer were most likely uncomfortable for the guinea pig upon injection. The burning sensation of these substances probably caused the animals to groom. It is possible that the grooming activities of the guinea pigs were picked up by the Med Associates System and that more grooming activity than actual tremor was monitored. However, this does not mean that pro-2-PAM failed to produce tremor or that the centrally acting antagonists did not

reduce the tremoregenic effect. Had a force plate actometer or a lighter load-cell transducer been used in this study, we may have detected tremor at higher frequencies in Experiment 1, and antagonized tremor at lower frequencies in Experiment 2.

Turning back to the acidity of pro-2-PAM and citrate buffer, we found that one of the biggest problems encountered throughout this experiment was repeated dosing with these substances. Some of the animals in the experiment chewed their toes off, indicating that they lost their sense of feeling when they groomed. They probably groomed excessively due to the stinging sensation at the injection site after pro-2-PAM or citrate buffer exposure. The loss of sensation was probably due to hitting a nerve upon injection. In order to avoid the animals chewing, it may be necessary to conduct further experiments studying the tremoregenic effect of pro-2-PAM without repeated dosing. However, before our lab replicates this study, it is necessary to acquire a different system to detect tremor.

Although tremor may not have been fully measured by the Med Associates system, we showed that pro-2-PAM's mechanism of action is most likely cholinergic because the centrally acting oximes, atropine sulfate and scopolamine HBr, successfully antagonized the toxic effects of pro-2-PAM in Experiment 2. Unlike the centrally acting anticholinergics, the peripherally acting anticholinergics did not reduce the tremoregenic effect of pro-2-PAM. These results were expected because pro-2-PAM crosses the BBB. Therefore, anticholinergic drugs that enter the brain would have more of an antagonizing effect versus anticholinergic drugs that do not enter the brain. Although atropine sulfate and scopolamine HBr reduced the tremoregenic activity of pro-2-PAM, we were limited in this study because we did not employ cholinesterase testing. Without such testing, we

do not know the extent to which cholinesterase inhibition occurred, nor do we know how well the antagonists worked to restore AChE levels. We could have conducted a cholinesterase activity analysis by collecting blood from the guinea pigs using the toenail clip method before and after pro-2-PAM exposure (Vallejo-Freire, 1951, as cited in Shih et al. 2011). However, we could not employ the cholinesterase test because the toe nail clip causes discomfort for the animal and may have increased the tremoregenic response after exposure to pro-2-PAM and the antagonists. We were only interested in the tremoregenic effect produced by drug intoxication; conducting toe nail clips throughout the study would have confounded the results.

Comparing the effects of atropine sulfate and scopolamine HBr in reducing the tremor produced by pro-2-PAM, a significant effect of dose and an interaction of drug by dose was evident. Thus, the centrally acting anticholinergics worked to reduce the tremorogenic effect produced by pro-2-PAM. A significant between subjects effect also occurred, showing us that scopolamine HBr appeared to antagonize tremor more than atropine sulfate. According to Kenley et al. (1982), drug delivery rates for the various routes of administration decrease in the order of IV > IP > SC > IM, so that drugs administered IV reactivate the greatest percentage of AChE. Taking this into account, scopolamine HBr should have worked better than atropine sulfate to reduce tremor because SC injections provide greater bioavailability than IM injections, allowing more of the drug to enter the affected area. Since scopolamine HBr was injected SC and atropine sulfate injected IM, scopolamine HBr worked better than atropine sulfate to reduce the tremorogenic effect of pro-2-PAM. Also, the highest dose of each drug antagonized tremor the most, which indicated that the high doses selected were adequate.

It may have been more beneficial for us to choose one route of injection for both atropine sulfate and scopolamine HBr. By injecting both drugs SC, increasing the bioavailability of each drug, we may have received different results. Perhaps atropine sulfate would have worked just as well as scopolamine HBr in antagonizing the tremorogenic effect of pro-2-PAM.

Turning to the behavior of the animals, we did not observe the animals as their tremor was quantified. Behavioral observations were performed in Fowler et al. (2001) and Gerhart et al. (1982) so that the frequency of tremor quantified could be associated with the animals' movement. We decided not to observe the animals because we did not want the system to pick up additional noise. Because we did not watch the animals as their tremor was assessed, we could not see what kind of tremor they produced after exposure to pro-2-PAM and the antagonists. Because the room was too small to incorporate video cameras to assess the animals' movement, we treated three separate groups of guinea pigs with one of the following treatment regimens: (1) 28.0 or 32.0 mg/kg of pro-2-PAM alone; (2) a high dose of scopolamine HBr (1.0 mg/kg) 15 minutes before exposure to 28.0 mg/kg of pro-2-PAM, and (3) a high dose of atropine sulfate (10.0 mg/kg) 15 minutes before exposure to 32.0 mg/kg of pro-2-PAM. These animals were not placed on the Med Associates System, but were observed in their home cages for one hour post treatment. The results showed that 57% of the animals treated with either dose of pro-2-PAM survived one hour post exposure with signs of tremor and convulsions. The results also showed that 83% of the scopolamine HBr animals survived and 75% of the atropine sulfate animals survived with either no signs of tremor, fasciculations, or convulsions. Because the animals displayed signs of fasciculations,

tremor, and convulsions after pro-2-PAM exposure alone or pre-treatment with centrally acting antagonists, it can be concluded that the same kind of behavior occurred while the animals were restrained on the Med Associates system. However, by taking actual observations during tremor assessment, we would have been able to pin point the exact blocks of time in which the most activity occurred. This would have helped us assess whether the Med Associates System was accurate in detecting actual tremor.

Despite the limitations that occurred throughout these studies, Experiment 1 characterized tremor produced by oxotremorine, physostigmine, and pro-2-PAM, and Experiment 2 successfully antagonized the tremor produced by pro-2-PAM. Future studies should utilize a new load cell-transducer to fully quantify the tremoregenic effect of pro-2-PAM in guinea pigs, as well as incorporate cholinesterase assays to further understand pro-2-PAM's putative mechanism of action.

