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Just Say "Nootropic": The Effects of Nicotine on Memory and Learning

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Abstract

This study investigated the effects of nicotine on memory and learning in adolescent and adult male Fischer-344 rats. Rats were given 0.2 mg/kg/day of either nicotine or saline chronically for 2 weeks and were tested in the Morris water maze as adolescents (Phase 1) and then again 4 months later as adults (Phase 2). There were 4 main groups: nicotine/nicotine, nicotine/saline, saline/nicotine, and saline/saline. In Phase 2 rats were tested for c-Fos and BrdU expression in the dentate gyrus of the hippocampus. Behavioral data indicated that as adults, rats given nicotine were significantly improved at the water maze task compared to rats given saline. Impairments of the S/N group suggest performance in the maze is state dependent upon the nicotine. An increase in c-Fos expression was seen in the saline rats, and no BrdU expression was seen in either group. The behavioral results imply that low doses of nicotine improve learning and memory in adult rats. This provides support for studies investigating nicotine as a therapeutic agent for diseases affecting cognition, such as Alzheimer's.

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For the past twenty years, nicotine and drugs that act as agonists to nicotinic receptors have been under investigation due to their cognitive enhancing properties and their potential for therapeutic use in certain neurodegenerative diseases, schizophrenia, and attention deficit hyperactivity disorder (Dunbar *et al.*, 2007; Levin & Rezvani, 2000; Rezvani & Levin, 2001; White & Levin, 1999, , 2004). At the present time, the drugs available to patients suffering from Alzheimer's disease and other diseases affecting cognition provide minimal relief of symptoms. Most of these drugs involve the indirect stimulation of cholinergic receptors, however studies have shown that direct stimulation of nicotinic receptors—via nicotine itself or nicotinic agonists—may have a more pronounced therapeutic effect (Ono, Hasegawa, Yamada, & Naiki, 2002; Rezvani & Levin, 2001). Thus, nicotine research has provided some promising results regarding possible therapies for such diseases.

Nicotine: Neural Mechanisms

Nicotine, the main psychoactive ingredient found in tobacco, mimics the effects of acetylcholine (ACh) at nicotinic ACh receptors (nAChRs). Like ACh, it modulates the signaling pathways of target neurons by stimulating the release of several neurotransmitters that are associated with memory, learning, and reward, including acetylcholine, glutamate, dopamine, norepinephrine, and serotonin (Fagen, Mansvelder, Keath, & McGehee, 2003; Levin, Limpuangthip, Rachakonda, & Peterson, 2006; Mansvelder, van Aerde, Couey, & Brussaard, 2006; Rezvani & Levin, 2001; Wang *et al.*, 2008). It has been confirmed by multiple studies that nicotine has a pronounced effect on

areas of the brain linked with reward, specifically the mesolimbic system, through the increased release of dopamine (Clarke, 1992; Di Chiara, Acquas, Tanda, & Cadoni, 1992). This is the essence of the controversy surrounding nicotine as a therapeutic drug: its stimulation of receptors that release reward-based neurotransmitters could lead to addiction, particularly if the dose is high and self-administration is possible (Merlo Pich, Chiamulera, & Carboni, 1999; Wang *et al.*, 2008).

Despite this controversy, addiction studies of nicotine provide some important information that leaves the door open for nicotine's use in diseases affecting cognition. The most common way people become addicted to nicotine is via cigarette smoking (Winger, Woods, & Hofmann, 2004). However, when nicotine is introduced into the body in this way, a large amount of nicotine reaches the brain extremely rapidly on inhalation. The immediate, semi-euphoric effect that results is what increases the likelihood for nicotine addiction (Winger *et al.*, 2004). Additionally, studies involving self-administration of nicotine in rats have shown that when rats are allowed to self-administer, they take in doses of nicotine on par with that of a cigarette smoker, which contributes to research labeling it as addictive (Abrous *et al.*, 2002). Evidence suggests that when nicotine is administered to animals (or humans) at a slow rate and in a controlled manner (not through cigarettes or tobacco), the possibility for addiction is much lower (Samaha, Yau, Yang, & Robinson, 2005). Thus, as long as the dose of nicotine is low and patients are on a well-defined schedule, it is less likely to produce an addiction and has the potential to be a safe therapeutic alternative.

Though nicotine is the main rewarding component in cigarettes and the reason smoking can be addictive, it is important to understand that the harmful effects of

smoking are largely due to the combination of an estimated 4000 constituents of cigarette smoke—not the nicotine itself (Winger *et al.*, 2004). When the smoke turns to vapor it has high amounts of toxic carbon monoxide, which adds to the harmful effects of cigarette smoking (Winger *et al.*, 2004). Because of the toxic nature of the ingredients in cigarettes and their reactions to being burned, it is no surprise that cigarette smoking can lead to serious illnesses such as coronary heart disease, emphysema and various forms of cancer (Winger *et al.*, 2004). Nicotine alone, especially when administered intravenously or by transdermal patches, does not cause these health problems (Winger *et al.*, 2004).

The action of nicotine in the brain relies upon nAChRs. These receptors are ligand-gated ion channels containing five subunits that form a circle around a central pore, and allow the influx of positively charged ions. Subunits can be either alpha, beta, gamma, or delta, but the subunits most connected to nicotinic involvement are alpha 4 beta 2 ($\alpha4\beta2$) subunits or alpha 7 ($\alpha7$) subunits (Hawi & Lowe, 2007; Winger *et al.*, 2004). When a nicotine molecule binds to a receptor, the receptor opens and allows positively charged ions to enter. This leads to an excitatory postsynaptic potential (EPSP), which increases the likelihood of neurotransmitter release from the responding cell. It is this potential for neurotransmitter release that leads to nicotine's cognitive enhancing effects. The makeup of the subunits of the receptor as well as the location of the receptor in the brain dictates the effect of the neurotransmitter release within the brain (Winger *et al.*, 2004).

Mouse studies of nicotine's effect on gene expression and receptors have reported that nicotine administration results in receptor upregulation in the nucleus accumbens (one area from which nicotine is known to stimulate the release of dopamine), as well as

in the hippocampus, which is highly involved in learning and memory (Wang *et al.*, 2008). Receptor upregulation is an increase in available receptors in the brain that can occur in response to a large amount of drug present in that brain area. In order to provide a place for the extra drug molecules to bind, receptors multiply—this results in an increased effect of the drug, and also leads to the potential for withdrawal symptoms if the drug is suddenly ceased. When receptors are exposed to a large amount a drug, however, receptors can become desensitized and receptor downregulation can occur as a compensatory mechanism (Benwell, Balfour, & Birrell, 1995).

With regard to nicotine's action upon certain receptors, much promising data has come from studies concerning nicotinic cholinergic receptors in rats and mice. The $\alpha 7$ and $\alpha 4\beta 2$ receptors, in particular, have been demonstrated to play a role in cognitive functioning (Levin, McClernon, & Rezvani, 2006). A study using $\alpha 7$- nAChR knockout mice determined that mice missing these receptors performed poorly on attentional tasks of cognitive function when compared to normal controls (Young *et al.*, 2007). Experiments performed with rats in a passive avoidance test also demonstrated that both chronic and acute nicotine administration had cognitive-enhancing effects as a result of $\alpha 7$-nAChR stimulation in the hippocampus (Uzum, Diler, Bahcekapili, Tasyurekli, & Ziylan, 2004). Wild-type mice with $\alpha 7$-nAChRs have also demonstrated more cell proliferation as a result of nicotine administration when compared to mice lacking these receptors (Koike *et al.*, 2004).

The Hippocampus

Multiple studies have shown that nicotine has a positive effect on memory and learning in humans and non-human animals, and one of the areas of the brain most

affected by nicotine administration is the hippocampus (Levin, Sledge, Baruah, & Addy, 2003; Wang *et al.*, 2008). The hippocampus plays a key role in learning and memory, particularly learning and memory involving spatial information (Izquierdo, 1995). Long Term Potentiation (LTP) is the mechanism that allows for the hippocampus' involvement in memory storage and memory retrieval (Izquierdo, 1995). LTP is an increase in synaptic effectiveness, which can result in changes in the synapses, an increase in synaptic firing and an increase in neurotransmission in the hippocampus (Schumann *et al.*, 2004). Spatial learning tasks such as the Morris water maze can increase LTP in the rat hippocampus, and it is possible that nicotine can also facilitate this process in conjunction with these tasks.

One group has proposed that glutamate release in the hippocampus is enhanced by nicotine. They examined the effect of nicotine and a glutamate agonist (dizocilpine) on spatial learning in the radial arm maze. When the rats were given dizocilpine in conjunction with nicotine, they displayed an impairment in working memory in the maze (Levin *et al.*, 2003). The dizocilpine was administered via a local infusion cannula in the ventral hippocampus, so the impairment in working memory was localized specifically to this brain area (Levin *et al.*, 2003). This result provides support for the notion that the hippocampus is an integral brain area in the formation of working memory, and that glutamate neurotransmission as a result of nicotine administration is central to the cognitive enhancing properties of nicotine.

Cognitive Enhancement in Humans

There have been numerous studies investigating nicotine's effect on human cognition. It has been shown that when regular smokers are deprived of cigarettes they

experience withdrawal symptoms that include cognitive deficits, such as decreased accuracy on tasks of performance, and a decrease in psychomotor skills (Hatsukami, Fletcher, Morgan, Keenan, & Amble, 1989). One study demonstrated that as little as 24 hours after cigarette cessation, reaction times and errors related to vigilance tasks were significantly increased (Hatsukami *et al.*, 1989). Though it is possible that the cognitive decline could have been due in part to the other symptoms of withdrawal from cigarettes (including headaches, nausea, etc.), it is likely that the decline was at least partially a result of the cessation of nicotine. In 24 hours the participants in the study were not reported to have any other noticeable withdrawal symptoms, which supports the notion that the removal of nicotine was a contributing factor to the decrease in performance on the cognitive tasks.

Due to the potential for cognitive decline following nicotine cessation in cigarette smokers, it is beneficial to investigate the effect of nicotine on adult non-smokers without any attentional or cognitive deficits—this research demonstrates nicotine's effectiveness in improving cognition in normal, healthy controls. Non-smokers are an ideal baseline in these types of studies because there is no possibility of withdrawal, which could skew the results. Additionally, regular smokers would have to change their smoking patterns for the study, which would introduce even more potential for variation in the results (Hatsukami *et al.*, 1989; Levin & Rezvani, 2000; Rezvani & Levin, 2001). Nicotine has been shown to improve the attention of non-smoking normal controls when administered through subcutaneous injections and transdermal nicotine patches, suggesting that it may also have a similar effect on patients with attentional disorders or other cognitive impairments (Levin, Conners *et al.*, 1998; Levin & Rezvani, 2000).

Alzheimer's Disease

Because nicotine enhances the cognition of human non-smokers, it has been of great interest to researchers whether it could have cognitive enhancing—and therefore therapeutic—effects on people with various cognitive ailments, including Alzheimer's disease. Alzheimer's disease (AD) is characterized by severe cognitive deficits, including impairments of memory and executive (higher-order) function (Nixon, 2002). Generally these deficits begin mildly, and become progressively worse as increased cell death leads to severe cortical atrophy (Nixon, 2002). The most widely understood neuropathology of post-mortem Alzheimer's brains includes the presence of neurofribrillary tangles and neuritic plaques (Buckner, Head, & Lustig, 2006; Nixon, 2002). The neuritic plaques are found largely in brain areas involved in learning and memory, such as the neocortex, the hippocampus, and the amygdala. This provides insight into Alzheimer's negative effect on cognition as the disease progresses (Nixon, 2002).

In 2002, Ono and colleagues demonstrated that nicotine interferes with the formation of in vitro amyloid plaques. This provides important information regarding the neuroprotective effects of nicotine that extend beyond nicotinic receptor upregulation, and supports the notion that nicotine can be used as a therapeutic agent in Alzheimer's disease. Studies have also shown that the neuropathology of postmortem Alzheimer's brains involves a significant decrease in nAChRs compared to that of normal ageing (Court *et al.*, 2001). Because nicotine administration results in an upregulation of nAChRs, this effect could help delay some of the more severe cognitive deficits of the disease by keeping the nAChR level in the brain stable and increasing neurotransmission.

Studies of nicotine's effects in humans are generally carried out using injections, nasal sprays, or transdermal nicotine patches. These methods of nicotine administration cause nicotine to reach the brain much more slowly and are less likely to lead to a nicotine addiction than smoking, in which nicotine reaches the brain extremely rapidly (Samaha *et al.*, 2005). The first study investigating the effects of intravenous nicotine administration on Alzheimer's patients used extremely small doses of nicotine, and found that even when the dose was as small as 0.25 μg/kg/min, AD patients showed decreased errors on a test of cognitive function (Newhouse *et al.*, 1988). In studies using acute nicotine doses of 0.4 to 0.8 mg, significant cognitive enhancement was demonstrated in normal controls as well as Alzheimer's patients on various tests of attention and cognitive function (Jones, Sahakian, Levy, Warburton, & Gray, 1992; Sahakian, Jones, Levy, Gray, & Warburton, 1989). More recently, however, the transdermal nicotine patch has been used in order to provide a larger dose and to avoid the necessity of injections. In a pilot study demonstrating the effects of the nicotine patch in AD patients, Wilson and colleagues (1995) demonstrated that the nicotine group (still receiving a moderate dose—0.9 mg/hr) showed improved learning. This was a result that had not been previously reported (early studies only showed improved attention), and it paved the way for further research in this area (Wilson *et al.*, 1995).

In 1999, White and Levin investigated the effect of a 5 mg transdermal nicotine patch when worn by patients with mild to moderate Alzheimer's disease, and found that the nicotine significantly improved patients' performance on a task of attention, but there were no significant differences relating to motor or memory function (White & Levin, 1999). Later, White and Levin (2004) performed a similar study using 5 mg transdermal

nicotine patches on patients with age-associated memory impairment (AAMI), which has milder cognitive deficits than AD. Using the same scale as in the previous study (the Conners' Continuous Performance Test), they were able to demonstrate the same increase in attention as a result of the nicotine administration in AAMI patients (White & Levin, 2004). Though in White and Levin's studies no significant effects were seen in memory, studies using nicotinic receptor agonists that report an improvement in declarative memory, as well as the Wilson et al. (1995) study that showed improved learning, provide evidence that nicotine can help alleviate the memory deficits found in AD and other diseases affecting cognition (Potter *et al.*, 1999).

Parkinson's Disease

In addition to nicotine's positive effect on patients suffering from diseases that primarily affect cognition, nicotine has also been shown to be therapeutic in Parkinson's disease (PD), a neurodegenerative disease characterized by impairments in motor coordination and function (Quik, 2004). The neuropathology of PD involves damage to dopaminergic nigrostriatal neurons. This leads to the motor deficits of the disease, which include tremor, rigidity, akinesia, bradykinesia, and gait disturbances (Mazziotta, Toga, & Frackowiak, 2000; Quik, 2004). Though not as prevalent as the motor symptoms, patients with Parkinson's disease can also suffer from dementia, which is one of the reasons nicotine has been investigated as a potential therapeutic agent (Quik, 2004). This branch of research was first investigated after a series of studies emerged suggesting that Parkinson's disease is less prevalent in cigarette smokers, and that smoking can potentially delay the onset of the disease (Fujita *et al.*, 2006; Quik, 2004). It was thought that the nicotine in cigarettes played a key role in this delay due to its stimulation of

dopaminergic nigrostriatal neurons, and its neuroprotective effects (Quik, 2004). In addition to this, a neuroimaging study comparing nAChR expression in normal controls vs. Parkinson's patients concluded that patients with PD display a significant decrease in nAChRs, and that nicotine could be beneficial to these patients as well as patients with other neurodegenerative disease because it stimulates these receptors (Fujita *et al.*, 2006).

A longitudinal study investigating the effects of high-dose nicotine administered via transdermal patches on Parkinson's patients confirmed the hypothesis that nicotine can be beneficial to such patients (Villafane *et al.*, 2007). Patients were given increasing daily doses of nicotine (5-105 mg/day) for 14 weeks, keeping the highest tolerated dose constant for at least 4 weeks before gradually decreasing the dose until the conclusion of the experiment at week 29. Though most patients experienced symptoms of nausea and insomnia, all patients demonstrated improved motor ability and required less of their usually prescribed dopaminergic drugs by the end of the study (Villafane *et al.*, 2007). The ideal daily dose was determined to be between 45 and 90 mg/day, and because this study was done over such a long duration, it provides convincing findings regarding nicotine's potential as a therapeutic agent for the motor symptoms of Parkinson's disease (Villafane *et al.*, 2007).

