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Effects of Nicotine and Anatoxin-A Exposures on the Operant Performance of Rats

Kimberly Ann Jarema
Western Michigan University

EFFECTS OF NICOTINE AND ANATOXIN-A EXPOSURES ON THE OPERANT PERFORMANCE OF RATS

by

Kimberly Ann Jarema

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ADVISOR: DR. ALAN D. POLING

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Kimberly Ann Jarema

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CHAPTER I

INTRODUCTION

Nicotine

Tobacco products are widely used by humans and the health problems associated with cigarette smoking and other forms of tobacco use are staggering (Julien, 1995). Although the reasons for human tobacco use were debated for many years, it is now clear that the underlying mechanism involves the positively reinforcing effects of nicotine. Humans (e.g., Henningfield & Goldberg, 1983), nonhuman primates (e.g., Goldberg, Spealman, & Goldberg, 1981), and rats (e.g., Shoaib & Stolerman, 1999) will all self-administer nicotine, and it is generally acknowledged that tobacco use in its various forms provides a means of self-administering the drug (Julien, 1995).

Despite the fact that nicotine can serve as a positive reinforcer, the drug is a poisonous alkaloid, and was one of the first known pesticides (Yamamoto, 1998). In the tobacco plant the highest concentration of nicotine is in leaves, where one small bite can result in death for an insect. Extracts from the tobacco leaf have long been recognized as effective insecticides that protected crops from leaf-eating insects (Crosby, 1966; Schmeltz, 1971). As early as 1690 European farmers documented using a plant spray made from tobacco extract to protect other kinds of plants; roughly 250 years later nicotine was commercialized as an insecticide in America (Schmeltz, 1971). Over the years nicotine's popularity as an insecticide decreased with the rise of synthetic

insecticides that were cheaper and more readily available (Yamamoto, 1998). Today, it has no commercial application as an insecticide (Yamamoto, 1998).

The neurochemical effects of nicotine are well established and involve the capacity of the drug to stimulate a class of cholinergic receptors termed "nicotinic." At low doses this stimulation results in: a slight increase in blood pressure and heart rate; a heightened sense of alertness, awareness and arousal; improved concentration, learning and short-term memory; and decreased anxiety and pain perception (e.g., Benowitz et al. 1989; Clarke, 1993; Girod et. al., 1999). At higher doses, when the receptors are stimulated too strongly, nicotine is a potent nerve poison that can cause headaches, giddiness, nausea, vomiting, impaired vision and hearing, mental confusion, rapid respiration, faintness, tremors, respiratory paralysis, convulsions and death (Schmeltz, 1971). The toxic effects of a large nicotine dose are noticed almost immediately and nicotine, for which the median lethal intravenous dose in humans is estimated to be 30-60 mg/kg, can cause death in 5 – 30 minutes (Schmeltz, 1971). This dose range is never approached by tobacco users and the harm associated with tobacco use primarily involves chronic effects on the pulmonary and circulatory systems, not acute toxicity.

In recent years, researchers have explored the use of nicotine as a possible treatment for several diseases. Levin (1992) reported that acute and chronic nicotine administration can enhance cognitive function in both humans and animals. Since then, studies have suggested that nicotine may help alleviate the cognitive impairments associated with aging, Alzheimer's disease, schizophrenia, attention-deficit/hyperactivity disorder, and Tourette's syndrome (e.g., Rezvani & Levin, 2001; Sanberg et al., 1997;

White & Levin, 2004). Despite these promising findings, nicotine is not yet recognized as an effective medication for any of these conditions.

Behavioral pharmacologists have examined nicotine as a positive reinforcer (e.g., Clark, 1969; Hanson et al., 1979; Stolerman, 1991) and as a discriminative stimulus (e.g., Chance et al., 1978; Craft & Howard, 1988; Rosecrans & Villanueva, 1991; Schechter & Rosecrans, 1972; Shoaib et. al., 1997; Stolerman, 1989). They also have examined its direct effects on schedule-controlled responding. The effects of nicotine on schedule-controlled responding are complex and difficult to summarize, although acute injections of moderate to high doses of nicotine frequently produce dose-dependent decreases in response rates under a variety of schedules (e.g., Clarke & Kumar 1983; Goldberg et al., 1989; Ksir, 1994; Morrison & Armitage, 1967). Low doses sometimes increase response rates, and there is some evidence that the effects of nicotine are rate- as well as dose-dependent (e.g., Morrison, 1967; Morrison & Armitage, 1967; Pradhan, 1970; Spealman et al., 1981; Stitzer et al. 1970).

Morrison (1967), for example, administered nicotine to rats responding under a variable-ratio (VR) 30 schedule of water reinforcement, and found that there was a slight increase in responding at 0.05, 0.1, and 0.2 mg/kg, and a slight decrease in responding at 0.4 mg.kg. Similar results were reported by Spealman et al. (1981), who studied the effects of nicotine in squirrel monkeys responding under a multiple fixed-interval (FI) 300-s fixed-ratio (FR) 30 schedule of food reinforcement. During the FI component, the lowest nicotine dose (0.01 mg/kg) had no effect on behavior, whereas intermediate doses (0.03, 0.1 and 0.3 mg/kg) increased responding, and the highest dose (1.0 mg/kg)

decreased responding. In the FR component, nicotine produced a dose-dependant decrease in responding. In addition to demonstrating how dose can influence the effects of nicotine on schedule-controlled responding, the Spealman et al. (1981) study also shows that the schedule under which behavior is maintained may influence nicotine's actions.

The schedule of reinforcement in effect is a powerful determinant of response rate, and response rate is known to modulate the effects of many drugs (e.g., Dews & Wenger, 1977; McKearney & Barrett, 1978). Ratio schedules, for example, typically produce high rates of behavior whereas interval schedules typically produce lower rates of behavior (Ferster & Skinner, 1957). In schedules that produce low baseline rates (e.g., long FI schedules), low nicotine doses often increase response rates (e.g., Morrison, 1967; Morrison & Armitage, 1967; Morrison & Stephenson, 1973; Pradhan, 1970; Spealman et al., 1981; Stitzer et al., 1970). These same doses may decrease high baseline response rates. Such an effect is illustrated in the Spealman et al. (1981) study described above, wherein a dose of nicotine that increased responding during the FI component decreased responding in the FR component. In contrast to these results, however, Morrison reported that nicotine increased the relatively high rates maintained under a VR schedule.

In addition, although VI schedules typically engender higher response rates than FI schedules of the same length, several studies have revealed similar effects of acute nicotine administrations (0.05 – 0.4 mg/kg) under VI 2-min and FI 2-min schedules, regardless of whether they were arranged singly or as components of a multiple schedule

(e.g., Morrison, 1967; Morrison & Armitage, 1967; Morrison & Stephenson, 1973; Pradhan, 1970). In these studies, nicotine increased response rates under both FI and VI schedules.

Goldberg et al. (1989) also reported that nicotine at doses of 0.01 - 0.03 mg/kg increased responding under the FI component of a multiple FI 5-min FR 20 schedule of food delivery, whereas 1.0 mg/kg reduced responding. However, Stitzer et al. (1970) reported that nicotine (0.05 - 0.4 mg/kg) produced dose-dependent decreases in the response rates of rats performing under an FI 88-s schedule of water reinforcement. The inconsistency of results observed under FI schedules suggests that the effects of nicotine on schedule-controlled responding may be influenced by a number of variables, even when overall response rates are relatively low.

As noted previously, under schedules that engender high response rates, low nicotine doses typically have no effect or slightly increase response rates, while higher doses decrease response rates in dose-dependent fashion (e.g., Goldberg et al., 1989; Morrison, 1967; Morrison & Armitage, 1967; Pradhan, 1970). This pattern of results is evident in experiments using FR 20 and 50 and VR 30 schedules, either alone or as components of a multiple schedule. Results showed that lower nicotine doses (0.05, 0.1, and 0.2 mg/kg) produced a slight increase in response rates, while higher doses (0.4 and 1.0 mg/kg) produced a decrease in response rates (Goldberg et al., 1989; Morrison, 1967; Morrison & Armitage, 1967). However, when examined under a multiple FR 20 Timeout (5 or 2.5 min) schedule of water reinforcement, nicotine at 0.2 mg/kg produced a slight decrease in responding during the FR 20 component (Pradhan, 1970). When

mice were trained to respond under an FR 25 schedule of food reinforcement, nicotine (0.2-1.6 mg/kg) produced a dose-dependent decrease in responding, with behavior almost completely suppressed at the highest dose (Hendry & Rosecrans, 1982). Findings obtained under ratio schedules, like those obtained under interval schedules, suggest that the effects of nicotine on schedule-controlled responding can be variable, although the factors responsible for the variability are not readily apparent. One factor that may influence results is the time when behavior is assessed relative to the time of drug injection. Some evidence suggests that nicotine may decrease responding relatively soon after administration, then subsequently increase it (e.g., Clarke & Kumar 1983; Goldberg et al., 1989; Ksir, 1994; Morrison & Armitage, 1967). If this is true, session length as well as presession injection interval could influence the overall effects of nicotine on schedule-controlled responding, and both of these variables vary substantially across studies.

Because tobacco is used chronically, researchers have examined how the effects of nicotine on schedule-controlled responding change with repeated exposures. In principle, changes in drug effects with repeated exposures can involve either tolerance or sensitization. Tolerance occurs when a given drug effect is reduced in magnitude as a function of repeated exposure, whereas sensitization occurs when a given drug effect is increased in magnitude as a function of repeated exposure (Poling & Byrne, 2000). Rightward and leftward shifts in the dose-response curve following repeated exposure provide evidence of tolerance and sensitization, respectively.

Domino and Lutz (1973) found that tolerance developed to the rate-decreasing effects of nicotine when rats, trained to respond under an FR 15 schedule of water reinforcement, were given 0.25 mg/kg twice each day (pre- and post- session) for 15 days. Following an initial decrease in response rates, responding gradually increased during repeated administration to baseline levels, indicating tolerance.

Hendry and Rosecrans (1982) trained mice to respond under an FR 25 schedule of food reinforcement. Initial nicotine administrations (0.2, 0.4, 0.8 and 1.6 mg/kg) decreased responding in a dose-dependent manner. Next, the mice received daily nicotine administrations (1.2 mg/kg) for 30 days. Response rates during repeated nicotine administration showed an initial decrease, then gradually increased and returned to baseline levels over the 30-day period. Additionally, the mice were again exposed to the same doses (0.2, 0.4, 0.8 and 1.6 mg/kg) originally administered and the dose-response curves for pre- and post- chronic administration were compared. Although the post-chronic curve still showed dose-dependent rate decreases, the rates at a given dose were much higher than when that dose was administered pre-chronically, indicating that tolerance did develop.

Villanueva et al. (1992) replicated the work of Hendry and Rosecrans (1982), only they used rats responding under a VI 15-s schedule of food reinforcement, with initial nicotine administrations of 0, 0.2, 0.4, and 0.8 mg/kg. Nicotine (0.8 mg/kg/day) was then chronically administered for 36 days. The results that Villanueva et al. (1992) reported were similar to those reported by Hendry and Rosecrans (1982), in that nicotine

initially produced dose-dependent decreases in responding, and tolerance developed to the rate-decreasing effects of the drug.