APPENDIX

Design of Tremor Characterization Experiment

Group	First	Second	Third	Fourth	Fifth	Animals
1	OXO	MINA	P2P	MID	PHYSO	4
2	PHYSO	OXO	MINA	P2P	MID	4
3	MID	PHYSO	OXO	MINA	P2P	4
4	P2P	MID	PHYSO	OXO	MINA	4
5	MINA	P2P	MID	PHYSO	OXO	4
					Total	20

Table 1: Oxotremorine (OXO), Physostigmine (PHYSO), Midazolam (MID), Pro-2-PAM (P2P), and MINA were presented in a Latin-square design across each of 5 treatment groups (n=4 guinea pigs) differing only with respect to order of drug presentation. For each drug, excluding oxotremorine and MINA, two doses were evaluated plus an appropriate vehicle control session.

Design of Antagonism Experiment: Pro-2-PAM

GP 1-6	GP 7-12	GP 13-18	GP 19-24
Atropine Sulfate	AMN	Scopolamine HBr	SMN
AMN	Atropine Sulfate	SMN	Scopolamine HBr

Table 2: A total of 24 animals received pro-2-PAM and a set of treatments. Guinea pigs one through six received pro-2-PAM to induce tremor and four different doses of atropine sulfate (across weeks) to antagonize the tremor. Upon completion of atropine sulfate assessment, an analogous assessment of atropine methyl nitrate (AMN) was conducted, concluding the participation of guinea pigs one through six. The next three groups of animals were treated similarly with a different order of presentation of the putative antagonists.

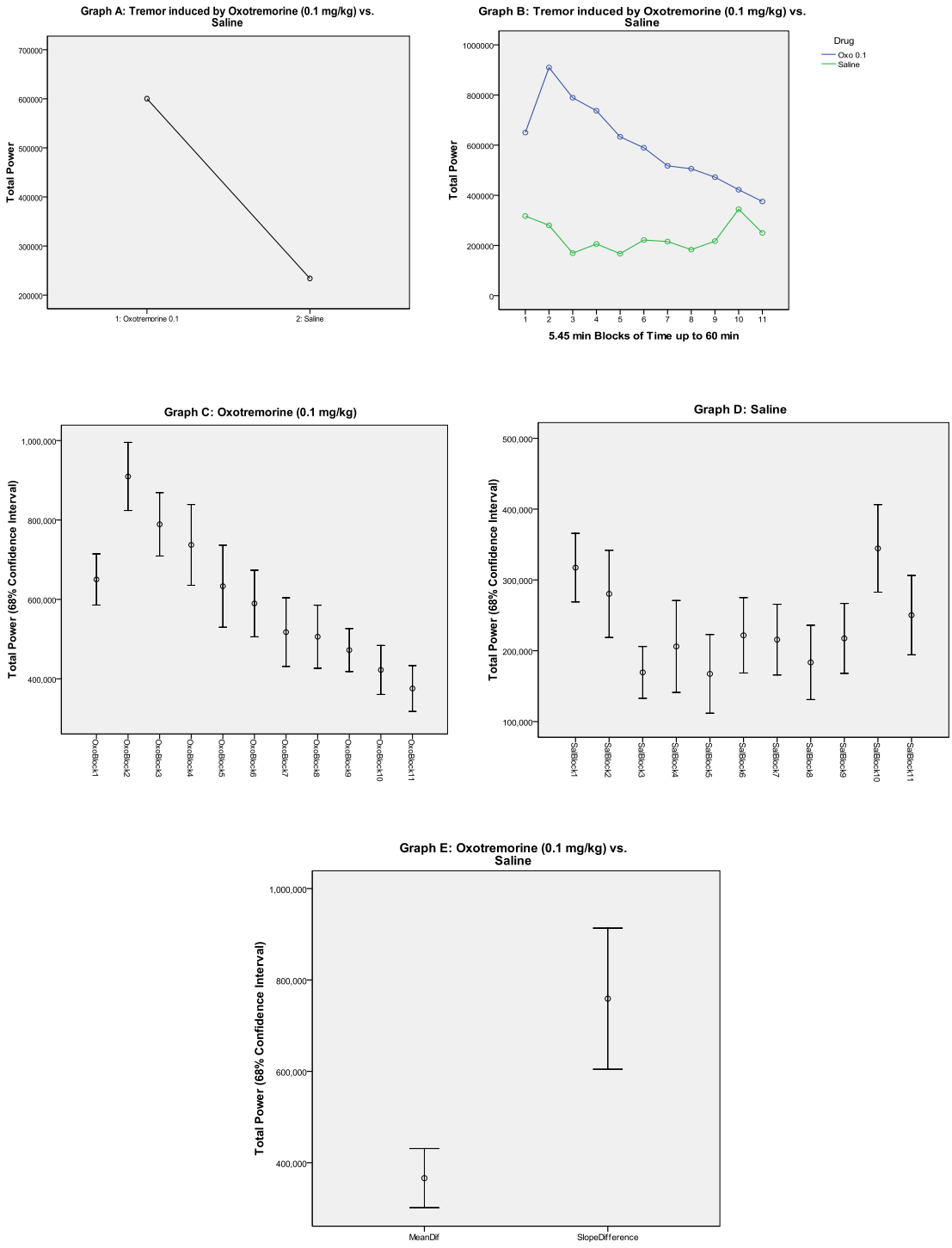
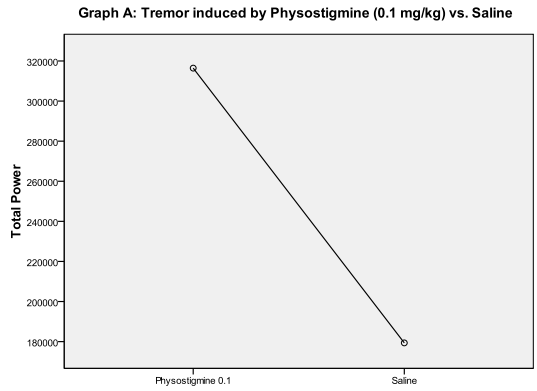
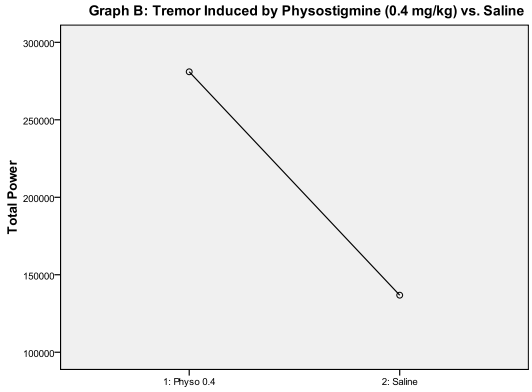
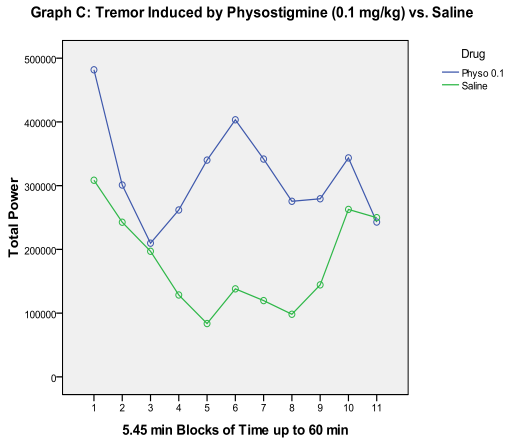


