Adolescent Smoking, Memory, and Brain Integrity using Magnetic Resonance Imaging in Rat Model

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By:

Sravya Malempati

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Approved:

__________________________
Elizabeth Ryder, Ph.D.
Department of Biology & Biotechnology
WPI MQP Advisor
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ABSTRACT

Nicotine exposure during the adolescent period can have critical consequences, particularly on already vulnerable individuals, such as those with Attention Deficit Hyperactivity Disorder. Using a rat model, nicotine’s effects on behavior and brain integrity were tested in two strains, the Sprague Dawley Rat and a model of ADHD, the Spontaneously Hypertensive Rat. For cognitive function, the main focus was memory through the use of novel object recognition paradigm. Brain integrity was observed using non-invasive methods of diffusion tensor imaging and magnetic resonance spectroscopy, applied as in humans. While both rat strains showed expected effects of nicotine on locomotion, no strong effects of the drug on novel object recognition were observed. SD rats presented a significant change in brain integrity due to nicotine exposure while SHR rats showed little effect of the drug on integrity. This may suggest that SHR rats are less sensitive to nicotine’s effects.
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Cigarette smoking, also referred to as "smoking," is the largest single risk factor for premature death, as well as the largest preventable risk factor for morbidity and mortality, in developed countries. Statistically speaking, approximately one fifth of the deaths in the United States are attributable to smoking; 28% of the smoking-attributable deaths involve lung cancer, 37% involve vascular disease, and 26% involve other respiratory diseases. Furthermore, more than 400,000 deaths per year and 30% of all cancers in the United States are attributable to smoking. The prevalence of current smoking among adults, defined as smoking daily or smoking on some days, is statistically significantly higher in those less than 65 years of age. Demographic and smoking prevalence trends show that the absolute number of current smokers in the United States, about 47 million individuals in 1995, will continue to increase, especially in those below the poverty threshold, in those with less than 13 years of education, and in those greater than or equal to 65 years of age. This suggests that smoking is and will be very detrimental to the population, but if reductions in smoking prevalence were to be observed, morbidity and mortality attributable to smoking would decline in the future (Bergen and Caporaso, 1999).

The addiction caused by cigarette smoking is the main cause of the increased morbidity and mortality of smokers. Daily smokers smoke cigarettes to maintain nicotine levels in the brain, primarily to avoid the negative effects of nicotine withdrawal and to modulate mood. For this reason, smokers of all ages have become dependent on the effects of nicotine. The addiction to nicotine results in the smoker becoming overly exposed to the many carcinogenic chemicals present in cigarettes, explaining the mortality of cigarette smokers. More importantly, current smoking in the United States is positively associated with younger age (Bergen and Caporaso, 1999).
Not only are adolescents associated with increased cigarette use, they have also reported more frequent daily use of marijuana than nonsmokers (Duhig et al., 2005). Both marijuana and nicotine are the substances of abuse used most commonly by adolescents, and co-occurring use of both substances is common as well (Rubinstein et al., 2014). Additionally, cigarette smoking is more common in adolescents with attention-deficit/hyperactivity disorder, or ADHD, than in healthy adolescents (Gray, et al., 2011). Not only do individuals with ADHD report earlier initiation of smoking, but they also report more difficulty quitting than individuals in the general population (Fuemmeler, 2007). Human studies have shown that a single dose of nicotine reduces cognitive deficits in non-smoking young adults with ADHD (Potter, 2007). These studies suggest that the nicotinic cholinergic system is significantly involved as a result of drug exposure (King, 2014). However, little is known about the relationship between cigarette and cannabis (marijuana) use trajectories in the context of treatment for ADHD (Gray et al., 2011). Therefore, the purpose of the conducted research is to understand the mechanisms by which the brains of ADHD adolescents are affected by nicotine exposure. Sprague Dawley rats, models of non-ADHD individuals, and Spontaneously hypertensive rats, models of ADHD were used for this project. Thus, to study the effects of drug exposure, the behavioral and associated neural effects of drug use is explored. Better understanding of these mechanisms will help to better understand the behavioral and neural effects of nicotine and/or marijuana use in adolescents with ADHD.
BACKGROUND