Researchers initially believed that frequent (e.g., daily) administrations were necessary for nicotine tolerance to develop. However, in recent years several studies (e.g., Stolerman et al., 1974; Miller et al., 2001; MacPhail et al., 2000) have shown that relatively infrequent administrations also result in tolerance. For example, Jarema et al. (2002) extended to schedule-controlled behavior the research conducted by Miller et al (2001), who demonstrated that tolerance developed to the locomotor effects of nicotine when the drug was given once a week. Jarema et al. used a multiple repeated acquisition-performance schedule to determine whether tolerance to a single dose of nicotine (0.6 mg/kg) would develop when that dose was administered weekly for 4 consecutive weeks. Initial nicotine administrations decreased both response rate and response accuracy, but tolerance developed rapidly to these effects. Similar effects were observed in both the repeated acquisition and performance components. The results of this pilot study are interesting, and one purpose of the present research was to examine further the effects of widely-spaced nicotine administration on operant behavior in rats. The second purpose was to compare the effects of nicotine to those of another drug with nicotinic cholinergic actions, anatoxin-a.

Anatoxin-a

In addition to studying the effects of nicotine, some researchers have examined the effects of other nicotine-like compounds, such as imidacloprid (Kagabu, 1997),

indoxacarb (Zhao et al., 1999), nornicotine and cotinine (Goldberg et al., 1989), and to a lesser extent those of the cyanotoxin, anatoxin-a, which is a nicotine agonist (e.g., Stolerman et al., 1992; Stevens & Krieger, 1991; Carmichael & Falconer, 1993).

Anatoxin-a is an alkaloid neurotoxin produced by several genera of cyanobacteria (Falconer, 1993). Cyanobacteria, commonly referred to as blue-green algae because of its color and similarity to algae, is typically found in warm, shallow, slow-moving or still freshwaters, although it can also be found in sea water (Chorus & Bartram, 1999). Warm, stagnant water rich in nutrients, such as lakes, ponds, roadside ditches, sewage lagoons and agricultural runoffs set the stage for the rapid growth of cyanobacteria often called a "bloom" (e.g., Carmichael, 1994; Paerl et al., 2001; Villatte et al., 2002). These blooms, also referred to as water or pond scum, often float on the water surface and are most common in late summer and early fall when water temperatures are 72-80°F (21-27°C) (Carmichael & Falconer, 1993). A cyanobacteria bloom may appear in as few as two days and typically lasts 1-2 weeks, however, successive blooms may overlap and appear as one continuous bloom (Crayton, 1993).

There are about 40 genera of cyanobacteria and less than half of them actually produce toxins (Carmichael & Gorham, 1981; Falconer, 1993). Additionally, in some of the toxin-producing genera the toxin levels are so low that they can be difficult to detect (Carmichael & Gorham, 1981; Falconer, 1993). Cyanobacteria toxins, also called cyanotoxins, are naturally produced poisons that are stored in the cells and typically not released into the water until the cells rupture or die (Carmichael, 1994). However, the most extreme poisonous effects are typically only experienced when the intact cell is

ingested because the toxin becomes diluted when released into the water. These toxins primarily attack the liver (hepatotoxins) and the nervous system (neurotoxins), or simply irritate the skin (Chorus et al., 2000).

Most of the time, cyanobacteria blooms have few harmful effects on plants or animals. Nonetheless, when animals (including humans) drink or swim in water where toxic blooms have formed, they sometimes experience cyanotoxin poisoning. Cyanotoxins are responsible for illness or death in cattle, horses, sheep, pigs, birds, dogs, rabbits, and small wild and domestic animals all over the world (e.g., Chorus et al., 2000; Codd et al., 1997; Edwards et al., 1992). Cases of cyanobacteria poisoning involving humans typically stem from recreational exposure, often including ingesting water, and result in mild discomfort such as skin and eye irritations, dizziness, fatigue, sore throat, dry cough, and headache (e.g., Chorus & Bartrum, 1999; Codd, 1984). In rare cases more serious symptoms such as, abdominal pain, nausea, vomiting, and diarrhea, blistering of the mouth, atypical pneumonia, and elevated liver enzymes in the serum have been reported (Chorus & Bartram, 1999). These symptoms may be due to exposure to the neurotoxin called anatoxin-a.

Anabaena, *Aphanizomenon* and *Oscillatoria* are three genera of cyanobacteria that produce the neurotoxin anatoxin-a (e.g., Carmichael, 1992; Duy et al., 2000). Neurotoxins are typically rapid-acting poisons where signs can be observed minutes after exposure and death may occur from 5 minutes to a few hours after exposure, depending on dose. They affect the nervous system by interfering with nerve impulse transmission

and can cause miosis, convulsions, tremor, seizures, and rigid paralysis (Patockaa & Stredab, 2002).

(+)Anatoxin-a is a nicotinic agonist that binds to and stimulates neuronal nicotinic acetylcholine receptors (e.g., Soliakov et al., 1995; Spivak et al, 1980). Exposure can occur through ingestion, inhalation, injection, or through the skin at high concentrations (e.g., Devlin et al, 1977; Patockaa & Stredab, 2002). The (+)anatoxin-a median lethal dose for mice is 386 μg/kg i.v. Although the median lethal dose for humans is not known, experts estimate it to be less than 5 mg, when ingested, for an adult male (Patockaa & Stredab, 2002). Anatoxin-a was once referred to as Very Fast Death Factor (VFDF) because signs can be observed within 5 minutes of exposure and death can occur within a few hours (e.g., Carmichael et al., 1979; Patockaa & Stredab, 2002). The symptoms of anatoxin-a poisoning follow a progression of muscle twitching and spasms, staggering, paralysis, convulsions, respiratory arrest, asphyxiation and lack of oxygen to the brain, and eventually death from suffocation (e.g., Carmichael, 1994; Carmichael & Falconer, 1993; Patockaa & Stredab, 2002). Small laboratory animals (e.g., rats and mice) typically exhibit gasping and sudden leaping movements before a sudden death, while larger animals (e.g., dogs) often collapse and quickly die (Carmichael, 2001; Smith & Lewis, 1987). There is no known treatment for anatoxin-a poisoning; however respiratory support may allow time for the toxin to leave the body and recovery to occur (e.g., Valentine et al., 1991; Codd et al, 1992).

To date there has only been one human case appearing to involve death from anatoxin-a poisoning. A 2003 article by Don Behm, in the Milwaukee Journal Sentinel,

stated that in July 2002 a healthy 17-year-old boy spent 15 minutes in a shallow golf-course pond with a friend in Dane County, Wisconsin, and died two days later. Prior to his death, the boy suffered from stomach cramps, vomiting, and uncontrollable diarrhea. He then went into shock and suffered a seizure before his heart failed. High levels of anatoxin-a were present in blood and tissue samples taken from this boy and his friend, who also suffered severe diarrhea and abdominal pain. The final autopsy report lists the likely cause of death as ingestion of toxic algae, which led to "acute diarrhea illness and subsequent death" (Behm, 2003).

Scientists have determined that anatoxin-a is a neurotoxin that primarily attacks the respiratory system, potentially paralyzing the lungs and sending the heart into arrest but they have not yet determined exactly how it kills (Campbell & Sargent, 2004). In animal studies death typically occurs within two hours of exposure to a toxic dose of anatoxin-a, so it puzzling that the Wisconsin boy survived for so long after exposure. It is perhaps for this reason that the cause of death is not listed as anatoxin-a poisoning.

In the last several years, anatoxin-a has been found in waters throughout the world. Many researchers are now studying this toxin for several reasons including determining the human health risks. The anatoxins also are being studied for their possible use as military weapons (Patockaa & Stredab, 2002), and, like nicotine, cyanobacteria toxins are being studied as possible treatments for Alzheimer's disease and other disorders (Carmichael, 1994). The majority of research has examined where anatoxin-a is found in nature, how it is formed, what it does in the brain, and its toxicity.

Very little is known, however, about its behavioral effects, especially at less-than-life-threatening doses.

Because of its similarity to nicotine, scientists have recently begun to examine the effects of anatoxin-a under behavioral procedures that have previously been used with nicotine. However, the literature is very limited. Stolerman, Albuquerque and Garcha (1992) appear to be the only researchers who have published research on the behavioral effects of anatoxin-a and its similarity to nicotine.

Stolerman et al. (1992) reported the locomotor effects of anatoxin-a in rats that were not previously exposed to nicotine (non-tolerant) and in rats that had previously received nicotine. Additionally, they reported the effects of anatoxin-a in a saline-nicotine drug discrimination procedure. They found that rats not previously made tolerant to nicotine showed a decrease in activity with increasing anatoxin-a doses, although there was a slight increase at the lowest dose, similar to the effects of low doses of nicotine. The activity-decreasing effects of anatoxin-a were substantially greater in rats previously made tolerant to nicotine than in rats not exposed to nicotine. The drug discrimination results indicated some similarities between anatoxin-a and nicotine, but mecamylamine did not block the effects of anatoxin-a. Mecamylamine typically blocks the effects of nicotine in a drug discrimination procedure (e.g., Clarke & Kumar, 1983; Reavill et al 1990).

The findings of Stolerman et al (1992) suggest that anatoxin-a, like nicotine, can have powerful behavioral effects. Even though this nicotinic agonist is similar to nicotine in many ways, differences in their effects are apparent. Nothing has been reported

concerning the effects of anatoxin-a on schedule-controlled responding. Therefore, it is of interest to compare its effects to those of nicotine in subjects responding under schedules of operant reinforcement. Moreover, nothing is known concerning the development of tolerance to anatoxin-a. The present study examined the pre- and post-chronic effects of the compound on schedule-controlled responding of rats.

Rationale for Studying Schedule-Controlled Behavior

Schedules of operant reinforcement are critical to understanding the behavioral effects of drugs (Branch, 1991). Dews (1955) published a seminal article demonstrating how the effects of pentobarbital on pigeon's key-peck responding depended on the schedule of reinforcement that maintained that key pecking. He showed how the same dose of pentobarbital would increase responding under a fixed-ratio (FR) schedule and decrease it under a fixed-interval (FI) schedule, illustrating that schedules of reinforcement are fundamental determinants of the behavioral effects of drugs. Subsequent studies have repeatedly confirmed that this is indeed true (e.g., Branch, 1991; Poling & Byrne, 2000).

The schedule chosen for the present study was a multiple variable-ratio 30 variable-interval 60-s (mult VR 30 VI 60-s) schedule of food reinforcement. Under a VI schedule the intervals between reinforcement opportunities vary in random or nearly random order (Ferster & Skinner, 1957). Therefore, under a VI 60-s schedule the opportunity for reinforcement will be presented, on the average, every 60 seconds. Because it is an interval schedule, one response is required after the time period has

elapsed before reinforcement. This schedule typically produces moderate and relatively constant response rates throughout the experimental session (Catania, 1992). Because of this characteristic, VI schedules have long been used to investigate the behavioral effects of drugs (Iverson & Lattal, 1991).

Under a VR schedule, reinforcement occurs after a given number of responses that vary unpredictably from one reinforcement to the next (Ferster & Skinner, 1957). Under a VR 60 schedule, for example, reinforcement will be presented, on the average, following every 60^{th} response. In the absence of drug, VR schedules typically engender brief post-reinforcement (pre-ratio) pausing, followed by relatively high-rate responding (Ferster & Skinner, 1957). Because rate of reinforcement and rate of responding are directly related under VR schedules, drug-induced rate decreases of the sort that acute injections of nicotine (and, by inference anatoxin-a) should inevitably lead to reinforcement loss relative to control (no drug) conditions. So long as some minimal rate of responding occurs, comparable rate decreases under VI schedules do not lead to reinforcement loss. In 1966, Schuster, Dockens, and Woods (1966) developed the reinforcement-loss hypothesis in an attempt to predict when tolerance would and would not develop to a drug's effects on operant behavior. They proposed that:

> Behavioral tolerance will develop in those aspects of the organism's behavioral repertoire where the action of the drug is such that it disrupts the organism's behavior in meeting the environmental requirements for reinforcement. Conversely, where the actions of the drug enhance, or do not affect, the organism's behavior in meeting reinforcement requirements, we do not expect the development of behavioral tolerance. (p. 181).

