LACK OF RESPIRATORY EFFECT OF NICOTINE APPLIED TO THE CAUDAL MEDULLARY RAPHÉ

by

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DEDICATION

Dedicated to my mother, Ms. Katrina Hicks,

whose words of encouragement inspired me to

continue the pursuit of my goals.

ACKNOWLEDGMENTS

I would like to first thank God for continuing to keep me focused and determined to reach my goal. Thanks to Dr. Bernard for continuing to work with me despite my persistent procrastination. Thank you for your patience and knowledge in helping me complete my research and thesis.

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To my friends - thank you so much for your continued support and not letting me quit and take the easy way out. I am always thankful to have friends like you.

Finally, saving the best for last, mom you know that I appreciate everything that you have done for me. Thank you for always being my rock and staying strong. Even when the storms kept on raging in my life, you kept me grounded and continued to pray. I love you and thank God for you everyday of my life. Thank you for guidance and support.

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ABSTRACT

LACK OF RESPIRATORY EFFECT OF NICOTINE APPLIED

TO THE CAUDAL MEDULLARY RAPHÉ

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Breathing is an involuntary and automatic process initiated by a network of neurons within the brainstem. These neurons are affected by a number of factors (mechanical, electrical and chemical) that help modulate the frequency and pattern of breathing. Three respiratory chemosensitive sites have traditionally been described on the ventral medullary surface. These sites are affected by the pH of the cerebrospinal fluid (CSF) bathing the brain and by chemicals dissolved within the CSF. More recently, additional sites have been described with chemosensitive activity and of these the medullary raphé has received the most attention.

Although the dangers of smoking are well known, people have continued with this habit. Of concern are women who smoke and of particular concern are pregnant women who continue to smoke during their pregnancy. This is of concern because of the many chemicals contained within

cigarette smoke, nicotine is particularly potent at affecting the health and well-being of the mother and fetus (and infant) because nicotine can enter the circulation within minutes of ingesting cigarette smoke. I am interested in how nicotine affects breathing. I therefore looked at the effects of nicotine on the caudal raphé, the raphé magnus.

Nicotine injected into the raphé magnus did not affect the animal's respiratory activity, even in pharmacological doses ($P > 0.05$). Neither respiratory frequency, amplitude, as well as inspiratory or expiratory time were affected. Administration of increasing levels of carbon dioxide before and after nicotine injections caused vigorous changes in respiratory activity. I tested the effectiveness of the drug by injecting it into a known nicotine-sensitive region (the caudal chemosensitive region) which resulted in respiratory stimulation. It appears that the raphé magnus is not responsive to nicotine. Immunohistomical studies should now be undertaken to determine if there are indeed nicotinic receptors at this location.

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CHAPTER 1

INTRODUCTION

1.1 Breathing in Mammals

Breathing is an involuntary activity that originates in the brain. Studies have shown that a network of respiratory-related neurons in the brainstem control inspiratory and expiratory movements. An important region in the brainstem containing several nuclei involved in respiratory control is the medulla oblongata. The medulla oblongata lies superior to the spinal cord, inferior to the pons and anterior to the cerebellum. It controls autonomic functions and relays nerve signals between the higher centers and the spinal cord. Functions of the medulla oblongata include relaying nerve messages from the forebrain to the spinal cord, processing inter-aural time differences for sound localization and controlling autonomic functions (such as the heart beating and breathing).

The ventral surface of the medulla oblongata contains some of the receptors responsible of detecting changes in the extracellular environment of the brain and adjusting breathing to maintain the pH within relatively narrow limits. These receptors have been localized to three areas on the ventral medullary surface which are also responsible for detecting changes in the PCO_2 of the cerebrospinal fluid bathing them as well as to changes in the pH of the blood perfusing these areas. It has been shown that an increase in PCO_2 and/or a decrease in pH lead to an increase in ventilation. Electrical stimulation of these areas also stimulates breathing (Brookhart, 1940; Fung and John,

1994). The mechanisms responsible for influencing the changes to these chemical stimulant are still not fully understood and research on these areas continue.

In addition to these superficial receptors, there are additional groups of cell bodies located deep within the brainstem that exert powerful influences on breathing. These neurons can be found in two groups of nuclei that lie within the medulla oblongata. They are the dorsal respiratory group (DRG) which contains inspiratory neurons (I neurons) and the ventral respiratory group (VRG) which contains neurons that control the muscles used for active expiration (E neurons) and for greater than normal inspiration (I^+ neurons). The I neurons control the external intercostal muscles and the diaphragm while the E neurons control the internal intercostal muscles, the scalenes and the sternocleidomastoid muscles. These neurons participate in a network that are thought to be the genesis for respiratory rhythm (Parks et al., 1989; Taylor and Lukowiak, 2000).

CO_2, and H^+ receptors create complicated synaptic interactions between neurons in the network which generates the rhythmic cycles of inspiration. Carbon dioxide is the most important chemical controller of ventilation and its concentration is controlled by central chemoreceptors located on the surface of the medulla oblongata. These chemoreceptors monitor cerebrospinal fluid (CSF) composition and respond to changes in the level of CO_2 in the CSF. H^+ levels are also well regulated via these central chemoreceptors ((Chitravanshi and Sapru, 1995; Hanson and Kumar, 1994).

Another region of the medulla oblongata that possess chemosensitive activity is the medullary raphé. It is exquisitely located along the midline of brainstem, extending from the pontine region, rostrally, to the spinal cord, caudally. The medullary raphé is divided into three areas - the

raphé magnus (RMa), the raphé pallidus (RPa) and the raphé obscurus (ROb) and all areas have demonstrated respiratory chemosensitivity. The raphé is also involved in the control of autonomic functions, which include respiration and blood pressure (Lovick, 1997). It possesses large amounts of serotonin containing neurons and is involved in sleep and wakefulness. It has been stated that neurons in the raphé begin to increase their activity just before you wake and slow their activity just before you fall asleep. These changes are also synchronous with the adjustments that occur with breathing during these periods.

1.2 Smoking and Breathing in Humans

Smoking has been a popular habit for a long time, even though the dangers of this activity have been well-documented. In the peripheral system, tar from the smoke can coat the alveoli of the lungs and increased the diffusion distance for O_2 and CO_2 to travel for gas exchange. The stickiness of the tar together with the loss of surfactant increases the work of breathing. This may lead to chronic obstructive pulmonary disease in which the lung has lost its elasticity and is not as compliant. The dust from the smoke can irritate the linings of the respiratory tree. In addition, the nicotine from the tobacco can render the cilia in the trachea ineffective, preventing them from moving small particulate matter out of the respiratory passages. Together, these disturbances lead to the traditional "smoker's cough" in which the chronic smoker frequently has to cough from the irritation and to remove particulate matter from the upper respiratory system (Hafström et al. 2005; Haglund 1993).

Nicotine can enter the blood within minutes after ingesting tobacco smoke and from there it can diffuse into the cerebrospinal fluid as well as enter the fetal circulation. This is of major

concern because there is a higher incidence of female smokers and hence an increase in the number of pregnant women who smoke. In effect, the fetus can be affected either directly from the mother's smoking or indirectly from second hand smoke (Barrantes, 1975; Hafström et al., 2005; Haglund, 1993; Milerad and Sundell, 1993).

1.3 Nicotine and Breathing in Humans

Nicotine is one of 4, 000 chemicals found in smoke from tobacco smoke. It is also one of the most active and well studied components of tobacco. Smokeless tobacco products such as snuff and chewing tobacco also contain many toxins as well as high levels of nicotine. Since nicotine was first identified in the early 1800's, it has been studied extensively and shown to have a number of complex and sometimes unpredictable effects on the brain and the body (Fewell and Smith, 1998). Nicotine has powerful pharmacologic effects (including increasing heart rate, heart stroke volume, and oxygen consumption by the heart muscle) as well as powerful psychodynamic effects (such as euphoria, increased alertness, and a sense of relaxation) (Fewell and Smith, 1998). When a person inhales cigarette smoke, the nicotine in the smoke is rapidly absorbed into the blood and starts affecting the brain within 7 seconds. The result is the release of the hormone adrenaline, the "fight or flight" hormone. Physically, adrenaline will increase a person's heart rate, blood pressure and restrict flow to the heart muscle. The smoker will experience rapid, shallow breathing. The nicotine molecule is very similar in shape to the neurotransmitter acetylcholine, which affects many bodily functions, including breathing, heart rate, learning and memory. Acetylcholine in turn also affects other neurotransmitters that have influence over appetite, mood, and memory. When nicotine gets into the brain, it attaches to nerve cells in places where acetylcholine would, creating the same

effects. In 1989, the U.S. Surgeon General issued a report that concluded that cigarettes and other forms of tobacco, such as cigars, pipe tobacco, and chewing tobacco, are addictive and that nicotine is the drug in tobacco that causes addiction.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Location of respiratory neurons in mammals

Three major neurological areas contribute to the generation of respiratory rhythm in mammals and all are located in the brainstem (Figure 2.1). The most rostral of the three major areas is the pontine respiratory group (PRG), which lies in the nucleus parabrachialis medialis and the Kölliker-Fuse nucleus of the dorsal lateral pons. This group of neurons receives afferent inputs from the vagal receptors related to lung volume and modulates respiratory frequency. The PRG is also thought to modulate the respiratory system's response to stimuli such as hypercapnia and hypoxia.

Another major neurological region with influence on respiration is the dorsal respiratory group (DRG), which lies bilaterally in the region of the nucleus tractus solitarius (NTS) in the dorsal medulla. It contains mainly inspiratory neurons, which project into the contralateral spinal cord (Levitzxy, 1999). The NTS is the primary site of termination for visceral afferent fibers of the ninth cranial nerve (glossopharyngeal) and the tenth cranial nerve (vagus). These nerves carry information about arterial Po_2, Pco_2, and pH from the carotid and aortic chemoreceptors, and information concerning the systemic arterial blood pressure from the carotid and aortic baroreceptors (Levitzxy, 1999). The DRG receives primary afferents from peripheral mechano and chemoreflexes and provides efferent connections to pre-motor neurons in the medulla and to spinal cord.

The third major region of respiratory neurons is a long column of cells, which lies in the ventral medulla, the ventral respiratory group (VRG). Expiratory neurons, many of which send efferents to motoneurons that control the resistance in the upper airways, are prominent most caudally in the VRG (Nattie 1999).

The system for generating the respiratory rhythm consists of respiratory neurons which are most dense in two specific areas of the pons and two regions of the medulla oblongata. Figure 2.1 shows the pons and medulla and the respiratory nuclei within each region. Neurons are situated adjacent to the midline and communicate with one another to form a neural networking which may be responsible for generating the respiratory rhythm.

Within the pons the cell bodies are situated in the nucleus parabrachialis medialis and Kölliker-Fuse nucleus. Together they form the pontine respiratory group (PRG). This region also contains the pneumotaxic center (Feldman, 1986; Lumsden, 1923). A second group of nuclei called the apneustic center is located just medial and caudal to the pneumotaxic center and is also intimately associated with respiration (Martini, 1998).