Schizophrenia

Schizophrenia, a type of recurring psychosis that can have detrimental effects on cognition, emotion, perceptions, and even motor function, is another illness that has long been known to have a therapeutic tie to nicotine (Kraly, 2006; Postma *et al.*, 2006). Schizophrenics often show much higher rates of cigarette smoking when compared to the general population, so studies involving nicotine and schizophrenia can be very useful in

order to better understand the disease (Postma *et al.*, 2006; Smith, Singh, Infante, Khandat, & Kloos, 2002). Schizophrenics have difficulty ignoring irrelevant sensory input, and often show impairment in acoustic prepulse inhibition (PPI). PPI is characterized by a decrease in the magnitude of response to a startling stimulus when the stimulus is preceded by a lower intensity, non-startling stimulus (Postma *et al.*, 2006). In schizophrenic patients, the reaction to the startling stimulus is not lessened by the prepulse, thus causing an overload of information that would not occur in normal patients (Kumari, Soni, Mathew, & Sharma, 2000).

Nicotine has been shown to enhance PPI in both normal controls and schizophrenic patients, and a functional magnetic resonance imaging (fMRI) study showed that the increase in PPI as a result of the nicotine appeared largely in the hippocampus (Postma *et al.*, 2006). In addition to improved PPI, nicotine administered via nasal spray has been demonstrated to improve cognition (through verbal memory tasks and spatial organization tasks) in schizophrenics and has also led to improved performance on oculomotor and attention tasks (Depatie *et al.*, 2002; Smith *et al.*, 2002). These results suggest that the increase in smoking in schizophrenic patients is possibly a form of self-medication, and provides evidence for a schizophrenia treatment involving another method of nicotine administration that does not include the added risks associated with cigarette smoking.

Attention Deficit/Hyperactivity Disorder

Because of the clear evidence that nicotine has a positive effect on attention, studies have also been carried out to demonstrate nicotine's effect on adults with Attention Deficit/Hyperactivity Disorder (ADHD). ADHD is characterized by symptoms

of inattention and hyperactivity that are severe enough to interfere with daily life (Sharkey & Fitzgerald, 2007). As would be expected, administration of nicotine to ADHD patients leads to improved performance on tasks of attention, such as the Conners' Continuous Performance Test (CPT), and has also been reported to decrease reports of depressed mood (Levin *et al.*, 1996; Rezvani & Levin, 2001). Nicotine patches administering 7 mg of nicotine to non-smoking ADHD patients showed an overall nicotine-induced improvement on a modified version of the Clinical Global Impressions (CGI) scale, as well as improving scores on the CPT (Levin *et al.*, 1996).

From the numerous studies investigating the administration of nicotine to people with diseases affecting different aspects of cognition, it can be inferred that nicotine can be extremely beneficial to people suffering from a variety of illnesses. In illnesses like Parkinson's disease, schizophrenia and ADHD, adult patients have been shown to self medicate with cigarettes, and evidence points to this being due to the nicotine they receive (Levin, McClernon *et al.*, 2006). Smoking, however, adds numerous risks due to the toxins found in the smoke that cause lung damage and cancer, as well as the addictive properties of nicotine when delivered in such high doses (Winger *et al.*, 2004). Thus, if nicotine can be administered in a more controlled and safe manner as a therapeutic agent, patients would not have to be subjected to the risks associated with cigarette smoking.

Animal Models and Working Memory

Due to experimental limitations in human nicotine studies, much of the preliminary and ongoing research regarding nicotine's effects on cognition has come from studies of non-human animals. Nicotine has been shown to have a significant effect on cognition in non-human animals in various behavioral tasks that demonstrate working

memory. Working memory is an updated term for short-term memory, and it involves the storage of information that is currently in use, or that is likely to be needed in a short amount of time (Hegarty & Waller, 2005; Schumann *et al.*, 2004). Working memory can include verbal (in humans), object, and spatial tasks, and it is relatively easy to test this type of memory function in lab animals using object recognition tasks, the Morris Water Maze, or the Radial Arm Maze (Hegarty & Waller, 2005; Schumann *et al.*, 2004). An example of nicotine's effect on working memory can be demonstrated by the performance of rats in an object recognition task: Rats that were given an acute dose of nicotine demonstrated improved working memory compared to normal controls due to a clear increase in the recognition of an old object vs. a new object (Puma, Deschaux, Molimard, & Bizot, 1999). In addition to this, zebrafish that were exposed to an acute dose of nicotine for 3 minutes showed increased performance in a spatial position discrimination task (Levin, Limpuangthip *et al.*, 2006).

One way to demonstrate working memory is to expose rats to the Radial Arm Maze (RAM), which consists of a maze with arms extending out from a center (the number of arms varies based on the study), and a reward at the end of each arm (usually a food treat). Rats should only venture down each arm once until all rewards are received, and any time a rat re-enters an arm, it is marked as an error. In this way, working memory can be assessed by the extent to which the rat remembers what arms it has already been down. In a study assessing the effects of acute and chronic nicotine on young and aged rats in the RAM, Levin and Torry (1996) found that the young rats treated with chronic nicotine showed an increase in working memory, but the aged rats did not show the same increase. There was no significant improvement in working

memory after acute doses of nicotine in the young rats, but there was a pronounced effect of the acute administration of nicotine in the aged group (Levin & Torry, 1996). These findings suggest that a chronic dose of nicotine may be more effective in cognitive enhancement in young rats, while aged rats may benefit more from acute doses administered immediately prior to a task (Levin & Torry, 1996). These findings contrast slightly with the findings of French and colleagues (2006), who tested aged rats with a chronic nicotine dose of 0.3 mg/kg in the RAM. They found nicotine significantly increased working memory in these rats, and suggested that the lower dose in aged rats helped improve performance due to their decreased metabolism of the nicotine (French, Granholm, Moore, Nelson, & Bimonte-Nelson, 2006). In the Levin and Torry (1996) study, the dose given to both groups was 0.5 mg/kg. It is possible that this dose was effective for the young rats, but too much for the old rats when administered chronically.

Another commonly used method to demonstrate increased cognition in rats exposed to nicotine is to test them in the Morris water maze (MWM) to determine their cognitive spatial abilities. The MWM is a large circular maze filled with opaque water, and rats (or mice) must locate a hidden platform using spatial cues surrounding the maze (Morris, 1984). At the start, rats are placed on the platform so they can become acclimated to the maze and exposed to the spatial cues that surround it. Then they must learn that there is no escape from the maze via the outside walls, and they must utilize the spatial cues around the maze to locate the platform. Full acquisition of the task occurs when the swim times decrease and level off, and remain low when the rats are placed at varying start locations around the maze (Morris, 1984). Studies have demonstrated that rats differ in performance in the Morris water maze based on age, gender, and sometimes

strain (Hansalik, Skalicky, & Viidik, 2006; Topic *et al.*, 2005). Age differences are apparent in that aged rats tend to have more difficulty in the acquisition of the water maze task compared to young rats, however according to a study by Topic and colleagues (2005), there is considerable variability due to individual differences. Thus, while most aged rats were slower than the young rats, there were some aged rats who had swim times that were on par with those of the youngest and fastest rats (Topic *et al.*, 2005). Early training, however, can decrease response times of aged rats compared to aged rats with no previous training, suggesting that the MWM is also a tool for assessing long-term memory retention (Hansalik *et al.*, 2006).

In nicotine studies examining spatial learning, the dose administered and length of administration prior to training are extremely important. A study using mice demonstrated that a nicotine dose of 0.7 mg/kg/day produced significant impairment in a water maze task, but 0.35 mg/kg/day administered intraperitoneally 5 days prior to training and throughout training itself produced significantly lower trial latencies (Bernal, Vicens, Carrasco, & Redolat, 1999). Thus, it is important not only to administer a low dose of nicotine, but also to administer it a few days prior to the task to allow the drug to take its full effect (Bernal *et al.*, 1999). The extra time needed is most likely due to the fact that receptor upregulation, which can enhance the effect the nicotine has on the brain, takes some time to occur. Nicotine doses as low as 0.2 mg/kg have been shown to have an effect on both young and older rats, and these studies have also utilized the technique of waiting a few days prior to the first acquisition trials in order for the drug to take effect (Socci, Sanberg, & Arendash, 1995). Using a dose of 0.2 mg/kg not only results in enhanced water maze acquisition in aged rats, but it also has been shown to improve

memory retention in young rats (Socci *et al.*, 1995).

It has also been reported that high doses of nicotine do not demonstrate nicotine's positive effects due to ACh receptor desensitization. Receptor desensitization occurs when a neurotransmitter binds to a receptor but the ion channel, through which positively charged ions would normally travel to lead to an EPSP, remains closed (Benwell *et al.*, 1995). Thus, though the neurotransmitter is bound to the receptor, an action potential does not occur and the neuron does not fire. Because of this desensitization (which occurs when rats are administered high levels of nicotine that are on par with the amount a regular smoker may receive, 1.0 and 4.0 mg/kg/day for this particular study), the effect of the nicotine on memory and learning is actually inhibitory (Benwell *et al.*, 1995). Lower doses of nicotine, for example, 0.25 mg/kg/day, did not show this desensitization, and thus still shows cognitive enhancement in spatial learning studies (Benwell *et al.*, 1995).

Long-Term Potentiation

Studies involving nicotine treatment in working memory tasks are also beneficial in that they can provide evidence for nicotine's inducement of Long Term Potentiation (LTP). As was mentioned earlier, LTP occurs in the hippocampus and results in an increase in synaptic firing and effectiveness (Schumann *et al.*, 2004). LTP is an important part of memory formation, and it has been shown to occur throughout the neuronal information pathways in the brain (Schumann *et al.*, 2004). This process also involves the upregulation of glutamate NMDA receptors, which have been shown to be modulated by nicotine in some studies of working memory in rats (Levin, Bettegowda, Weaver, & Christopher, 1998).

One study looked at nicotine's effect on LTP in the rat hippocampus by assessing its therapeutic effects in rats suffering from working memory impairments. The working memory impairments were induced by stress, and the subcutaneous administration of 1 mg/kg of nicotine prevented the memory impairments associated with the stressor (Aleisa, Alzoubi, Gerges, & Alkadhi, 2006). The nicotine served to normalize the LTP that had been reduced in the stressed rats treated with saline. This suggests that nicotine does have neuroprotective effects regarding memory and learning, so much so that it can counteract the impact of a stressor in rats (Aleisa *et al.*, 2006). The control rats in the study that were not exposed to the stressor but received nicotine treatment did not show any learning or memory improvement, however this could be due to the extremely high dose (1 mg/kg) of nicotine that was administered. It has already been demonstrated that high doses can show water maze impairment, so it is possible that such a high dose is only necessary to reverse the effects of cognitive impairment, not to enhance the performance of otherwise normal rats (Bernal *et al.*, 1999).

It has also been demonstrated that nicotine can attenuate working memory impairments caused by dizocilpine, an NMDA glutamate receptor antagonist, providing more evidence for nicotine's neuroprotective effects (Levin, Bettegowda *et al.*, 1998). NMDA glutamate receptors are known to be upregulated as a result of LTP, so the attenuation of these impairments could be due to an increase of LTP as induced by the nicotine. Because the nicotine stimulated the release of glutamate, it was able to counteract the drug and reverse the working memory impairments, though it did not increase performance in the radial arm maze. When the dose of nicotine (0.4 mg/kg) was given without the dizocilpine, an increase in maze performance was also not detected—it

is possible that this dose was too high to produce positive effects, as it has been demonstrated that a dose of 0.7 mg/kg in mice actually causes impairments in spatial learning tasks (Bernal *et al.*, 1999; Levin, Bettegowda *et al.*, 1998). In a later study involving dizocilpine and nicotine, the nicotine dose of 0.4 mg/kg led to inconclusive results regarding its neuroprotective effects. This suggests that 0.4 mg/kg may be at some kind of dose threshold where the effects of the nicotine switch from positive to negative (Levin *et al.*, 2003).

Neurogenesis

In past studies demonstrating nicotine's attenuation of working memory impairments, a case has been made that nicotine can be neuroprotective, meaning it minimizes the damage done by certain drugs or events by stimulating the release of neurotransmitters such as glutamate or dopamine (Levin, Bettegowda *et al.*, 1998). Despite this, a study has indicated that nicotine actually decreases neurogenesis in the brain when self-administered by rats (Abrous *et al.*, 2002). However, in this study, the self-administration aspect led rats to have plasma nicotine levels that were extremely high and equivalent to those of smokers—the rats in cognitive enhancement studies as well as humans in controlled nicotine patch studies do not receive such high levels of nicotine (Abrous *et al.*, 2002). It has already been reported that such high nicotine levels desensitize important receptors involved with the release of both glutamate and dopamine, neurotransmitters essential to memory and learning (Benwell *et al.*, 1995). For this reason, it makes sense that large amounts of nicotine may actually decrease neurogenesis in the brain.

Studies employing the use of bromodeoxyuridine (BrdU) are essential in

demonstrating the effects of spatial learning tasks on neurogenesis, and can indicate whether nicotine has any effect on the cell proliferation. When injected intraperitoneally, BrdU incorporates into the DNA of all cells undergoing synthesis at the time of learning. The BrdU stays in these cells and can be detected post-mortem through immunohistochemical procedures (Cooper-Kuhn & Kuhn, 2002). It has been demonstrated that an increase in newly-generated cells in the hippocampus occurs due to the process of learning itself—not just training (Dalla, Bangasser, Edgecomb, & Shors, 2007). Even when the task was not hippocampus dependent, rats that "learned well," that is, that demonstrated a high acquisition of the task after its completion, showed increased cell proliferation compared to rats that did not learn well, thus emphasizing the importance of learning rather than training (Dalla *et al.*, 2007). BrdU studies have also clearly shown that neurogenesis decreases significantly from adolescence to adulthood, though neurogenesis in the hippocampus is still apparent in adults as a result of certain tasks involving learning (He & Crews, 2007). Though the cell growth seen as a result of the Morris water maze task is generally attributed to the learning that occurs during the late phase of this task, there is also evidence that exercise can increase cell proliferation as well (van Praag, Kempermann, & Gage, 1999). This suggests that tasks involving exercise, such as the Morris water maze, could be even more indicative of cell proliferation in the hippocampus.

Morris water maze studies involving BrdU injections can provide useful results regarding neurogenesis in the hippocampus as a result of the learning that occurs during the task. In one Morris water maze BrdU study, the early phase of learning, in which there is a rapid improvement in performance, did not produce significant cell

proliferation, while the late phase of learning, during which the highest point of performance is reached, demonstrated a significant increase in the number of newly generated cells (Dobrossy *et al.*, 2003). This was thought to potentially be due to the stress of the early phase of learning, in which rats were becoming acclimated to the task—for this reason, perhaps, neurogenesis was dampened at that time (Dobrossy *et al.*, 2003). Neurogenesis in the late phase, it was hypothesized, could indicate that the dentate gyrus of the hippocampus was available for the integration of new information and the storage of new memories at that phase of learning (Dobrossy *et al.*, 2003).

Studies using lower doses of nicotine in tasks that are known to produce neurogenesis in the brain, particularly in the hippocampus, have shown that low doses of nicotine do not have a detrimental effect on neurogenesis (Scerri, Stewart, Breen, & Balfour, 2006). In accordance with the findings of previous studies, Scerri and colleagues (2006) concluded that a nicotine dose of 0.4 mg/kg per day had an adverse effect on neurogenesis (as demonstrated by a BrdU investigation), but rats receiving 0.25 mg/kg per day showed an increase in cell proliferation in response to the Morris water maze task. This study provides further evidence that learning associated with the Morris water maze task can be demonstrated clearly in the dentate gyrus of the hippocampus, and that nicotine may have a positive impact on the cell proliferation that occurs there (Scerri *et al.*, 2006).

c-Fos Expression

In addition to increased cell proliferation, it has been widely reported that nicotine produces an increase in c-Fos expression in various areas of the brain (Guan, Kramer, Bellinger, Wellman, & Kramer, 2004; McCormick & Ibrahim, 2007; Panagis, Nisell,

Nomikos, Chergui, & Svensson, 1996; Salminen, Lahtinen, & Ahtee, 1996). c-Fos is a protein that indicates neuronal activation, and it has often been studied in relation to stress (Kovacs, 1998; McCormick & Ibrahim, 2007). In a study combining stressors with nicotine treatment in adolescent male and female rats, it was found that males showed an increase in c-Fos in the hypothalamus while females did not, and chronic nicotine groups showed more c-Fos expression than did acute nicotine groups (McCormick & Ibrahim, 2007). From these results, it can be hypothesized that nicotine must be chronically present in the system in order to have an effect on c-Fos expression in the brain. It has also been found that nicotine significantly increases c-Fos expression in brain areas specifically related to stress, such as the paraventricular hypothalamic nucleus and the supraoptic nucleus (Salminen *et al.*, 1996).

The rate of nicotine delivery can also have an effect on c-Fos expression (Samaha *et al.*, 2005). In one study, rats were infused with either 25 or 50 µg/kg nicotine in a time span of 5, 25, or 100 seconds. The results showed that c-Fos expression and receptor sensitization were highest when the infusion rates were fastest. High administration rates are thought to be central to the addictive properties of nicotine, and perhaps the increase in c-Fos expression is a result of the rapidly increasing occupancy of nicotinic receptors that occurs when nicotine is delivered at a fast rate (Samaha *et al.*, 2005).