The present study examined whether differential tolerance developed under a multiple schedule with VR and VI components. A multiple schedule comprises two or

more independent schedules that alternate throughout the session, with each schedule correlated with a different stimulus (Catania, 1992). Multiple schedules are popular in pharmacology research because they provide a way for researchers to collect data from two different behavioral measures (schedules) in a single experiment. Two different schedules were arranged in the present study to increase the amount of information generated regarding the drugs of interest, and to ascertain whether the type of schedule arranged modulated tolerance to infrequent administrations of nicotine and anatoxin-a. Based on prior studies, we expected that both drugs would decrease responding under both the VR 30 and VI 60-s schedules. Such an effect would necessarily reduce the frequency of reinforcement under the VR schedule, but not under the VI. In many, but not all, prior studies, and consistent with the reinforcement-loss hypothesis, tolerance developed quicker or to a greater extent under schedules where the initial effect of the drug was reinforcement loss rather than reinforcement gain or no change in reinforcement frequency (e.g., Cornfield-Sumner & Stolerman, 1978).

The present experiments systematically compared the effects of acute and episodic exposures to nicotine and to anatoxin-a, a nicotine-like compound. Two separate three-phase studies were conducted, one with nicotine and one with anatoxin-a. The studies were equivalent, save for the drug administered. In the first phase, we accomplished two objectives. First, we determined acute dose-effect relations. Second we compared the changes that occurred with weekly dosing over a four- week period, and determined whether tolerance developed. The second phase was similar to the first in that weekly administrations were given over four consecutive weeks. However, in Phase II

we wanted to determine not only if tolerance would develop, but also if it would be sustained over a time period of three weeks between injections. In the third Phase we addressed the question of whether behavioral or pharmacological tolerance developed to the effects of nicotine and anatoxin-a.

Experimenters studying repeated nicotine exposure have found that tolerance will often develop to nicotine's depressant effects and sensitization to its stimulant effects (e.g., Clarke & Kumar, 1983; Miller et al., 2001; Reid et al., 1996; Walter & Kuschinsky, 1989). Moreover, early research suggested that several weeks of administration were necessary before tolerance to nicotine would develop (Mattila and Saarnivarra, 1967). Since the majority of research on sensitization and tolerance to nicotine involves locomotor activity as a measure of behavior, it will be only briefly mentioned here. Instead, the main focus of this paper is on the effects that chronic nicotine and anatoxin-a exposure have on schedule-controlled responding.

Researchers studying the effects of chronic nicotine administration, by measuring locomotor activity as a measure of behavior, have found that tolerance can develop with daily (e.g., Stolerman et al., 1974), twice-weekly (e.g., Morrison & Stephenson, 1973) and weekly (e.g., Miller et al., 2001) administrations, shown by an increase in activity with subsequent nicotine administrations. With regard to schedule-controlled responding, when nicotine is administered chronically, at doses that tend to initially decrease responding, there is often an increase in responding following the subsequent (repeated) administrations (e.g. Domino & Lutz, 1973; Hendry & Rosecrans, 1982; Jarema et al., 2002; Villanueva et al., 1992).

CHAPTER II

EXPERIMENT 1

Materials and Methods

Subjects

Eighty-eight experimentally-naïve adult male Long-Evans rats (Charles River, Raleigh, NC), approximately 90 days old at experiment inception, were maintained at 350g via daily food restriction (Purina Rat Chow, St. Louis, MO) and served as subjects.

Upon arrival the rats were given time to acclimate to the housing colony and reach their target weight of 350 g. They were fed *ad libitum* until they approached that weight and then were switched to a weight-maintenance program where their daily food allotment was gradually reduced and regulated so they maintained a weight of 350 g (Ali et al., 1992). The rats were on this weight-maintaining feeding schedule for the duration of the experiment.

The rats were housed individually in 19.5 x 45.5 x 25.0 cm hanging plastic cages, with pine shaving bedding, in a temperature- (21-23°C) and humidity- (50-55%) controlled colony. A lighting schedule of 12-hr light and 12-hr dark was in effect (light on at 6:00 am) with water available *ad libitum*. During the experiment proper, sessions were conducted Monday through Friday during the light cycle. Rats were transported to the laboratory for daily testing in individual plastic cages, with filter tops, measuring 15.5 x 27.5 x 15.0 cm.

Apparatus

Behavioral sessions were conducted in commercially available operant test chambers (Coulbourn Instruments, Inc., Lehigh Valley, PA) positioned inside sound-attenuating enclosures (Ralph Gerbands Co., Arlington, MA) and ventilated by a fan. The inside of the test chamber measured 30 cm wide x 24 cm deep and 31 cm tall. A grid floor was raised 3.5 cm from the bottom of the chamber to allow for a collection pan and to keep the inside of the chamber clean. The front and back sides of the chamber were made of clear plastic while the right (component panel), left, top and bottom sides were metal. The front side opened down to allow access into the chamber. Each chamber was equipped with one response lever located on the right side of the component panel, 5 cm above the grid floor. Lever operation required a minimum downward force of 0.25 N. A set of triple-cue lights was located 3.5 cm directly above the lever. A pellet trough, into which 45-mg food pellets (P.J. Noyes Co. Inc., Lancaster, NH) could be dispensed from a dry-food feeder, was located to the left of the lever and 1.5 cm above the grid floor. The trough contained an overhead cue light that was briefly illuminated during food-pellet delivery. A Sonalert tone generator was situated 16 cm above the lever and was activated briefly (100 msec) after each response. A houselight, darkened only during food delivery, was situated at the top center of the component panel. Experimental events and data collection were controlled by a Digital Equipment Corporation (Maynard, MA) PDP 11/73 computer, programmed with the SKED-11 system (Snapper et al., 1982).

Behavioral Procedure

Subjects were initially trained to lever press during one 8-hour overnight training session. This session comprised three successive schedules. First, a variable-time 60-s (VT 60-s) schedule was in effect for 60 food pellet presentations. Under this schedule, food was delivered on average every 60 s, regardless of the rat's behavior. In addition, conditions were arranged such that, if the lever was pressed 20 times, the schedule immediately shifted to a fixed-ratio (FR) 1. Under this schedule, every response produced a food pellet. If the lever was not pressed a minimum of 20 times, the VT 60-s schedule continued until 60 minutes had elapsed, at which time the schedule changed to an FR 1. Upon completing 60 responses under the FR 1 schedule, the value was increased to FR 2, which remained in effect until the rat emitted another 60 responses, at which time the session ended. If all three schedule requirements were not met, the session ended after eight hours had elapsed.

Handshaping and FR training were conducted during the days following the overnight session for those rats that did not acquire the lever-press response. If they still were not pressing the lever after two additional training days, food pellets were crushed and placed on the lever. On a few occasions the rats also needed to be trained to eat from the food cup. In this situation, the crushed food pellets were not only placed on the lever but on the edge of the food cup as well.

Upon completion of the training procedure, each rat was exposed to a variable-ratio (VR) schedule of food reinforcement during daily 23-min sessions. Rats were first exposed to a VR 3, then to VR 5, VR 10, VR 20, and VR 30-response schedule of

reinforcement. The rate at which the ratios were increased was based on each rat's individual performance. Under the VR schedule, food was delivered following completion of a varying number of responses, with the mean ratio requirement equal to the specified schedule. Thus, on average, every 30th response produced food under the VR 30 schedule.

When rate of responding under the VR 30 schedule was stable (no visible trend) for at least six days, the terminal schedule, a multiple VR 30 variable-interval (VI) 60-s schedule (mult VR 30 VI 60-s), was introduced. Under this schedule the two components, VR 30 and VI 60-s, alternated in 2-min blocks with sessions always starting with the VR component. Under the VI 60-s schedule, food became available on average once every 60-s, and was delivered dependent on a lever press. The triple-cue lights served as a discriminative stimulus for the VR component while the house light was the discriminative stimulus for VI responding. That is, the triple-cue lights were illuminated only during VR 30 components and the house light was illuminated only during VI 60-s components. During food delivery the feeder light was the only light illuminated as the cue lights and house light were briefly darkened. No lights were illuminated during 5-s blackout periods between components. Each daily session lasted 45-48 minutes and ended after completion of the final VI component.

Pharmacological Procedure

Subjects were exposed to the mult VR 30 VI 60-s schedule of food reinforcement until there were no visible trends in response rates across 10 consecutive sessions (i.e.,

performance was stable). Thereafter, each subject received subcutaneous injections of either isotonic saline or nicotine 5-min prior to testing. (-)-Nicotine hydrogen tartrate (Sigma-Aldrich, St. Louis, MO) was dissolved in isotonic saline solution and prepared at an injection volume of 1 ml/kg. Doses are expressed as total salt weights. Subjects were tested Monday through Friday with dosing on Wednesdays. Doses and pre-session injection intervals were based on previous work by MacPhail et al. (2000) and Stolerman et al. (1974).

Phase I

Rats were divided at random into 6 groups (n=8) and received weekly injections of either saline or nicotine (0.125, 0.3, 0.6, 1.2, 1.8 mg/kg) for 4 weeks. An ED_{50} was next derived (by linear interpolation) from the week 1VR response-rate (percent-of-control) data and used during the second and third phases.

Phase II

Rats were divided at random into 4 groups (n=8) and received injections of the nicotine ED_{50} (0.73 mg/kg), derived from Phase I, once a week for four weeks. Group 1 (NNNN) received nicotine injections each week for four weeks (a replication of the pharmacological procedures in Phase 1). Group 2 (NVVN) received nicotine on the first and fourth weeks only, with saline-vehicle injections during the middle two weeks. Group 3 (VVVN) received vehicle injections for the first three weeks and nicotine during the last week only. Group 4 (VVVV) received vehicle injections during all four weeks.

Phase III

Eight rats were given weekly injections of saline and the nicotine ED_{50} (0.73 mg/kg), derived from Phase I, for four weeks. During the first three weeks each rat received a saline injection before the session and nicotine after the session. During the fourth week nicotine was given before the session and saline was not administered.

Results

Phase I

Figure 1 shows the dose-response data for variable-ratio response rates (VRrsp), variable-ratio reinforcement rates (VRrnf), variable-interval response rates (VIrsp) and variable-interval reinforcement rates (VIrnf) when nicotine was administered on 4 weekly occasions. Following the initial administration, nicotine produced dose-dependent decreases in response rates and reinforcement rates in both components of the multiple schedule, although no nicotine dose completely suppressed behavior. Statistical analysis by Repeated Measures Analysis of Variance (ANOVA) showed a significant effect (for all statistical tests, significance is defined at $p < 0.05$) of drug dose on all four dependent measures during the nicotine administration ($Pr>F = 0.0001$ for VRrsp, VRrnf, VIrnf and $Pr>F = 0.0002$ for VIrsp). Post-hoc (Tukey) analyses revealed a statistically significant difference between vehicle and the three highest doses (0.6, 1.2 and 1.8 mg/kg) for all dependent variables. The two lowest doses (0.125 and 0.3 mg/kg) were not significantly different from vehicle, with the exception of VIrsp where 0.3 mg/kg nicotine differed significantly vehicle.

Subsequent nicotine administrations show a diminished effect indicating substantial tolerance. Visual inspection of the data in Figure 1 clearly illustrates the reduced effect of each nicotine dose across subsequent weekly administrations. The biggest changes occurred from the first to the second administration.