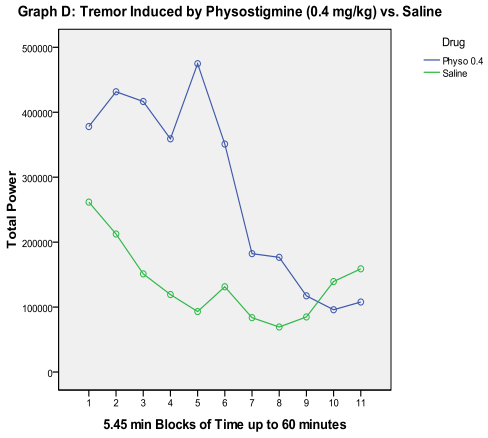
Figure 1: Graphs A and B show that 0.1 mg/kg of oxotremorine induced more tremor in the animals versus saline. The error bars associated with 0.1 mg/kg of oxotremorine and saline are shown in Graphs C and D, respectively. Graph E shows that the mean and

slope difference were significant, indicating there was a difference in the tremor produced by oxotremorine and saline.









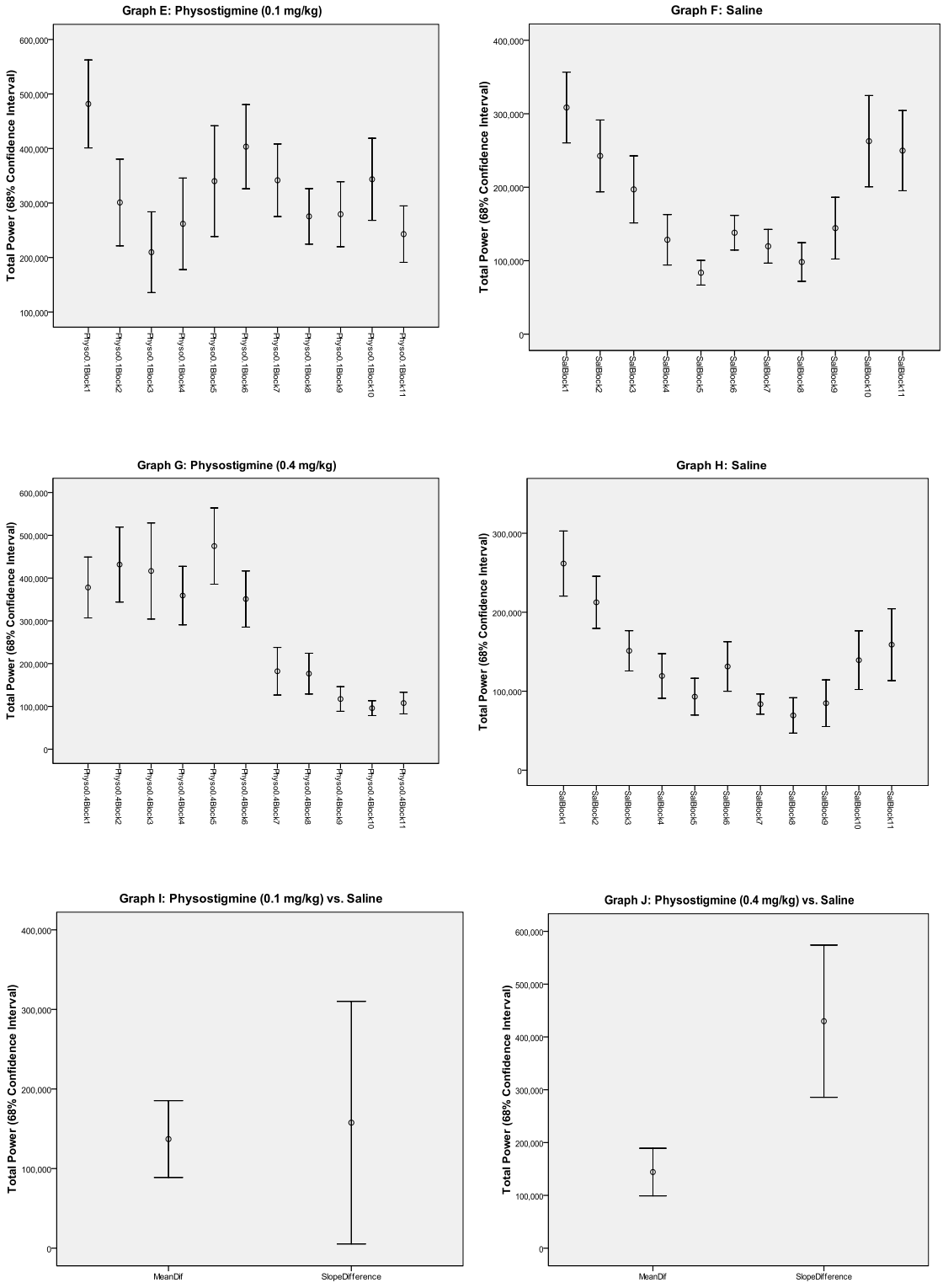


Figure 2: Graphs A and C show that 0.1 mg/kg of physostigmine induced more tremor in the animals versus saline; Graphs B and D show that 0.4 mg/kg of physostigmine induced