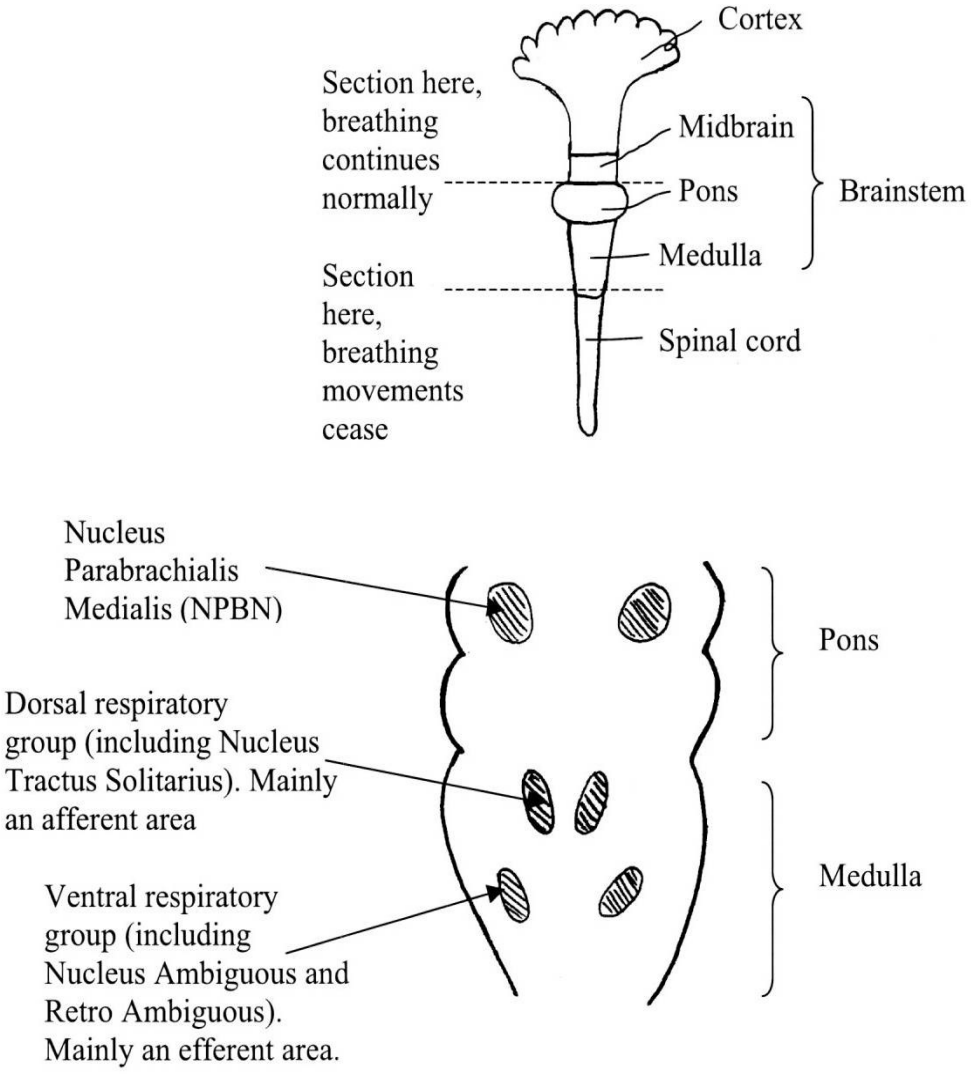

Figure 2.1: Respiratory nuclei in the pons and medulla sectioned at various levels in the brainstem of anaesthetized animals.

Within the medulla oblongata can also be found clusters of nuclei. The dorsal respiratory group (DRG) lie in the ventrolateral nucleus tractus solitarius (NTS) whereas the ventral respiratory group (VRG) lie within the nucleus retroambiguus of the ventrolateral medulla (Bianchi et al. 1995; Ramirez et al. 1998; Richter et al. 1997; J. Smith et al. 1989).

The pneumotaxic and apneustic centers are adjacent nuclei that attenuate the output of the respiratory rhythm complexes. Generally, the respiratory rate can be modified by these centers. In quiet respiration, the apneustic neurons are constantly stimulated and send projections to the DRG on the same side of the medulla, resulting in an increase in the intensity of inspiration over a period of two seconds. The apneustic center is then inhibited by signals from the pneumotaxic center ipsilaterally and this instigates passive or active exhalation. Thus, the apneustic center stimulates and prolongs inspiration while the pneumotaxic center inhibits inspiration and in so doing prevents overinflation of the lungs. The way to understand the impact of these connections is view an increase in pneumotaxic activity as increasing the frequency of respiration by decreasing the duration of inspiration, whereas a diminishing pneumotaxic output slows respiratory frequency but augments the depth of respiration because the apneustic centers are more active (Martini, 1998).

The DRG influences the inspiratory phase of respiration. It receives input from the apneustic center, as well as signals from chemoreceptors and baroreceptors. It also receives signals that travel from stretch receptors in the airways through the vagus nerve. The DRG then directs efferent signals to the phrenic motor nucleus which then relays the information to the phrenic nerve. The phrenic nerve innervates the external intercostal muscles and the diaphragm. Contraction of these muscles

cause the inspiratory phase of breathing. This network operates during all respiratory cycles, whether quiet or forced (Silverthorn, 1998).

The VRG is different in that it is inactive during normal quiet respiration. Instead, it modulates inspiration and expiration during forced respiration. This area consists of cell bodies that also conduct information to the phrenic motor nucleus. From the phrenic motor nucleus the information is relayed to the phrenic nerve which innervate other complementary respiratory muscles necessary for forceful exhalation and maximum inhalation (see Figure 2.1) (Martini, 1998).

2.2 Central Chemoreception

CO_2 sensitive central and peripheral chemoreceptors are thought to provide ongoing and rapid feedback to the brainstem respiratory control system concerning the levels of CO_2 in arterial blood and in the alveolar gas exchange spaces. In the brainstem, perfusion of arterial blood is from the ventral surface by penetrating vessels of the basilar artery and is varied in nature. CO_2 is rapidly hydrated in physiological solutions to carbonic acid, which dissociates into a proton and bicarbonate ions. Increases in CO_2 are rapidly associated with decreases in pH. In addition, CO_2 is extremely soluble in tissue and blood, which gives it a high diffusion capability. Thus, it seems that any site within the brainstem could supply easy and rapid access to blood CO_2 levels. Early researchers attempted to localize regions sensitive to CO_2 on or near the VSM by the direct application of acidic artificial CSF, resulting in the widely accepted description of the chemosensitive areas in adult cats (Figure 2). Efforts to reveal a widespread distribution of chemoreceptors in the brainstem included the use of small acetazolamide injections used as a probe to search the medullary regions. As a result, chemoreceptive sites have been located along the ventral medulla within 800μm of the

surface, near the NTS, near the locus coeruleus, dorsally located but without a known role in respiratory control, the medullary raphé , and the VRG region (Nattie, 1999), which supports the hypothesis of widespread central chemoreception.

Dr. Dee Silverthorn states in her book that the chemoreceptors are strategically associated with the arterial circulation. If the rate of carbon dioxide production by the cells exceeds the rate of carbon dioxide removal by the lungs, ventilation is intensified to match carbon dioxide removal to production.

Three respiratory chemoreceptive sites have been identified on the brainstem. Rostrally is area-M, caudally is area-L and between these two areas is area-S (Figure 2.1). Areas L and M are sensitive to changes in the chemical environment in which they are subjected whereas Area-S does not show much sensitivity.

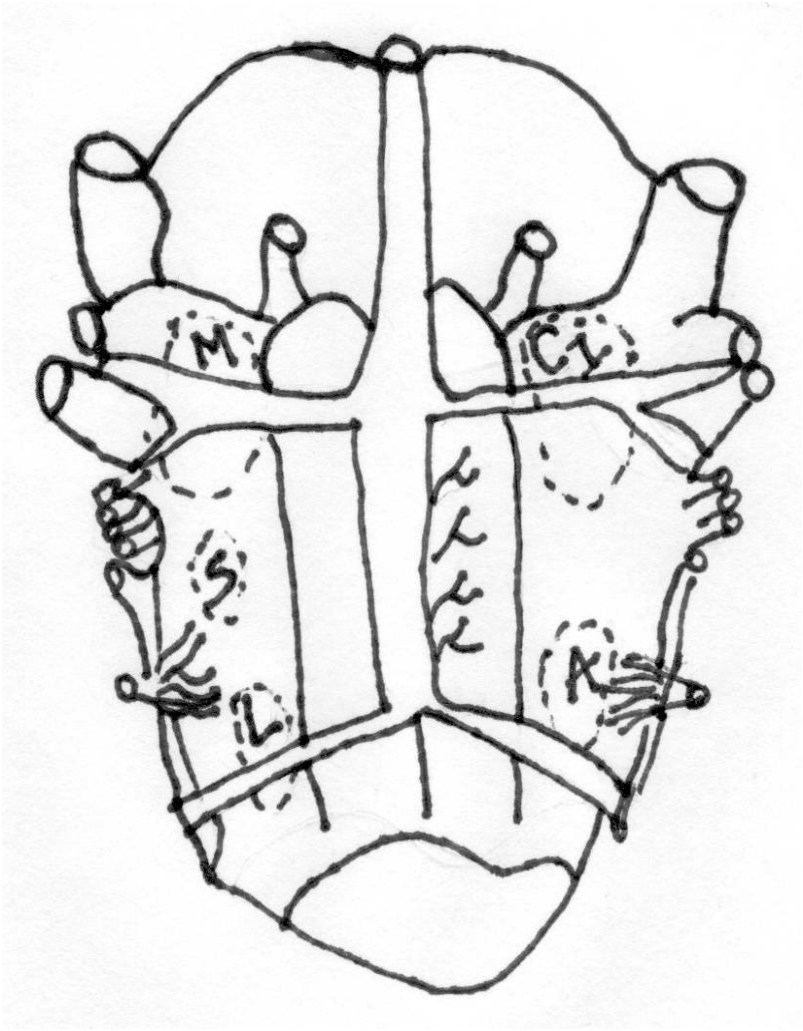

Figure 2.2: Diagram of the ventral brainstem of an adult rat showing the three chemosensitive regions. M, S and L refer to the superior, middle and caudal respiratory chemosensitive areas.

2.3 The Medullary Raphé

While the respiration centers and regions synchronize breathing, there are other regions of the medulla involved in respiration that have become of great interest to researchers. One of these regions is the medullary raphé. The medullary raphé complex consists of three major regions – the raphé magnus, the raphé obscurus, and the raphé pallidus. The raphé pallidus and obscurus are positioned in the posterior and anterior midline at the middle to rostral medullary sites. The raphé magnus is rostral to the pallidus and obscurus and extends rostrally into the caudal pons. The locations of these regions of the medullary raphé is shown in Figure 2.3.

Studies conducted on the medullary raphé revealed that serotonergic and non-serotonergic neural units located here are responsible for varied firing over the sleep/wake cycle (Heym et al. 1982). Chemical and electrical stimulation of these nuclei have also established the medullary raphé's role in homeostatic mechanisms such as cardiorespiratory processes, blood pressure (Adair et al., 1977; Bernard et al., 1996; Bernard, 1998; Cabot et al., 1979; Gereau IV and Conn, 1995; Haselton et al., 1988; Haxhiu et al., 1996; Richerson, 1995; Saxena and Villalon, 1990), blood circulation rate (Blessing and Nalivaiko, 1999), sensory and motor reactions (Jacobs and Fornal, 1997), nociception due to heat exposure (Leung and Mason, 1999) chemoreception (Bernard et al., 1996), thermoregulation (Berner et al., 1999; Morrison et al., 1999), and phrenic nerve responses (Bernard, 1998; Haxhiu et al., 1996). Since the raphé extends through the midline of the medulla, it is well positioned to coordinate and integrate varied sensory information, including respiration. Not studies to date have been conducted on the effects of nicotine in the medullary raphé.