Vehicle control values remained stable across all 4 weeks, demonstrating that performance did not shift simply as a function of the injections. Baseline values (data not shown) also remained both stable and comparable to vehicle control values.

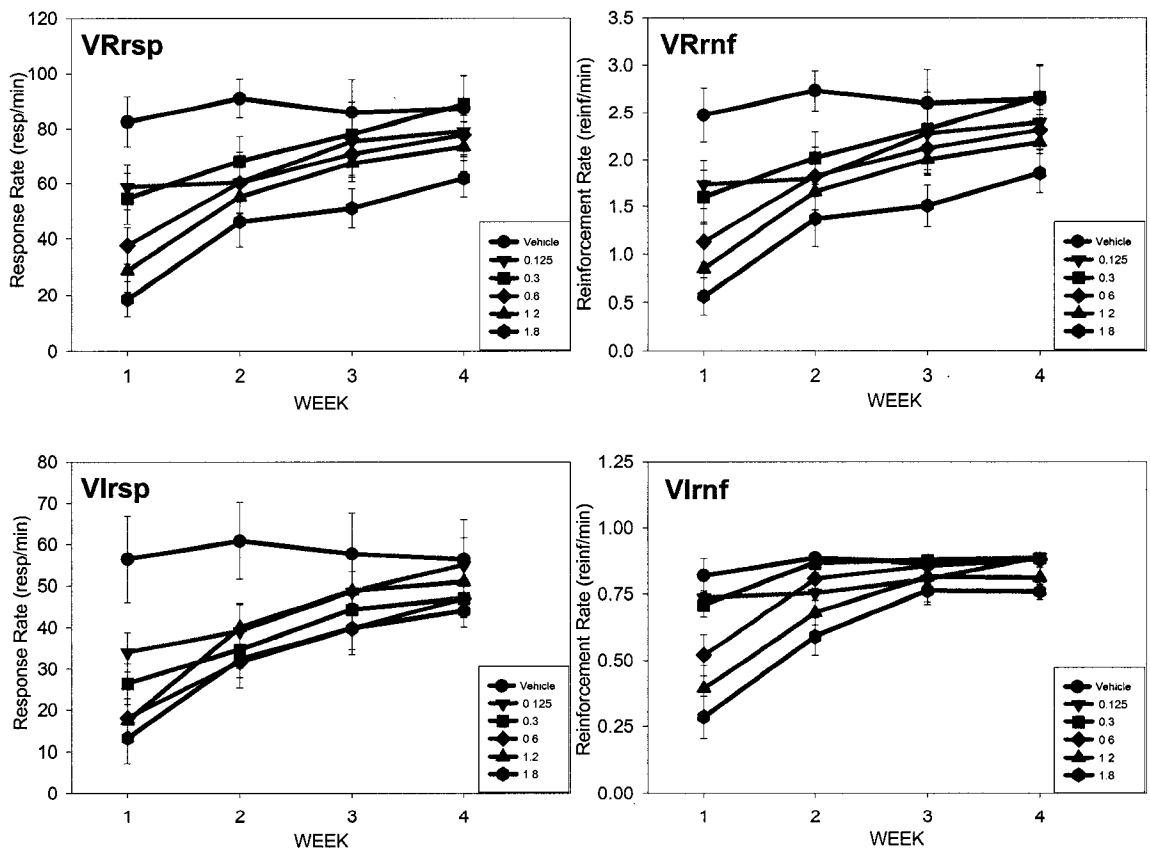

Figure 1: VR and VI response and reinforcement rates following Phase I vehicle (saline) or nicotine (mg/kg). Each symbol represents mean ± SEM of eight rats.

Estimated ED_{50} values for each exposure to nicotine were calculated for each dependent measure to quantify changes in drug effects with repeated exposure (Table 1). Estimated ED_{50} values for all four dependent variables increased across exposures to nicotine for response and reinforcement rates in the variable-ratio (VR) component, indicating tolerance. In the variable-interval (VI) component, ED_{50} values steadily rose across the first three exposure, indicating the progressive development of tolerance. There was, however, no increase from the third to the fourth exposure, indicating that the greatest change in the ED_{50}s occurred between the first two weeks with effects lessening between the third and fourth weeks.

Table 1

Estimated ED_{50} Values for Nicotine

	VRrsp	***VRrnf***	***VIrsp***	***VIrnf***
Week 1	0.73 mg/kg	0.20 mg/kg	0.72 mg/kg	1.23 mg/kg
Week 2	1.97 mg/kg	1.96 mg/kg	4.49 mg/kg	3.00 mg/kg
Week 3	2.55 mg/kg	2.44 mg/kg	7.06 mg/kg	9.56 mg/kg
Week 4	3.36 mg/kg	3.28 mg/kg	6.60 mg/kg	5.77 mg/kg

Pairwise comparisons were made using contrast statements calculated for each between-week comparison, at every dose, for all four dependent measures (Tables 2-5). Results revealed a statistically significant effect, expressed by the shaded areas, in the first 3 weeks (between weeks 1&3 or weeks 2&3) for all doses in each dependent measure, except 0.125 mg/kg in VIrnf, where the only significant contrast statement at the lowest dose was between weeks 1 & 4.

VRrsp contrast statements (Table 2) indicated significant differences between weeks 1 and 3 for 0.3, 0.6, 1.2, and 1.8 mg/kg ($Pr>F = 0.0002, 0.0131, 0.0026$ and 0.0002, respectively) and between weeks 2 and 3 for 0.125 mg/kg ($Pr>F = 0.0128$).

Table 2

Contrast Statements for Nicotine Phase I VRrsp

Dose	*Weeks 1&2*	*Weeks 1&3*	*Weeks 1&4*	*Weeks 2&3*	*Weeks 2&4*	*Weeks 3&4*
0.125	0.8449	0.1933	0.13	0.0128	0.0768	0.7418
0.3	0.0213	0.0002	0.0014	0.0165	0.0117	0.0636
0.6	0.0546	0.0131	0.0031	0.0109	0.0001	0.046
1.2	0.0053	0.0026	0.0085	0.0851	0.1349	0.5605
1.8	0.0002	0.0002	0.0001	0.3286	0.0112	0.0722

VRrnf contrast statements (Table 3) indicated significant differences between weeks 1 and 3 for 0.3, 0.6, 1.2, and 1.8 mg/kg (Pr>F = 0.0008, 0.0142, 0.0032 and 0.0001, respectively) and between weeks 2 and 3 for 0.125 mg/kg (Pr>F = 0.0112).

Table 3

Contrast Statements for Nicotine Phase I VRrnf

Dose	*Weeks 1&2*	*Weeks 1&3*	*Weeks 1&4*	*Weeks 2&3*	*Weeks 2&4*	*Weeks 3&4*
0.125	0.8053	0.1596	0.1166	0.0112	0.0774	0.7504
0.3	0.0234	0.0008	0.0019	0.0136	0.0094	0.0581
0.6	0.0576	0.0142	0.0035	0.0142	0.0003	0.0391
1.2	0.0073	0.0032	0.01	0.1302	0.173	0.5667
1.8	0.0003	0.0001	0.0001	0.39	0.0136	0.0786

VIrsp contrast statements (Table 4) indicated significant differences between weeks 1 and 3 for 0.3, 0.6, 1.2, and 1.8 mg/kg (Pr>F = 0.0001, 0.0004, 0.0009 and 0.0001, respectively) and between weeks 2 and 3 for 0.125 mg/kg (Pr>F = 0.0066).

Table 4

Contrast Statements for Nicotine Phase I VIrsp

Dose	*Weeks 1&2*	*Weeks 1&3*	*Weeks 1&4*	*Weeks 2&3*	*Weeks 2&4*	*Weeks 3&4*
0.125	0.3619	0.0521	0.0441	0.0066	0.0403	0.3987
0.3	0.0003	0.0001	0.0011	0.0012	0.0061	0.3352
0.6	0.0103	0.0004	0.0002	0.0295	0.0001	0.0375
1.2	0.0046	0.0009	0.0176	0.0451	0.2902	0.7927
1.8	0.0001	0.0001	0.0001	0.0836	0.0389	0.3403

VIrnf contrast statements (Table 5) indicated significant differences between weeks 1 and 3 for 0.3, 0.6, 1.2, and 1.8 mg/kg (Pr>F = 0.0047, 0.0019, 0.0011 and 0.0001, respectively) and between weeks 1 and 4 for 0.125 mg/kg (Pr>F = 0.0009).

Table 5
Contrast Statements for Nicotine Phase I VIrnf

Dose	*Weeks 1&2*	*Weeks 1&3*	*Weeks 1&4*	*Weeks 2&3*	*Weeks 2&4*	*Weeks 3&4*
0.125	0.8416	0.5247	0.0009	0.1612	0.1468	0.4277
0.3	0.0056	0.0047	0.0055	0.4052	0.2756	0.262
0.6	0.0009	0.0019	0.0018	0.0009	0.0515	0.3086
1.2	0.0041	0.0011	0.0071	0.0045	0.1337	0.9554
1.8	0.0003	0.0001	0.0001	0.0052	0.0127	0.8742

In summary, detailed analysis of the four data sets as a function of the number of weekly exposures to nicotine confirms that tolerance developed to the effects of the drug on both response rate and reinforcement rate, and under both VI and VR schedules.

Phase II

Figure 2 shows VR and VI response rates and reinforcement rates during Phase II. Vehicle (VVVV) controls, represented by the inverted triangles, remained stable across all four weeks of dosing. The only nicotine dose (0.73 mg/kg) used during phase II was a derived ED_{50} value, based on the results from phase I. The group that was a replication of phase I (NNNN) represented by the circles in Figure 2, produced similar results as in phase I. There was a decrease in response rates and reinforcement rates in both components of the multiple schedule following initial administration. Effects then lessened across weekly nicotine administrations.

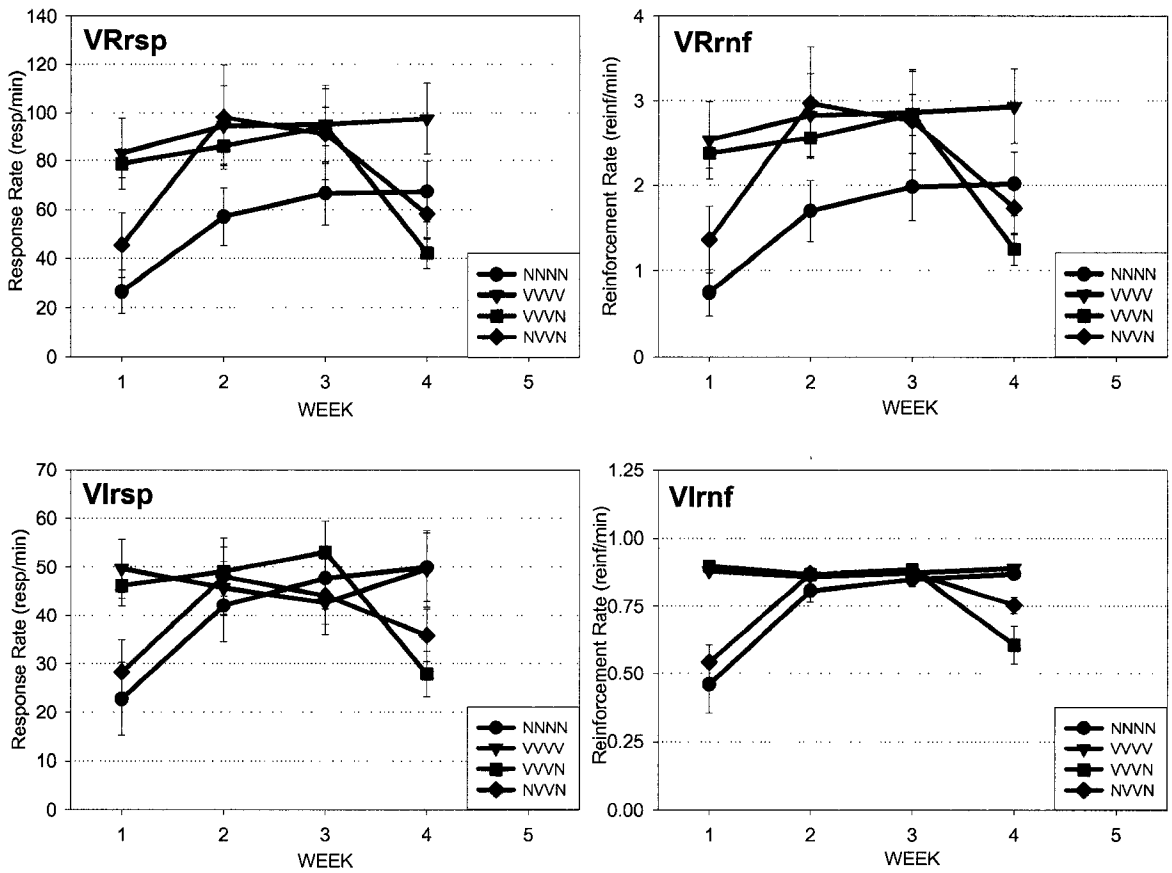

Figure 2: VR and VI response and reinforcement rates following Phase II vehicle (saline) and nicotine (0.73 mg/kg) injections. Each symbol represents mean ± SEM of eight rats.