more tremor in the animals versus saline. The error bars associated with 0.1 mg/kg of physostigmine and its saline control are shown in Graphs E and F, respectively; the error bars associated with 0.4 mg/kg of physostigmine and its saline control are shown in Graphs G and H, respectively. Graph I shows that the mean difference was significant, but the slope difference was not significant between 0.1 mg/kg of physostigmine and its saline control. Because the mean difference was significant, there was a difference in the tremor produced by 0.1 mg/kg of physostigmine and its saline control. Graph J shows that the mean and slope difference were significant, indicating there was a difference in the tremor produced by 0.4 mg/kg of physostigmine and its saline control.

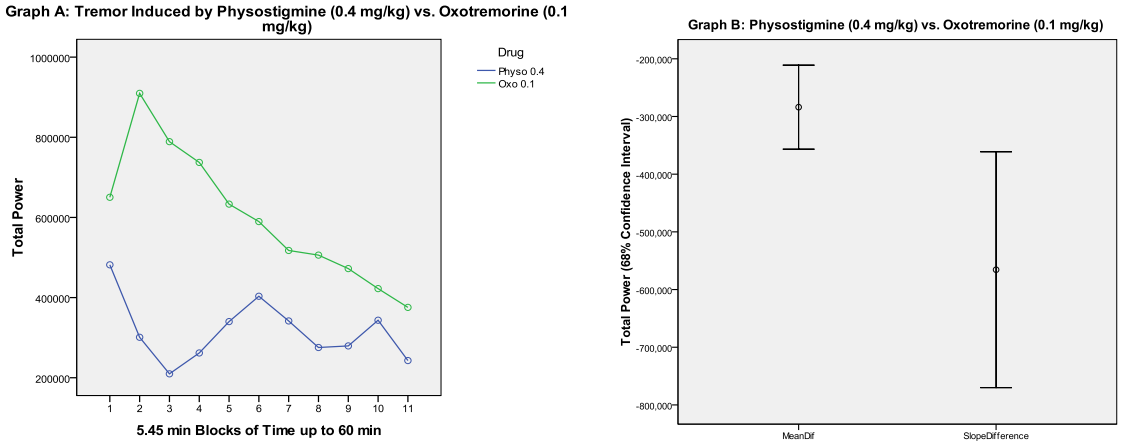


Figure 3: Graph A shows that 0.4 mg/kg of physostigmine induced more tremor than 0.1 mg/kg of oxotremorine. Graph B shows that there was a significant mean difference and slope difference between 0.4 mg/kg of physostigmine and 0.1 mg/kg of oxotremorine.

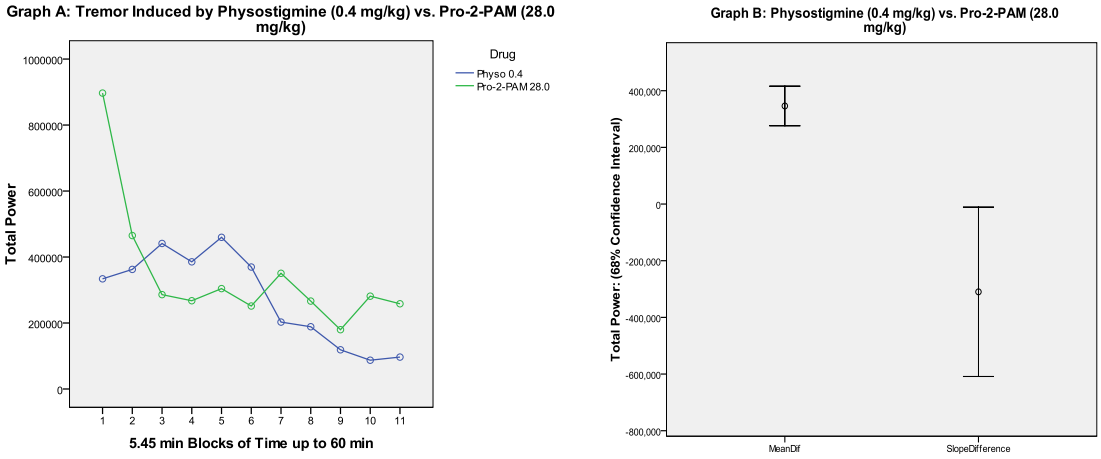


Figure 4: Graph A shows that there was no significant effect of drug in comparing 0.4 mg/kg of physostigmine and 28.0 mg/kg of pro-2-PAM, but there was a significant effect of block and a significant interaction of drug by block. Graph B shows that although there was no significant slope difference between 0.4 mg/kg of physostigmine and 28.0 mg/kg of pro-2-PAM, there was a significant mean difference between these drugs. However, it is impossible to tell which drug produced more tremor because no significant effect of drug occurred.