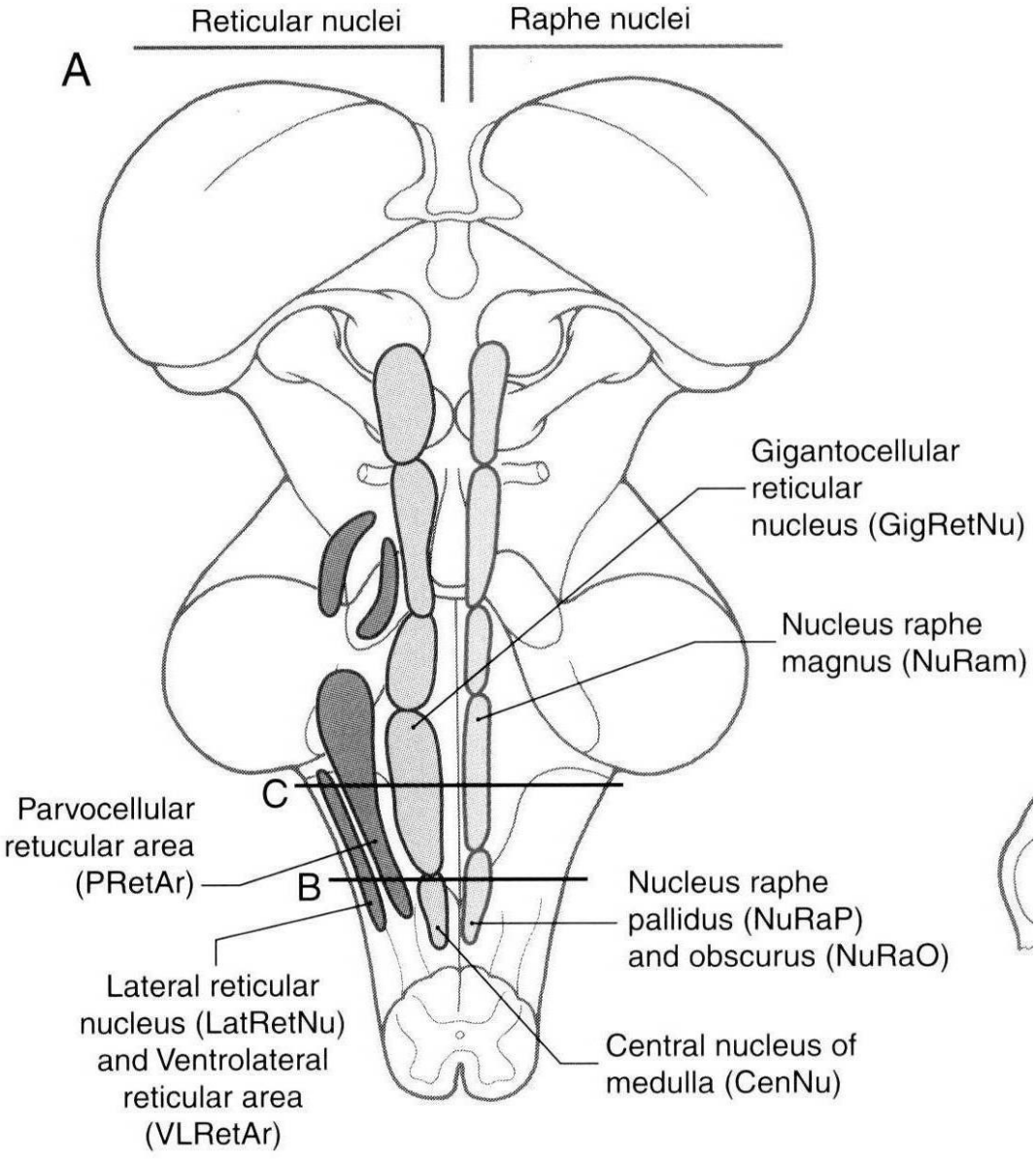

Figure 2.3: Medullary and reticular nuclei in the brainstem.

2.4 Brain-lung Interaction and Breathing

The lungs are located within your chest cavity inside the rib cage. They are made of spongy, elastic tissue that stretches and constricts as you breathe. The airways that bring air into the lungs (the trachea and bronchi) are made of smooth muscle and cartilage, allowing the airways to constrict and expand. The lungs and airways bring in fresh, oxygen-enriched air, and expel carbon dioxide made by your cells. They also help in regulating the concentration of hydrogen ion (pH) in your blood. When you inhale, the diaphragm and intercostal muscles contract and expand the chest cavity. This expansion lowers the pressure in the chest cavity below the outside air pressure. Air then flows in through the airways (from high pressure to low pressure) and inflates the lungs (Kandall and Gaines, 1991). When you exhale, the diaphragm and intercostal muscles relax and the chest cavity gets smaller. The decrease in volume of the cavity increases the pressure in the chest cavity above the outside air pressure. Air from the lungs (high pressure) then flows out of the airways to the outside air (low pressure). The cycle then repeats with each breath (Smith et al., 2003).

Many studies have proven that there are several other chemicals that affect breathing. Some of these chemicals are acetylcholine, serotonin, glutamate, caffeine, apomorphine, and nicotine (Kahn et al. 2002). Nicotine is a tertiary amine consisting of a pyridine and a pyrolidine ring. There are two stereoisomers of nicotine: (S)-nicotine is the active isomer which binds to nicotinic cholinergic receptors and is found in tobacco. During smoking, some racemisation takes place, and a small quantity of (R)-nicotine, a weak agonist of cholinergic receptors, is found in cigarette smoke. Of all the chemicals found in the smoke of tobacco products such as cigarettes and cigars, nicotine is the primary component in tobacco that acts on the brain. Smokeless tobacco products such as

snuff and chewing tobacco also contain many toxins as well as high levels of nicotine. Since nicotine was first identified in the early 1800's, it has been studied extensively and shown to have a number of complex and sometimes unpredictable effects on the brain and the body (Fewell and Smith, 1998). Nicotine has powerful pharmacologic effects (including increasing heart rate, heart stroke volume, and oxygen consumption by the heart muscle) as well as powerful psychodynamic effects (such as euphoria, increased alertness, and a sense of relaxation) (Fewell and Smith, 1998). Cigarette smoking is the most prevalent form of nicotine addiction in the United States. Most cigarettes in the U.S. market today contain 10 milligrams (mg) or more of nicotine. Through inhaling smoke, the average smoker takes in 1 to 2 mg of nicotine per cigarette. Nicotine is absorbed through the skin and mucosal lining of the mouth and nose or by the linings of the lungs. Depending on how tobacco is consumed, nicotine can reach peak levels in the bloodstream and brain rapidly. Cigarette smoke is composed of volatile and particulate matter. Some 500 gaseous compounds including nitrogen, carbon monoxide (CO), carbon dioxide (CO_2), ammonia, hydrogen cyanide and benzene have been identified in the volatile phase which accounts for about 95% of the weight of cigarette smoke; the other 5% is accounted for by particulates. There are about 3,500 different compounds in the particulate phase, of which the major one is the alkaloid, nicotine. Other alkaloids include nicotine, anatabine and anabasine 37. The particulate matter without its alkaloid and water content is called tar. Many carcinogens, including polynuclear aromatic hydrocarbons, N-nitrosamines and aromatic amines, have been identified in cigarette tar (Neff et al., 2004). Cigarette smoking, for example, results in the rapid distribution of nicotine throughout the body and can reach the brain within 10 seconds of inhalation (Fewell and Smith, 1998).

If a woman is pregnant and smokes the nicotine will easily cross the placenta and will be found in the fetal cord blood in concentrations equal to or greater than that in maternal blood. Nicotine in the womb may slow or even stop the firing of respiratory nerves that triggers breathing (Fewell and Smith, 1998). The American Lung Association has stated that smoking during pregnancy is estimated to account for 20 to 30 percent of low-birth weight babies, up to 14 percent of preterm deliveries, and some 10 percent of all infant deaths (Fewell and Smith, 1998). Smoking during pregnancy causes as many as 141,000 tobacco induced miscarriages, 61,000 low-birth weight infants, 26,000 infant admissions to neonatal intensive care units, and 2,200 deaths from SIDS annually. Sudden Infant Death Syndrome (SIDS) is the sudden death of an infant under 1 year of age which remains unexplained after a thorough case investigation including performance of a complete autopsy, examination of the death scene, and review of the clinical history (Iback and Stalhandske, 2003). During a convening of expert panelists by the National Institute of Child Health and Human Development in 2002 it was stated that SIDS was a major cause of death of infants from 1 month to 1 year of age, with most deaths occurring between 2 and 4 months (Iback and Stalhandske, 2003). SIDS is the third ranking cause of death between one month and one year of age. Sudden infant death syndrome kills an estimated 4,500 babies in the United States each year. Nearly 90% of the deaths occur in the first six months of life, most of them in cold-weather months. Native American and African American infants were 2 to 3 times more likely to die from SIDS than white infants. SIDS is thought to occur during sleep or during the transition from sleep to wakefulness (Iback and Stalhandske, 2003). While boys are affected more than girls, risk factors include babies who sleep on their stomachs (up to 4 months of age), soft bedding in the crib (up to

1yr of age), premature births, a history of a sibling who had SIDS, illegal drug use in mom, teen mother, short intervals during pregnancies, later prenatal care, poverty, and smoking (Iback and Stalhandske, 2003). Babies whose mothers smoked during pregnancy were five times more likely to die from sudden infant death syndrome than babies of mothers who did not smoke. Infants whose mothers smoke can develop hypoxia and ischemia. Hypoxia is the deficiency in the amount of oxygen reaching body tissues and ischemia is a decrease in the blood supply to a body organ, tissue, or part caused by constriction or obstruction of the blood vessels (Roy and Sabherwal 1994). Smoking during pregnancy is a major risk factor for SIDS and can also be related to respiratory tract infections. Infants that succumb to SIDS typically experience severe bradycardia that precedes or is accompanied by a centrally mediated life-threatening apnea (Bozarth et al., 1998). Dr. Peter Fleming of the University of Bristol said that every hour spent each day in a room where people smoke, the risk increases 100%. If an infant spends four hours per day in such a room, he or she is four times as likely to die of SIDS as a child not exposed to tobacco smoke. Infants breathing the smoke of others in the same house were found to be 31/2 times more likely to die of SIDS than youngsters not exposed to smoke in their homes (Bozarth et al., 1998).

The respiratory system is a way that smokers have been infusing nicotine into their bodies for millions of years. In tobacco smoke nicotine has been studied and found to be very addictive. When a woman who is pregnant smokes the nicotine gets into her blood and is transferred to the placenta which then finds itself in the fetus. The nicotine is now in the blood of the fetus which then travels in the cerebrospinal fluid it enters into the brain tissue and takes affect. With so many cases of SIDS arising many wonder if this may be affected by nicotine. Studies have shown that centrally

injected nicotine has been proven to initiate cardiovascular response, via central nicotinic mechanisms (Hellsrtöm-Lindahl and Court, 2000; Zhang et al., 1994). These are the regions in which nicotinic receptors, responsible for the cardiovascular responses, are located have not been fully investigated. Research done by Kubo and Misu has indicated that injections of nicotine on the cisterna magna produced an initial rise in blood pressure, followed by a fall, in rats (Kubo and Misu 1981). I feel that by studying nicotine on the portion of the brain that is related to respiration, blood pressure and the medulla oblongata, I plan to see if this is a possible effect.

Table 2.1: Effect of selected drugs on respiratory activity

Drug	Concentration	Excitatory/ Inhibitory	Exogenous/ Endogenous
Nicotine	62mM	Excitatory	Exogenous
Caffeine	0.05 ml/10 g	Excitatory	Exogenous
Acetylcholine	11 mM	Excitatory	Endogenous
Apomorphine	0.1-0.4 mg/kg	Inhibitory	Exogenous
Glutamate	10,100,1000 mM	Excitatory	Endogenous

CHAPTER 3

SPECIFIC AIMS

The goal of this research was to determine if nicotine, when injected into raphé magnus, stimulated breathing in Sprague-Dawley rats. This was determined by micro-injecting different concentrations of nicotine into the medullary raphé.