Nicotine can also cause Fos increases in areas of the brain specifically related to dopamine transmission, such as the nucleus accumbens and the caudate-putamen. In a study looking at both c-Fos and ΔFosB (another member of the Fos family that stays in the brain longer and has been linked to drug addiction), nicotine was shown to increase both Fos types in these dopaminergic brain areas, providing further support for nicotine's

role in increasing dopamine transmission (Marttila, Raattamaa, & Ahtee, 2006). The increase that nicotine causes in locomotor activity, also known as behavioral sensitization, has also been related to an increase in Fos in the nucleus accumbens, the striatum, and the ventral tegmental area, again providing support for nicotine's role in the dopamine release process, and providing further evidence for its rewarding properties (Shim *et al.,* 2001).

Increased c-Fos expression in nicotine studies is also dependent on the nicotine dose used. One study found that a nicotine dose of 2 mg/kg showed the most dramatic expression of c-Fos in the brain, while the lower doses of 0.5 mg/kg and 1mg/kg showed minimal increase in c-Fos (though these doses did still produce an increase when compared to normal controls) (Ren & Sagar, 1992). Other studies have shown an increase in c-Fos expression in their brain areas of interest (for example, the hypothalamus, the visual pathways, the striatum) using doses of 0.4 mg/kg, (Shim *et al.*, 2001), 0.5 mg/kg (McCormick & Ibrahim, 2007), and 1mg/kg (Salminen *et al.*, 1996). Though these doses are slightly higher than those used in spatial learning studies, the fact that they produce c-Fos expression suggests that lower doses may still lead to an increase in expression when compared to normal controls.

The fact that chronic nicotine shows an increase in c-Fos in certain brain areas provides support for the idea that neurons may undergo changes in gene expression due to nicotine exposure (Ren & Sagar, 1992). This alteration of neuronal gene expression, which could potentially be long-term, would be beneficial to people suffering from diseases that affect cognition because it could mean an increase in nAChrs, which would increase their cognition and potentially delay the onset of cognitive decline (Ren &

Sagar, 1992).

The purpose of the current study was to assess the effects of a chronic dose of nicotine on the ability of rats to acquire the Morris water maze task in adolescence and adulthood. Previous studies of chronic nicotine administration have indicated that doses around 0.2 mg/kg serve to increase cognitive functioning, and that higher doses run the risk of adversely affecting maze performance (Bernal *et al.*, 1999; French *et al.*, 2006; Socci *et al.*, 1995). For this reason, we used 0.2 mg/kg as our dose for rats of all age groups.

Because most of the previous studies involve specifically young or aged rats, this study was unique in that it examined the effects of nicotine on the same rats in adolescence (Phase 1) and adulthood (Phase 2). The schedule of nicotine was also varied based on the groups. There were four main groups: rats that received nicotine in Phase 1 during adolescence and nicotine again in Phase 2 when they reached adulthood (N/N), rats that received nicotine in Phase 1 and saline in Phase 2 (N/S), rats that received saline in Phase 1 and nicotine in Phase 2 (S/N), and rats that received saline in Phases 1 and 2 (S/S). In Phase 1, rats were trained in the Morris water maze while receiving either 0.2 mg/kg of nicotine or saline for 2 weeks, and then nicotine or saline administration was ceased until four months later. At this time, drug treatment was resumed based on the group, and Morris water maze testing was repeated in the same fashion. Additionally, Bromodeoxyuridine (BrdU) injections were introduced into the study during the last two days of Phase 2 acquisition in order to view possible neurogenesis as a result of the nicotine exposure and water maze training (Scerri *et al.*, 2006).

Though this study involved nicotine cessation after a 2-week period of chronic

nicotine administration, the rats were not expected to experience serious withdrawal symptoms. A study investigating withdrawal from chronic nicotine showed that even after an extremely high dose (22.2 mg), withdrawal symptoms in adolescent rats were minimal, even after the immediate introduction of a nicotinic agonist (Wilmouth & Spear, 2006). Adult rats showed minor withdrawal symptoms that abated shortly after nicotine cessation (Wilmouth & Spear, 2006). This finding, coupled with the fact that the dose was significantly higher in these studies, suggests that the rats in the present study were not in danger of severe withdrawal symptoms in the interim between phases.

The purpose of the varying nicotine schedules was to determine whether learning was state dependent (for the N/N group), whether early experience had a greater effect on task acquisition (for the N/S group), or whether the effect of nicotine was more pronounced in older rats (for the S/N group). The rats that received saline in both phases served as a control for this experiment. It was expected that, in both phases, nicotine rats would perform better than saline rats in the Morris water maze. Due to the previous training that the rats received in Phase 1, it was expected that reaction times would be faster overall in Phase 2 during the re-acquisition of the task, however it was hypothesized that rats receiving nicotine in Phase 2 would re-acquire the task faster than rats not receiving nicotine, and that rats receiving nicotine in both phases would be better at the task overall (Hansalik *et al.*, 2006).

It was also expected that rats in all groups would show an increase in cell proliferation in the hippocampus due to the water maze task, and that the Phase 2 nicotine rats would have more BrdU expression than the saline rats due to previous studies suggesting nicotine is neuroprotective and involves nAChR upregulation (Dobrossy *et*

al., 2003; Wang *et al.*, 2008). We also tested for c-Fos in the hippocampus following the last day of training in the second phase of testing for all rats. Based on previous studies indicating that chronic nicotine at doses of 0.5 mg/kg to 2 mg/kg increase c-Fos expression in certain brain areas, we expected c-Fos levels to be elevated in rats receiving nicotine in Phase 2 (McCormick & Ibrahim, 2007).

We specifically looked at c-Fos expression in the dentate gyrus of the hippocampus. A previous study examining nicotine's effect on c-Fos in the pigeon brain reported the hippocampus to be one of the greatest sites of c-Fos expression, however to our knowledge a study investigating c-Fos expression in the rat hippocampus following nicotine exposure and a spatial learning task has not previously been attempted (Chadman, Woods, & Stitzel, 2007). For this reason, the present research provides important results regarding the effect of nicotine on c-Fos in the hippocampus and the implications this has regarding spatial learning. A significant c-Fos increase in the hippocampus may provide even further evidence that nicotine has a significant effect on cell growth and receptor upregulation in this brain area, as c-Fos is generally an indicator of the beginnings of neuronal change and activity (McCormick & Ibrahim, 2007; Ren & Sagar, 1992).

Method

Subjects

Subjects included 50 male Fisher-344 rats born at the Charles River Laboratories in Stoneridge, NY, USA. Rats from all cohorts were 6 weeks old on arrival. The study began 2 weeks later, at which time rats in Cohort 1 (numbers 1-16) weighed between 160 g and 185 g, rats in Cohort 2 (numbers 17-35) weighed between 155 g and 180 g, and rats

in Cohort 3 (numbers 36-50) weighed between 150 g and 180 g. Five months later during Phase 2 of the experiment, Cohort 1 weighed between 305 g and 340 g, and Cohort 2 weighed between 300 g and 345 g, with one rat weighing 276 g.

Rats were housed 3 or 4 to a clear plastic cage measuring 19 x 9 x 9 in. They were given regular shavings, water, and rat chow. Some of the rats from Cohorts 1 and 2 were given wet food along with the dry food a few months into the study due to slightly decreased weight that may have been the result of a mouth infection. Rats were also given a square red Plexiglas tube to use as a shelter.

Procedures were approved by the Connecticut College Animal Care and Use Committee in accordance with the guidelines of the USDA and the Office of Laboratory Animal Welfare.

Apparatus

Alzet osmotic mini-pumps (model 2004, Braintree Scientific, Braintree, MA) were used to administer a chronic dose of 0.2 mg/kg of nicotine or saline per day for a 2-week period.

Activity levels were recorded in an activity box measuring 42 x 45 cm on the inside and 11 cm high, with a normal rat cage, measuring 42 x 22 cm and 21 cm high, inside of it. The normal cage was made of transparent plastic and had a metal lid and wood shavings for bedding. The activity box surrounding the cage was made of metal and measured the activity of the rat. There was a light beam on one side of the cage projecting to the other side, which measured beam crossings (the amount of times the rat interrupted the light). This provided a quantitative measurement of the rat's movements.

Spatial learning was tested using a Morris water maze. The maze was a large white plastic tub that was 4 ft in diameter. The water level was kept at 13 in, with the platform approximately 0.5 in below the water level. The platform was a tall Plexiglas tube with a petri dish fitted to the top. A white washcloth was attached to the top of the platform with a rubber band. The platform was kept stationary, 11 in from the right side of the maze, using a weight at the base. The water was made opaque with white tempera paint, which was mixed thoroughly in the water prior to each acquisition and test day. The water was kept at 20 °C during all experimental trials.

The maze was in a rectangular shaped room with white walls and various stationary visual cues surrounding it. The visual cues included rectangular pieces of construction paper with different shapes (circles, a star, stripes) of contrasting color on top of them. The metal temperature gauge and hose, as well as the open door and the experimenter's position also acted as visual cues, and remained constant during the experiment. The maze was 10.5 in away from the two longer walls, and 17 in away from the shorter wall farthest from the door to the room.

Procedure

Rats were allowed to acclimate to their new surroundings for one week prior to being handled and weighed for the first time. The rats were handled for approximately 3 minutes each on at least two separate occasions prior to their first day of surgery. Weight measurements (in grams) were taken two days prior to surgery, as well as the day of surgery in all cases, in order to ensure the rats were gaining weight at a normal pace and to make the measurement of the necessary anesthetic as accurate as possible. Activity levels were taken on the day of the last surgery for all groups in order to determine

possible changes in locomotion in the nicotine rats. The early phase of acquisition trials in which Cohorts 1 and 2 (these two cohorts make up what is later referred to as the dual-phase cohort) were first exposed to the experimental procedure was considered Phase 1. Cohort 1 initially contained 16 rats, however due to some complications with the pumps the data from 2 of the rats was not used. Cohort 2, containing rats that arrived one week after Cohort 1 with all of the same specifications, included 18 rats. One rat died during the surgical procedure, so its data was not used in the experiment. At this time, rats were given either nicotine or saline for the experimental procedure. See Table 1 for a description of the drug treatments and number of subjects in each group.

During Phase 2 of testing, rats in the dual-phase cohort were broken up into four groups, N/N, N/S, S/N, and S/S, depending on what drug treatment they received in Phases 1 and 2 (see Table 1). The purpose of the N/N rats was to indicate whether acquisition of the procedure was state-dependent. The N/S rats were to demonstrate whether early experience had a greater effect on task acquisition than the nicotine itself. The S/N rats were to demonstrate whether the effect of the nicotine would be more pronounced in older rats, and the S/S group acted as a control for the three other groups.

The 16 rats in Cohort 3 (the single-phase cohort) contained the same specifications as all previous rats, and underwent the same procedure, with 8 receiving nicotine pumps and 8 receiving saline pumps. One rat died during the procedure, leaving 7 nicotine rats and 8 saline rats. After one phase of acquisition and test trials, these animals were sacrificed and their brains were examined for neurogenesis (as evidenced by BrdU) and c-Fos in the hippocampus. In this way they acted as a control for age, as their results were expected to provide insight into the c-Fos and BrdU expression that

might occur in adolescent rats as a result of nicotine exposure. They did not undergo a second testing phase.

Approximately 24 hours prior to the surgery the pumps were set up following the procedure as outlined by the Alzet company. The nicotine was mixed using 0.2859 grams of nicotine per 3 ml of saline. This concentration allowed the animals to receive 0.2 mg/kg per .29 µl nicotine base per day, while the control animals would receive an equal volume of vehicle (saline) per day. In order to fill the pumps, the empty pump was first weighed along with the flow moderator. Solution was drawn into a 1 ml syringe and a 27 gauge filling tube (provided by the Alzet company) was fit to the end of it. The empty pump was held upright and filled slowly, ensuring there were no air bubbles in the pump. When the solution appeared at the outlet of the pump, the filling tube was removed and excess solution was wiped off. The flow moderator was then inserted until it was flush with the pump, and the displaced solution was wiped off. The pump was then weighed again, ensuring that the difference in the weight from the empty pump gave the proper net solution of the pump, 30 µl. Pumps were then incubated in sterile saline and allowed to equilibrate at 37 °C overnight.

On the day of surgery, all animals were weighed in order to ensure an accurate dosage of anesthetic. The anesthetic was prepared by mixing 1.95 ml of xylazine and 1.8 ml of ketamine. In order to determine the specific amount of anesthetic necessary for each rat, 1.25 ml was multiplied by the most recent weight measurement in kilograms. The result, usually hovering around 0.2 ml, was administered intraperitoneally and the rats were put in a covered cage as the drug took effect. Once the rat was asleep, a small area between the shoulder blades was shaved, and a vertical incision was made in the

skin. The pump was then inserted subcutaneously. The wound was then closed with two metal clips, and the rat was placed in another covered cage until the anesthetic wore off. This procedure was repeated for each rat.

During the first surgeries for Cohort 1, the pumps were inserted with the top of the pump facing the wound. Due to the complications of a few of the pumps coming out because of excessive scratching, it was determined that the release of nicotine may have been irritating the wound. Thus, all other surgeries were conducted by inserting the top of the pump first.

Two days after the surgery all rats were weighed to ensure they did not lose a significant amount of weight as a result of the drug. The rats were given 7 days prior to the acquisition trials for the drug to begin to take effect. After this time had elapsed, all rats were tested in the Morris water maze.

Testing began daily at 4:00 pm, and usually lasted between 1.25 and 2 hours. Prior to the first trial on Day 1 of acquisition, all rats were placed on the platform for 60 seconds so they could become acclimated to their surroundings. During the acquisition trials, rats were positioned at start location 1 (see Figure 1) and a time measurement was taken with a stopwatch to measure how long it took them to reach the platform. If any rat failed to reach the platform after 60 seconds of swimming during any given trial, the rat was removed from the maze and placed on the platform for another 60 seconds.

Acquisition involved five trials from start location 1 per day for five consecutive days. Rats were tested by cage, so trials were alternated between 4 rats at a time in order to avoid fatigue from completing multiple trials per rat in succession. After the fifth day of acquisition trials there were two days without any trials, followed by one day of test

trials (see Table 2). During the testing day, the first trial was at the familiar start location (start location 1), and the remaining 4 trials began at varying start locations around the maze (see Figure 1 for a diagram of the maze and start locations). These start locations remained constant throughout the study.

It should be noted that during the first day of acquisition trials for Cohort 1 in Phase 1 of testing, 10 acquisition trials were taken per rat (as opposed to 5). It was determined that this number was too high, as latencies began to increase toward the end of the acquisition period. The increase was most likely due to fatigue, and thus all acquisition days following that first day involved only five trials, and only the first five trials from acquisition day 1 for Cohort 1 were used in the data analysis.

After the week of testing was over, rats were weighed, activity levels were recorded in an activity box for five minutes, and then they were anesthetized using the same procedure as outlined previously. The pumps were removed and the rats from the dual-phase cohort were kept until Phase 2 of testing, which occurred four months later. These rats were weighed once a week in between testing phases.

Acquisition and testing for the single-phase cohort and Phase 2 of the dual-phase cohort were subject to a slightly different procedure. Surgery was performed on these rats as per usual, and acquisition trials began one week following the procedure. The procedure for acquisition trial days 1-4 were identical to those of Phase 1 of the dual-phase cohort, however after the fourth day one day was skipped, and the last day of acquisition was on the sixth day of the experiment. The test trials were on the day immediately following the last day of acquisition (day 7), in order to allow for perfusion of rats that coincided with the test trials and the injection of BrdU (see Table 2 for the full

experimental schedule). This change in procedure was due to complications with the BrdU, however it was not expected that any significant difference would arise due to this change. The order in which the trials occurred and the small timing changes of the testing trials were not expected to have any effect on the ability of the rats to perform the task and to recall it during testing.

BrdU was introduced during Phase 2 acquisition trials for the dual-phase cohort, and during the acquisition trials of the single-phase cohort. BrdU was mixed in a concentration of 25 mg/ml in physiological saline. It was necessary to warm the solution in order for the BrdU to dissolve. Once the BrdU was in solution, rats were weighed, the proper amount for injection (2 ml/kg) was calculated, and rats were injected at roughly 4:30 pm during the free day between acquisition days 4 and 5. The second BrdU injection occurred 30 minutes prior to acquisition trials on acquisition day 5, which was one day prior to test trials. Two rats from the dual-phase cohort did not receive the BrdU injection, as they were aggressive and difficult to inject.