Drug Addiction

Drug addiction has been described as a complex and chronic disease process occurring in the brain (Gupta and Kulhara, 2007). The behavioral abnormalities that occur as a result of drug addiction develop gradually and progressively during a course of repeated exposure to a drug of abuse, and can persist for months or years after discontinuation of drug use (Nestler, 2004). The common finding in drug addiction is that once specific regions in the brain are exposed to the drug, the mesolimbic dopamine system is activated, and the system reinforces both pharmacological and natural rewards. The mesolimbic dopamine system consists of dopaminergic neurons in the ventral tegmental area (VTA) and their axonal projections to terminal fields in the nucleus accumbens (NAc) and the prefrontal cortex (Fig. 1; Gupta and Kulhara, 2007). Cannabinoids, a group of active compounds found in cannabis, as well as nicotine, increase extracellular dopamine (DA) release at the projection areas of the midbrain reward pathway (Mao and McGehee, 2009). When repeatedly administered, these effects are enhanced so that re-exposure to the drug, weeks to months later, produces greater dopaminergic and behavioral activation than seen initially. This long-term enhancement in the ability of such drugs to activate DA neurotransmission and elicit appetitive behaviors is termed sensitization. Sensitization may be a contributing factor to the initiation, maintenance, and escalation of drug use that is characteristic of the transition from casual experimentation with drugs to drug craving and abuse in humans (Vezina et al., 2007). Figure 1 below displays the effects of nicotine and cannabis in the dopaminergic system.
Figure 1. Interactions of reinforcing drugs with the mesoaccumbens dopamine (DA) system, focusing on the ventral tegmental area (VTA) (Wolf, 2003). Nicotine acts via the nACHRs to activate the release of the excitatory neurotransmitters, glutamate and dopamine. Cannabinoids, which arise as a result of the administration of marijuana, act to inhibit the release of GABA which in turn disinhibits the release of dopamine. Additionally, opioids and ethanol play a role in the dopaminergic system by inhibiting GABA release as well, disinhibiting dopamine release.

Acutely, all reinforcing drugs increase DA transmission in this system (Fig. 1).

The nicotine acetylcholine receptors (nACHRs) play an important role in the dopaminergic system. Consistent nicotine exposure ultimately influences neuronal activity and behavior through its effects on nACHRs. Due to nicotine exposure, these nicotinic receptors are activated and result in excitatory transmission. Activation of nACHRs enhances glutamate release and contributes to long-term potentiation (LTP) induction at that synapse. In the continued presence of nicotine, nACHRs show strong desensitization through chronic exposure to the drug. Activation and desensitization of nACHRs due to prolonged exposure of nicotine
contribute to nicotine's effects on DA neuron excitability and drug addiction (Mao and McGehee, 2009).

The route of drug administration in the dopaminergic system is similar with both nicotine and marijuana use. Nicotine and marijuana both function similarly in the reward pathway to increase the release of dopamine and enhance its addictive properties. Therefore, changes to brain structure and function are not an effect of only nicotine exposure. Marijuana also is a contributing factor to these changes. Studies of marijuana users have indicated that volumes of several brain areas are smaller in heavy marijuana users, particularly the hippocampus area (Filbey et al., 2015). The effects of marijuana on hippocampal volume lead to poorer episodic memory performance. Thus, marijuana use has a detrimental effect on memory, attention, and learning. Similarly, nicotine exposure is associated with impaired working memory, attention, and verbal abilities. Therefore, in addition to structural changes, exposure to marijuana and nicotine are also associated with declines in cognitive function (Filbey et al., 2015). However there have been studies that show there are beneficial effects of nicotine on ADHD individuals (Potter, 2007).

**Increased Cigarette Use in ADHD Individuals and Adolescents**

Studies suggest that there is a developmental risk for substance use problems and disorders in individuals with ADHD, a common childhood disorder associated with many behavioral problems in adolescence and adulthood. Children with this disorder experience "significant behavioral functioning difficulties in adolescence and early adulthood (Looby, 2008). The disorder involves high levels of "impulsivity, hyperactivity, and inattention." These three words that describe ADHD are based on observations of how children with the disorder behave: impulsivity refers to thoughtless actions, hyperactivity signifies restlessness and excess
of movement, and inattention involves a disorganized style preventing sustained effort. The core problems of ADHD can persist over time and cause impairment in the development of children. Studies that have followed diagnosed schoolchildren over periods of 4 to 14 years have found that when compared to people of the same age who have not had mental health problems, the children with ADHD show "persistence of hyperactivity and inattention, poor school achievement, and a higher rate of disruptive behavior disorders." Severe levels of hyperactivity and impulsivity make children more likely to show substance misuse in later adolescence and adult life (NCC, 2009).