This research focused on the hypothesis that the injection of increasing concentrations of nicotine in the medulla raphé will stimulate breathing. In order to accomplish the aims of this study, the effects of the increasing concentrations of nicotine injections into the medulla raphé of the anesthetized, unidirectionally, ventilated, catherized rats instrumented to measure phrenic nerve activity (an index of the animals' breathing), expired carbon dioxide and blood pressure were investigated.

CHAPTER 4

MATERIALS AMD METHODS

4.1 Animal Acquisition and Care

Adult male Sprague Dawley rats were obtained from the UTA animal care facility. Rats were housed in The University of Texas at Arlington animal facilities room; 3 rats per cage and had access to food and water ad lib. The light/dark cycle was 12/12 and the humidity in the room was kept at 40%.

4.2 Surgical Procedure

Adult male Sprague Dawley rats (300-450g) were used in all experiments. Rats were anesthetized with isofluorane 2-3% in oxygen before cannulating the femoral artery and vein for blood pressure measurement and drug injections, respectively. To do this an incision was made along the medial surface of the hind leg. The outer lying muscles were carefully separated to expose both artery and vein. A small incision was made in the artery and the vein, into which the catheters were inserted into both. After securing the catheters with sutures, an initial dose of 5mL of buffered chloralose and urethane solution was introduced to the rat. This mixture was used to replace the isofluorane. Additional anesthetic was given to the animal during the experiment as needed. The arterial catheter was connected to a blood transducer. Body temperature was maintained at 37 ± 0.5 $^{\circ}$C, using a heating pad and a heating lamp controlled by a temperature indicating controller (YSI Indicating Controller, Model 73A) that measured the body temperature via a rectal probe. The

trachea was cannulated and the animal was artificially ventilated (Columbus Instrument, Columbus, OH) with 100% O_2 to maintain an end-expired carbon dioxide (P_ECO_2) concentration of ≈ 5%. Bilateral thoracotomies and vagatomies were also performed to allow the lungs to expand freely in the thoracic cavity and to prevent feedback from peripheral receptors, respectively.

The dorsal surface of the brainstem was exposed by a craniotomy. To expose brainstem, the head was first secured in a stereotaxic unit (Stoelting, Wood Dale, IL). The tissue and the bones covering the brain were removed. The incision was closely monitored for any bleeding. Bleeding was controlled using cotton-tipped applicators, bone wax, gel foam, and cautery.

The phrenic nerve was exposed and the central cut end placed on bipolar silver electrodes and kept from drying by insulating and protecting it with Wacker sil-gel. The end-tidal CO_2 from the trachea was sampled and measured with a CO_2 analyzer (IITC Life Science, Capstar 100, Ardmore, PA), and manually adjusted by changing the rate and volume of the ventilator. Saline was infused through the arterial catheter helped prevent blood from clotting in the catheter (0.01ml/min) via a persistalic pump. Blood pressure pulses, CO_2 levels, raw and integrated PNA ran through a PCM recording adapter (A.R. Vetter, Model 3000A, Rebersburg, PA) and stored on VCR tapes for later analysis. The signals were also displayed on a computer monitor using a data acquisition system (Dataware Technologies, Longmont, CO).

4.3 Experimental Setup

After surgery, the animal was instrumented to continue with the experiment. The head of the animal was stabilized in a stereotaxic apparatus. This same apparatus was used to record and position the pipette to make the injections.

End expired carbon dioxide was measured by sampling the air the animal breathe via the tracheal cannula. Blood pressure was measured via a catheter inserted into the femoral artery. The catheter was kept open by infusing small amounts of normal buffered saline into the blood. Phrenic nerve activity was recorded by placing the central cut end of the phrenic nerve on bipolar silver electrodes. The signal from the nerve was amplified, filtered, full-wave rectified and displayed on a computer monitor. Both raw and modified signals were recorded for later use.

Rectal temperature of the animal was kept constant via a heating pad on its dorsal surface and by a heating lamp on its ventral surface. Heating lamp was controlled via a rectal probe. The animal was continuously monitored for signs of consciousness and pain. Additional doses of anesthetic were given to prevent the animal from waking up. These were so noted on the recordings.

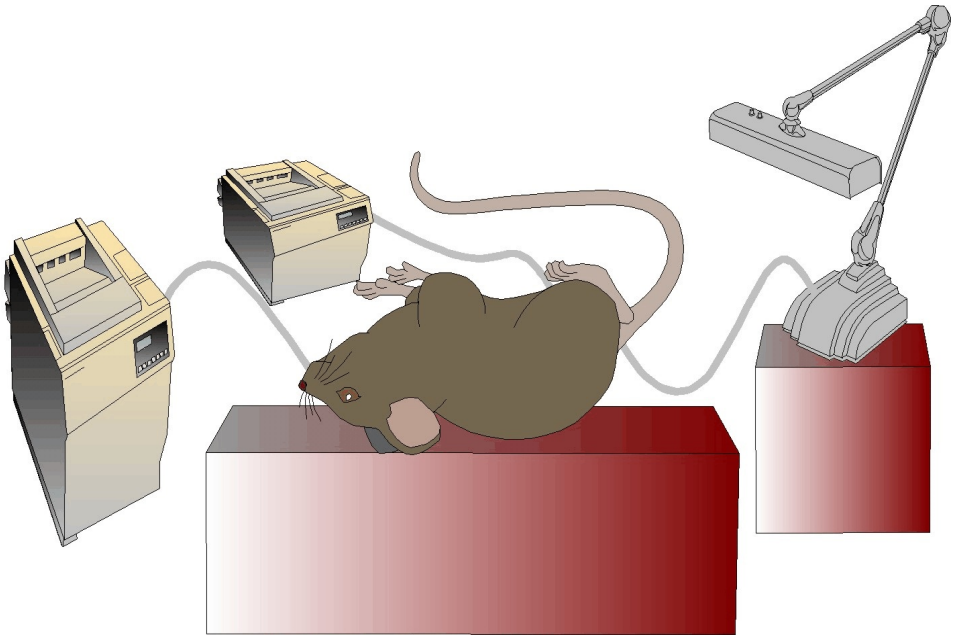

Figure 4.1: Diagram of experimental setup for rats.

4.4 Experimental Protocol

Each animal served as its own control. Recordings of the initial control measurements for blood pressure and phernic nerve activity were made for at least 10 minutes before any injections were made. Injections of mock cerebrospinal fluid (mCSF) or nicotine (Sigma, St. Louis, MO) were made using a picrospritzer (General Valve, Fairfield, NJ) and a double barreled pipette. The pipette was pulled to a tip diameter of approximately 10 μm using a Flaming/Brown Micropipette Puller (Sutter Instrument, Model P-87). The injection volume was calculated by measuring the diameter of the spherical droplet ejected using the equation for a sphere. A micromanipulator was used to position the pipette tip for all injections, which were made at a depth of 1.0 to 2.0mm below the surface and close to the midline of the ventral surface of the medulla starting at the intersection of the basilar and vertebral arteries and proceeding rostral in 1mm increments. Each experimental animal received at least two micro-injections of mCSF and/or nicotine. The first injection was 50-100nl of mCSF and the second injection was 50-100nl of nicotine. After each micro-injection cardiorespiratory activities were recorded for at least 30 minutes. The injections for the dose response curve, (0.0mM, 10mM, 100mM, and 1000mM nicotine), were made 1mm rostral of the intersection of the basilar and vertebral arteries and 1.5mm deep.

All animals were tested for their responsiveness to CO_2 before, after and sometimes during the above mentioned procedure. All animals responded to CO_2. We did not make injections within 5 minutes of administering additional anesthetic.

4.5 Microinjections

Injections of mock cerebrospinal fluid (mCSF) were made using a picrospritzer (General Valve, Fairfield, NJ) and a double barreled pipette. The pipette was pulled to a tip diameter of ~ 10 μm using a Narishige Scientific Instrument (Tokyo, Japan). Mock CSF was made fresh before each experiment. The composition (mM), consisted of NaCl -126.0, KCl -3.0, $MgCl_{2-}$ 0.8, $NaHCO_3^-$ - 26.2, and dextrose- 2.6. The mCSF was bubbled with 5% CO_2 in air to help maintain the pH of 7.4 and kept at 37˚C before the addition of calcium chloride (1.3mM). Three concentrations of nicotine were used (10, 100, 1000 mM) by dissolving the appropriate quantities in mCSF. This solution was bubbled like the mCSF with 5% CO_2 to again maintain a pH level of 7.4. Two injections of different solutions with microspheres (Polysciences, Warrington, PA) were made at each injection site. Different colored microspheres were put into each solution which enabled the injection site to be viewed under a fluorescence microscope for verification of the injection and to calculate the injection volume.

The volume of the injections were calculated by measuring the diameter of the spherical droplet ejected using the mathematical equation of a sphere. The other method used for volume estimation was to measure the area containing the fluorescent beads in each brain section then multiplying by 20 μm to determine the actual volume. Rats were routinely tested for their responsiveness to inspired CO_2 by increasing the end-tidal P_{CO2} (PET_{CO2}) from 35 to 63 Torr in 7 Torr increments. The end-tidal CO_2 was held constant for 3 minutes after each level. Before any injection was made into the medullary raphé the rat was given at least 30 min to rest after the CO_2 test.

A micromanipulator was used to position the pipette tip at the intersection of the basilar and vertebral arteries and was then used as a visual reference for determining the injection sites. Two or more injections were made in the raphe magnus. If the rat responded to the first injection, then both blood pressure and phrenic nerve activity were allowed to return to baseline before a second injection was made. If there was no response after 5 min had elapsed another injection was made at the same location and at least 10 min was allowed to elapse before an injection was made at a different location. A non-respiratory site that was chosen to be injected as another control, was the olivary nucleus. This site was chose because it is in close proximity to the raphé and it does not control any respiratory activity.

4.6 Morphology

At the ending of each experiment the rat was given an overdose of anesthetic followed by an injection of saturated KCL. The brainstem was carefully removed and placed in a cryostat (Leica, CM 1800) at - 25˚C for 10 hr before being sectioned into 20 μm slices and placed on glass slides for observation. Sections were examined with a fluorescence microscope (Olympus, BH-2) to determine the locations of the microspheres. Slides with the largest injection area were stained with cresyl violet (Sigma-Aldrich,) and compared to the atlas of Paxinos and Watson (Paxinos and Watson, 1986) to determine the injection site more precisely.

Table 4.1: Composition on mock cerebrospinal fluid

Constituent	Moles/Liter
NaCl	1.220
KCl	0.050
$MgSO_4$	0.055
CaCl	0.065
$NaHCO_3$	0.210

4.7 Data Analysis

Data were collected in 5 minute bins, and the bins for at least the control, maximal and minimal values were used for analysis. Variables presented here are as follows: mean arterial blood pressure (MABP), heart rate (HR), lung pressure, bout duration, and episodic frequency. The MABP and HR were calculated from the real time arterial pressure tracings. The lung pressure, bout duration, and episodic frequency were all calculated from the lung pressure tracings. A ONE WAY ANOVA and a student's T-test were used to determine if any variable changed significantly. All values are presented as mean ± S.E.M. and were considered significant at the $P < 0.05$ level.

CHAPTER 5

RESULTS

Microinjections were made in the medullary raphé and on the caudal chemosensitive region. The pipettes were tested before and after each injection to determine the volume of fluid injected. Phrenic nerve activity and blood pressure were monitored and recorded.