Rats were perfused 90 minutes after their last test trial in order to preserve the brains to look at c-Fos and incorporation of BrdU in the hippocampus. This process involved euthanizing the rat and perfusing the body with the fixative 4% PFA, then removing the brain and soaking it in the fixative overnight. The following day, the brain tissue was transferred to a 30% sucrose mixture. Using a cryostat, the brain tissue for each rat was then frozen and sliced at 25μm through the dentate gyrus of the hippocampus. The sections of brain tissue were then placed in a preserving solution, and 3 slices from each rat were selected to be stained and examined further for c-Fos content. A separate set of sections was selected to be stained for BrdU incorporation. The

sections of tissue were chosen based on their representation of the dentate gyrus. A rat brain atlas was utilized to determine which sections of the brain tissue were to be examined (Paxinos & Watson, 1998). One rostral (AP –3.30 mm in reference to Bregma), middle (AP –2.12 mm in reference to Bregma) and caudal (AP –3.6 mm in reference to Bregma) section was taken for each rat to represent different sections of the hippocampus (Paxinos & Watson, 1998).

c-Fos Immunohistochemistry

The c-Fos staining procedure involved washing the selected pieces of tissue for 30 minutes and the placing them in a solution containing the 1^0 antibody (rabbit anti-Fos polyclonal IgG, Santa Cruz Lot #c270) that binds to segments of the c-Fos protein. The concentration was 1:8000, diluted in 50 ml of blocking solution (98.75mL 0.01 M PBS, 1 ml normal goat serum, 1 g bovine serum albumin, 0.25 ml 30% Triton X100). They were left in this solution overnight, and then rinsed and placed in a solution containing biotinylated 2^0 antibodies (goat anti-rabbit polyclonal IgG, Jackson Laboratories) that recognize the 1^0 antibody. This concentration was 1:200 diluted in 50 ml of blocking solution. Two hours later the tissue was rinsed again and avidin-biotin molecules complexed with horseradish peroxidase diluted in 50 ml of 0.01 M PBS were added. These molecules bind to the biotin molecules on the antibodies. After one hour enhanced DAB (Diaminobenzidine enhanced with nickel and cobalt) diluted in 50 ml of 0.1 M PB was added, which interacted with the peroxidase to form a precipitate that allows for the visualization of the c-Fos location.

After being left to dry overnight, the slides were coverslipped and viewed under a microscope. A 240 x 152 µm portion of the dentate gyrus was viewed in each section of

tissue for each rat. The dimensions of this area were kept constant across rats. For the middle and caudal sections, the portion of the dentate gyrus that was measured for c-Fos content was the hilus. For the rostral sections, the lower portion of the dentate gyrus was measured, and this location was kept constant across caudal sections. Only the darkest particles in each section were counted, ensuring that only Fos-positive nuclei were being measured.

BrdU Immunohistochemistry

The BrdU staining procedure was performed as outlined in the kit from Chemicon International. The procedure first involved the deparaffinization of the control tissue included with the kit. This involved soaking the paraffinated slides in xylene, and then washing them in 100%, 90%, 80%, and 70% ethyl alcohol for 3 minutes each. Afterwards tissue was washed in PBS for 3 minutes. Control slides and experimental slides were soaked in Quenching Solution (dilute 30% hydrogen peroxide 1:10 in methanol) for 10 minutes, and then washed with PBS for 2 minutes. Two drops of 0.2% Trypsin Solution were added to control slides and slides were incubated at room temperature for 10 minutes. Slides were then washed for 3 minutes in distilled water. Denaturing Solution was added to all slides and they were allowed to incubate at room temperature for 30 minutes, followed by 2 PBS washes for 2 minutes each. Blocking solution was then added to each slide, and they incubated for 10 minutes. Slides were blotted dry on a paper towel before the Detector Antibody was added, and then were left to incubate for 60 minutes. Following incubation slides were washed twice with PBS, 2 minutes per wash.

Streptavidin-HRP Conjugate was then added to each slide and slides were

incubated at room temperature for 10 minutes. This was followed by two 2-minute washes in PBS. DAB concentrate was added to slides at a concentration of 1 µl for every 29 µl of Substrate Reaction Buffer. Slides were allowed to incubate for 10 minutes and were washed with distilled water for 2 minutes. Hematoxylin Counterstain was added to all slides and left to incubate for less than a minute. Slides were briefly washed with distilled water and then incubated in PBS for 1 minute until the color turned blue. Slides were washed for 2 minutes in distilled water. To prepare for coverslipping, slides were incubated in 90% ethanol for 30 seconds, 100% ethanol for 30 seconds, and xylene for 30 seconds. Slides were coverslipped and left to dry overnight.

Results

Weight/activity level analysis

Weights and activity levels were monitored throughout the study to determine if the administration of chronic nicotine led to significant weight loss or increased locomotion. One-way ANOVAs were conducted for the weights of the dual-phase cohort after they had been exposed to nicotine or saline for 14 days in Phase 1 and in Phase 2. The results for these rats were not significant, indicating that after 14 days of nicotine administration in both phases, no significant differences arose between the weights of the saline and nicotine rats. The single-phase cohort also did not demonstrate significant differences between the weights of rats receiving nicotine and rats receiving saline.

One-way ANOVAs were also conducted for the activity levels of the dual-phase cohort (in Phases 1 and 2) and the single-phase cohort after they received nicotine or saline for 14 days to view potential increases in locomotion as a result of nicotine administration. No significant results were found between the activity levels of the

nicotine or saline rats in any cohort. This shows that the nicotine did not have a significant effect on the locomotion of the rats during the experiment.

Spatial learning analysis

Single-phase cohort

A repeated measures analysis using a 2 (group) x 5 (days) mixed design ANOVA was conducted with trial totals across the 5 days of acquisition. Trial totals were calculated by adding the latencies of trials 1 through 5 for each rat in each day. The independent variable was the drug condition and the dependent variables were the trial latencies.

The effect of condition across days using trial totals was not significant, indicating that condition had no significant effect on trial totals across days 1 through 5. However, an effect of training could be found by comparing the trial latencies across days for all rats, regardless of drug condition. In the analysis of trial totals, a significant effect of training was seen across the days of the acquisition period, $F(4, 52) = 21.49$, $p < .001$. This shows that the rats learned the task due to an overall decrease in trial latencies.

Performance between groups on each acquisition day was assessed using a 2 (group) x 5 (trials) mixed design analysis of variance (ANOVA), which was conducted with repeated measures across the 5 trials per day. The effect of condition (nicotine or saline) on trial latencies for each individual day was not significant, indicating no significant difference between rats treated with nicotine and rats treated with saline. In the analyses of individual days, however, a significant effect of training was found for acquisition days 1 ($F(4, 52) = 4.18$, $p = .005$), and 3 ($F(4, 52) = 3.18$, $p = .02$), and effects for days 2, 4, and 5 were not significant (see Figure 2 for a graph of mean trial latencies

in acquisition days 1-5 that demonstrate the learning of the task). This indicates that the majority of learning occurred across trials on days 1 and 3, during the earlier days of acquisition.

A one-way ANOVA was conducted for test trial totals (the sum of all test trials for each rat), and a separate ANOVA was conducted for test trial averages (the sum of all test trials for each rat divided by the number of trials). The effect of condition for the trial totals and averages were not significant, however the means for saline (M = 30.02) and nicotine (M = 26.52) rats indicate a trend toward significance (see Figure 3 for a graph of test trial averages at each start location). Additionally, five one-way ANOVAs were conducted for each test trial (each test trial was at a different start location, and there were 5 in total) in order to view possible effects of condition. Effects of condition for test trials 1 through 5 were not significant, indicating that the nicotine did not produce a significant decrease in trial latencies in adolescent rats.

Dual-phase cohort

Phase 1

Statistical analyses for Phase 1 acquisition were conducted using the same procedure as outlined for the single-phase cohort. A repeated measures analysis using a 2 (group) x 5 (days) mixed design ANOVA was conducted with trial totals across the 5 days of acquisition. Trial totals were calculated by adding the latencies of trials 1 through 5 for each rat in each day. Again, the independent variable was the drug condition and the dependent variables were the trial latencies.

The results for this group of adolescent rats in acquisition coincided with those of the single-phase cohort adolescent rats. The effect of condition across days using trial

totals was not significant, confirming that nicotine did not have a significant effect on trial totals across acquisition days 1 through 5. The effect of training for trial totals across days 1 through 5 was significant, $F(4, 116) = 57.54$, $p < .001$, indicating learning due to a decrease in latency totals across acquisition days in Phase 1 acquisition.

Performance between groups on each acquisition day was assessed using a 2 (group) x 5 (trials) mixed design ANOVA, which was conducted with repeated measures across the 5 trials per day. The effect of condition (nicotine or saline) on trial latencies for individual days 1, 2, 4, and 5 were not significant, indicating the nicotine did not have a significant effect in the adolescent rats in Phase 1 of acquisition. There was a significant effect of condition seen for Day 3, however, indicating a decrease in trial latencies in the nicotine treated rats, $F(4, 116) = 2.56$, $p = .04$. Additionally, there was a significant effect of training in day 1, $F(4, 116) = 7.22$, $p < .001$, and day 2, $F(4, 116) = 2.95$, $p = .02$, indicating learning via a decrease in latencies across trials in both conditions. The effects of training on days 3, 4, and 5 were not significant, coinciding with the results from the single-phase cohort suggesting that the bulk of learning occurs during the early days of acquisition (see Figure 4 for a graph of mean trial latencies in Phase 1 acquisition days 1-5).

A one-way ANOVA was conducted for test trial totals, and a separate ANOVA was conducted for test trial averages. Effects for trial totals and averages were not significant, however the averages for trial totals in the saline ($M = 26.42$) and nicotine ($M = 21.31$) conditions indicate a clear trend toward significance (see Figure 5 for a graph of Phase 1 test trial averages). Additionally, as in the single-phase cohort, five one-way ANOVAs were conducted for each test trial in Phase 1. Effects of condition for trials 1

through 5 were not significant, indicating that the nicotine did not produce a significant difference in latencies in each trial.

Phase 2

Statistical analyses for Phase 2 acquisition were conducted in the same fashion as in Phase 1 and the single-phase cohort. A repeated measures analysis using a 2 (group) x 5 (days) mixed design ANOVA was conducted with trial totals across the 5 days of acquisition. Trial totals were calculated by adding the latencies of trials 1 through 5 for each rat in each day. The independent variable was condition and the dependent variables were the trial latencies.

In the analysis of trial totals, an effect of condition across days was not seen, indicating that nicotine did not have a significant effect on trial totals across acquisition days 1 through 5. A significant effect of training was seen for trial totals across days, $F(4, 116) = 9.86$, $p < .001$. This demonstrates an effect of the task, as latencies in both conditions decreased as a result of training.

The performance between groups on each acquisition day was assessed using a 2 (group) x 5 (trials) mixed design ANOVA, which was conducted with repeated measures across the 5 trials per day. An effect of condition (nicotine or saline) for individual days 1 through 5 was not seen, indicating that in the second phase of acquisition, condition did not have a significant effect on trial latencies. The effects of training for day 1, $F(4, 116) = 8.86$, $p < .001$, and day 2, $F(4, 116) = 4.82$, $p < .001$, were significant, indicating a decrease in latencies due to learning of the task (see Figure 6 for a graph of Phase 2 acquisition trials as compared to Phase 1 acquisition trials). The effects of training for

days 3, 4, and 5 were not significant, indicating again that the bulk of learning occurred in the early days of acquisition.

A paired samples t-test was conducted to determine differences between the first trial in Phase 1 acquisition and the first trial in Phase 2 acquisition. This provided insight as to whether previous training had a significant effect on trial latencies in Phase 2 acquisition, regardless of group. The difference between the two trials was significant, $t(1, 30) = 9.39$, $p < .001$, indicating that trial latencies were significantly decreased in Phase 2 acquisition when compared to Phase 1 (this result is also demonstrated in the graphs in Figure 6). For Phase 2 acquisition, a one-way ANOVA was also conducted to view possible differences in trial latencies across the four drug treatment groups, N/N, N/S, S/N, or S/S. The independent variable was the drug treatment group and the dependent variables were the trial latencies. Effects of group for days 1 through 5 were not significant, indicating no significant differences between groups in the Phase 2 acquisition trials.

Similar to the test trial analyses conducted in the single-phase cohort and Phase 1, a one-way ANOVA was conducted for test trial totals, and a separate ANOVA was conducted for test trial averages. The independent variable was condition (rats that received either nicotine or saline in Phase 2), and the dependent variables were trial latencies. The effect of condition on trial totals was significant, $F(1, 29) = 9.16$, $p = .005$, demonstrating that the nicotine treated adult rats had lower overall trial latencies compared to saline treated rats. A significant effect of condition was also seen in trial averages, indicating lower trial latency averages in nicotine rats than in saline rats, $F(1, 29) = 9.16$, $p = .005$ (see Figure 7 for a graph of Phase 2 test trial averages). This shows

that the nicotine rats demonstrated increased learning of the task when compared to saline rats.

Additionally, five one-way ANOVAs were conducted for each test trial in Phase 2, with condition (nicotine or saline) as the independent variable and trial latencies as the dependent variable. Significant effects of condition were found for trial 1, which had the same start location as the acquisition trials, $F(1, 29) = 6.19$, $p = .019$, trial 4, $F(1, 29) = 6.59$, $p = .016$, and for trial 2, $F(1, 29) = 4.12$, $p = .05$. This indicates that in these testing trials, the nicotine had a positive effect on the cognition of the older rats. The effects in trials 3 and 5 were not significant. These results, coupled with the results from the test trial totals and test trial averages, demonstrate that nicotine administration led to lower trial latencies in adult rats.

A one-way ANOVA and LSD follow-up tests were then conducted to view differences between the four drug treatment groups, N/N, N/S, S/N, and S/S, in the Phase 2 test trials. The independent variables were the four groups with varying drug conditions, and the dependent variables were the trial latencies. Trial totals for each group were calculated, and these scores were used in the ANOVA. A significant effect of group was found for trial totals, $F(3, 27) = 3.03$, $p = .047$. Least Square Difference (LSD) post-hoc tests conducted at a significance level of $p < .05$ confirmed that groups receiving nicotine responded more quickly in the MWM due to decreased trial latencies. LSD tests indicated that for trial totals, the N/N group was significantly faster than the N/S group (mean difference = 8.57) and the S/N group was significantly faster than the N/S group (mean difference = 9.31).

Five one-way ANOVAs were conducted using the four groups as the independent

variables and the test trial latencies as the dependent variables, in order to view differences between groups in individual trials. The effects of the groups in individual trials 1 through 5 were not significant. However, LSD tests indicated a significant difference between the N/N group and S/S group in Trial 1 (mean difference = .81) and Trial 4 (mean difference = 2.08), as shown in Figure 8. Though the effects of these were not significant in the ANOVA, the significant post-hoc test provides more support for the result that nicotine rats in Phase 2 were faster than saline rats. For a graph demonstrating the differences between the N/N, N/S, S/N, and S/S test trial averages, see Figure 9.

A one-way ANOVA was conducted for Phase Differences, which was the difference in latencies for the four groups (N/N, N/S, S/N, S/S) between Phase 1 and Phase 2. A near-significant effect of condition was found, $F(3, 27) = 2.93$, $p = .051$, and no significant effect was seen for age alone. This indicates an effect of the nicotine as the rats aged, however the lack of a significant result for age alone suggests that due to the previous training, there was no significant difference between test trial latencies in Phase 2 versus Phase 1. LSD post-hoc tests indicated a significant difference between the S/N and N/S groups, showing that the rats receiving saline in Phase 1 and nicotine in Phase 2 (S/N) had significantly lower trial latencies than rats receiving nicotine in Phase 1 and saline in Phase 2 (N/S) (mean difference = 16.36).

c-Fos Analysis

Separate one-way ANOVAs were conducted for Phase 2 of the dual-phase cohort and the single-phase cohort to analyze c-Fos expression in the dentate gyrus of the hippocampus. Here, the independent variables were the drug conditions and brain sections, while the dependent variable was c-Fos content.

For Phase 2, three one-way ANOVAs were conducted across condition (nicotine or saline) on c-Fos expression separately for each section of the hippocampus. The three sections of interest were the rostral, middle, and caudal portions of the dentate gyrus. A significant effect of condition was seen in the caudal section, $F(1, 26) = 10.43$, $p = .003$. Means indicate that the rats receiving saline in Phase 2 ($M = 14.47$) had higher c-Fos content in this section than did the rats receiving nicotine in Phase 2 ($M = 9.23$). Effects for the rostral and middle sections were not significant, indicating the largest c-Fos difference occurred in the caudal hippocampus section. See Figures 10, 11, and 12 for examples of rostral, middle, and caudal sections of the dentate gyrus for both saline and nicotine rats. A one-way ANOVA conducted for the single-phase cohort revealed no significant effect of condition across nicotine and saline rats in any brain section, indicating that the nicotine did not have an effect on c-Fos content in the adolescent rats.