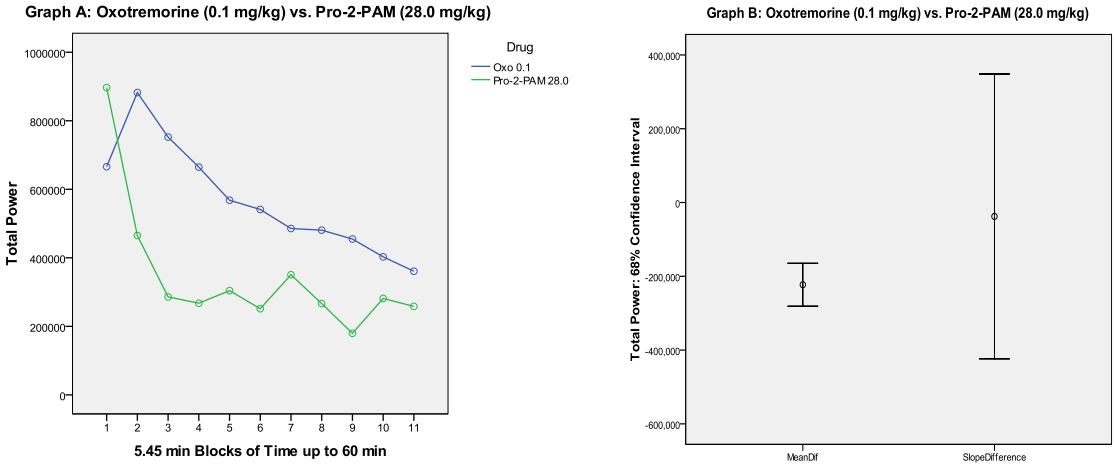


Figure 5: Graph A shows that 0.1 mg/kg of oxotremorine induced more tremor than 28.0 mg/kg of pro-2-PAM. Graph B shows that although there was no significant slope

difference between 0.1 mg/kg of oxotremorine and 28.0 mg/kg of pro-2-PAM, there was a significant mean difference between these drugs.

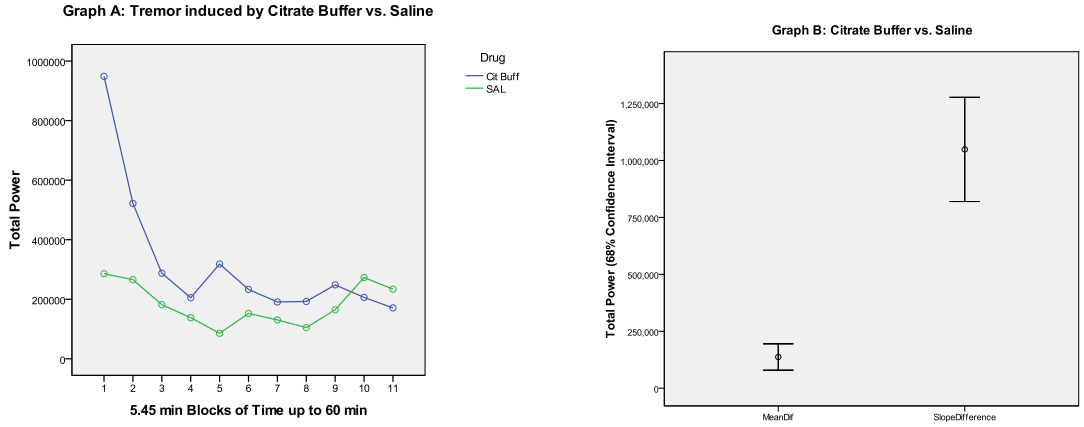


Figure 6: Graph A shows that citrate buffer induced more activity on the Med Associates System than saline. Graph B shows that a significant mean and slope difference occurred for both citrate buffer and saline.

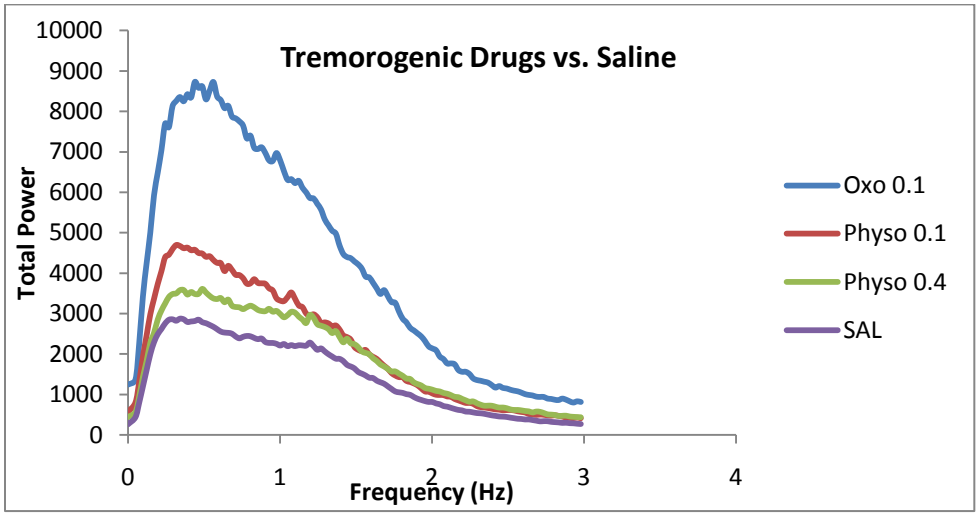


Figure 7: On average, oxotremorine had the highest peak amplitude over the 60 minute period versus both doses of physostigmine (0.1 and 0.4 mg/kg) and saline. For the tremoregenic drugs, tremor was prominent at approximately 0.5 Hz, fell at approximately 1.5 Hz, and was non-existent at 3 Hz.