5.1 Effect of Carbon Dioxide on Phrenic Nerve Activity

Rats were tested for their responsiveness to increasing levels of inspired carbon dioxide before, after and sometimes during an injection. A representative response curve is displayed below in Figure 5.1 where phrenic nerve activity is shown in the upper panel and inspired CO_2 levels are shown in the lower panel. In this experiment during the control period the rat inspired 4% CO_2. As the level of inspired CO_2 was increased to 6, 8 and 9%, the amplitude of the PNA increased as well. All rats used for this report showed a similar response profile to increasing levels of CO_2.

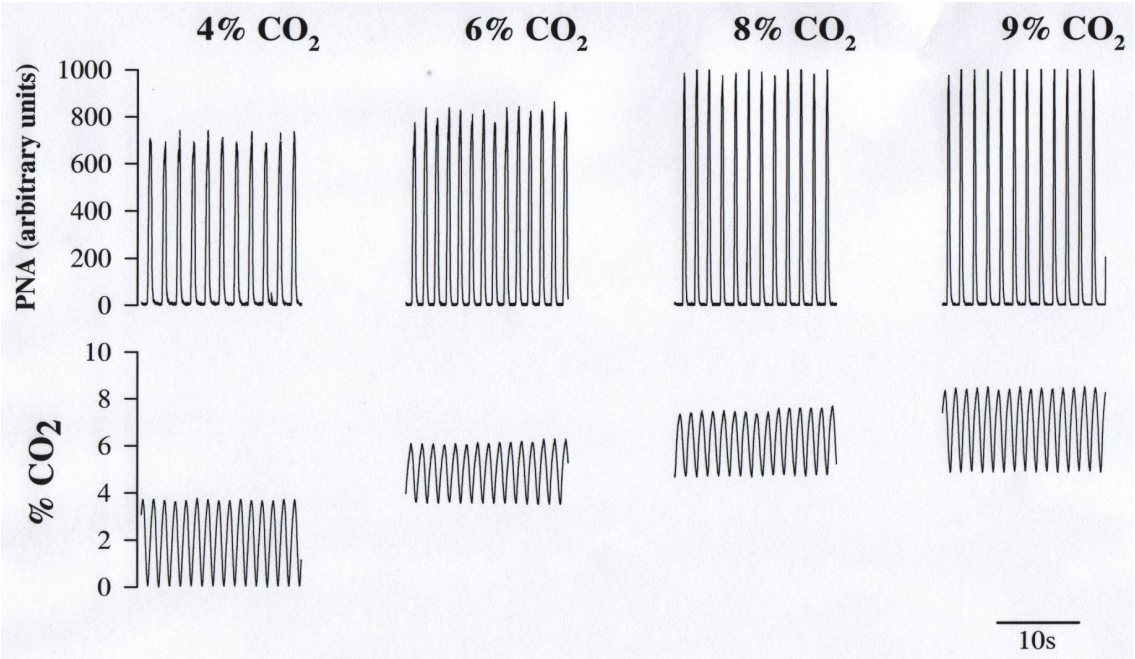

Figure 5.1: Response of phrenic nerve activity to increasing levels of inspired carbon dioxide. Phrenic nerve activity is shown in the upper panel and the levels of inspired carbon dioxide are show in the lower panel. As CO_2 levels rise there is an increase in phrenic nerve activity.

A summary of the response to the above intervention is shown in Figure 5.2. The data is represented as the ratio of change. The ratio of change was calculated by dividing the response by the control - in this case by the values at 4% CO_2, therefore each animal served as it own control. Figure 5.2 shows the change in phrenic nerve activity (amplitude, frequency and ventilation; y-axis) in response to increasing levels of carbon dioxide (x-axis). Phrenic amplitude is represented by the solid lines and closed circles; phrenic frequency is represented by closed triangle and dashed line; while phrenic ventilation (the product of amplitude and frequency) is represented by the solid square and the dashed and dotted line.

The first point was taken by dividing the control by itself. This signifies the control or the baseline condition. An increase in the ratio above this level (above 1) signifies an increase in activity while a decrease from this ratio signifies a decrease in activity. Hence, in this figure phrenic amplitude, frequency and ventilation ratios increased from 1 to 1.174, 1.273, and 1.494 respectively, when the CO_2 level was 6%. The ratio of change in phrenic amplitude continued to increase to 1.348 and 1.391 for 8 and 9% CO_2, respectively. On the other hand, the ratio of change for phrenic frequency decreased to 1.091 at 8% and stayed the same at 9% CO_2. The ventilatory ratio of change decreased followed by a small increase (4.470 and 1.518, respectively).

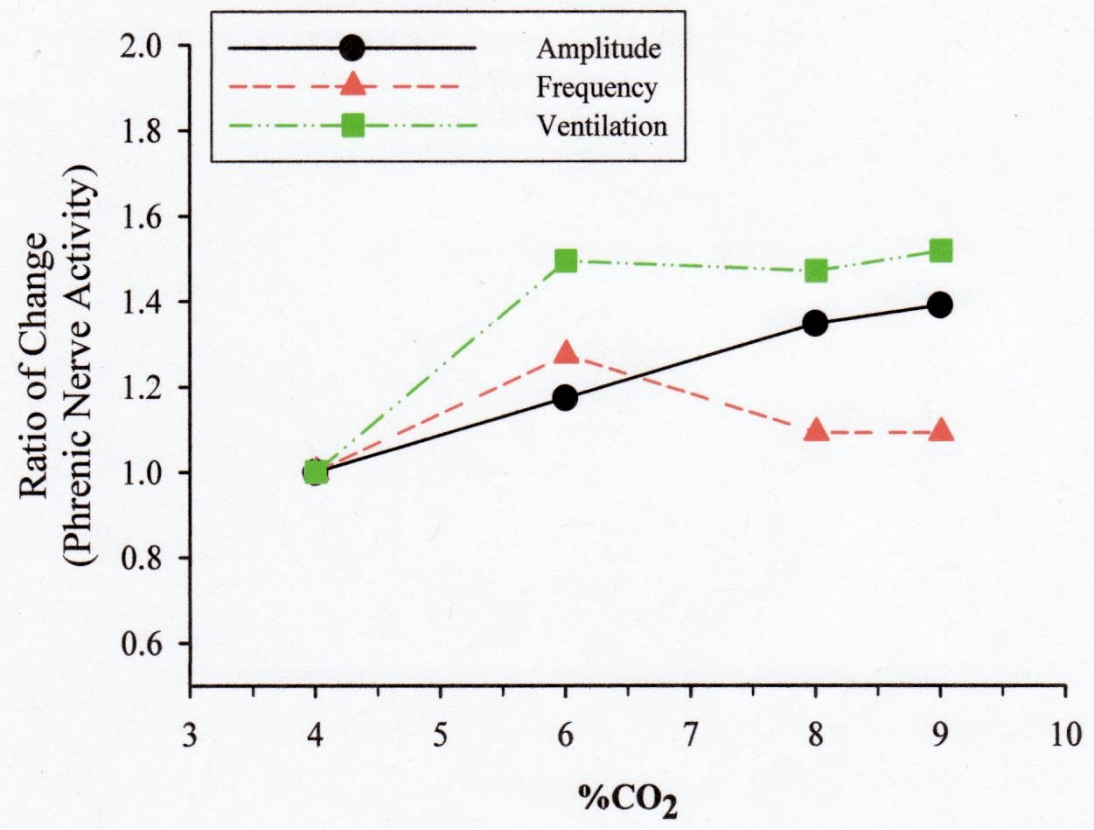

Figure 5.2: Ratio of change of phrenic nerve activity to changes in inspired CO_2.

5.2 Effect of Nicotine Applied to the RMg on Phrenic Nerve Activity

Injections of nicotine (10, 100, 1000 mM) were made in the raphé magnus while the caudal chemosensitive area received only 100mM nicotine. Injections of nicotine in the raphé magnus did not significantly ($p > 0.05$) affect phrenic nerve activity, whereas injections on the ventral surface of the medulla oblongata at the caudal chemosensitive site caused a vigorous respiratory response ($p < 0.05$).

Figure 5.3 shows the original trace for the time course of the effect of 100mM nicotine injected 1.5mm beneath the ventral medullary surface and 1mm rostral of the intersection of the basilar and vertebral arteries. Blood pressure recordings are shown in the upper panel while phrenic nerve activity is shown in the lower panel. Blood pressure was control so that this would not affect the animal's breathing.

Phrenic nerve activity was recorded continuous for the first 5 minutes (upper panels). We did not observe any change in phrenic nerve activity during this time. We then recorded for one minute, every 5 minutes for the next15 minutes. For the next 30 minutes we recorded for 1 minute every 10 minutes.

We observed a small, but not significant decrease in phrenic nerve activity during the last 25 minutes of recording. In this case both amplitude and frequency (and hence ventilation) decreased. Another injection made at this site did not affect phrenic nerve activity either.

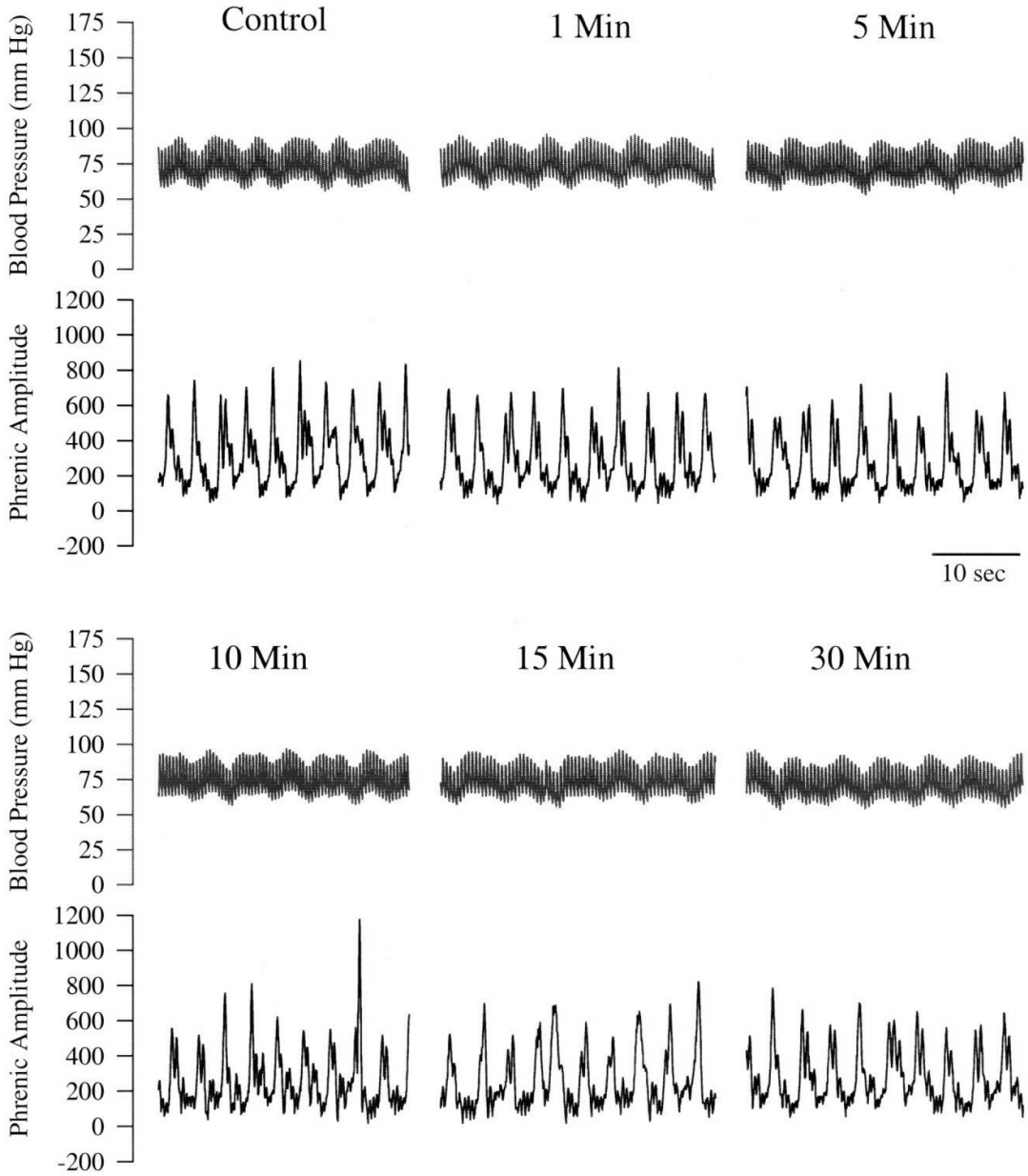

Figure 5.3: Effects of 100 mM nicotine microinjected into the raphé magnus on phrenic nerve activity. 50nl of nicotine was injected at a site 1mm rostral of the intersection of the basilar and vertebral arteries and 1.5 mm deep. Over the course of the recording PNA was unaffected, even after 40 min.