Three one-way ANOVAs and LSD post hoc tests at significance level $p < .05$ were also conducted in Phase 2 to view differences between the N/N, N/S, S/N, and S/S groups in the three hippocampus sections. Here, the independent variables were the four groups, and the dependent variable was the c-Fos content. Again, the effect of group for the caudal section was significant, $F(3, 24) = 4.14$, $p = .017$, indicating significantly higher c-Fos expression in the saline rats. LSD post-hoc tests indicated that S/S rats had significantly more c-Fos than N/N rats (mean difference = 6.08), and than S/N rats (mean difference = 7.00). No significant effects of group were seen for the rostral and middle sections.

To view differences relating to age between the Phase 2 rats and the single-phase cohort, three 2 (age) x 2 (condition) ANOVAs were conducted for the three hippocampus

sections of interest. Phase 2 rats were considered adults and single-phase cohort rats were adolescents, and they compared by condition (nicotine or saline). Again, a significant effect of condition was seen in the caudal section of the hippocampus, $F(1, 37) = 8.22$, $p = .007$, indicating the saline rats showed more c-Fos expression than the nicotine rats. Significant effects for age were seen in the rostral section, $F(1, 37) = 5.34$, $p = .027$, and the caudal section, $F(1, 37) = 5.78$, $p = .021$, indicating that adolescent rats showed less c-Fos expression than adult rats. The effect of age in the middle section indicated a trend toward significance, $F(1, 37) = 3.52$, $p = .069$.

BrdU Analysis

No BrdU expression was demonstrated in the dentate gyrus of the hippocampus in the rostral, middle, and caudal sections examined for the single-phase and dual-phase cohorts.

Discussion

The hypothesis that rats receiving nicotine would be significantly improved in the test trials of the Morris water maze task when compared to rats receiving saline was partially supported: The adult rats demonstrated a clear improvement in the task after nicotine exposure, while the adolescent rats did not demonstrate such improvement. As expected, in Phase 2 testing the N/N rats were significantly improved at the task when compared to the three other groups, and the S/N rats were improved compared to the S/S and N/S groups that received saline in Phase 2. The hypothesis that previous training in the Morris water maze would lead to lower trial latencies overall in Phase 2 of acquisition was supported, as all rats (regardless of drug condition) demonstrated much faster reaction times than they did in Phase 1. However, the hypothesis that nicotine rats in

Phase 2 of acquisition would re-acquire the task faster than the saline rats was not supported, indicating that the nicotine did not have an effect on the re-acquisition of the task from the same start location.

The hypothesis regarding c-Fos in the dentate gyrus of the hippocampus was not supported, as we expected to see an increase in c-Fos in the nicotine rats. There was, however, a significant increase in c-Fos in the dentate gyrus of the rats that received saline in Phase 2, as the S/S group had a much higher amount of c-Fos than the N/N and S/N groups. Additionally, it was found that adult rats demonstrated more c-Fos expression in the hippocampus than adolescents from the single-phase cohort. The hypothesis that there would be an increase in BrdU in the dentate gyrus of the nicotine rats was not supported, as we were not able to obtain any BrdU expression in our tissue. This could have been due to a number of methodological factors, which will be expanded upon later.

Behavioral Effects of Nicotine

In adolescent rats, contrary to our original hypothesis, there were no significant differences between the nicotine and saline rats. These rats were approximately two months old, and still at an early stage of development. It is possible that the lack of a significant difference in the acquisition and test trials for the adolescent rats (in both the single-phase cohort and Phase 1 of the dual-phase cohort) is due to the fact that a nicotine dose of 0.2 mg/kg/day is not enough to demonstrate such an effect in rats of this age. It has already been demonstrated that adolescent rats metabolize drugs faster than adult rats, so it is possible that in order to see a change in trial latencies, adolescent rats would need to be administered a higher dose of nicotine (French *et al.*, 2006). This conclusion

coincides with a previous study that gave adolescent and aged rats a high nicotine dose of 0.5 mg/kg/day, but demonstrated an improvement in the working memory of the adolescent rats only (Levin & Torry, 1996). This provides evidence for a possible dosing threshold in both adolescents and adult rats.

In Phase 2 of testing, when the rats in the dual-phase cohort were approximately 6 months old and thus considered adults, complete support of our hypothesis was demonstrated. The results from the test trials for this phase, in which rats were to find the hidden platform from varying start locations, demonstrated a clear decrease in trial latencies in the nicotine rats as compared to the saline rats. This follows from studies that have demonstrated nicotine as having a pronounced effect on aged rats (French *et al.*, 2006; Socci *et al.*, 1995). Additionally, previous studies have demonstrated the importance of allowing a few days for the drug to take effect before starting acquisition trials (Bernal *et al.*, 1999). Because the rats in this study were given a week prior to acquisition to acclimate to the drug, it can be concluded that the effects of the nicotine in the Morris water maze in Phase 2 of testing were due to the nicotine itself and not some other factor. It can also be concluded that the improvement in the Morris water maze task among adult rats receiving nicotine was not due to an increase in locomotion as a result of the nicotine, as the results demonstrate that nicotine did not have an effect on the activity levels of the rats. This provides more evidence that the nicotine served to increase the cognition of the rats, and not merely to increase their locomotion.

Our study is unique in that the rats in the dual-phase cohort were given varying drug schedules that allowed for four separate groups to be examined—this allows us to draw conclusions regarding the level of impact the nicotine has on the rats' ability to

learn the Morris water maze task. In Phase 2, our hypothesis was supported because we found that the N/N rats had significantly lower latencies in the test trials than the N/S rats and the S/S rats. This provides evidence that learning in the Morris water maze was state-dependent on the nicotine—when rats received nicotine in Phase 1 and then nicotine again in Phase 2, they performed very well in the test trials in Phase 2. When rats received nicotine in Phase 1 and saline in Phase 2, they demonstrated impairment when compared to all other rats (including the S/S group). This suggests that when rats are deprived of nicotine and then faced with a task they learned while under the influence of nicotine, the absence of nicotine leads them to perform worse on the task than they would have if they had never received nicotine in the first place. In Phase 2 of this study, however, the rats were faced with the same exact task under identical conditions. It is possible that if faced with a slightly different task, or the same task in a different environment, the deficits would not be as pronounced. Thus, the extent to which learning is state-dependent on nicotine is an area that lends itself to further study.

These results also demonstrate the importance of previous training in conjunction with the drug treatment, as the re-acquisition of the task in Phase 2 demonstrated lower latencies overall than did the acquisition in Phase 1. Clearly the previous training helped the animals learn the task, most likely due to Long Term Potentiation (LTP) in the hippocampus. Because the training led to an increase in neuronal firing and expression, the rats were able to perform better during the second phase of acquisition. This follows from previous research that highlights early training as an indicator of long-term memory—because the rats remembered the task from the previous acquisition phase, they were able to re-acquire it much more quickly in Phase 2 (Hansalik *et al.*, 2006).

Because the nicotine did not produce a significant difference in acquisition trials in Phase 2, it can also be concluded that the previous training in the maze from that start location is what allowed the rats to recall the task much more quickly than they did in Phase 1. Additionally, the test trial latencies in Phase 2 did not differ overall from test trial latencies in Phase 1—thus, the main difference in latencies from one phase to the next was seen in the acquisition trials. The real difference between nicotine and saline rats was seen in the Phase 2 test trials, in which the rats had to remember the task relying only on spatial cues. In this instance, it is clear that the nicotine improved the spatial learning and memory retention in the rats.

Neuronal Effects of Nicotine

Initially, we hypothesized that there would be an increase in c-Fos expression in the adult rats in Phase 2 as a result of the nicotine expression. Though this hypothesis was not supported, a significant result in the opposite direction was found. The saline rats in Phase 2 demonstrated an increase in c-Fos in the dentate gyrus of the hippocampus when compared to nicotine rats. Though this did not follow with our hypothesis regarding c-Fos as an indicator of neuronal change, it follows from previous research investigating c-Fos as an indicator of stress (Kovacs, 1998). Previous research demonstrating an increase in c-Fos as a result of nicotine exposure has shown that higher doses (of up to 2 mg/kg) of nicotine lead to this expression (McCormick & Ibrahim, 2007; Ren & Sagar, 1992). Because the dose used in the present study was quite low (0.2 mg/kg), it is possible that this was not enough nicotine to produce a c-Fos increase in the nicotine rats. That being said, the increase in c-Fos in saline rats could instead be due to the increased amount of stress the saline rats were under. Because the results show that

saline rats demonstrated significantly higher latencies in the test trials of the MWM task, it is possible that being in the maze for a longer period of time led to more stress in these rats when compared to the nicotine rats that were in the maze for much shorter periods of time. This follows from studies examining the effects of swim stress on rats—rats forced to swim (usually for 15 minutes) with no escape platform show high stress levels as indicated by increased c-Fos expression in the brain (Lino-de-Oliveira *et al.*, 2006).

Though it seems this will often be a confound in an experiment like this, it would be possible to use a different Morris water maze method that would eliminate the possibility of increased stress in the rats with higher trial latencies. In this experiment, the platform was present at all times during the test trials. A future experiment could employ the use of a camera suspended over the maze during test trials—the platform would be removed, and the amount of time the rats spend in each quadrant would be assessed instead. The amount of time spent in the quadrant that contained the platform during acquisition would be the measure of spatial learning. This way, all rats would be in the maze for the same amount of time (generally 60 seconds), and increased stress as a result of higher swimming times would not potentially skew the c-Fos results.

Though we expected to see an increase in cell proliferation in the dentate gyrus of the hippocampus as evidenced by BrdU, no such staining was obtained. Due to a number of studies demonstrating an increase in neurogenesis in the hippocampus as evidenced by BrdU expression after nicotine administration and spatial learning, the lack of BrdU staining is most likely because of a problem with our procedure (Dalla *et al.*, 2007; Dobrossy *et al.*, 2003). It is possible that the number of injections was not enough to lead to significant BrdU staining in the hippocampus. Previous studies employing the use of

BrdU generally use three or more injections of 50 mg/kg BrdU, or they use one or two injections of an extremely high concentration of BrdU (Dalla *et al.*, 2007; He & Crews, 2007; Scerri *et al.*, 2006). In this study, we only injected the rats twice with a concentration of 50 mg/kg BrdU, and it is possible that in order for the nicotine or training to have an impact on BrdU expression we would have needed more injections.

In addition to administering more BrdU injections, it would be beneficial in the future to modify the injection schedule so the timing coincides with the time when the most learning takes place. A previous study divided the learning process in the Morris water maze into an early phase, in which most of the learning occurs, and a late phase, in which the latencies decrease and level off (Dobrossy *et al.*, 2003). It was found in the study that the early phase of learning did not produce significant cell proliferation potentially due to increased stress of a new task, but that the late phase of learning did demonstrate increased cell proliferation as evidenced by BrdU (Dobrossy *et al.*, 2003). However, the study employed fewer acquisition trials per day, and they only looked at acquisition (what they termed "learning") trials. The timing of our injections was right before the last day of acquisition and before the first day of testing—it is possible that because at this point the rats had already significantly learned the task, there were not enough newly-formed cells to demonstrate BrdU incorporation in the hippocampus. It can also be argued that the later acquisition trials in our study were less hippocampal-dependent because the rats had already been exposed to a number of trials from the same start location. If this is the case, it makes sense that there was no BrdU expression in the hippocampus after these trials. Instead, administering injections during the first acquisition days in which the bulk of the learning occurs would most likely lead to an

increase in BrdU expression.

Due to our knowledge of the effects of nicotine at the neural level, it can be concluded that despite the lack of c-Fos and BrdU expression in the nicotine rats, the nicotine still had an effect on the nAChRs in the rats' brains. The hippocampus is known to be highly involved in memory and learning due to LTP, which is an increase in synaptic effectiveness. Because the behavioral data demonstrated that the adult rats treated with nicotine were significantly improved in the Phase 2 test trials, we can reach the conclusion that the nicotine was binding to nAChRs and stimulating the release of neurotransmitters in the brain. This led to an increase in neuronal firing, as indicated by the enhancement in their cognition as related to the Morris water maze task.

The results of this study follow from previous studies investigating the effects nicotine has on the hippocampus. It has been shown that nicotine facilitates synaptic activity and can increase LTP in the hippocampus via tasks involving memory and learning (Rezvani & Levin, 2001). Nicotine's positive effect on LTP has also been supported by studies investigating nicotine as a neuroprotective agent in rats that have been exposed to significant stressors (Aleisa *et al.*, 2006). In the case of Alesia and colleagues (2006), nicotine was shown to normalize LTP and counteract working memory impairments that were induced by stress. Nicotine has also been shown to attenuate working memory deficits as induced by certain drugs, again providing evidence that nicotine is neuroprotective and plays a significant role in LTP in the hippocampus (Levin, Bettegowda *et al.*, 1998).

While there is significant evidence that nicotine leads to increased neuronal firing and LTP in the hippocampus as a result of tasks involving memory and learning, it is

possible that the site of neuronal change could be elsewhere in the brain, or in addition to these hippocampal effects. Studies have shown that nAChRs are also present in other brain areas, such as the amygdala, the prefrontal cortex, the thalamus, and the motor cortex (Levin, McClernon *et al.*, 2006; Mansvelder *et al.*, 2006). It would be beneficial to investigate these brain areas with respect to nicotine exposure and working memory tasks, as this would provide a more complete view of the neural mechanisms of nicotine.

Limitations

The limitations of this study mostly involved the newness of the procedure. The osmotic mini pumps had never been used in this laboratory before, and due to some complications in the first round of surgeries a few rats lost their pumps. This was most likely due to poor placement of the pumps, as the first batch had the releasing end of the pump facing the wound. The excessive scratching that led to the loss of the pumps could have been due to the nicotine aggravating the wounds before they healed. After the first round of surgeries all other surgeries were completed without incident, providing further evidence that the pump placement was the reason for the excessive scratching and subsequent pump loss.

The use of BrdU was another limitation in this study. It did not immediately go into solution, so based on the findings of previous studies we heated the BrdU significantly in order to help it dissolve (Hume & Keat, 1990). We later found, however, that heating BrdU might actually decrease the likelihood that it will incorporate into new neurons. Despite this, the previous literature that used the heating technique did still see BrdU expression—thus, it was most likely a problem with the timing of our injections that led to the lack of expression (Hume & Keat, 1990). The results from the present

study suggest that the ideal time to inject BrdU is when learning is most evident, which would have been during days 1 and 2 of the acquisition trials.

Another potential limitation in this study could have been in the use of the Morris water maze. There are a few different ways the Morris water maze can be used to demonstrate spatial learning, and the procedure used in this study may be unusual compared to other, more widely used procedures. Because the rats were to be exposed to the maze four months after Phase 1 of testing, we wanted to ensure that the rats learned the task as well as possible during Phase 1. For this reason, we chose to have five days of acquisition, with five trials from the same start location each day. As the results indicate, the rats learned the spatial cues by the end of acquisition trials in both Phase 1 and 2. It can be argued that by the end of the acquisition trials the task was no longer hippocampal-dependent because the start location remained the same; however the good performance of the rats in the test trials indicates that the hippocampus was still highly involved in the task.

There have been previous studies using the Morris water maze as both a hippocampal-dependent and independent task. It has been found that when the task involves spatial learning, such as in a traditional Morris water maze study in which rats must locate a hidden platform, it is dependent upon the hippocampus (Teather, Packard, Smith, Ellis-Behnke, & Bazan, 2005). This has been evidenced by an increase in c-Fos in this brain area after such a task (Teather *et al.*, 2005). When a task is due to a stimulus-response "habit," such as in a Morris water maze task in which the platform is visible, the task can be completed independent of the hippocampus, with more reliance on the dorsal striatum. In the case of habitual learning, the increase in c-Fos is seen in the

dorsal striatum, and not the hippocampus (Teather *et al.*, 2005). Based on the findings of this study, it is possible that the later acquisition trials in the present study were more habitual, and that they relied on the dorsal striatum more than the hippocampus. Yet these findings still provide support for the test trials as being hippocampal-dependent because the rats had to find the hidden platform from unfamiliar start locations around the maze.

Many Morris water maze studies also employ the use of a video camera in order to assess the amount of time rats spend in each quadrant when the platform is removed (Bernal *et al.*, 1999; Socci *et al.*, 1995). Because this kind of technology was not available to our laboratory at the time of Phase 1 acquisition and testing, we chose to keep the platform in the maze for all parts of the experiment. Therefore, it was necessary to have all acquisition trials begin at the same start location, and to test the learning of the task using 5 different start locations around the maze. Although this is a more traditional method of assessing Morris water maze spatial learning, it still yielded significant results and demonstrated that, after 5 days of acquisition and 1 test day at varying start locations, rats learned the task as evidenced by a significant decrease in trial latencies (Morris, 1984).