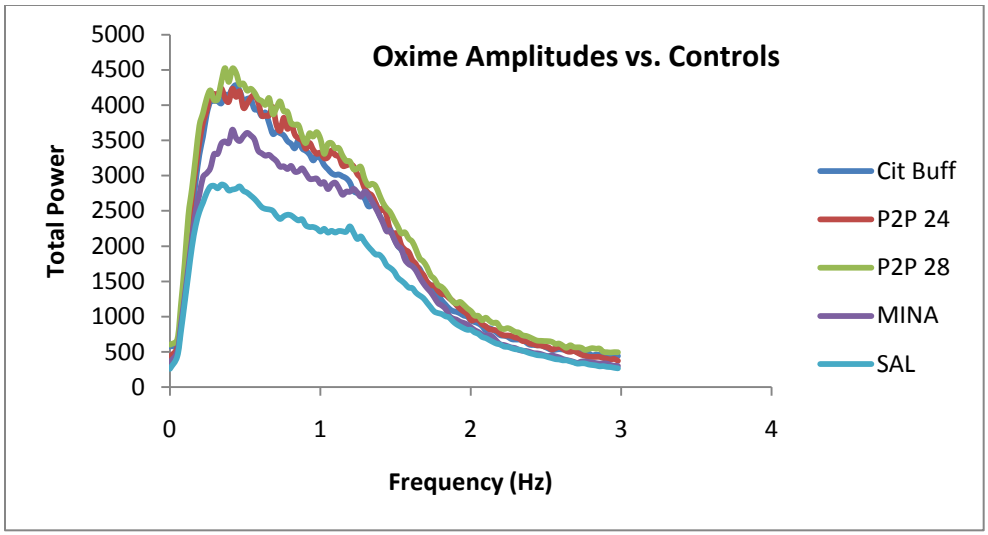


Figure 8: On average, citrate buffer induced more tremor than both doses of pro-2-PAM (24.0 and 28.0 mg/kg), and saline. There was no significant difference between the tremor produced by both doses of pro-2-PAM (24.0 and 28.0 mg/kg), even though the figure shows that 28.0 mg/kg produced more tremor than 24.0 mg/kg of pro-2-PAM. It also looks like both doses of pro-2-PAM produced more tremor than 200.0 mg/kg MINA, but this cannot be concluded because there was no significant difference between MINA and both doses of pro-2-PAM. On average, saline produced the least tremor. For the oximes, tremor was prominent at approximately 0.5 Hz, fell at approximately 1.5 Hz, and was non-existent at 3 Hz.

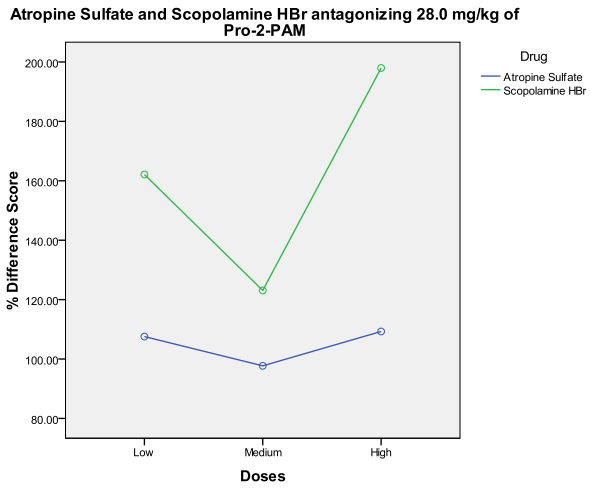


Figure 9: All doses of scopolamine HBr antagonized the tremor produced by 28.0 mg/kg of pro-2-PAM more than all doses of atropine. There was no significant difference between the atropine doses (low: 2.0 mg/kg; medium: 4.0 mg/kg; high: 10. mg/kg), but there was a significant difference between the high dose of scopolamine HBr (1.0 mg/kg) and the medium (0.3 mg/kg) and low (0.1 mg/kg) doses. Overall, the high dose of scopolamine HBr antagonized the tremor produced by 28.0 mg/kg of pro-2-PAM more than the low and medium doses of scopolamine HBr.

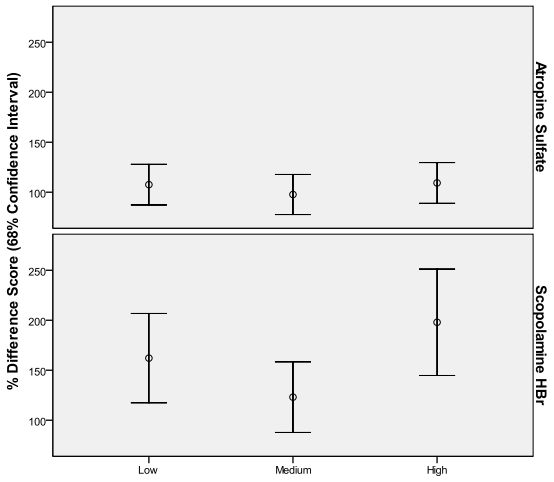


Figure 10: Different animals were exposed to each dose of atropine sulfate and scopolamine HBr, and a significant between subjects effect occurred. There was no

significant difference between the atropine doses (low: 2.0 mg/kg; medium: 4.0 mg/kg; high: 10.0 mg/kg), but there was a significant difference between the scopolamine hydrobromide doses (low: 0.1 mg/kg; medium: 0.3 mg/kg; high: 1.0 mg/kg). The high dose of scopolamine HBr antagonized the tremor produced by 28.0 mg/kg of pro-2-PAM more than the low and medium doses of scopolamine HBr.

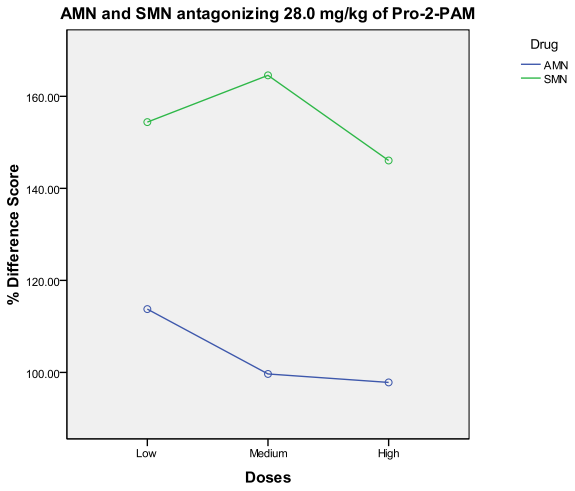


Figure 11: Different animals were exposed to each dose of AMN (low: 2.0 mg/kg; medium: 4.0 mg/kg; high: 10.0 mg/kg) and SMN (low: 0.1 mg/kg; medium: 0.3 mg/kg; high: 1.0 mg/kg), and a significant between subjects effect occurred. SMN appeared to antagonize the tremor produced by 28.0 mg/kg of pro-2-PAM more than AMN, but because no significant effect of drug occurred, this cannot be concluded.