Response and reinforcement rates for the group that received nicotine the first and fourth weeks only (NVVN) are slightly higher during week 4 than during week one, indicating that some tolerance developed. There is about a 25% increase in all dependent variables (27% for VRrsp, 25% for VRrnf, 26% for VIrsp and 28% for VIrnf) from week 1 to week 4 in NVVN, indicating slight tolerance.

Looking at the first nicotine injection only, it appears that the three additional weeks of testing did not influence the effects of nicotine on response and reinforcement rates for the group that received nicotine during the last week only (VVVN). During

week 4, when nicotine was first administered to the VVVN group, the rates were not significantly different from the groups (NNNN and NVVN) that first received nicotine during week 1. When expressed as a percent of the baseline control (data not shown), NNNN (47% for VRrsp, 34% for VRrnf, 50% for VIrsp and 53% for VIrnf) and NVVN (47% for VRrsp, 46% for VRrnf, 52% for VIrsp and 61% for VIrnf) during week 1 and VVVN (43% for VRrsp, 43% for VRrnf, 49% for VIrsp and 69% for VIrnf) during week 4, all produced similar results.

Additionally, these percent of baseline control values add support to our derived ED_{50} from phase I. The two groups that received nicotine during week 1 (NNNN and NVVN) both show a 53% decrease from baseline responding following their first nicotine injections, while the group that received nicotine for the first time during week 4 (VVVN) showed a 57% decrease. Even though these values are slightly higher than 50% it still shows that our derived dose of 0.73 mg/kg was very close to an ED_{50}. This comparison is only made in the VRrsp component because that is the only dependent measure used to calculate the derived ED_{50} from the Phase I data

Phase III

The third phase was included to determine whether tolerance was due to behavioral or pharmacological variables. Vehicle injections were given before each weekly test session and nicotine was given after each weekly test session during the first 3 weeks. In week 4, nicotine was administered for the first time before the weekly test session. The only nicotine dose (0.73 mg/kg) used during phase II was the derived ED_{50} value based on the results from phase I.

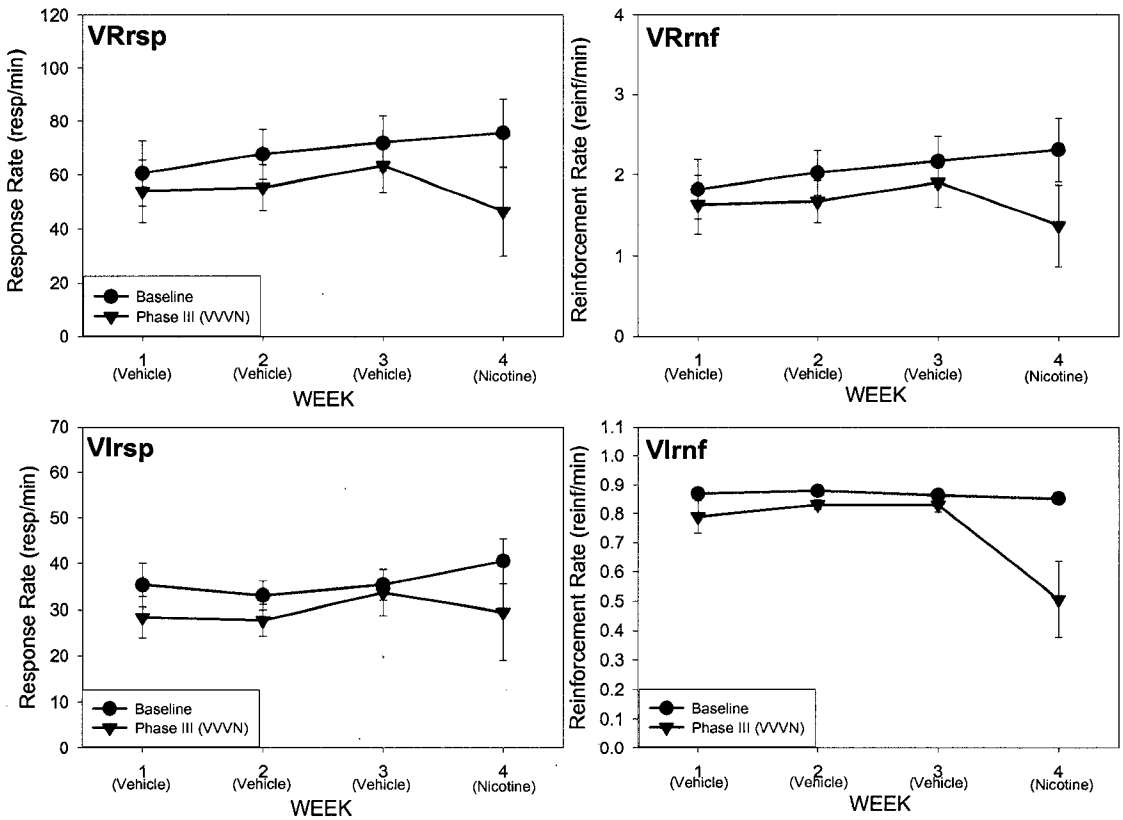

Figure 3: VR and VI response and reinforcement rates following Phase III vehicle (saline) or nicotine (0.73 mg/kg) injections. Baseline values were determined by averaging data from the day before injections for all rats. Each symbol represents the mean ± SEM of eight rats.

Figure 3 shows VR and VI response rates and reinforcement rates following Phase III saline and nicotine injections. Baseline values (day before each injection day) are represented by the circles, and Phase III test data are represented by the inverted triangles. During the first 3 weeks, when vehicle was given before the session, and nicotine after the session, both response and reinforcement rates were very consistent, indicating that vehicle injections did not affect performance. In week 4, when nicotine was given before the session, there were small decreases (27% for VRrsp, 28% for

VRrnf, 13% for VIrsp and 39% for VIrnf) in response and reinforcement rates relative to those obtained in week 3, when nicotine was not administered before the session.

These phase III results indicate that both pharmacological and behavioral tolerance occurred. There was a change in rates from the first 3 weeks, when vehicle was given before the session, to the 4th week, when nicotine was given before the session, so we know that tolerance that developed was not purely pharmacological (i.e., due to drug exposure *per se*). Moreover, this change was not statistically significant ($Pr>F = 0.5754$ for VRrsp, 0.5399 for VRrnf, 0.9116 for VIrsp, and 0.0253 for VIrnf) for most of the variables, so it is not solely behavioral tolerance. The only statistically significant result was for VIrnf ($Pr>F = 0.0253$) and post-hoc (contrast) statements revealed that the significance was between weeks 2 and 4.

Discussion

Weekly administration of nicotine (0.125, 0.3, 0.6, 1.2 and 1.8 mg/kg) produced behavioral tolerance over a period of four weeks during Phase I. Initial nicotine administrations (Week 1) decreased response rates and reinforcement rates in generally a dose-dependent manner in both the VR and VI components of the multiple schedule, although no dose completely suppressed behavior. Each subsequent weekly nicotine administration resulted in slightly higher response and reinforcement rates, indicating that tolerance developed. These results are similar to previous findings under conditions where nicotine was administered less frequently than once per day (Jarema et al., 2002; MacPhail et al., 2000; Miller et al., 2001; Stolerman et al., 1974).

During the second phase, when nicotine administrations with the derived ED_{50} dose (0.73mg/kg) were separated by a 3-week period, some tolerance also developed. Results indicate about a 25% increase, from the first to the fourth week, in response rates for both the VR and VI components, suggesting that tolerance will develop to nicotine when the period between administrations is greater than one week. Although previous studies have shown that tolerance can develope to nicotine when the drug is administered once a week, the results of Phase II appear to be the first demonstration of tolerance when the drug is administered less often than that. In addition, the group in Phase II that was a replication of the conditions in Phase I produced similar results as in Phase 1, providing additional support for the Phase I findings.

Phase III was included in this experiment as a way to examine whether the tolerance was more behavioral or pharmacological in nature. Pharmacological tolerance means the tolerance is the result of exposure to a drug *per se*, whereas behavioral tolerance means that tolerance is the result of performing the behavior of interest in the drug state. Behavioral tolerance is evident when a drug produces smaller effects following chronic exposure in animals that have repeatedly performed the task of interest in the presence of the drug than in other animals that have had comparable drug exposure, but have not performed the task in the presence of drug. Unfortunately, the data from Phase III were not nearly as orderly as those for the first two phases and therefore are difficult to interpret. They suggest, however, that some degree both behavioral and pharmacological tolerance developed to the effects of nicotine.

In all, the present findings indicate that intermittent acute (episodic) nicotine administrations can result in the development of tolerance. Additional testing should extend these findings by examining different reinforcement schedules and different time periods between injections, and by determining the extent to which performing the task of interest in the presence of drug influences tolerance. Further research should also determine whether tolerance develops to other nicotine-like compounds such as the chloronicotinyl insecticide imidacloprid (Kagabu, 1997).

CHAPTER III

EXPERIMENT 2

Materials and Methods

Subjects

One hundred and four experimentally naïve adult male Long-Evans rats (Charles River, Raleigh, NC), approximately 90 days old at experiment inception, were maintained at 350 grams via daily food restriction (Purina Rat Chow, St. Louis, MO) and served as subjects.

Upon arrival the rats were given time to acclimate to the housing colony and reach their target weight of 350 grams. They were fed *ad libitum* until they approached that weight and then were switched to a weight-maintenance program where their daily food allotment was gradually reduced and regulated so they maintained a weight of 350 grams (Ali et al., 1992). The rats were on this weight-maintaining feeding schedule for the duration of the experiment.

The rats were housed individually in 19.5 x 45.5 x 25.0 cm hanging plastic cages, with pine shaving bedding, in a temperature- (21-23°C) and humidity- (50-55%) controlled colony. A lighting schedule of 12-hr light and 12-hr dark was in effect (light on at 6:00am) with water available *ad libitum*. During the experiment proper, sessions were conducted Monday through Friday during the light cycle. Rats were transported to

the laboratory for daily testing in individual plastic cages, with filter tops, measuring 15.5 x 27.5 x 15.0 cm.