This study may have also been limited by low statistical power. Though it began with 50 subjects and an even number of nicotine and saline rats in each group, complications with the surgeries and pumps caused these numbers to decrease, and ultimately the data from four subjects were lost before the close of the experiment. Though the results from the test trials were significant, it is possible that any differences in the adolescent rats in Phase 1 were too subtle to be demonstrated by the small number

of rats used in each group. Additionally, the complicated nature of the study design led us to perform many statistical analyses, which could have contributed to the possibility of Type I error. Despite this, the conclusions made were based largely on the results of tests using trial totals and averages, and the separate ANOVAs conducted were simply to view potential differences within the days of acquisition, and within the individual test trials. It may be necessary in the future, however, to alter the experimental design to allow for fewer statistical analyses to avoid this potential problem.

Implications

The results of this study lead to a number of implications that can be translated to human studies of nicotine's cognitive-enhancing properties. The finding most relevant to human research is that nicotine did enhance the cognition of the adult rats. This conclusion provides continuing support the use of nicotine as a therapeutic alternative for people suffering from diseases affecting cognition, including Alzheimer's disease. It also paves the way for future studies in this area that will give us even more information about the cognitive enhancing properties of nicotine, and how it can be best utilized for treatment of such disorders.

The fact that we tested adult rats that were 6 months old as opposed to "aged" rats that were up to 24 months old could be a potential limitation of this study because it makes it difficult to compare to previous literature. Most studies examining the differences between young and aged rats use aged rats because it is easier to relate to human studies of elderly people, particularly ones with age-associated memory impairments. The fact that we used adult rats, however, is also beneficial because of the lack of research done on rats of this age group—we were able to demonstrate that adult

rats benefited from a small amount of nicotine, which provides important results regarding a potential dosing threshold between adolescent and adult rats. Previous studies have only been able to demonstrate that adolescent rats need a higher dose than aged rats—the current study is able to narrow that age gap, and it suggests that there is a point between the age of 2 months and 6 months where the dose of nicotine can be decreased dramatically.

This translates to human studies involving nicotine by suggesting that if adults are facing a disease affecting their cognition, they do not need a dose as high as an adolescent might need in order to delay the onset of this disease. This could dramatically decrease the potential dangers of employing the use of nicotine patches in adult patients, because they would not be exposed to extremely high doses of nicotine that could lead to adverse side effects or increased likelihood of addiction. Instead, future studies of nicotine's use in adults facing cognitive impairments could begin using a relatively low dose of nicotine—if that does not produce the desired effects, then a higher dose might be tested. But the fact that the adult rats in this study benefited from the same low dose that has been shown to improve cognition in aged rats provides evidence that adult humans may also benefit from the same dose as the elderly (Socci *et al.*, 1995). Human studies of nicotine employing the use of nicotine patches delivering 0.9 mg/kg per hour (considered a moderate dose) to patients in their late 70's have demonstrated a clear improvement on tasks of cognition (Wilson *et al.*, 1995). Based on the results of the current study, it would most likely not be necessary to give an adult a higher dose than that in order to improve cognition.

The results regarding the dose in this study are also important in demonstrating

that cigarette smoking is not a safe therapy to use for people with diseases affecting cognition. Though it has been hypothesized that many patients with schizophrenia and ADHD self-medicate with cigarettes to obtain nicotine, the results of this study imply that this practice can actually have adverse effects upon the patients due to the very high dose of nicotine and the other harmful ingredients in cigarettes (Levin, McClernon *et al.*, 2006; Winger *et al.*, 2004). Cigarette smoking can actually impair cognition because the non-nicotine ingredients can restrict blood flow to the brain and lead to cell death. Also, the very high amount of nicotine in cigarettes can actually decrease cognition due to receptor desensitization (Winger *et al.*, 2004). Thus, nicotine should really be administered by itself and in a low dose to decrease the potential negative side effects of the drug.

It can also be concluded from the current study that the performance of the adult rats in the Morris water maze was state-dependent on the nicotine. This means that after being exposed to nicotine in conjunction with the spatial learning task, it was necessary to provide nicotine to the rats in any future exposures to the same task. This is why the group that received nicotine in Phase 1 was impaired in Phase 2 when they received saline—their learning of the task was dependent on the nicotine, and thus their trial latencies without it were much higher than rats in all other groups. It is possible, however, that the detriment in the N/S group might not have been evident if they were exposed to a different task, or the same task under new circumstances. In the current study, it is not possible to separate the state-dependent effect from the potential long-term benefits because we used the same spatial learning task to assess memory retention in both phases.

Despite the current data regarding the state-dependent effects of nicotine, it is not

likely that nicotine cessation would produce such profound deficits in humans after learning a task. In a study examining the potential cognitive deficits due to withdrawal from the nicotine in cigarettes, impairments on tasks of cognition were seen a few hours following cessation of the drug (Hatsukami *et al.*, 1989). However, it was mentioned that other studies using similar methods demonstrated that test scores on tasks of cognition after quitting were higher than scores obtained when the same participants were engaged in cigarette smoking (Hatsukami *et al.*, 1989). This suggests that the decline in the study done by Hatsukami and colleagues (1989) may not have been wholly due to the nicotine cessation, but also due in part to lasting negative effects of the combination of ingredients in cigarettes. Additionally, the study did not test their participants beyond 24 hours after quitting—this would be crucial for conclusions that nicotine cessation produces long-term cognitive deficits. Because this was not done, it is not possible to make this kind of assertion.

In order to further examine whether cognitive tasks are state-dependent on nicotine in humans, it would be necessary to perform follow-ups to the studies on nicotine's effect on human cognition. There have been a number of studies demonstrating that a chronic (or even acute) dose of nicotine improves memory and learning in Alzheimer's patients, however it would be beneficial to re-examine these patients after a significant period of time with the same tasks to see if the absence of nicotine has a negative effect on their ability to perform the task (Jones *et al.*, 1992; Sahakian *et al.*, 1989; Wilson *et al.*, 1995). This would provide more concrete evidence for the state-dependent effects of nicotine in humans.

The conclusion from the current study that c-Fos was increased in the

hippocampus potentially due to the elevated stress levels of the saline rats has interesting implications for human studies. It suggests that humans who are facing the beginning stages of cognitive decline could be under significantly more stress than people not undergoing the same decline. Though more research would need to be done in this area, it can be hypothesized that if humans are given nicotine to enhance their cognition at these beginning stages, they may be under much less stress because they would not have the same level of difficulty performing everyday tasks. Additionally, this provides some evidence that perhaps nicotine has anxiolytic effects. This is a result that would benefit from further study, because it provides more support for the development of a therapeutic treatment for such diseases that enhances cognition and delays the onset of marked cognitive decline.

Future Studies

Because this is a preliminary study, it lends itself to replication on a larger scale. Increasing the number of subjects in each group would be extremely beneficial, as the increase in power would potentially demonstrate subtle changes between groups, particularly in the adolescent rats. There were many trends toward significance, and it is assumed that with more subjects, there would be more clear indications of significance. Replicating this study would also be beneficial as many of the problems encountered would be anticipated and resolved before they occurred. It is now known how to properly perform the surgeries, so this would not be a complication, and the administration of BrdU would be handled differently. More injections of BrdU would be administered, and the timing of these injections would be altered so that the rats would be injected during the first two acquisition days. These days were when the bulk of the

learning occurred, so it is most likely that injections during these two days would lead to an increase in BrdU expression in the hippocampus.

This study also paves the way for further research in this area. We demonstrated that a nicotine dose of 0.2 mg/kg showed a significant decrease in the trial latencies of adult rats in the Morris water maze, but this dose did not demonstrate a decrease in latencies in adolescents. In the future, this experiment could be replicated by giving a group of adolescent and adult rats a higher dose of nicotine (for example, 0.4 mg/kg), in order to determine whether this dose improves the performance of the adolescents, and whether it impairs the performance of the adults.

Because the results regarding c-Fos expression in the dentate gyrus of the hippocampus were unexpected, further research regarding nicotine's effect on c-Fos expression in the rat brain would be beneficial to our understanding of this relationship. In order to demonstrate whether the increased c-Fos expression was due to the stress of having higher trial latencies, it would first be useful to look at c-Fos as a result of Morris water maze training alone—then the results could be separated into rats with longer latencies and rats with shorter latencies, to see if the rats that spent more time in the maze had more c-Fos expression. It would also be beneficial to examine c-Fos expression in the rat hippocampus as a result of nicotine when stress is not a factor. This would simply involve administering nicotine for a certain length of time and then examining c-Fos expression in the hippocampus after that time. If there is c-Fos present in the nicotine rats, it can be concluded that nicotine increases c-Fos expression when stress is not a factor.

Because the present study examined the hippocampus only, it would certainly be

beneficial to examine other brain areas that are linked both with memory and learning, and also with stress. This would be useful to provide conclusions regarding the effects of the nicotine at the neural level. Because nicotine can lead to increased attention in humans, it has been proposed that nicotine may also have an effect on the prefrontal, parietal, and occipital cortex, as well as the amygdala, and the hippocampus (Mansvelder *et al.*, 2006). Additionally, the thalamus and the motor cortex have also been shown to demonstrate a high number of nAChRs, which could also have implications regarding nicotine's effect on the human as well as the rat brain. Replicating this study and examining c-Fos or BrdU expression in some of these brain areas could be extremely beneficial for this field of research, as the largest amount of work has been done with the hippocampus. Paying closer attention to the brain areas about which less is known regarding nicotine's effects could lead to some important conclusions that could be carried over to human studies of nicotine. Examining nicotine's effect on the motor cortex could be particularly interesting when testing rats in motor tasks like the Morris water maze, because if nicotine stimulates neuronal growth in this brain area it could have a significant impact on the ease with which the rats acquire the task.

Conclusions

The current study provides support for the use of nicotine as a therapeutic agent in diseases affecting cognition. Because of nicotine's direct stimulation of nAChRs in the human brain, it would be a more effective alternative to the current treatments available for diseases like Alzheimer's, which provide minimal symptom relief. Though a considerable amount of information is known about nicotine's effects on the hippocampus, it is important that future studies examine nicotine's effects on different

brain areas in order to provide a more comprehensive view of its neural mechanisms. Once this knowledge is attained, it will hopefully lead the use of nicotine as a therapeutic drug—this has the potential to dramatically increase the quality of life for people suffering from diseases affecting their cognition.

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Table 1

Drug Treatment Groups

Group	Phase 1	Phase 2	Number of Subjects
N/N	Nicotine	Nicotine	7
N/S	Nicotine	Saline	7
S/N	Saline	Nicotine	9
S/S	Saline	Saline	8

Table 2

Experiment Schedule

Experimental Day	1	2	3	4	5	6	7	8
Phase 1	A	A	A	A	A	-	-	T
Phase 2 (and single-phase cohort)	A	A	A	A	-	A	T	

Note. A = Acquisition day, T = Test day, dash (-) = no trials

Figure Caption

Figure 1. Morris water maze start locations and platform placement.

Figure 2. Mean trial latencies for single-phase cohort acquisition.

Figure 3. Mean trial latencies for single-phase cohort test trials.

Figure 4. Mean trial latencies for Phase 1 acquisition.

Figure 5. Mean trial latencies for Phase 1 test trials.

Figure 6. Mean trial latencies for Phase 2 acquisition compared to mean trial latencies for Phase 1 acquisition.

Figure 7. Mean trial latencies for nicotine and saline rats in Phase 2 test trials.

Figure 8. Mean trial latencies for N/N and S/S rats in Phase 2 test trials.

Figure 9. Mean trial latencies for N/N, N/S, S/N, and S/S in Phase 2 test trials.

Figure 10. Photograph of c-Fos expression in saline and nicotine rats in the rostral section of the dentate gyrus (arrows point to examples of c-Fos particles).

Figure 11. Photograph of c-Fos expression in saline and nicotine rats in the middle section of the dentate gyrus.

Figure 12. Photograph of c-Fos expression in saline and nicotine rats in the caudal section of the dentate gyrus.

Figure 1.

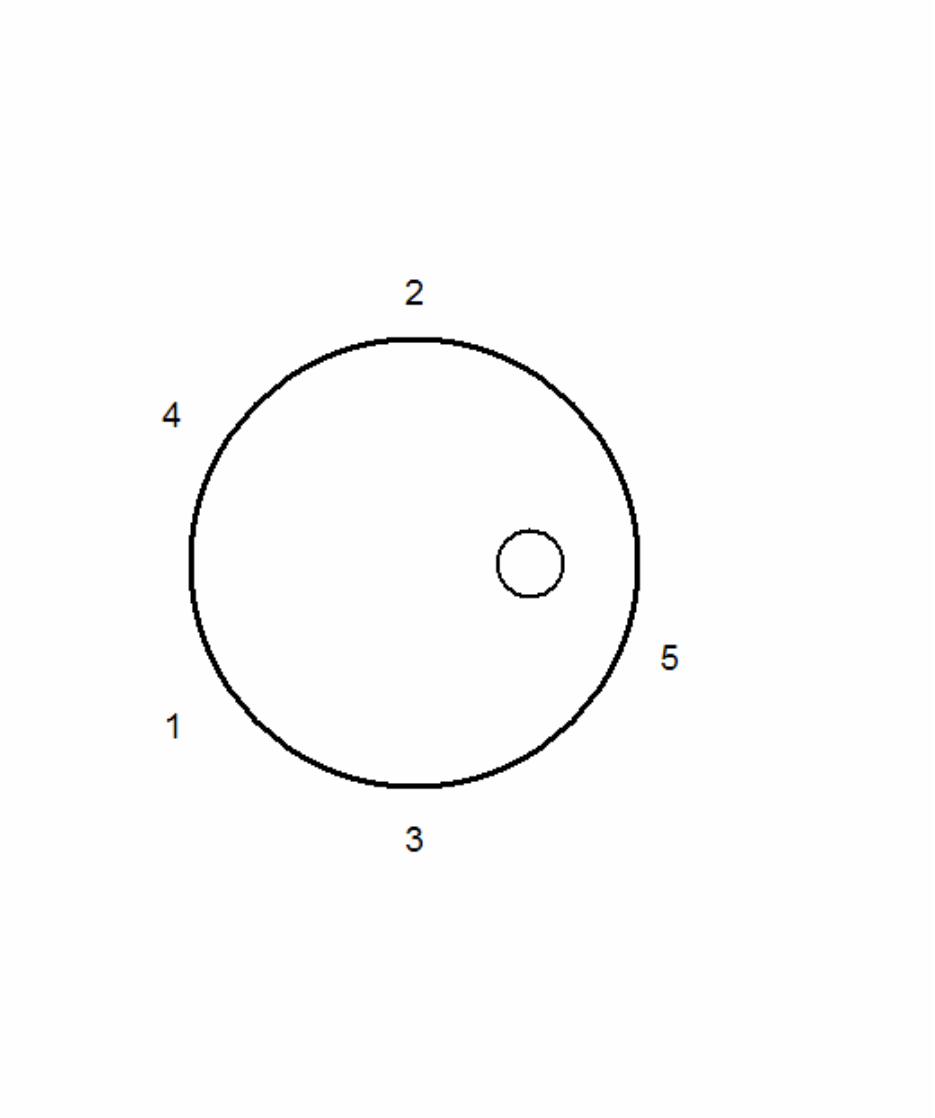
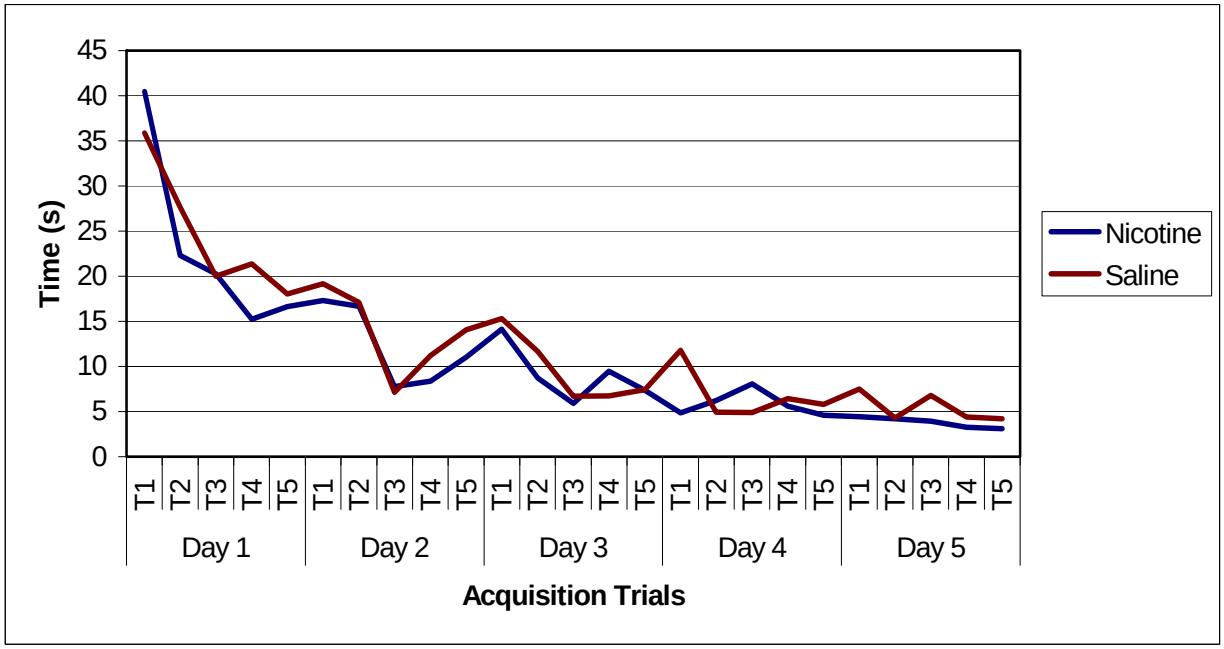


Figure 2.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Day 4
Day 5
Acquisition Trials
Nicotine
Saline

Figure 3.