Figure 5.4 shows the time course for the ratio of change for an injection of 100mM nicotine injected into the medullary raphé. During the first 5 minutes there was no change in phrenic nerve frequency, however there was a small decrease in phrenic nerve amplitude which also caused ventilation to decrease as well. 10 minutes after the injection saw an increase in phrenic nerve amplitude and a slight decrease in phrenic nerve frequency which was reflected in an increase in ventilation from the previous value. The last two points saw an increase in frequency and a decrease in amplitude. Finally both frequency and amplitude decreased.

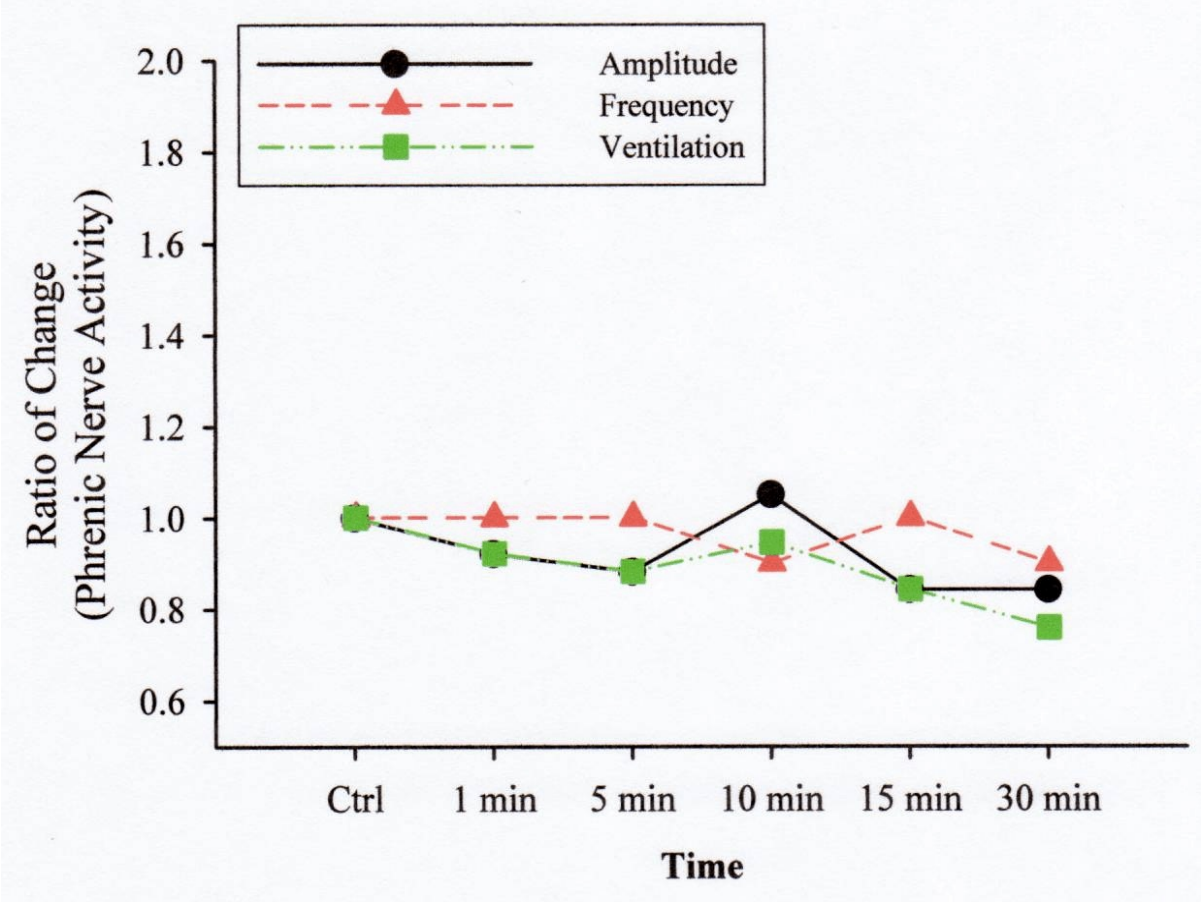

Figure 5.4: Time course of ratio of change in phrenic nerve activity to a single injection of nicotine made in the raphé magnus.

The time course for the summary of eight injections of 100mM nicotine made into the raphé magnus is shown in Figure 5.5. During the time course of this intervention there was no significant change in phrenic nerve activity.

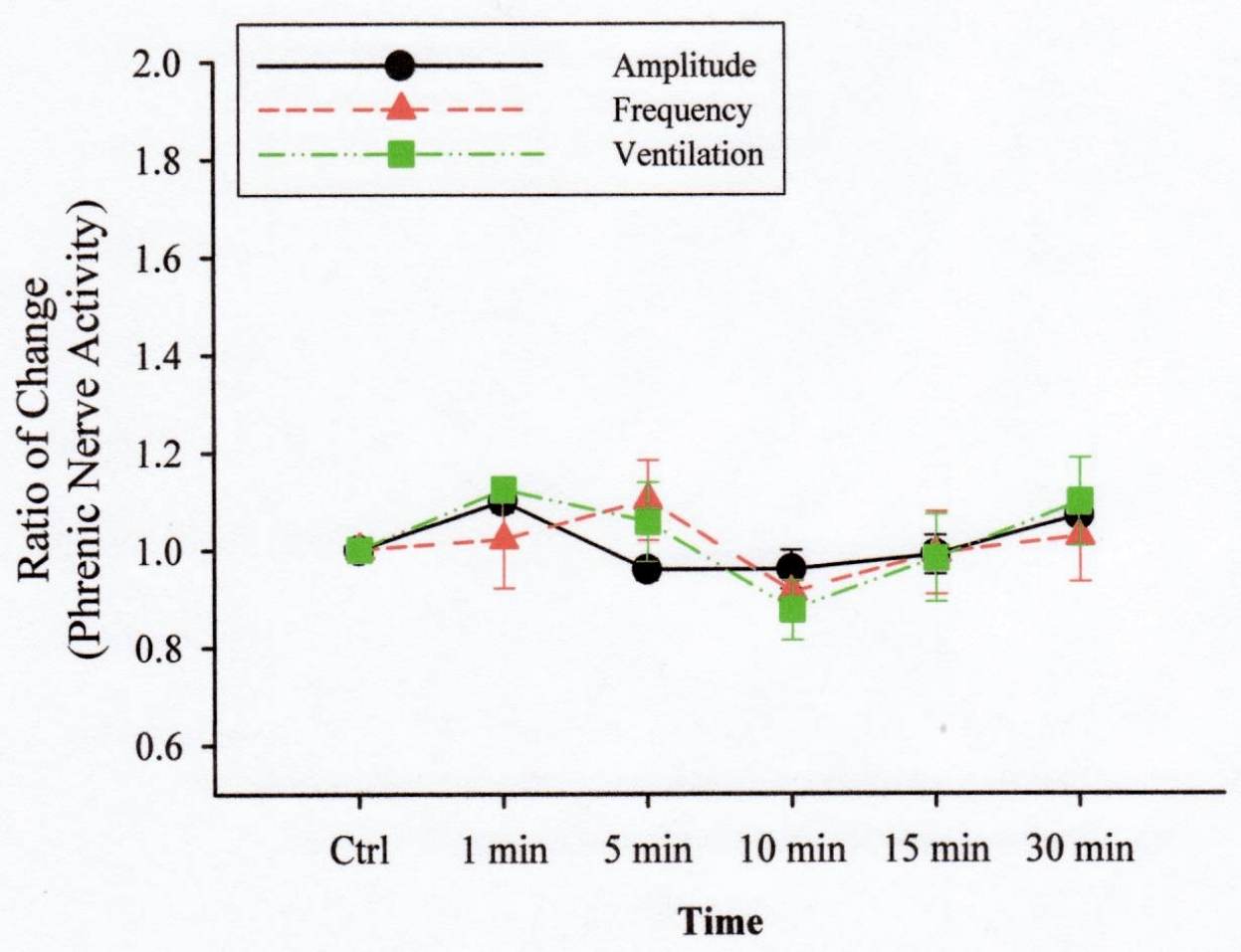

Figure 5.5: Summary of graph showing the time course of the ratio of change in phrenic nerve activity when nicotine was injected into the raphé magnus.

A dose response curve was plotted for the ratio of change to different concentrations of nicotine. The control solution used was the mCSF into which the appropriated amount of nicotine was dissolved. We first injected 100mM nicotine and made eight such injections into the raphé magnus. 5 injections of 1000mM nicotine was made into the raphé magnus while two injections of 10mM were made. Since the large doses of the drug did not affect phrenic nerve activity we did not believe that additional injections of 10mM nicotine would have changed the outcome.

Overall there was no change in activity for the different doses of the drug. At 100mM nicotine phrenic amplitude, frequency and ventilation were 1.013 ± 0.041, 0.979 ± 0.096, 0.992 ± 0.011, respectively and for 1000mM they were 1.063 ± 0.072, 1.013 ± 0.090, 1.076 ± 0.100. Figure 5.6 shows these changes and shows that phrenic nerve activity was unaffected by increase doses of nicotine.