One of the most commonly used treatments that is very effective for ADHD symptoms is stimulant medication. In 2004, it was reported that 74% of American children with a diagnosis of ADHD receive stimulant treatment. However, there eventually was a concern about whether these medications were contributing to later substance use, particularly due to the fact that children with ADHD appear to already have been at increased risk for substance use disorders (Looby, 2008). Individuals with ADHD are known to initiate smoking at a younger age, possess increased risk for developing nicotine dependence, and have a more difficult time quitting compared to the general population (Gray et al., 2005). Substance use disorders are often seen in adolescents and young adults who were diagnosed with ADHD as children (Looby, 2008).

Marijuana and nicotine use occurs individually or combined as the use of each drug fosters initiation, escalation, and prolonged, problematic use of the other. While overall rates of tobacco use and co-use with marijuana are lower in adolescents compared with adults, most addicted adults develop nicotine dependence during adolescence. Therefore, adolescence is a critical period to study the effects of nicotine and/or marijuana use (Rubinstein et al., 2014).
Adolescence is a period of transition from childhood to adulthood marked by characteristic behavioral changes, including risk-taking, novelty-seeking, and peer associations that are thought to ease successful transition to independence and autonomy in adulthood. It is a period of enhanced clinical vulnerability to nicotine, and it involves a profound reorganization of brain regions necessary for mature cognitive and executive function, working memory, reward processing, emotional regulation, and motivated behavior. Nicotine acetylcholine receptors (nACHRs) regulate critical facets of brain maturation and any disruption in this system during adolescence due to drug exposure has unique consequences on adolescent development, on the development of the brain to be specific (Yuan et al., 2015). Thus, it is critical to focus the study of the effects of nicotine and/or marijuana use in the adolescent period.

**Significance of the Lab Rat**

To conduct this significant research on ADHD effectively, studying behavior is crucial, and rats are uniquely suited for these studies because of the database available in this species for assisting in experimental design, implementation, and interpretation of results (Cady, 1990). Furthermore, as a model of human disease, the rat offers many advantages over the mouse and other organisms in medical research. Primarily, the physiology of the rat is closely related to that of humans and is easy to monitor. Additionally, the rat is an excellent model for stroke and hypertension (Iannaccone, 2009).

Additionally, in studies of cognition and memory, the rat is superior to other models because the physiological systems involved in learning and memory have been so extensively studied in this animal. Also, the rat is more intelligent than the mouse and thus is capable of learning a wider variety of tasks that are important to cognitive research. The size of the animal enhances its use as a disease model not only because of the ability to perform surgical procedures
but also because of the proportional size of important substructures in organs. The size affects how much of the organ is involved in the experimental lesion, and it also allows the experimenter to determine the distance effects of drug administration to specific anatomical areas (Iannaccone, 2009). Furthermore, the rats are needed due to the secondary measures of this study which include nicotinic receptor measurements. Measuring the receptor entails post-mortem studies on the rat's brain which cannot be done in a clinical research setting.

Spontaneously Hypertensive (SHR) strain is chosen in particular as a model of ADHD. The strain is derived from the Wistar-Kyoto strain by selective breeding for arterial hypertension (Vendruscolo, 2009). Hyperactivity alone is insufficient for the animal to qualify as a model of ADHD. Based on a wider range of criteria-behavioral, genetic, and neurobiological—the SHR rat obtained from Charles River, Germany constitutes the best validated animal model of ADHD at the moment (Aase, et al., 2009). When compared with other strains, SHR rats show a sustained attention deficit, hyperactivity in some situations, and motor impulsiveness. Additionally, they are novelty seekers and risk-takers (Vendruscolo, 2009).