Apparatus

Behavioral sessions were conducted in commercially available operant test chambers (Coulbourn Instruments, Inc., Lehigh Valley, PA) positioned inside sound-attenuating enclosures (Ralph Gerbands Co., Arlington, MA) and ventilated by a fan. The inside of the test chamber measured 30 cm wide x 24 cm deep and 31 cm tall. A grid floor was raised 3.5 cm from the bottom of the chamber to allow for a collection pan and to keep the inside of the chamber clean. The front and back sides of the chamber were made of clear plastic while the right (component panel), left, top and bottom sides were metal. The font side opened down to allow access into the chamber. Each chamber was equipped with one response lever located on the right side of the component panel, 5 cm above the grid floor. Lever operation required a minimum downward force of 0.25 N. A set of triple-cue lights was located 3.5 cm directly above the lever. A pellet trough, into which 45-mg food pellets (P.J. Noyes Co. Inc., Lancaster, NH) could be dispensed from a dry-food feeder, was located to the left of the lever and 1.5 cm above the grid floor. The trough contained an overhead cue light that was briefly illuminated during food-pellet delivery. A Sonalert tone generator was situated 16 cm above the lever and was activated briefly (100 msec) after each response. A houselight, darkened only during food delivery, was situated at the top center of the component panel. Experimental events and

data collection were controlled by a Digital Equipment Corporation (Maynard, MA) PDP 11/73 computer, programmed with the SKED-11 system (Snapper et al., 1982).

Behavioral Procedures

Subjects were initially trained to lever press during one 8-hour overnight training session. This session comprised three successive schedules. First, a variable-time 60-s (VT 60-s) schedule was in effect for 60 food pellet presentations. Under this schedule, food was delivered on average every 60 s, regardless of the rat's behavior. In addition, conditions were arranged such that, if the lever was pressed 20 times, the schedule immediately shifted to a fixed-ratio (FR) 1. Under this schedule, every response produced a food pellet. If the lever was not pressed a minimum of 20 times, the VT 60-s schedule continued until 60 minutes had elapsed, at which time the schedule changed to an FR 1. Upon completing 60 responses under the FR 1 schedule, the value was increased to FR 2, which remained in effect until the rat emitted another 60 responses, at which time the session ended. If all three schedule requirements were not met, the session ended after eight hours had elapsed.

Handshaping and FR training were conducted during the days following the overnight session for those rats that did not acquire the lever-press response. If they still were not pressing the lever after two additional training days, food pellets were crushed and placed on the lever. On a few occasions the rats also needed to be trained to eat from the food cup. In this situation, the crushed food pellets were not only placed on the lever but on the edge of the food cup as well.

Upon completion of the training procedure, each rat was exposed to a variable-ratio (VR) schedule of food reinforcement during daily 23-min sessions. Rats were first exposed to a VR 3, then to VR 5, VR 10, VR 20 and VR 30-response schedule of reinforcement. The rate at which the ratios were increased was based on each rat's individual performance. Under the VR schedule, food was delivered following completion of a varying number of responses, with the mean ratio requirement equal to the specified schedule. Thus, on average, every 30^{th} response produced food under the VR 30 schedule.

When rate of responding under the VR 30 schedule was stable (no visible trends) for at least six days, the terminal schedule, a multiple VR 30 variable-interval (VI) 60-s schedule (mult VR 30 VI 60-s), was introduced. Under this schedule the two components, VR 30 and VI 60-s, alternated in 2-min blocks with sessions always starting with the VR component. Under the VI 60-s schedule, food became available on average once every 60-s, and was delivered dependent on a lever press. The triple-cue lights served as a discriminative stimulus for the VR component while the house light was the discriminative stimulus for VI responding. That is, the triple-cue lights were illuminated only during VR 30 components and the house light was illuminated only during VI 60-s components. During food delivery the feeder light was the only light illuminated as the cue lights and house light were briefly darkened. No lights were illuminated during 5-s blackout periods between components. Each daily session lasted 45-48 minutes and ended after completion of the final VI component.

Pharmacological Procedures

Subjects remained on the mult VR 30 VI 60-s schedule of food reinforcement until there were no visible trends in response rates across 10 consecutive sessions (i.e., performance was stable). Thereafter, each subject received subcutaneous injections of either isotonic saline or anatoxin-a 5-min prior to testing. (+)-Anatoxin-a fumarate (Sigma-Aldrich, St. Louis, MO) was dissolved in isotonic saline solution and prepared at an injection volume of 1 ml/kg. Doses are expressed as total salt weights. Subjects were tested Monday through Friday with dosing on Wednesdays. Doses and pre-session injection intervals were based on Stolerman et al. (1974) and pilot work in our laboratory.

Phase I

Rats were divided at random into 6 groups (n=8) and received weekly injections of either saline or anatoxin-a (50, 100, 150, 200, 250 μg/kg) for 4 weeks. After the first two injections we observed that 250 μg/kg completely suppressed behavior. Because 200 μg/kg nearly suppressed behavior when initially administered, we decided that no useful information would be gained from additional injections of the 250 μg/kg dose and therefore it was discontinued. In addition, visual analysis of the dose-response curve indicated a substantial decrease in response rates from the 50 to the 100 μg/kg doses. Therefore, we decided to test two additional doses (75 μg/kg and 125 μg/kg) wit two additional groups of rats (n=8). Each of these doses was given once a week for four weeks as described above.

An ED_{50} was next derived (by linear interpolation) from the initial VR response-rate (percent-of-control) data using 50, 75, 100, 150 and 200 μg/kg; this ED_{50} was used

during the second and third phases of the experiment. It is important to note that two doses (125 μg/kg and 250 μg/kg) were not used in calculating the ED_{50}. The higher dose (250 μg/kg) was not included because it initially completely suppressed responding. Moreover, visual inspection of the effect of the 125 μg/kg dose led us to conclude it was an anomaly, although no cause for it was apparent. In any case, data obtained at this dose were not used in calculating the ED_{50}. Had those data been used, the ED_{50} would have been 102 μg/kg, 10 μg/kg higher than the ED_{50} dose with those data excluded (92 μg/kg), which was used in Phases II and III.

Phase II

Rats were divided into 4 groups (n=8) and received injections for 4 weeks. Group AAAA received anatoxin-a injections each week for 4 weeks (a replication of the pharmacological procedures in phase I). Group AVVA received anatoxin-a on the first and fourth weeks only, with saline-vehicle injections during the middle two weeks. Group VVVA received vehicle injections for the first three weeks and anatoxin-a during the last week only. Each of these groups received 92 μg/kg anatoxin-a. Group 4 VVVV received vehicle injections during all four weeks.

Phase III

Eight rats were given weekly injections of saline and the anatoxin-a ED_{50} (92 μg/kg), derived from Phase I, for four weeks. During the first three weeks each rat received a saline injection before the session and anatoxin-a after the session. During the fourth week anatoxin-a was given before the session and saline was not administered.

Results

Phase I

Figure 1 shows dose-response curves for variable-ratio response rates (VRrsp), variable-ratio reinforcement rates (VRrnf), variable-interval response rates (VIrsp) and variable-interval reinforcement rates (VIrnf) when anatoxin-a was administered on 4 weekly occasions. Following the initial administration, anatoxin-a produced dose-dependent decreases in response rates and reinforcement rates in both components of the multiple schedule. Although the two lowest anatoxin-a doses (50 and 75 μg/kg) only produced slight decreases from baseline, the next highest dose (100 μg/kg) produced a significant effect. The two highest doses (150 and 200 μg/kg) strongly reduced response and reinforcement rates, and the slight differences between them are not statistically significant for any dependent variable. Statistical analysis by Repeated Measures Analysis of Variance (ANOVA) showed a significant effect (for all statistical tests, significance is defined at $p < 0.05$) for all four dependent measures during the first week of anatoxin-a administration ($Pr>F = 0.0001$ for VRrsp, VRrnf, VIrsp and VIrnf). Post-hoc (Tukey) analyses revealed a statistically significant difference between vehicle and the three highest doses (100, 150 and 200 μg/kg), but not between vehicle and the lowest doses (50 and 75 μg/kg), for all dependent measures.

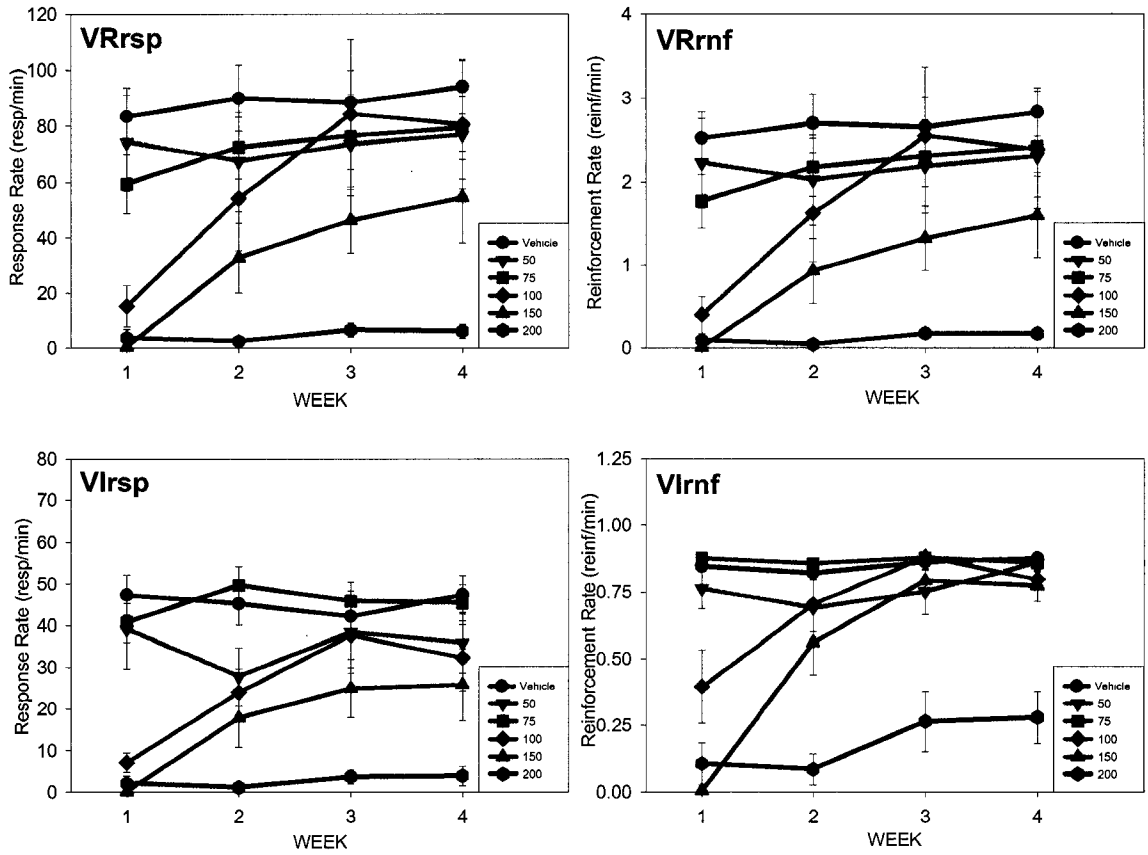

Figure 4: VR and VI response and reinforcement rates following Phase I vehicle (saline) or anatoxin-a (μg/kg). Each symbol represents mean ± SEM of eight rats.