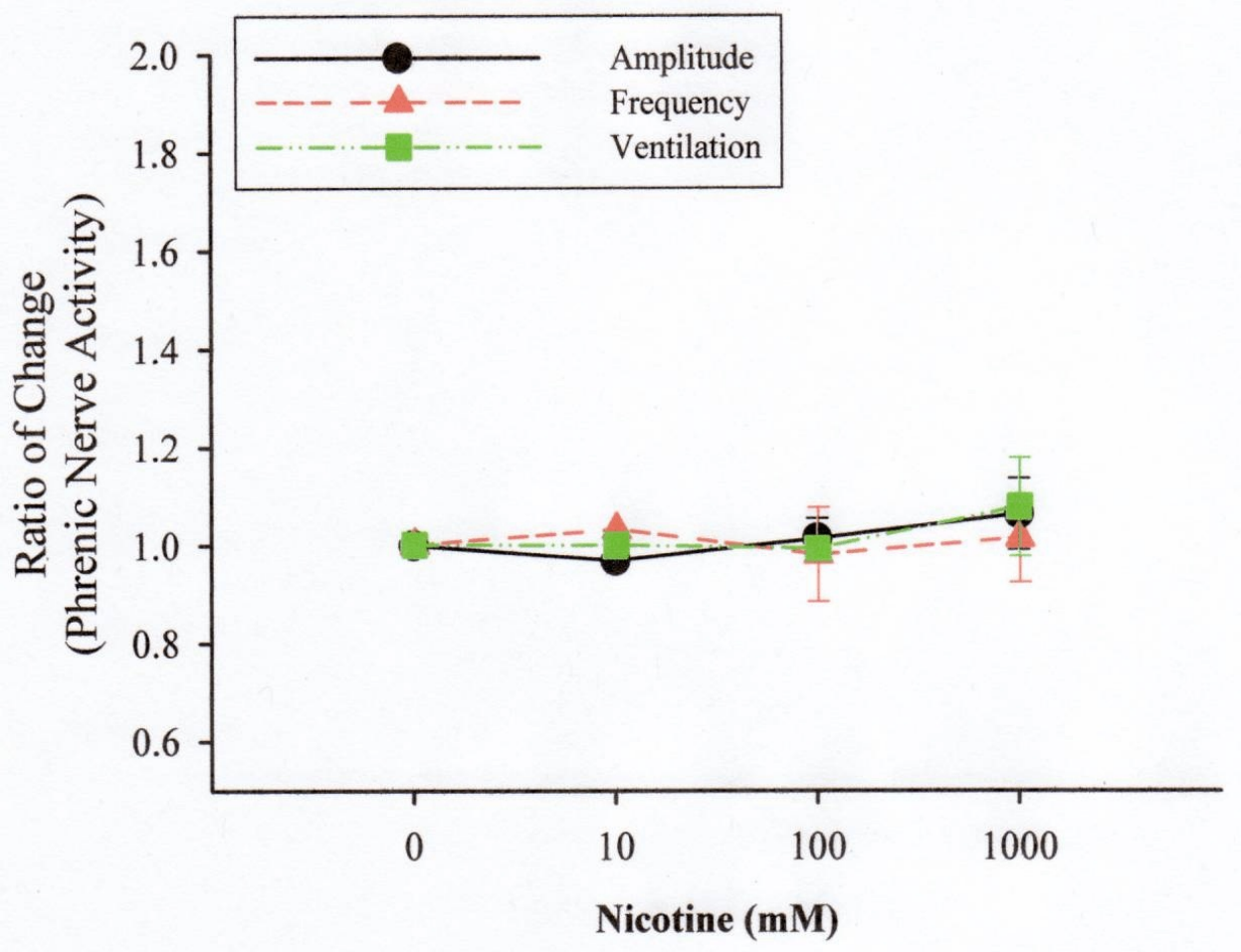

Figure 5.6: Dose response curve for the ratio of change in phrenic nerve activity for different concentrations of nicotine injected into the raphé magnus.

5.3 Effect of Nicotine Applied to Area-L on Phrenic Nerve Activity

Nicotine injections into the raphé magnus did not stimulate breathing, even at high pharmacological doses (1000mM). We did not know if it was the drug, the technique or the personnel that may have been inhibiting a response because we anticipated a vigorous response to nicotine at this location. To eliminate the causal effect of the above, we decided to test our protocol on another site, known to be responsive to nicotine (Dev and Loeschcke, 1979b; Feldberg, 1976; Trouth et al., 1982).

Figure 5.7 shows the response of the original trace of phrenic nerve activity of a rat to an injection of 100mM nicotine (50-100 nl) made unilaterally onto the caudal chemosensitive area (Area-L). End tidal CO_2 was held constant and blood pressure was unchanged during the procedure. Phrenic amplitude almost doubled in this instance.

This animal was previously subjected to injections of mCSF and 100mM nicotine in the raphé magnus. There was no change in phrenic activity after 30 minutes from each intervention. The pipette was then removed, placed on the surface of Area-L and subjected to the nicotine. The animal almost immediately responded. The surface was washed with mCSF and the animal allowed to recover. Another injection at the same site was made using mCSF. There was no change in activity. On the other hand, inspired CO_2 cause an increase in phrenic nerve activity.

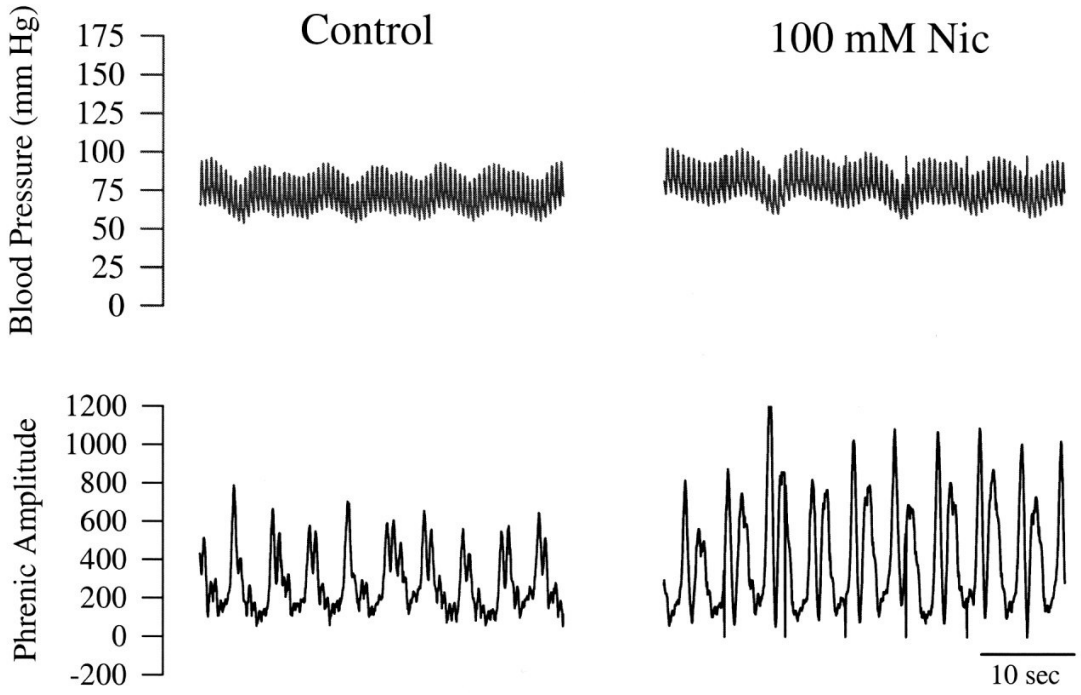

Figure 5.7: The effect of nicotine (100mM) applied to the caudal chemosensitive area on phrenic nerve activity.

The ratio of change for Figure 5.7 is shown in Figure 5.8. It reflects a 71% (1.708) increase in phrenic nerve amplitude, an 11% (1.111) increase in phrenic nerve frequency and a 90% (1.898) increase in phrenic nerve ventilation.

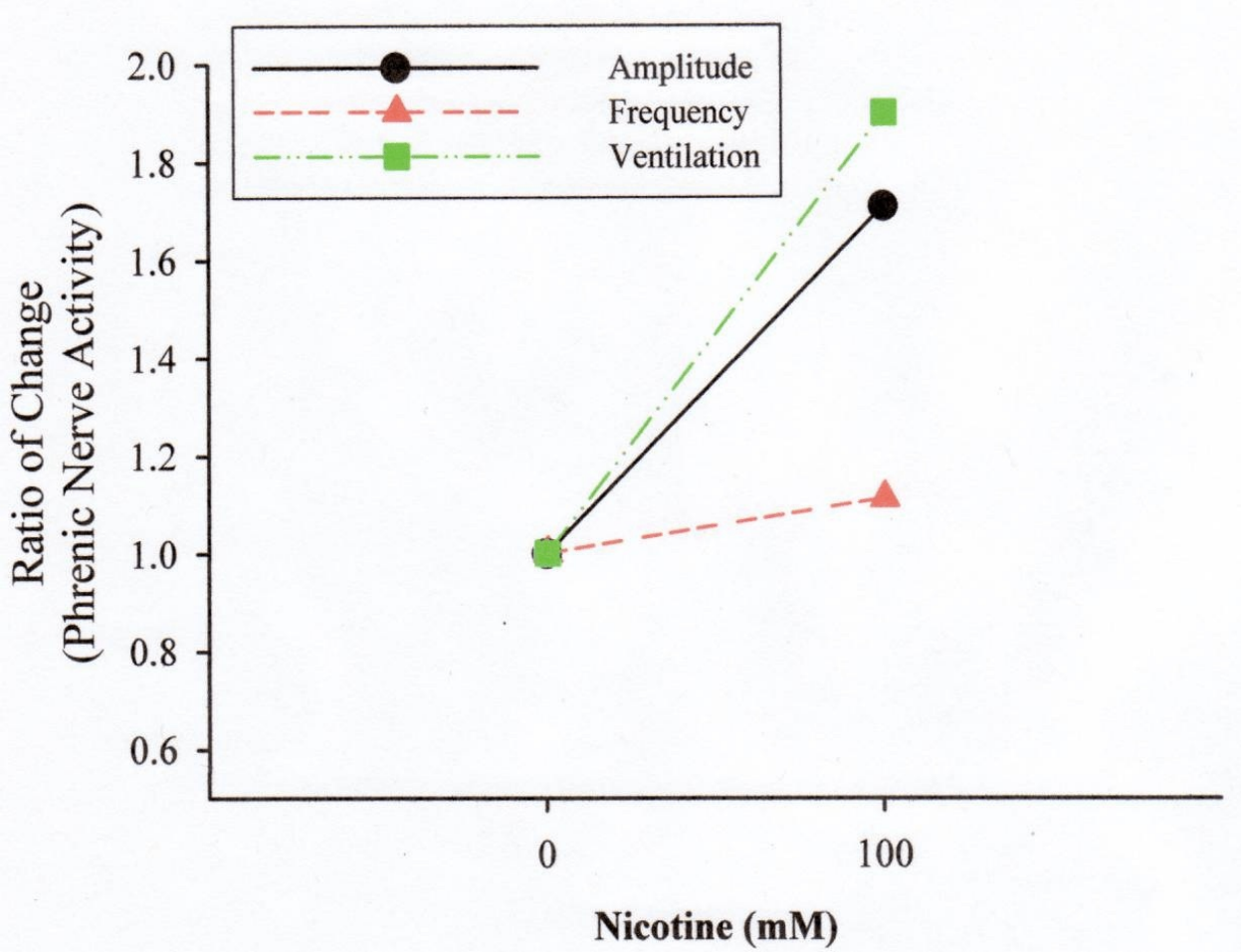

Figure 5.8: Ratio of change in phrenic nerve activity to a single injection of nicotine unto the caudal chemosensitive area.

We made four (4) injections of 100mM onto Area-L in three (3) rats. In one rat we made an injection on each side. Each injection was preceded by an equivalent volume of mCSF. The surface was washed with mCSF after each injection, even after the mCSF injections. We allowed the animal to fully recover before making a second injection.