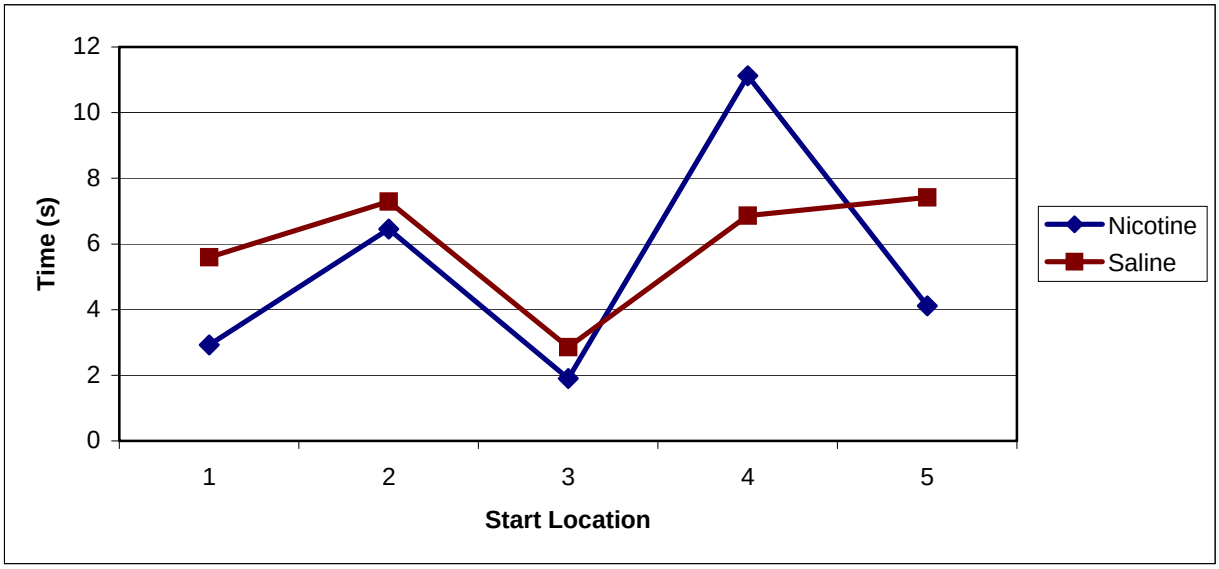
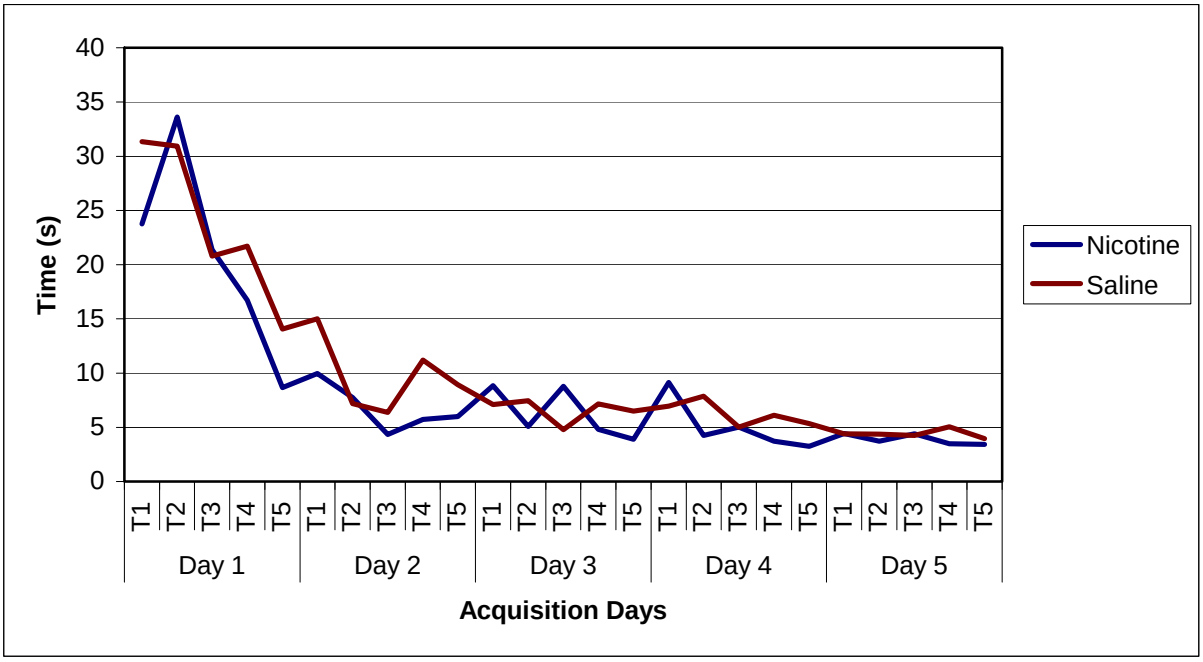


Figure 4.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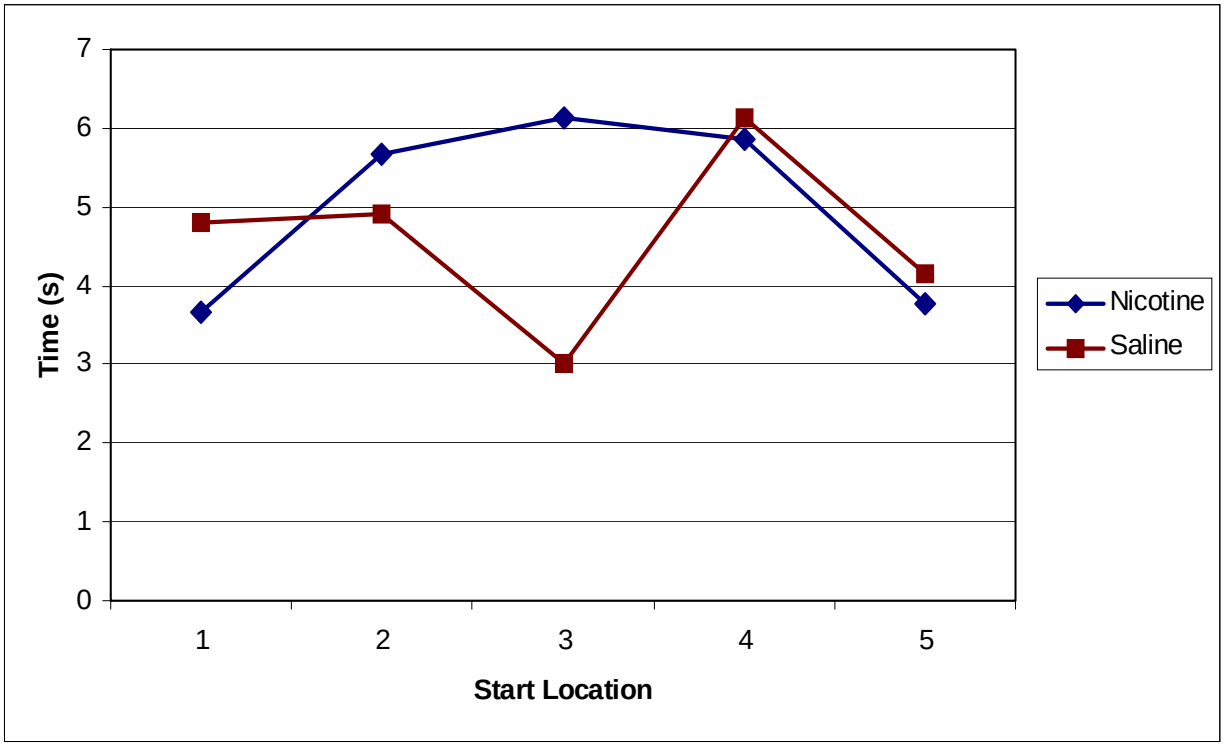
Day 5
Acquisition Days
Nicotine
Saline

Figure 5.



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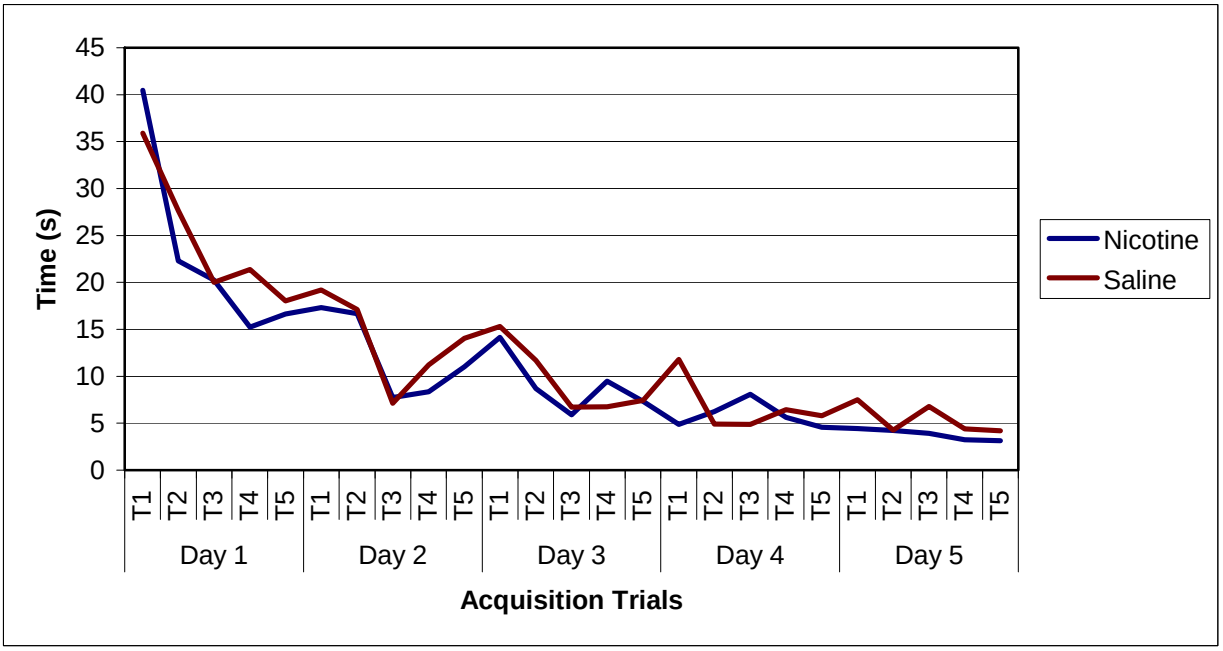
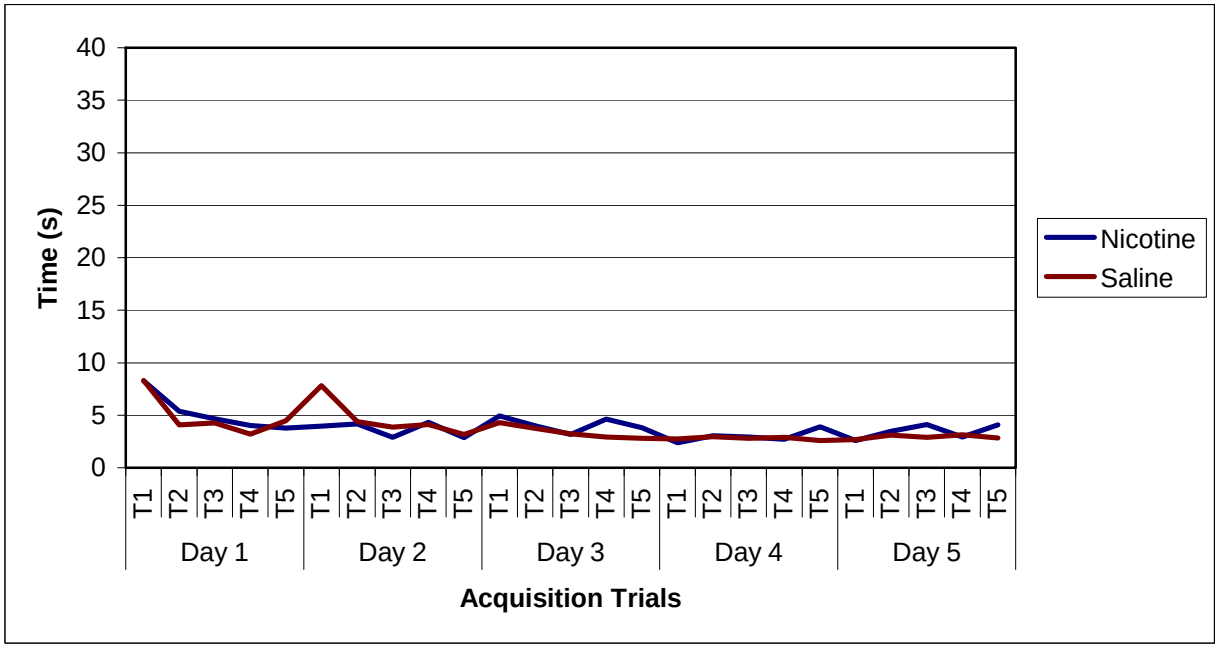


Phase 1 Acquisition Trials



Phase 2 Acquisition Trials

Figure 7.

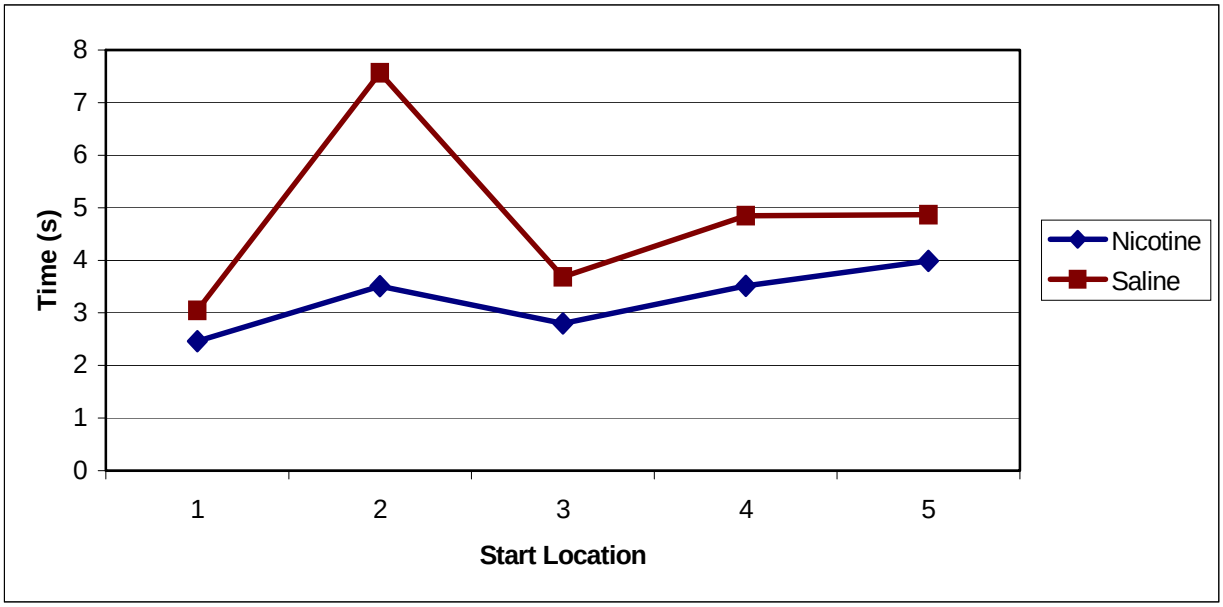


Figure 8.