Subsequent anatoxin-a administrations show a diminished effect, indicating substantial tolerance, for most of the doses. Visual inspection of the data in Figure 1 shows that for the most part the biggest change in effect occurred from the first to the second exposure to the compound. Two anatoxin-a doses, 50 μg/kg and 200 μg/kg, represented by the squares and circles, respectively, did not show a substantial change across the four weeks of testing. The highest dose (200 μg/kg) completely suppressed behavior in the first 2 weeks with just a slight increase during weeks three and four.

Additionally, 75 μg/kg actually produced rates equal to or greater than baseline values in both measures of VI performance.

Vehicle control values remained stable across all 4 weeks, demonstrating that performance did not shift simply as a function of the injections. Baseline values (data not shown) also remained both stable and comparable to vehicle control values.

Estimated ED_{50} values for each exposure to anatoxin-a were calculated for each dependent measure to quantify changes in effects of the compound with repeated exposure (Table 6). Consistent with the graphic analysis, systematic changes in the estimated ED_{50}s are indicative of tolerance. Table 6 shows an increase in the estimated ED_{50} values for response and reinforcement rates in the VR and VI components across the first three weekly anatoxin-a administrations, indicating tolerance. The rates did not increase further between weeks 3 and 4; in fact, some decreases were apparent.

Table 6

Estimated ED_{50} Values for Anatoxin-a

	VRrsp	***VRrnf***	***VIrsp***	***VIrnf***
Week 1	91.94 μg/kg	90.24 μg/kg	94.69 μg/kg	116.53 μg/kg
Week 2	118.56 μg/kg	117.49 μg/kg	121.21 μg/kg	156.31 μg/kg
Week 3	147.02 μg/kg	145.50 μg/kg	150.39 μg/kg	203.58 μg/kg
Week 4	134.54 μg/kg	133.63 μg/kg	130.08 μg/kg	166.40 μg/kg

Pairwise comparisons were made using contrast statements calculated for each between-week comparison, at every dose, for all four dependent measures (Tables 7-10). Results revealed a statistically significant effect, expressed by the shaded areas, in the first 3 weeks (1&3 or 2&3) for the three middle doses in each dependent measure, except 75 μg/kg in VIrsp where there were there was no statistically significant difference between any of the weeks. There was not a substantial change across the 4 weeks for the

lowest (50 μg/kg) or highest (200 μg/kg) doses. However, there was a significant change between the second and fourth weeks for the 200 μg/kg dose.

VRrsp contrast statements (Table 7) indicated significant differences between weeks 1 and 3 for 75, 100 and 150 μg/kg (Pr>F = 0.0029, 0.0380 and 0.0262, respectively).

Table 7

Contrast Statements for Anatoxin-a Phase I VRrsp

Dose	*Weeks 1&2*	*Weeks 1&3*	*Weeks 1&4*	*Weeks 2&3*	*Weeks 2&4*	*Weeks 3&4*
50	0.6131	0.9467	0.7978	0.5798	0.3867	0.5035
75	0.0512	0.0029	0.0295	0.3872	0.3327	0.5993
100	0.1109	0.038	0.0221	0.0706	0.1101	0.6156
150	0.0881	0.0161	0.0224	0.0715	0.093	0.6133
200	0.6174	0.6058	0.8222	0.2804	0.0651	0.9732

VRrnf contrast statements (Table 8) indicated significant differences between weeks 1 and 3 for 75, 100 and 150 μg/kg (Pr>F = 0.0037, 0.0364 and 0.0244, respectively).

Table 8

Contrast Statements for Anatoxin-a Phase I VRrnf

Dose	*Weeks 1&2*	*Weeks 1&3*	*Weeks 1&4*	*Weeks 2&3*	*Weeks 2&4*	*Weeks 3&4*
50	0.6168	0.9087	0.8202	0.6278	0.4126	0.4884
75	0.0401	0.0037	0.0256	0.3662	0.2765	0.4912
100	0.1051	0.0364	0.022	0.0688	0.1298	0.05224
150	0.1051	0.0244	0.0268	0.0777	0.0914	0.488
200	0.5117	0.8401	0.7433	0.3259	0.0544	0.7093

VIrsp contrast statements (Table 9) indicated significant differences between weeks 1 and 3 for 100 and 150 μg/kg (Pr>F = 0.0045 and 0.0272, respectively).

Table 9

Contrast Statements for Anatoxin-a Phase I VIrsp

Dose	*Weeks 1&2*	*Weeks 1&3*	*Weeks 1&4*	*Weeks 2&3*	*Weeks 2&4*	*Weeks 3&4*
50	0.1597	0.9399	0.6528	0.1704	0.1607	0.5501
75	0.0719	0.1836	0.4142	0.3863	0.355	0.9229
100	0.0206	0.0045	0.0138	0.0622	0.2931	0.0569
150	0.104	0.0272	0.0315	0.0666	0.068	0.4463
200	0.658	0.4392	0.7591	0.223	0.0979	0.9501

VIrnf contrast statements (Table 10) indicated significant differences between weeks 1 and 3 for 100 and 150 μg/kg (Pr>F = 0.0090 and 0.0001, respectively), and between weeks 2 and 3 for 75 μg/kg (Pr>F = 0.0475).

Table 10

Contrast Statements for Anatoxin-a Phase I VIrnf

Dose	*Weeks 1&2*	*Weeks 1&3*	*Weeks 1&4*	*Weeks 2&3*	*Weeks 2&4*	*Weeks 3&4*
50	0.6182	0.9266	0.1798	0.6571	0.1947	0.2027
75	0.0862	0.7586	0.6062	0.0475	0.945	0.5489
100	0.0666	0.009	0.0143	0.1167	0.4159	0.0274
150	0.0219	0.0001	0.0001	0.0596	0.0803	0.2893
200	0.7604	0.2729	0.3527	0.2058	0.0257	0.9353

In summary, detailed analysis of the four data sets as a function of the number of weekly exposures to anatoxin-a confirms that tolerance developed to the effects of the compound on both response rate and reinforcement rate, and under both VI and VR schedules.

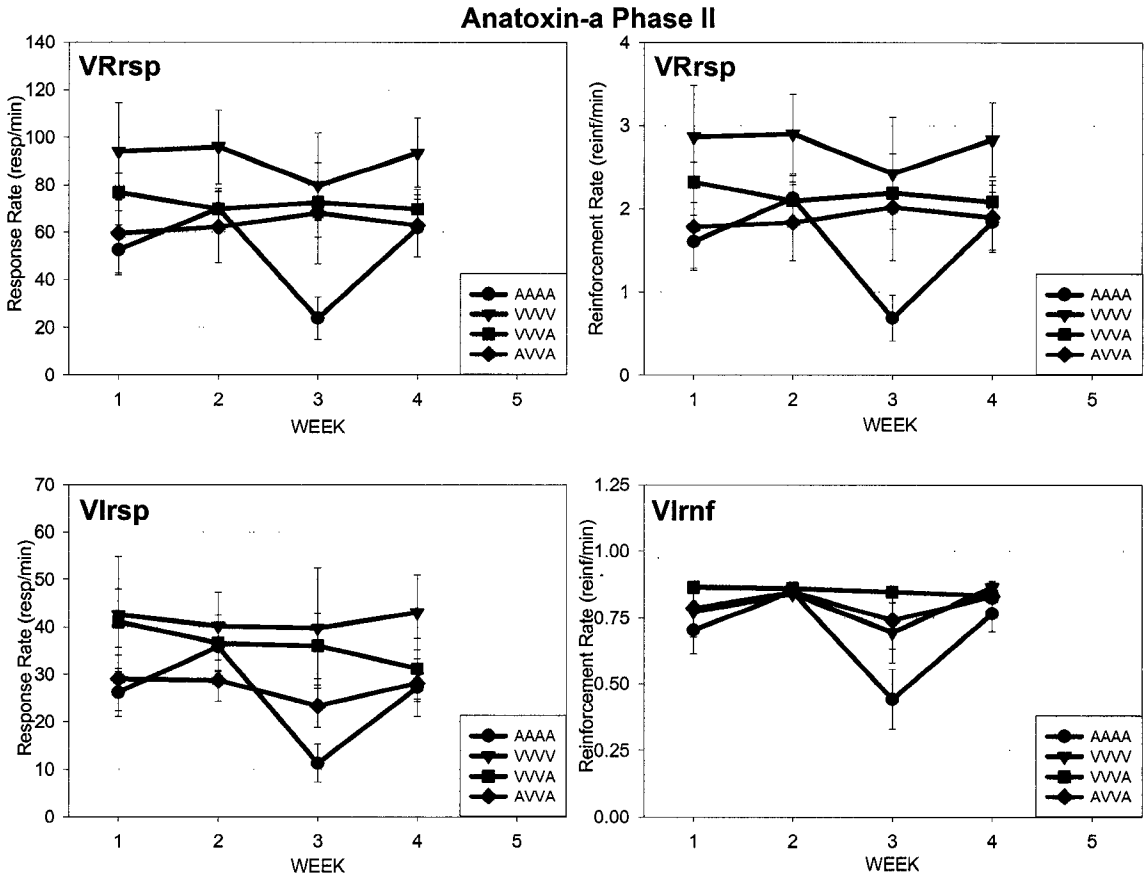

Figure 5: VR and VI response and reinforcement rates following Phase II vehicle (saline) and anatoxin-a (92 μg/kg) injections. Each symbol represents mean ± SEM of eight rats.

Phase II

The only anatoxin-a dose (92 μg/kg) used during phase II was a derived ED_{50} value, based on the results from phase I. The group that was a replication of phase I (AAAA), represented by the circles in figure 4, produced results that differed in some regards from those of phase I. There was a decrease in response rates and reinforcement rates in both components of the multiple schedule following initial administration, and the effects then lessened between the first and second weekly administrations, as they did

in Phase I. However, during the third weekly administration for AAAA, VR and VI response and reinforcement rates sharply decreased to nearly 25% below those obtained the week before. Group VVVV also showed a slight decrease in response and reinforcement rates during the third week for all components except VIrsp. No known equipment or experimenter error can account for this decrease. The effect of anatoxin-a on all rates increased again in week 4 but they were still slightly lower than in week 2. Vehicle (VVVV) rates during week 4 were similar to those observed during the first two weeks.

Response and reinforcement rates for the group that received anatoxin-a in the first and fourth weeks only (AVVA) did not change significantly over the four weeks, except for the unexplainable decrease during week 3, indicating that tolerance did not develop. There is about a 5% increase (5% for VRrsp, 6% for VRrnf, and 5% for VIrnf) from week1 to week 4 in AVVA, suggesting that no tolerance occurred. There was actually a 3% decrease from week 1 to week 4 for VIrsp.

A comparison of the first anatoxin-a administration (AAAA) and the vehicle control (VVVV) show almost a fifty percent reduction (44%) for VRrsp, supporting our derived ED_{50} value. This comparison is only made in the VRrsp component because it is the only one used to calculate the derived ED_{50} from the Phase I data. Group AVVA, which also received anatoxin-a during the first week, was not significantly different from AAAA, and VVVA was not statistically significant from VVVV during week 1.

Looking at the first anatoxin-a injections only, the rates were not significantly different for groups AAAA and AVVA, which first received anatoxin-a during week 1.

When expressed a percent of the baseline control (data not shown), results for AAAA (68% for VRrsp, 68% for VRrnf, 73% for VIrsp and 82% for VIrnf) are very similar to those for AVVA (68% for VRrsp, 69% for VRrnf, 71% for VIrsp and 90% for VIrnf) during week 1 administration. However, for the group (VVVA) that first received the anatoxin-a during week 4, the percent of the baseline control values (90% for VRrsp, 89% for VRrnf, 101% for VIrsp and 95% for VIrnf) are much higher than those observed in the two groups (AAAA and AVVA) that first received anatoxin-a during week 1. In fact, group VVVA didn't seem to be affected by anatoxin-a injections at all.