Figure 5.9 shows the cumulative response from all 4 nicotine injections. In all four cases, the animal responded to the injection. On average, phrenic amplitude increased by 47% (1.466 ± 0.101), phrenic frequency increased by 26% (1.256 ± 0.125) and phrenic ventilation increased by 84 % (1.843 ± 0.110)

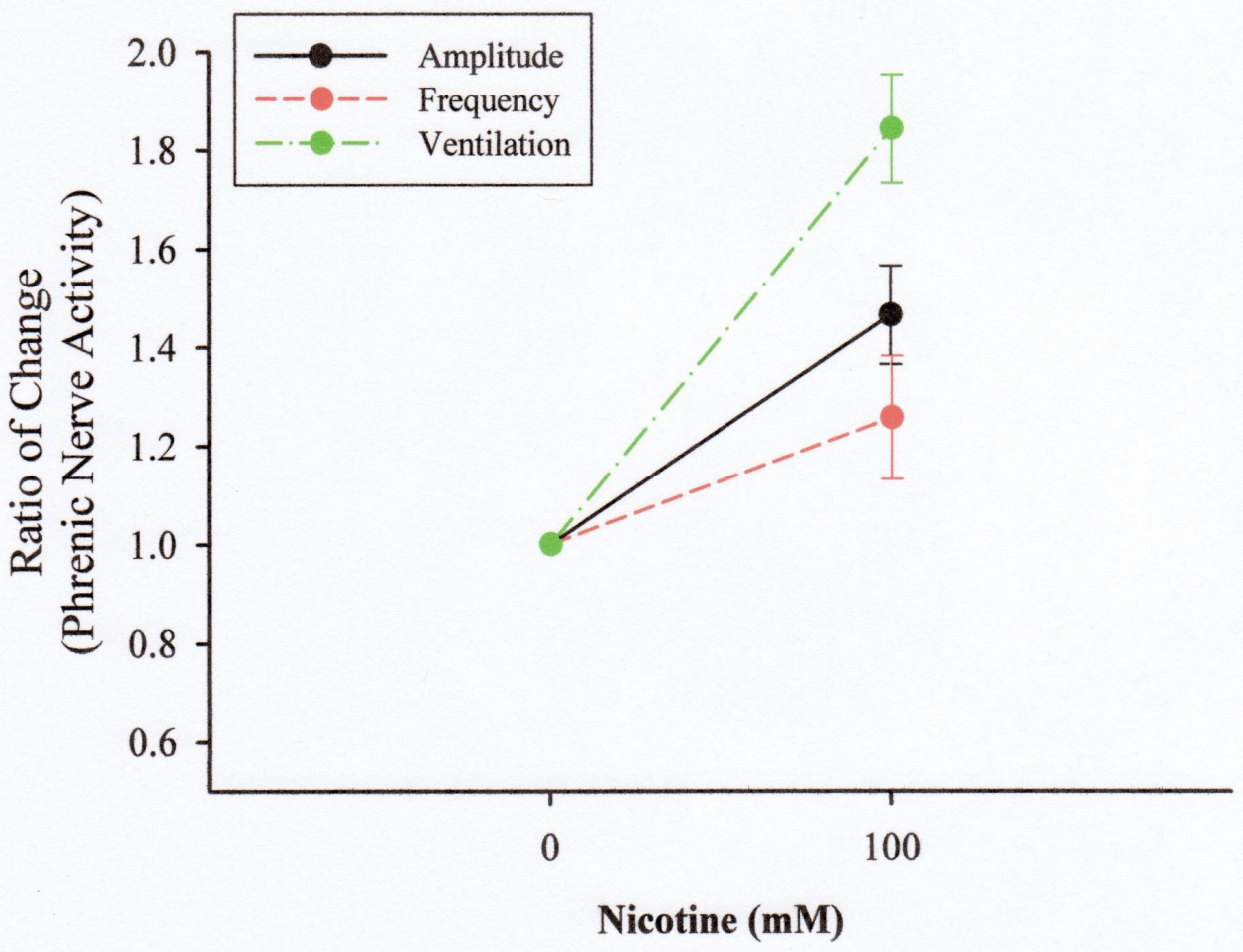

Figure 5.9: Summary graph showing the ration of change to 4 injections of 100mM nicotine made in the caudal chemosensitive area.

CHAPTER 6

DISCUSSION

6.1 Discussion of Results

Nicotine is an excitotoxic and addictive chemical found in cigarettes and tobacco products. It is not naturally found in the body. The major route of entry for nicotine into the body is by cigarette smoking or by the use of tobacco products. Once ingested, nicotine easily crosses the mucosal membranes of the respiratory and gastrointestinal system and enters the blood. Because it is highly lipophilic, it easily crosses other membrane boundaries and quickly appears in the cerebrospinal fluid via the blood brain barrier or the fetal blood via the placenta. Of course, once in the fetal blood it can gain access to the cerebrospinal fluid of fetal brain and may then affect breathing.

As early as 1934 the effects of cholinergic agents on the brain were being investigated (Dikshit, 1934). By1939, Pitts and colleagues had begun work on localizing regions on the brainstem with respiratory function (Pitts et al., 1939b; Pitts et al., 1939a) and by 1941, Brookhart was using electrical stimuli to further delineate sites on and in the brainstem with respiratory related functions (Brookhart, 1940). In the 1950's scientists were beginning to understand that there may be specific regions within the brainstem that are specifically designed to control breathing (Amoroso et al., 1951; Leusen, 1954; Loeschcke and Katsaros, 1959; Ngai and Wang, 1957; Schaefer, 1958). The central control centers for respiration were localized to the medulla oblongata and several sites

along the surface of this region were identified. In addition, deeper sites were also observed to be respiratory chemosensitive as well (Chai and Wang 1962; Gill 1963; Hugelin and Cohen 1963; R.A. Mitchell et al. 1963b; R.A. Mitchell et al. 1963a; Robert A. Mitchell 1966; Stahlman 1963; C. Trouth, Ovid 1969). Finally, electrical stimulation was used to more finely delineate these sites of respiratory chemosensitivity. Three respiratory chemosensitive sites were described and they appear in Figure 2.2. Later other deeper sites were described which included the medullary raphé (Adair et al., 1977).

Dev and coworkers (Dev and Loeschcke, 1979a, 1979b; Fukuda and Loeschcke, 1979) were one of the first to state that a cholinergic mechanism was involved in respiratory chemosensitivity at the medulla oblongata. During the next decade reports began linking the incidence of sudden infant death syndrome with respiration (Loeschcke, 1982; Shannon and Kelly, 1982; Mellins and Haddad, 1983; Parks et al., 1989) and changes in breathing via CSF acidosis were shown to be mediated through cholinergic mechanisms (Burton et al., 1989). During this time it became clear that there were other regions of central respiratory chemosensitivity apart from those regions on the ventral medullary surface. These regions included the lateral pons (Johnston and Gluckman, 1989), the fastigial nucleus (Williams et al., 1989), and the medullary raphé (Richerson, 1993; Bernard et al., 1994).

We do not as yet know the cause of sudden infant death syndrome (SIDS). Environmental factors that increase the risk for death have been identified and include bedding on which the infant sleeps, cold weather and reduced birth weight. Two other important risk factors include prone sleeping and exposure to cigarette smoke (Machaalani and Waters, 2006).

With the knowledge that the medullary raphé is intimately involved in the regulation of cardiorespiratory functions, it would be a reasonable conclusion that damage or impairment of this region may lead to some form of respiratory and/or cardiovascular difficulties. Also, since nicotine has been show to be in fetal blood, and hence fetal brain, it is with these factor that we chose to examine the effects of nicotine on neurons in the caudal medullary raphé.

6.2 Positive Effects of Nicotine

The effects of nicotine on the body is not all negative. There have been reports on the beneficial effects of nicotine such as the property of enhancing attention (Stolerman et al., 1994; Newhouse et al., 2004). In addition, Hahn et al. (2007) demonstrated that nicotine can enhance the visuospatial attention by deactivating areas of the resting brain default network (Hahn et al., 2007). These suggest that nicotine may have potential therapeutic effects in chronic disease states in which there is a dysfunction in attention (Levin and Rezvani, 2002).

6.3 Location of Nicotine Injections in the Raphé Magnus

We used the atlas of Paxinos and Watson to position the micropipette in the raphé magnus (Paxinos and Watson, 1986). The range of the depth for the tip of the micropipette was 1.0 to 2.0 mm beneath the ventral surface of the brainstem and lateral, but adjacent to the basilar artery. Microscopic observation of slides from the brainstem revealed that we were on target, that is, the injection sites were all located within the raphé magnus.

A diagram showing the location of the injection sites is presented in Figure 6.1. Injections were confined to the right side of the basilar artery and extended 1.1 to 1.75 mm from the ventral medullary surface. We did not observe any injection site outside the raphé magnus. The size of the

dots do not correspond to the size of the injections and are presented as a representation of the approximate locations of each injection. We did not calculate the injection volume from the injection sites, but relied on the measured diameter of the injection volume immediately prior to each injection for our determination of injection volume.

Bernard (1998) in a previous study found that different regions in the medullary raphé affected cardiorespiratory activity differently when stimulated with injections of glutamate (Bernard, 1998). Some regions affected respiratory output, while others affected cardiovascular output, while still others affected both respiratory and cardiovascular outputs. We could not tell whether the sites we stimulated fell into the region that only affect cardiovascular activity, however, from the data obtained this seems unlikely. We did not detect blood pressure changes during the course of our experiments when nicotine was inject into the raphé magnus. The fact that we neither saw a change in respiration or cardiovascular activity remains an enigma that demands further study.

One other point that must be made is this. Our injections were confined to a region beginning at the intersection of the vertebral and basilar arteries and extending to a location only 2 mm rostral. We do not believe that injections made more rostral than ours would change the outcome of our results.

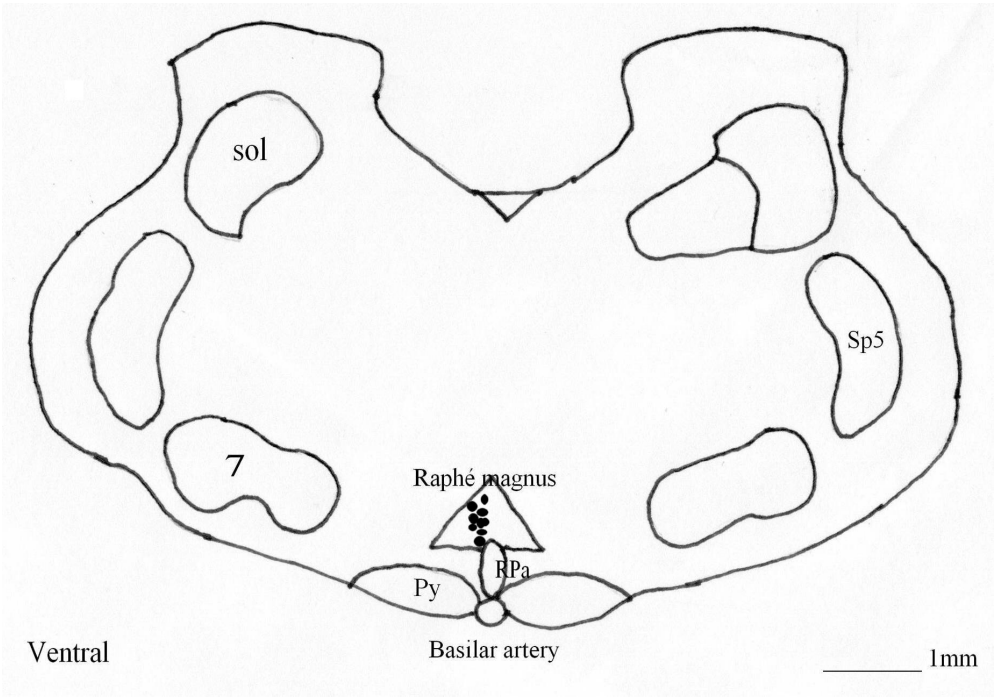

Figure 6.1: Cross-section of the medulla oblongata showing injection sites (black dots) in the raphé magnus. All injection sites analyzed were located within the raphé magnus. 7 - facial nucleus; Py - pyramidal tract; Rpa - raphé pallidus; sol - nucleus solitary tract; Sp5 - spinal trigeminal tract.

6.4 Future Direction

It remains worthwhile to continue to explore the central regulatory influences of the medullary raphé on the control of the respiratory and cardiovascular activity in mammals. I believe that examining the raphé magnus for cholinergic receptors and more specifically, nicotinic cholinergic receptors would be a logical step to explore.

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Nicole Bates graduated from Grambling State University in May 2003 with a Bachelor's degree in biology. As a graduate student Nicole maintained a 3.6 GPA, while teaching 7th grade math at Byrd Middle School.

Nicole's plans for the future is to begin a career in forensics as a DNA analyst working for the FBI. Nicole resides in Grand Prairie with her dog Neiko.