**Magnetic Resonance Imaging (MRI)**

The brain integrity of the rats can effectively be studied using the method of functional magnetic resonance imaging. With this method, the brain activity of fully conscious rats can be measured. Functional MRI (fMRI) allows the experimenter to measure neuronal activation and to study separate regions of the brain. Along with studying the activity of different brain regions and tissues using MRI, the chemical composition of the tissues can be studied using one of the tools of MRI called magnetic resonance spectroscopy (MRS). The main use of MRS is the chemical analysis of tissues, and it has been successful in assessing the condition of the brain in regards to the level of metabolites. As an example, MRS can determine if the level of certain
metabolites has increased or decreased as a result of drug exposure. Additionally, enzyme defects in muscle are easily detected by MRS (Cady, 1990).

In addition to MRS, Diffusion Tensor Imaging (DTI) is another tool of MRI used to study the integrity of the brain. DTI is a promising method for characterizing microstructural changes in neuropathology. It may be used to map and characterize the three-dimensional diffusion of water as a function of spatial location. Additionally, estimates of white matter connectivity patterns in the brain from white matter tractography may be obtained with the DTI tool. One measure of DTI is the fractional anisotropy, or the FA (Alexander, et al., 2007). The FA is a measure of the degree of directionality of diffusion. Its values range from 0, which signifies no directional dependence of the diffusion - or isotropic diffusion, to 1, which signifies diffusion along a single direction - or anisotropic diffusion (Schimrigk, et al, 2007). See figure 2 below.

**Figure 2. Ellipsoids showing isotropic and anisotropic directionalities of diffusion.** As the FA value approaches 0, the more isotropic the direction of diffusion. As the FA value approaches 1, the more anisotropic the direction of diffusion.

The equation used to find the FA value is shown below in figure 3.
Equation for calculating FA value.

FA is calculated from the eigenvalues of the diffusion tensor (fig 4). Eigenvalues represent the different DTI measures: mean diffusivity (MD), axial diffusivity (AD), and radial diffusivity (RD) (Alexander, et al., 2007).

\[
FA = \sqrt{\frac{1}{2} \sqrt{\frac{(\lambda_1 - \lambda_2)^2 + (\lambda_2 - \lambda_3)^2 + (\lambda_3 - \lambda_1)^2}{\lambda_1^2 + \lambda_2^2 + \lambda_3^2}}}
\]

Eigenvalues required to calculate the fractional anisotropy.

If all the eigenvalues are equal, which happens for isotropic diffusion, as in free water, the FA is 0. If the FA reaches its maximum value of 1, the diffusion is confined to one direction alone (Alexander, et.al., 2007). The FA value of the hippocampus region was one of the DTI measures that served as the main focus of this study. Together with MRS, DTI is an important tool of MRI that is an effective method to study the separate regions of the brain and to gain a deeper understanding of the effects of nicotine on the integrity of the brain.

PROJECT GOAL

As a first step toward looking at the effects of nicotine, the goal of this research project was to study the effect of nicotine exposure on behavior and cognition. The two rat models used were the Sprague Dawley (SD) and Spontaneously Hypertensive (SHR) rats. The SD rats were used as the control and the SHR rats were used as an ADHD model. Each strain was divided into two drug groups: a saline group and a nicotine group. Using two tools of MRI, DTI and MRS, white matter integrity and levels of brain metabolites were assessed in response to acute and
chronic nicotine exposure. In addition to brain integrity, the effect of nicotine on memory and behavior was examined and compared between the SD and SHR rats. It was hypothesized that the SHR strain would exhibit a difference in behavior and brain integrity as well as an improvement in cognition due to nicotine exposure.

METHODS

Habituation

The animals, during their awake hours, were handled for approximately ten to fifteen minutes per cage. In addition to handling, the rats were given an injection of saline each day.

Acclimation

For each rat, Lidocaine was applied on the nose and in front of the ears. After thirty minutes of letting the cream settle in, the rat was anesthetized. While maintaining constant exposure to the anesthesia, the rats' fore and hind paws were taped using medical tape. After taping the fore and hind paws, a headpiece was placed onto the head of the rat, with its sides resting in front of the ears. Once the headpiece was in place, the rat was placed into a head tube. Using a metal rod, the teeth were placed at a bar in such a way that the rat could bite on the bar. Once its teeth were situated, a nosepiece was taped onto the rat's nose. Then, the screws attached on the side of the head tube were screwed into the sides of the headpiece to keep the rat's head in place. Once the rat was situated into the head tube, the head tube containing the rat was taped into a longer tube. Another cylindrical tube was placed over the long tube. After the rat was completely restrained in this way, it was placed into a room that simulated the sounds of an MRI machine for the required amount of time. Each day, the rat was placed in the room fifteen minutes longer than the previous day. The maximum time the rat was left in the room was ninety minutes.
It was important that the amount of anesthesia given was sufficient only for the purpose of restraining the rat so that the anesthesia could eventually wear off during acclimation. The reason for this is because the point of acclimation is to eventually be able to take an MRI brain scan of a conscious rat.