One concern with these percent control values is that they do not support our derived ED_{50}. The two groups that received anatoxin-a during week 1 (AAAA and AVVA) both show only a 32% decrease from baseline responding, in VRrsp, following their first anatoxin-a injections, and the group that received anatoxin-a for the first time during week 4 (VVVA) showed only a 10% decrease. These values are too far from 50% to be considered an accurate ED_{50} value and thus we must conclude that our derived dose of 92 μg/kg is too low. This comparison is only made in the VRrsp component because that is the only dependent measure we used to calculate the derived ED_{50} from the Phase I data.

Phase III

During phase III, vehicle injections were given before each weekly test session and anatoxin-a was given after each weekly test session during the first 3 weeks. In week 4, anatoxin-a was administered for the first time before the weekly test session. The only

anatoxin-a dose (92 μg/kg) used during phase II was the derived ED50 value based on the results from phase I.

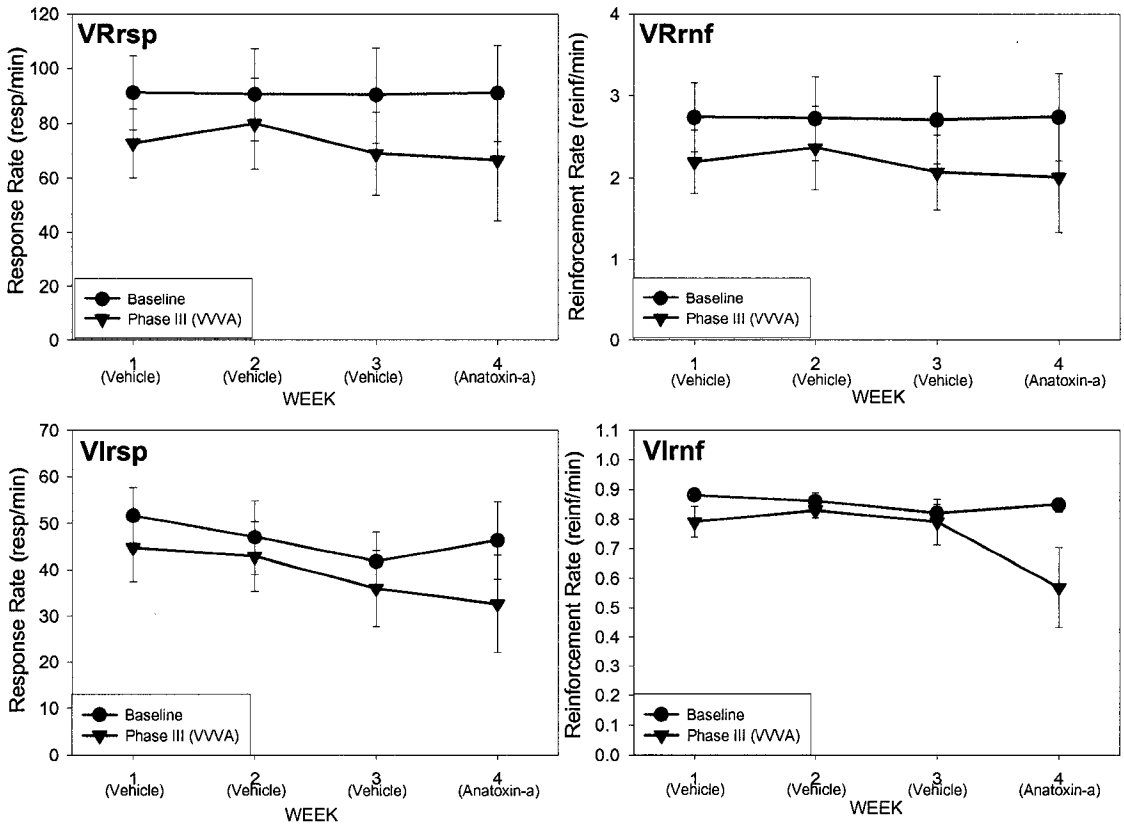

Figure 6: VR and VI response and reinforcement rates following Phase III vehicle (saline) or anatoxin-a (92 μg/kg) injections. Baseline values were determined by averaging data from the day before injections for all rats. Each symbol represents mean ± SEM of eight rats.

Figure 3 shows VR and VI response rates and reinforcement rates following Phase III saline and anatoxin-a injections. Baseline values (day before each injection day) are represented by the circles and Phase III test data are represented by the inverted triangles. During the first 3 weeks, when vehicle was given before the session, and anatoxin-a after the session, both response and reinforcement rates were very similar

indicating that the vehicle injection itself did not affect performance. In week 4, when anatoxin-a was given before the session, there was only a very small decrease (3% for VRrsp, 3% for VRrnf, 9% for VIrsp and 28% for VIrnf) in performance levels relative to week 3, when anatoxin-a was not administered before the session. There was only a very small change in VI rates from week 3, when vehicle was given before sessions, and the 4th week, when nicotine was given before the session, so any tolerance that occurred was not purely pharmacological (i.e., due to drug exposure per se). Moreover, this change was not statistically significant ($Pr>F = 0.7769$ for VRrsp, 0.8172 for VRrnf, 0.3366 for VIrsp and 0.1291 for VIrnf) so the tolerance was not solely behavioral. However, it does appear in phase III, as with phase II, that our derived ED50 value for anatoxin-a was too low.

Discussion

Weekly administration of (+)anatoxin-a (50, 75, 100, 150, and 200 μg/kg) produced behavioral tolerance over a period of four weeks during Phase I. Initial (+)anatoxin-a administrations (Week 1) decreased response rates and reinforcement rates in generally a dose-dependent fashion under both the VR and VI components of the multiple schedule. Each subsequent weekly (+)anatoxin-a administration resulted in slightly higher response and reinforcement rates, indicating tolerance. Additionally, the two highest does (150 and 200 μg/kg) nearly suppressed all behavior during the first week, but behavior emerged in the later weeks indicating that tolerance will develop to (+)anatoxin-a even at doses high enough to nearly eliminate behavior after initial

exposure. No prior studies have examined tolerance to anatoxin-a, but the results from the initial administration are similar to findings reported by Stolerman et al. (1992), who found that anatoxin-a decreased activity in dose-dependent fashion.

During the second phase, when (+)anatoxin-a administrations with the derived ED_{50} value (92 μg/kg) were separated by a 3-week period, tolerance did not develop. Results showed that there was not a significant change in response rates for either the VR or VI components over the 4-week period. One possible explanation for this phenomenon is that our derived ED_{50} value simply wasn't high enough. There were also puzzling results with the group that was a replication of the conditions in Phase I. These results did not resemble the results in Phase I. Initial administration did produce a decrease in behavior, and during the second week response rates were greater than the first. However, during the third week there was an unexplainable sharp decrease in rates for all components of the multiple schedule. Given these puzzling findings, the results of Phase II should be replicated before strong conclusions are drawn concerning the effects of administering anatoxin-a less frequently than once a week.

The results of Phase III do not clarify whether behavioral or pharmacological tolerance develops to anatoxin-a. The only real effect of (+)anatoxin-a administration occurred in the VIrnf component, where there was a slight decrease in reinforcement rate. These results suggest that the derived ED_{50} value was too low. Phase III needs to be replicated, using a higher dose of anatoxin-a.

The present results do clearly indicate that tolerance will develop with weekly (+)anatoxin-a administration. They do not, however, indicate whether tolerance will

develop with less-frequent anatoxin-a injections, or whether the tolerance that develops is pharmacological, behavioral, or a combination of the two. The derived ED_{50} seems to have been an ineffective dose, and therefore a higher dose may have produced different results. Only one published study has examined the behavioral effects of (+)anatoxin-a (Stolerman et al., 1992), and further studies, including replications of the conditions of Phase II and Phase III with higher doses, are needed to clarify how tolerance develops to this compound. Research should also explore whether racemic anatoxin-a produces results similar to those of the (+) isomer.

CHAPTER IV

GENERAL DISCUSSION

Weekly administration of both nicotine and (+)anatoxin-a produced behavioral tolerance over the course of four weeks. The two highest doses of (+)anatoxin-a nearly suppressed all behavior after the first administration, but then behavior emerged during subsequent treatments, indicating tolerance could develop even to relatively high doses. It remains to be determined whether tolerance will develop to similar severely disruptive nicotine doses, which were not examined in the present study.

With both nicotine and anatoxin-a, a similar degree of tolerance was observed under the VI and VR schedules, even though the degree of initial reinforcement loss (relative to baseline levels) was greater under the latter schedule. Thus, the present findings indicate that relative reinforcement loss did not modulate the development of tolerance to either compound. Some degree of initial reinforcement loss did, however, occur under both schedules, thus the present findings are consistent with the reinforcement-loss hypothesis as initially proposed by Schuster et al. (1966).

At the completion of Phase I it appeared as though tolerance developed similarly to (+)anatoxin-a and nicotine. This did not appear to be the case in Phase II. When administrations were separated by a 3-week period, some tolerance developed to nicotine, but not to (+)anatoxin-a. However, it appears that the derived ED50 for (+)anatoxin-a used in Phase II was too low, and therefore comparisons of the effects of nicotine and anatoxin-a based on these data are suspect. Further research should be conducted to

assess whether tolerance develops to anatoxin-a when it is administered less frequently than once a week.

In general, prior studies have suggested that there are similarities between nicotine and (+)anatoxin-a with respect to their neurochemical actions (e.g., MacCallan et al., 1988; Thomas et al., 1993; Wonnacott et al., 1991) and the current results suggest that there also are similarities in the behavioral effects of the two compounds with both acute and weekly administrations. Further testing is needed, however, to fully ascertain the extent of these similarities.

APPENDIX A

IACUC Approval

IACUC LAPR APPROVAL SHEET

Navy 160

LAPR #: 04-07-001 **PRINCIPAL INVESTIGATOR: Robert MacPhail**

PROJECT TITLE: Systematic Changes and Behavioral Effects of Acute and Episodic Nicotine and Imidacloprid Exposures (Rat)

DIVISION/BRANCH: NTD/NBTB **MAIL DROP:MD-74B** **PHONE #: 541-7833**

ALTERNATE CONTACT: Kimberly Jarema **PHONE #: 541-2299**

REQUIREMENTS/RESTRICTIONS

- Please be advised that the animals ordered under this LAPR are intended to be used only for this project.
- This LAPR must be updated annually. This can be accomplished by submitting an amendment sheet on :

  First Year Due Date: **July 31, 2002** Second Year Due Date:**July 31, 2003**
- All LAPR'S are required to be completely renewed every three (3) years. This LAPR comes due on:**July31,2004**
- Please include a copy of this approval sheet with all future animal orders to ensure that there are no delays in your animal orders.

This is to inform you that your LAPR has been approved by the Institute Animal Care & Use Committee

Veterinarian: B Ann Hobbs Date: 7/9/01

IACUC Chair: C.J. Bowen Date: 7/9/01

IACUC Member: Karen H. Brock Date: 7/9/01

IACUC Member: Deborah James Date: 7-9-01

IACUC Member: C Moore Date: 7-9-01

IACUC Member: Ben Michael Date: 7-10-01

IACUC Member:_____ Date:_____

IACUC Member:_____ Date:_____

IACUC Member:_____ Date:_____

IACUC Member:_____ Date:_____

IACUC Member:_____ Date:_____

See Backside For Special Requirements and/or Restrictions

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