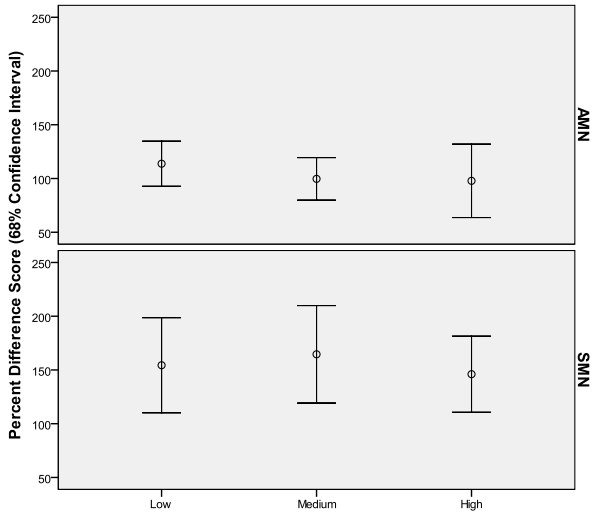


Figure 12: No significant effect of drug occurred when all doses of AMN (low: 2.0 mg/kg; medium: 4.0 mg/kg; high: 10.0 mg/kg) were compared to all doses of SMN (low: 0.1 mg/kg; medium: 0.3 mg/kg; high: 1.0 mg/kg). Thus, it cannot be concluded that either drug antagonized the tremorogenic effect of 28.0 mg/kg of pro-2-PAM.

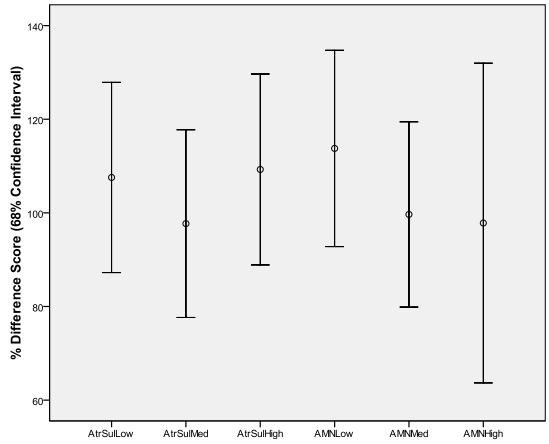


Figure 13: No significant effect of drug occurred for all doses of atropine sulfate (low: 2.0 mg/kg; medium: 4.0 mg/kg; high: 10.0 mg/kg) versus all doses of AMN (low: 2.0 mg/kg; medium: 4.0 mg/kg; high: 10.0 mg/kg). Thus, it cannot be concluded that either drug antagonized the tremorogenic effect of 28.0 mg/kg of pro-2-PAM.

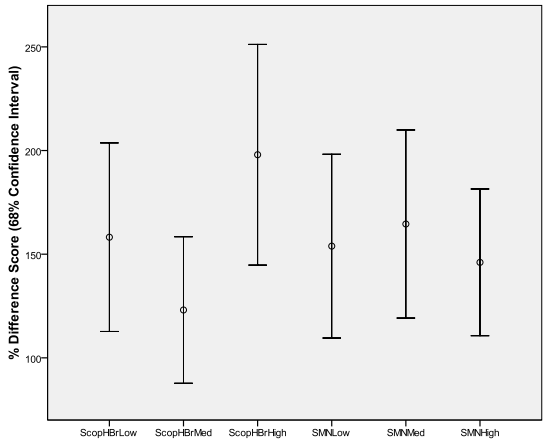
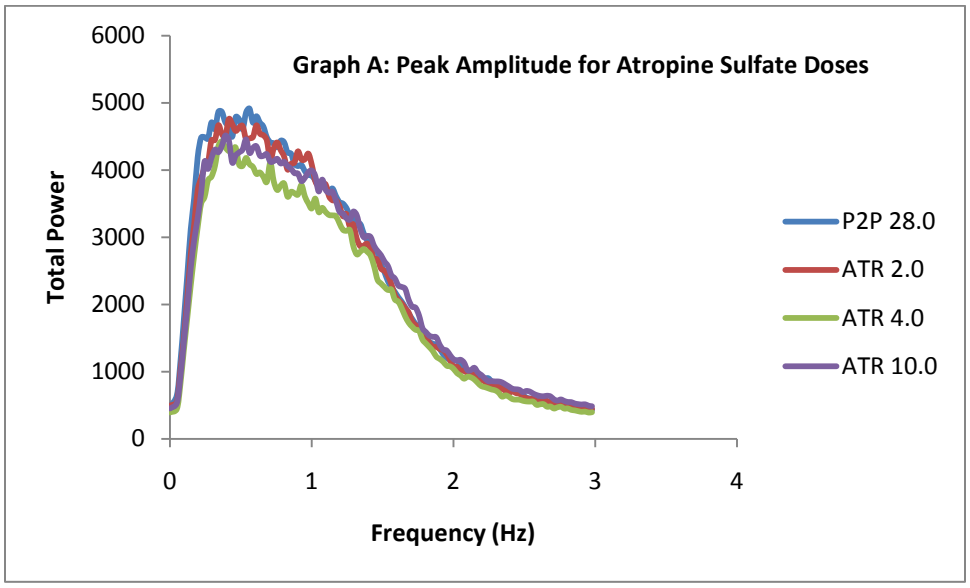


Figure 14: No significant effect of drug occurred for all doses of scopolamine HBr (low: 0.1; medium: 0.3; high: 1.0 mg/kg) versus SMN (low: 0.1; medium: 0.3; high: 1.0 mg/kg).