**Drug Administration**

Material used to make nicotine were an 18 gauge needle, 5mL syringe, 0.1M NaOH, pH slips, and solid nicotine. 20mL of saline was added to a conical tube. Then 0.008 of solid nicotine was measured in a balance. The nicotine was added to the saline, resulting in a nicotine solution. Adding nicotine created a very acidic solution. Therefore, NaOH was added until the solution reached a pH of approximately 7. The drug injection was administered to be 1mL/1kg for each rat.

**Behavior Test #1: Open field Sensitization**

Each rat was weighed to measure the dose of the saline and nicotine that was to be administered each day. After the drug injection, the rat was placed in an open field for thirty minutes. EthovisionXT, a video tracking software used to analyze behavior, movement, and activity of an animal ("Video Tracking Software", n.d.), was used to record the total distance moved of each rat. On the first day, each rat was given a saline injection as a baseline. Following baseline behavior, the rats were divided into two drug groups. Those in the saline group received an injection of saline each day, while those in the nicotine group received nicotine each day. The amount of drug administered was 1mL/1kg. For example, if the rat weighed 120grams, it would receive an injection of 0.12mL of drug. Each rat was exposed to its respective drug for seven consecutive days. On the first and seventh day, a novel object recognition test was performed.
Behavior Test #2: Novel Object Recognition Test

The Novel object recognition test involved the familiarization phase and the novel object recognition (NOR) phase. For the familiarization phase, which lasted five minutes, four of the same objects were placed in the open field at set locations (figure 5).

![Figure 5. Schematic representing the familiarization phase.](image)

Figure 5. Schematic representing the familiarization phase. The purple diamonds represent the four objects used during familiarization phase. The schematic also shows the locations that were used for the objects.

The rat was then placed in the open field to familiarize itself with the objects for five minutes.

Video scoring was performed where a specific button would be pressed to make a record of each time the rat explored the object. After a 45-50min delay that followed the familiarization phase, the rat was put through a novel object recognition (NOR) test which also lasted five minutes. One of the schematics used during NOR is shown below in figure 6.
Figure 6. Schematic used during novel object recognition (NOR) test phase. The schematic above shows the objects used during NOR test phase. The purple diamonds represent the same objects used during the familiarization phase while the triangles represent novel objects. The top diamond represents a familiar object in a familiar location (same object and location used in the familiarization phase) while the bottom diamond represents familiar object in a novel location. The orange triangle represents novel object in a familiar location while the green triangle represents novel object in a novel location.

This test involved four objects, two from the familiarization phase and two new objects. Two objects were placed in the same location as the familiarization phase, and the other two objects were placed in new locations. Therefore, four scenarios were tested: exploration of a familiar object in a familiar location, familiar object in a novel location, novel object in a familiar location, and a novel object in a novel location. Once again, the exploration of each object was recorded using EthovisionXT. Video scoring was also performed for this phase where a specific button corresponding to each object would be pressed to make a record of each time the rat explored the object.

RESULTS

Nicotine sensitization has crucial effects on the brain and behavior. Using Sprague-Dawley rats as a control model and Spontaneously hypertensive rats as an ADHD model, several experiments were conducted where the rats in each model were divided into two groups: a saline group and a nicotine group. The amount of drug administered was 1mL/1kg. The experiments tested the behavior, cognition, and brain integrity of the rats to observe any changes from
nicotine exposure. The hypothesis was that nicotine would have a beneficial effect on ADHD symptoms in the SHR strain.

**Magnetic Resonance Spectroscopy (MRS)**

To analyze any change within the metabolites in the hippocampus region of the brain, an MRS scan was performed. In Figure 7 below, a brain scan of an SD rat is shown.