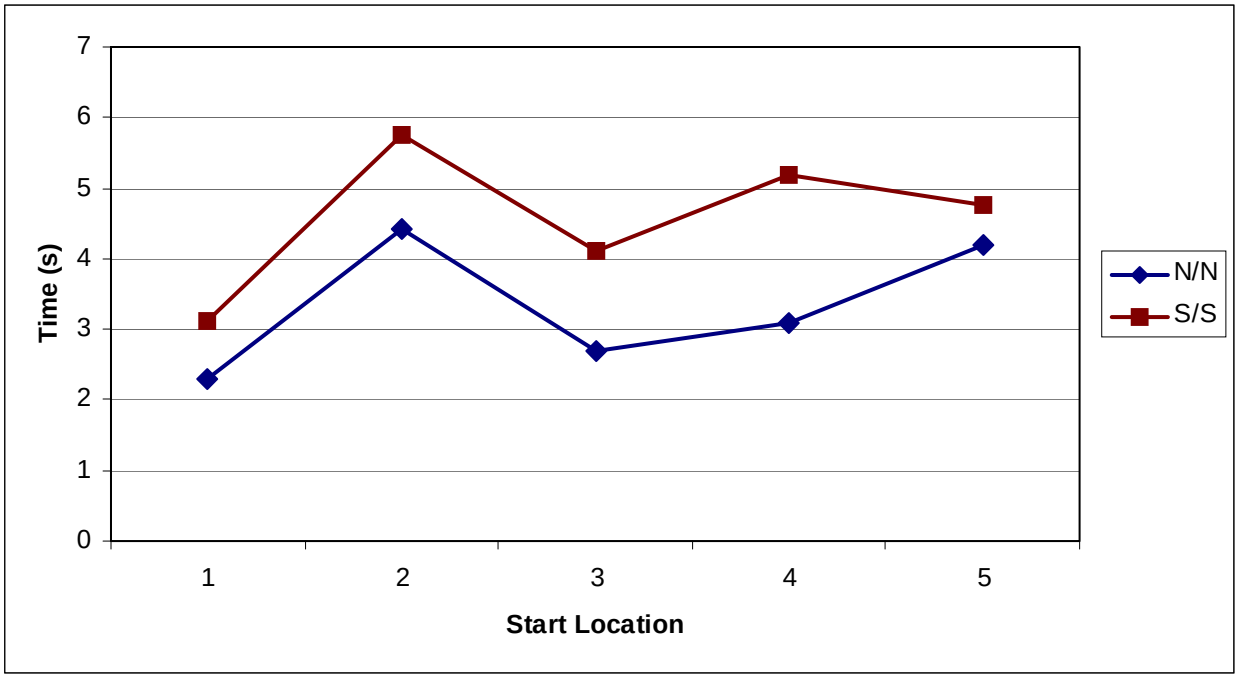


Figure 9.

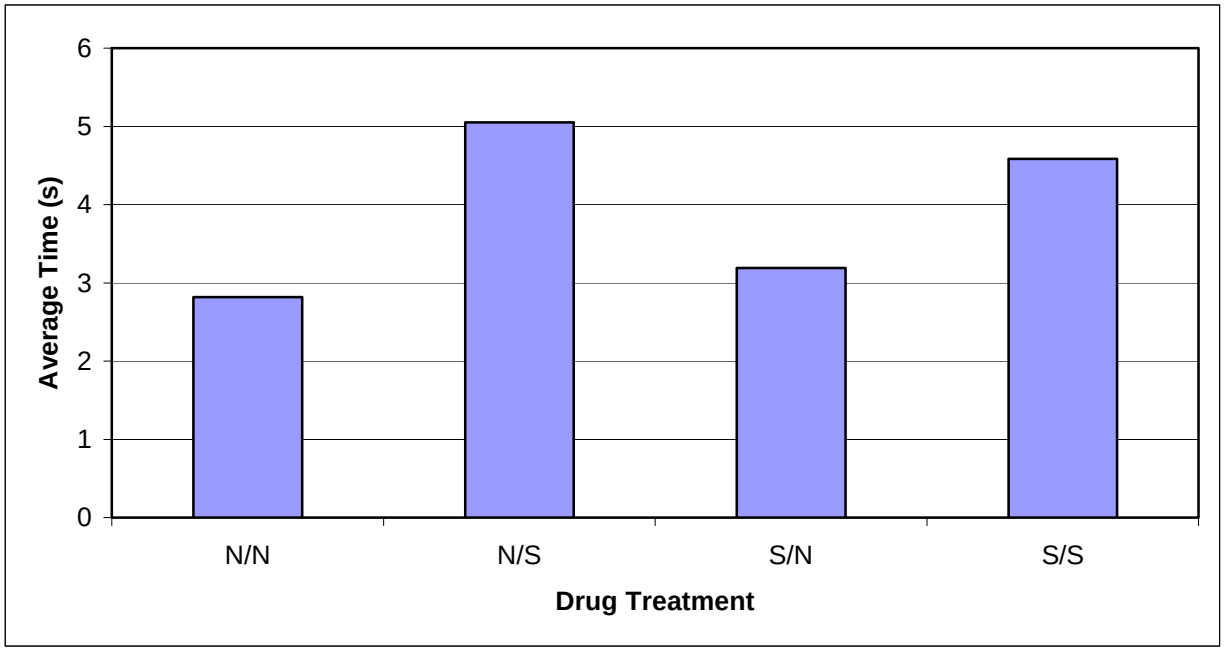
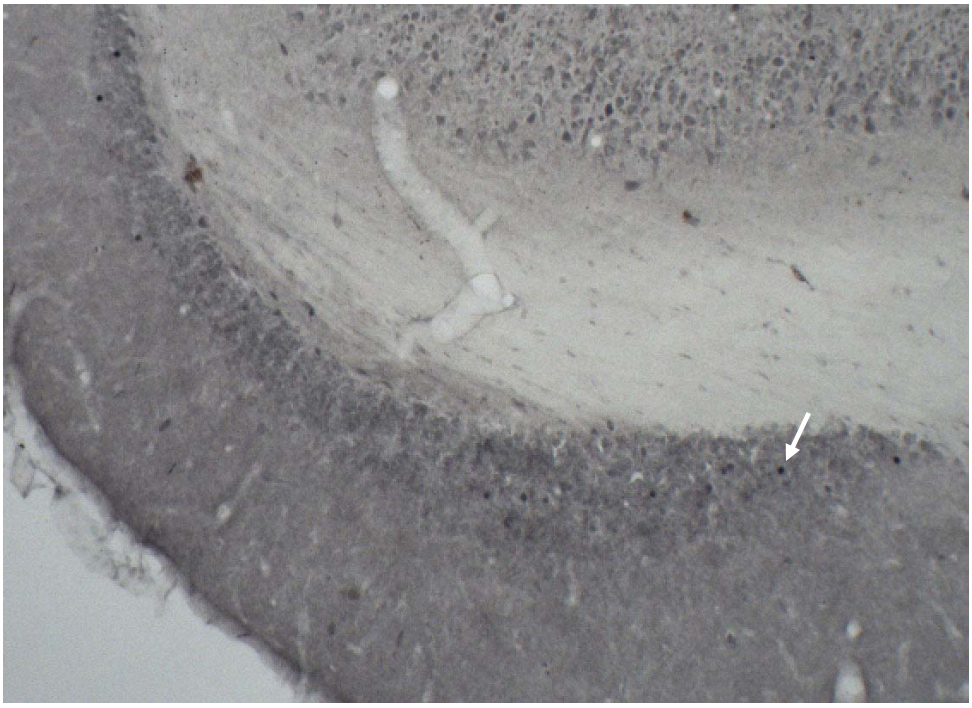
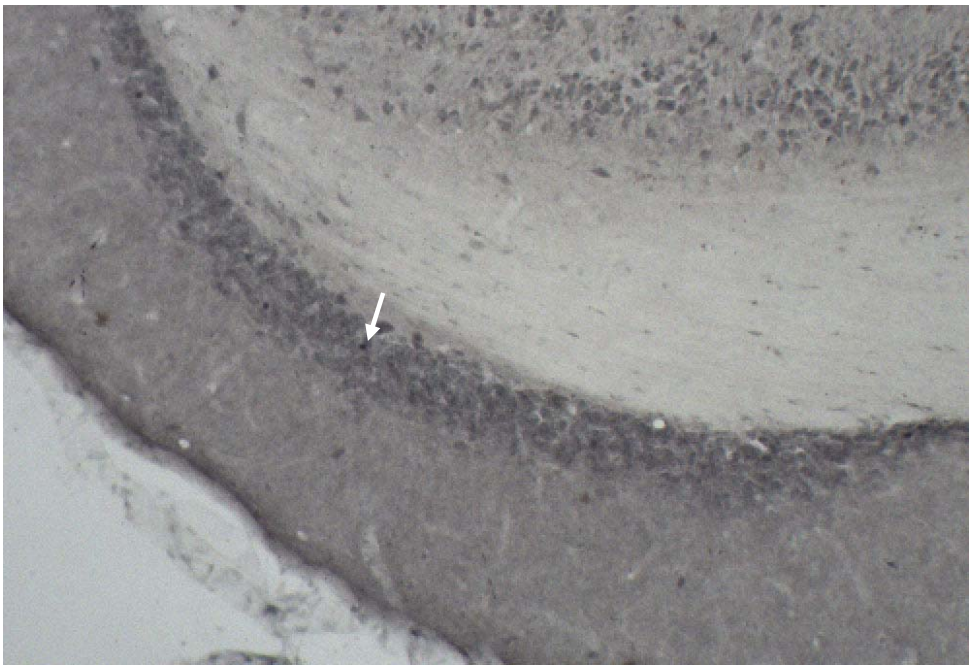


Figure 10.

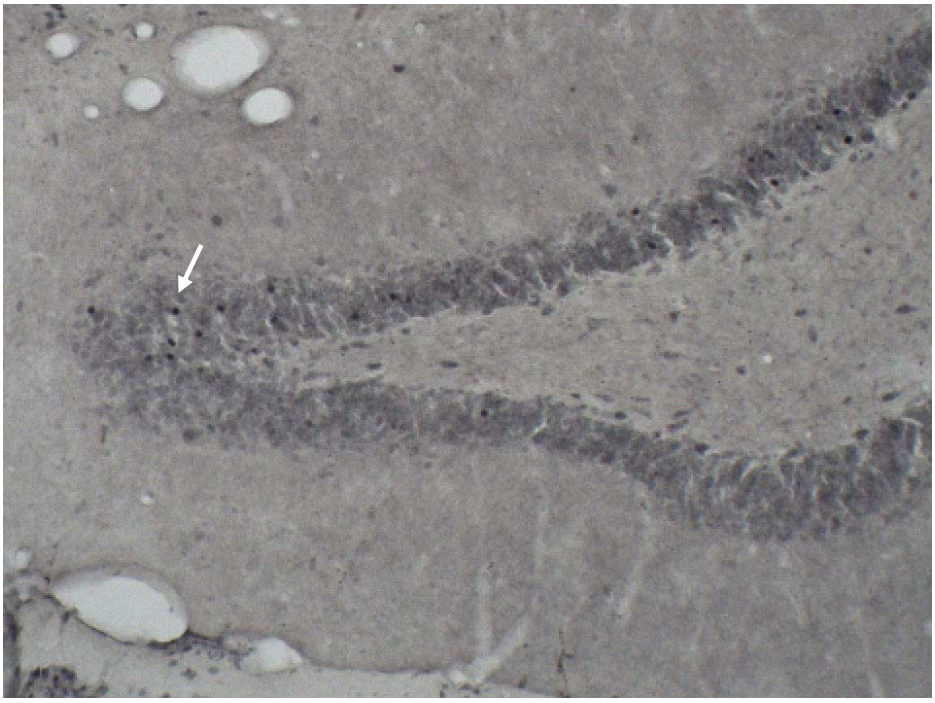


Saline



Nicotine

Figure 11.



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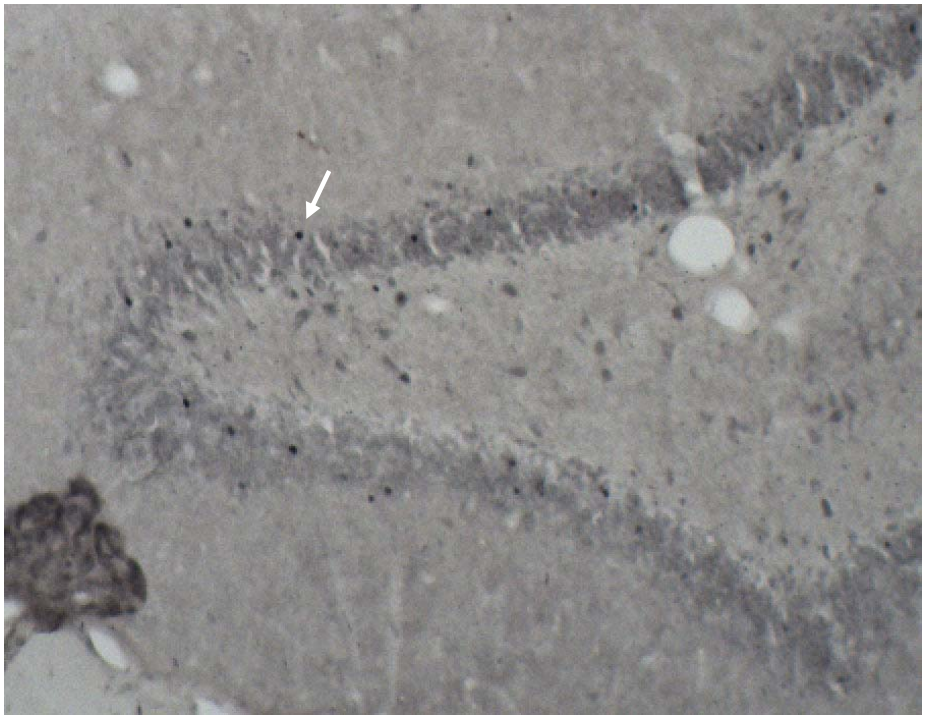
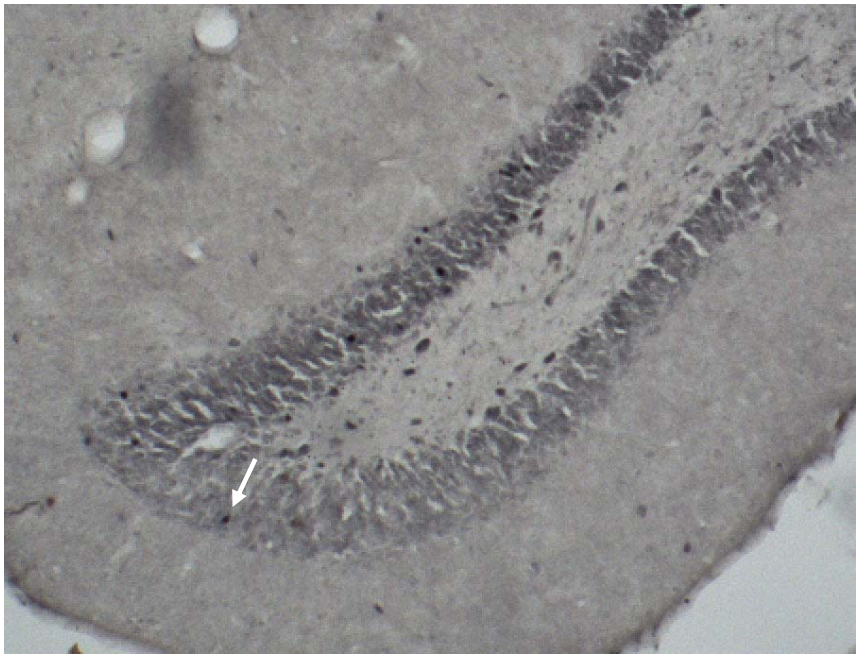


Nicotine

Figure 12.



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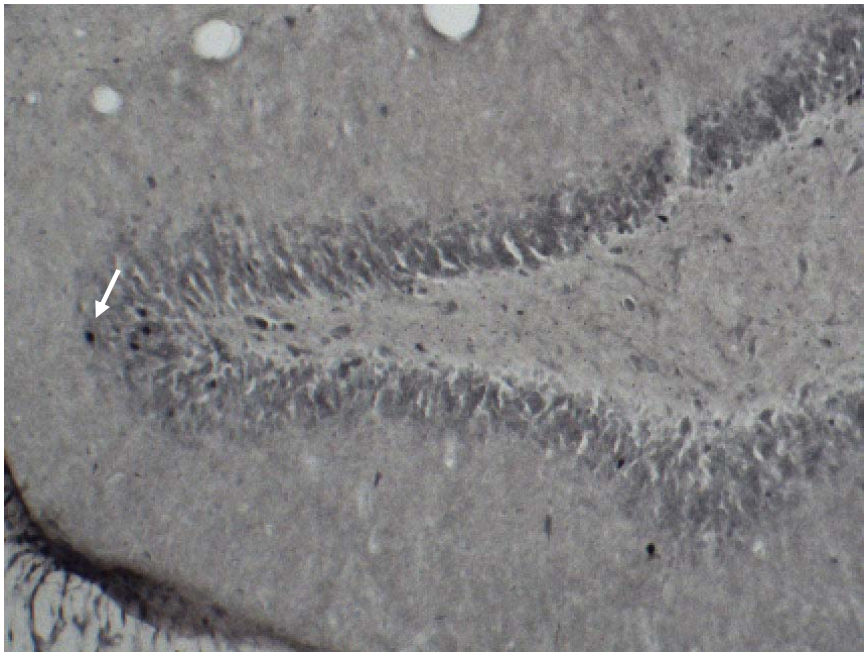

Nicotine