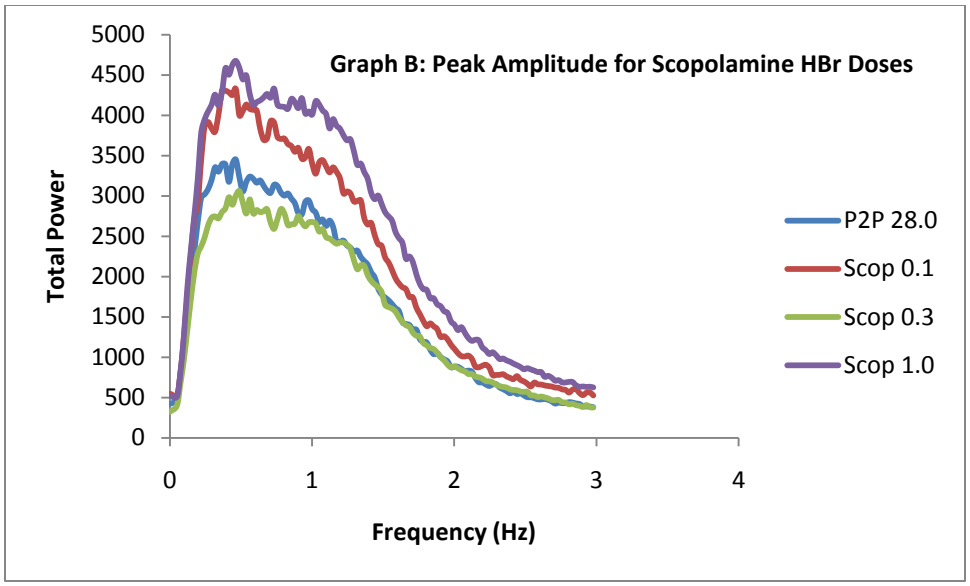
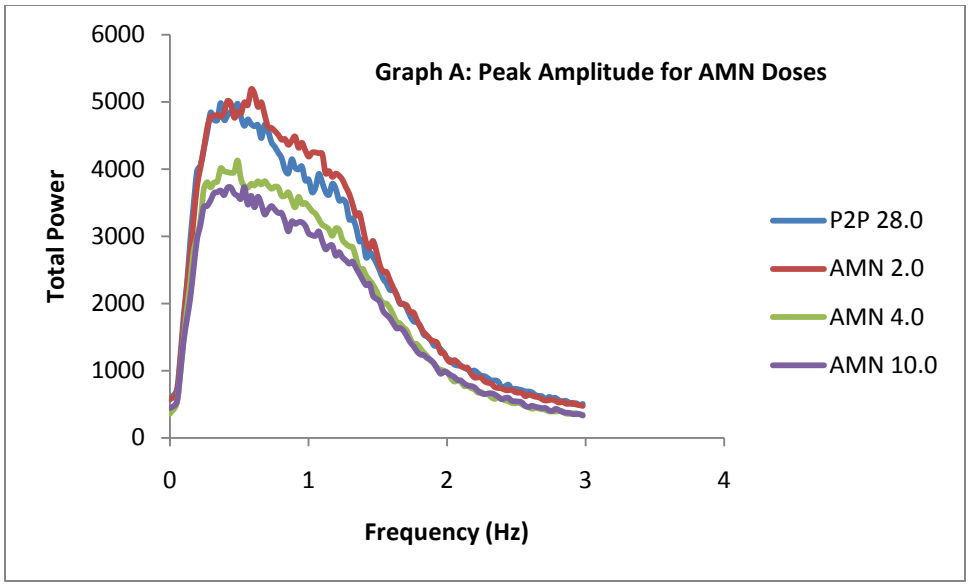


Figure 15: Graph A compares each dose of atropine sulfate in antagonizing 28.0 mg/kg of pro-2-PAM. The statistics showed that none of the atropine sulfate doses were significantly different from one another as seen by the overlapping amplitudes in Graph A. The most tremor occurred between 0 and 1 Hz, fell at 2 Hz, and was non-existent at 3 Hz. The statistics also indicated that 1.0 mg/kg of scopolamine HBr antagonized the tremor produced by 28.0 mg/kg of pro-2-PAM more than the other doses of scopolamine HBr (0.1 and 0.3 mg/kg), as seen in Graph B. Tremor appeared to occur between 0 and 1.5 Hz, fell at 1.5 Hz and was non-existent at 3 Hz.



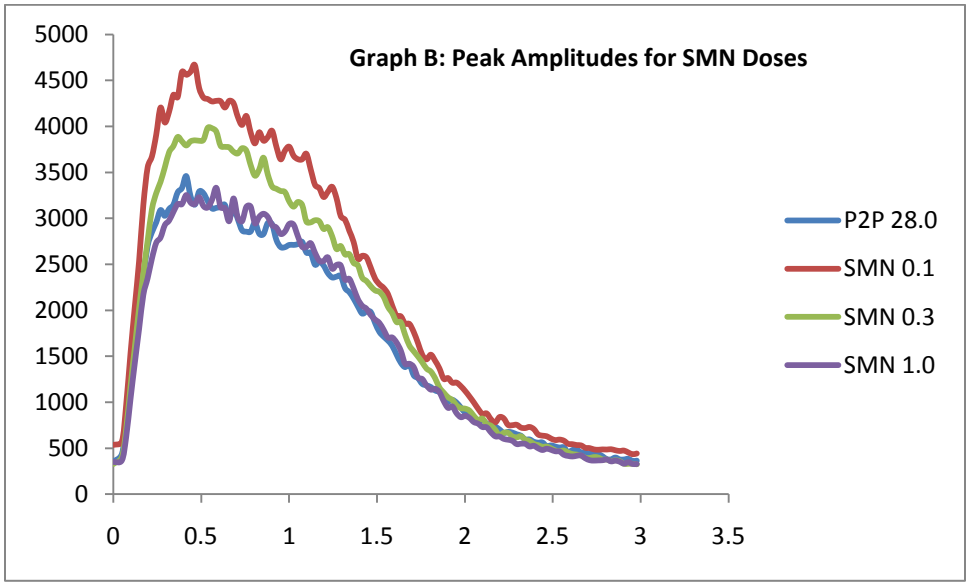


Figure 16: There was no significant difference between the AMN and SMN doses tested in this study. Graph A shows that for 28.0 mg/kg of pro-2-PAM, tremor occurred between 0 and 1.5 Hz, fell at 1.5 Hz, and was non-existent at 3 Hz. Similarly, Graph B shows that for 28.0 mg/kg of pro-2-PAM and the SMN doses working against pro-2-PAM, tremor occurred between 0 and 1.5 Hz, fell at 1.5 Hz, and was non-existent at 3 Hz.

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