**Figure 7. Representative MRS of SD rat showing dorsal hippocampus (top) and spectrum of metabolites (bottom).** The top of the figure shows three slices of the hippocampus region (outlined by the red rectangle). The bottom of the figure shows the different levels of sample metabolites in that particular region. Ins, inositol; tCho, choline; tCr+PCr, creatine+phosphocreatine; tNAA, N-acetylaspartate. Error bars show standard deviation of peaks for all slices measured.

The spectrum above shows the levels of metabolites in the highlighted region of the brain, the hippocampus. Different metabolites of hippocampus region were analyzed in order to see whether there were changes in the levels within each group of both strains (Figure 8).
Figure 8. Levels of brain metabolites. Levels of metabolites in the saline and nicotine groups of the SD strain (left) and the SHR strain (right). The mean and SEM of each metabolite is graphed. *, p<0.05; **, p<0.01. SD: n=3, saline; n=3, nicotine. SHR: n=9, saline, n=8; nicotine, n=9.

The figure above shows that the nicotine group of the SD strain displays a significant reduction in many metabolites compared to the saline group while the SHR strain shows a significant difference in only one metabolite between drug groups. These results may suggest that SHR rats are less sensitive to nicotine's effects. Also, it is important to consider that the sample size of each drug group in the SD strain is 3 while the sample size of each drug group in the SHR strain is 8 or 9. The small sample size within the SD strain could have an effect in the results seen in figure 8.

**Diffusion Tensor Imaging (DTI)**

Another tool of MRI in addition to MRS that was used to study the integrity of the brain was DTI. The brain scan of each of the three regions of the hippocampus was analyzed to determine the fractional anisotropy (FA) value for each drug group of each strain (Figure 9).
Figure 9. Hippocampal FA of both strains. Graph shows the FA value of the rostral, intermediate, and caudal segments of the hippocampus for each drug group of the SD and SHR strains. Each bar for each drug group represents one of the three slices of the hippocampus region. Strain x Drug: $F_{(1,11)} = 12.5, p = 0.005$. n~4 for each group.

The SD strain shows an increase in FA value in the nicotine group as compared with the vehicle group, while the SHR strain shows no significant difference between groups. These results suggest that a structural change in the brain has occurred in the hippocampus of SD but not SHR rats in response to nicotine exposure.

Effect of drug exposure on locomotion

In order to test whether nicotine sensitization caused a change in locomotion, the total distance moved during the open field sensitization behavior test was measured for both the saline and nicotine groups of each strain (Figure 10). Each group of the SD and SHR strains received either saline or nicotine for seven straight days, and then the rats were individually placed in an open field for thirty minutes. Distance moved was normalized over baseline (pre-drug exposure) in order to adjust for activity differences among animals.
Figure 10. **Ambulation in the open field (30’).** Graph shows the locomotion of the saline and nicotine groups of both strains after seven days of drug administration. The total distance moved in thirty minutes was measured each day. These data were normalized for each rat to its baseline movement. Mean ± SEM are graphed for each day. Distance ratio = total distance moved / baseline distance moved. (SD: n=9, saline; n=10, nicotine; SHR: n=10, saline; n=10, nicotine). Nicotine increased ambulation (main effect of Drug, F(1,35) = 10.2, p = 0.003. There was also a differential strain effect according to day [Time x Strain, F(4.535,210) = 3.0, p = 0.017, with SD > SHR on day 5 p= 0.009, all others >0.2). Other effects were not significant (p ≥0.15).

The locomotion of the saline group in both strains is relatively constant over time, as expected (Figure 10). However, there is an effect of drug over time in the nicotine group of the SD strain where locomotion increases, while there is only an effect of drug and not time within the SHR strain.

**Effect of drug exposure on memory**

In addition to distance, the impact of nicotine on memory was tested. It was hypothesized that the nicotine group would exhibit differences in memory function compared to the saline group in both strains. To test memory function, the novel object recognition test was used in which each rat was familiarized with certain objects in certain locations and was later introduced to two familiar objects and two novel objects in either familiar or novel locations. The frequency
of exploration of each object was measured to determine how much the rat remembered the 
objects from its familiarization phase. It was hypothesized that the novel object in a novel 
location would be explored the most, and, thus, the frequency of exploration for that object would be the highest. Frequency of exploration was defined as how many times the rat attended to the object during a trial of five minutes.

Figure 11 below shows the frequency of exploration of all four objects within both the 
SD and SHR strains.

![Graph showing frequency of exploration based on object identity and location in the SD and SHR strains.](image)

**Figure 11. Frequency of exploration based on object identity and location in the SD and SHR strains.** Graph shows the number of times each object was explored. The data acquired from day 7 is analyzed. The mean and SEM for each group is graphed. The four conditions seen on the graph from left to right are fI_fL, familiar identity in a familiar location; fI_nL, familiar identity in a novel location; nI_fL, novel identity in a familiar location; and nI_nL, novel identity in a novel location. SD: n=8, saline; n=10, nicotine. SHR: n=10, saline; n=10, nicotine. Effect of strain, * p<0.05; effect of drug and effect of strain*drug not significant (p>0.05). Other effects not significant p>0.05.

During day 7 of testing, the frequency of exploration in the SHR strain is significantly lower than that of the SD strain in both saline and nicotine groups. There is similarity between the saline and nicotine group of both strains in that the novel object identity regardless of location appears to be explored slightly more frequently. However, there is no significant difference in exploration of novel and familiar objects as well as location from this analysis.
In addition to the frequency of exploration, the time spent exploring the objects was analyzed to determine the effect of nicotine exposure on memory. The four conditions (fI_fL, fI_nL, nI_fL, and nI_nL) were analyzed in different ways to determine the impact of object identity versus object location. Two methods were used to assess the impact of only novel object identity. In this analysis, time spent exploring an object out of the total time spent exploring in the open field was measured, rather than simply the number of times an object was explored.

First, only objects in familiar locations were considered, and the time spent exploring the novel object was calculated. The total time spent exploring the novel object in a familiar location was divided by the total time spent exploring both novel and familiar objects in the familiar location. The result was multiplied by 100 to acquire the time spent exploring the novel object as a percentage (figure 12). If novel and familiar objects were explored equally, then this percentage of exploration should be about 50%.
Figure 12. Percentage of investigation of a novel object within familiar locations in the SD and SHR strains. The duration (in seconds) of exploration during day 7 was analyzed within the saline and nicotine groups of both strains. The percentage of exploration was calculated from duration of exploration of novel object in a familiar location divided by the sum of duration of exploration of both novel and familiar objects in a familiar location. The mean and SEM are graphed for each group. Effect of drug, p=0.136; effect of strain, p=0.272; effect of strain*drug, p=0.389.

The saline and nicotine groups of the SD strain remains relatively similar. Although the percentage of investigation seems to have increased in the nicotine group of the SHR strain, there is no statistical significance. Additional testing will need to be conducted to analyze this further.

Second, both locations, familiar and novel, were looked at to determine percentage of exploration of a novel object in those two locations within all four objects. The total time spent in seconds exploring the novel object in a familiar location and novel object in a novel location was divided by the total time spent exploring all four objects. The result was multiplied by 100 to acquire the time spent exploring the novel objects in the two different locations as a percentage (figure 13).
Figure 13. Percentage of exploration of novel object from sum of all four objects in the SD and SHR strains. Graph shows the percentage of exploration of the saline and nicotine group of both strains. The percentage was calculated from the total time spent in seconds exploring the novel object in both the familiar and novel location divided by the total time spent in seconds exploring all four objects multiplied by 100. Graph shows data acquired during day 7 of NOR testing. The mean and SEM is graphed for each group. Effect of drug, p=0.669; effect of strain, p = 0.679; effect of strain*drug, p = p=0.773;

There is similarity between the saline and nicotine groups of both the SD and SHR strains. However, since there is no statistical significance, additional testing is required for further analysis.

In addition to novel object, the percentage of exploration in the novel location was analyzed. The total time spent in seconds exploring the familiar object in a novel location was divided by the total time spent exploring both the familiar object in a familiar location and familiar object in a novel location. The result was multiplied by 100 to acquire the time spent exploring the familiar object in the novel location as a percentage. See figure 14 below.
Figure 14. Novel location exploration within familiar objects in the SD and SHR strains. The duration (in seconds) of exploration during day 7 was analyzed. The percentage of exploration was calculated from duration of exploration of familiar object in a novel location divided by the sum of exploration of familiar objects in a familiar location and novel location. The mean and SEM are graphed for each group. Percentage of exploration of familiar object in a novel location. Effect of drug, p=0.092; effect of strain, p=0.720; effect of strain*drug, p=0.124.

It seems that the saline and nicotine groups within the SHR strain are exhibiting a difference in object exploration. However, there is no statistical significance, and further testing is required.

The final method of analysis that shows the amount of exploration in the novel location is shown in figure 15 below. The total time spent in seconds exploring the familiar object in a novel location and novel object in a novel location was divided by the total time spent exploring all four objects. The result was multiplied by 100 to acquire the time spent exploring the novel locations as a percentage. See figure 15 below.
Figure 15. Percentage of exploration in a novel location within all four objects in the SD and SHR strains. Graph shows the percentage of exploration in a novel location during day 7 of testing. The percentage was calculated from the time spent in seconds exploring the familiar and novel object in a novel location divided by the time spent in seconds exploring all four objects multiplied by 100. Graph above shows mean and SEM of the saline and nicotine groups of each strain. Effect of strain, p=0.912; effect of drug, p=0.785; effect of strain*drug, p=0.921.

There seems to be no differences within either strain in this analysis. There is no statistical significance here, but further testing will help to determine the effect of nicotine exposure on ADHD adolescents.

**Adult Locomotion**

To test for any withdrawal effects of adolescent nicotine exposure, locomotion and memory were analyzed for the saline and nicotine groups of the SD strain in the adult phase. After approximately two months of drug withdrawal, the rats were placed in the open field again to observe any changes in locomotion (Figure 16).
Figure 16. Total distance moved within the SD strain (30'). Graph shows the total distance moved for thirty minutes within the saline and nicotine group of the SD strain. Data is acquired from one day of testing. The mean and SEM is graphed. T-test, p=0.715

There is no effect of drug withdrawal during locomotion in the adult phase of the SD strain.

Once the SHR strain is tested during the adult phase, behavior test results from both strains can be compared, and a conclusion can be made about the withdrawal effects of nicotine.

**Adult Novel Object Recognition Test**

To test whether the memory has been affected due to nicotine withdrawal, the SD adult rats were placed in the open field for the NOR test. One analysis from the data is shown below in figure 17.
Figure 17. Percentage of exploration of novel object within familiar locations within the SD strain. Graph shows the total time spent exploring novel object in a familiar location divided by the following two scenarios: familiar object, familiar location and novel object, familiar location and then multiplied by 100. Data is acquired from one day of NOR testing. Data shows that the saline group exhibits some discrimination of the objects which is significant. T-test of both groups: p = 0.396

There seems to be no significant difference between the two groups during object exploration.

However, with further testing involving SHR adult strain, a conclusion can be made about whether or not there are significant withdrawal effects within the SD and SHR strains.

Another analysis that shows the importance of the novel object is shown below in figure 18.
Figure 18. Novel object identity percentage of investigation from sum of all objects within the SD strain. Graph shows the percentage of exploration within the SD rats from the saline and nicotine groups. The percentage was calculated from the time spent in seconds exploring the novel object in both the familiar and novel location divided by the time spent in seconds exploring all four objects multiplied by 100. Data is acquired from one day of NOR testing. T test: $p=0.5560$

Again, there is no significant difference between groups in this analysis, and testing of the SHR adult strain is required for a more genuine analysis.

In addition to analyzing the exploration of novel object, exploration in a novel location was also graphed. One analysis showing the importance of novel location is shown below in figure 19.
Figure 19. Percentage of exploration in novel location with familiar objects within the SD strain. Graph shows the total time spent exploring the familiar object in a novel location divided by the two scenarios: familiar object, familiar location and familiar object, novel location and then multiplied by 100. Data is acquired from one day of NOR testing. The mean and SEM of the saline and nicotine group is graphed. T-test: p=0.419

There is no identifiable difference between the groups when analyzing preference of novel location during object exploration. Further testing is required to determine the effect of drug withdrawal in novel location exploration.

The final analysis used to analyze the amount of exploration in a novel location is shown in figure 20 below.
Figure 20. Percentage of exploration of novel object location from sum of all objects within the SD strain. Graph shows the percentage of exploration of a familiar and novel object in a novel location. Percentage was calculated from total time spent in seconds exploring the familiar and novel object in the novel location divided by the total time spent in seconds exploring all four objects. T-test: $p=0.285$. Again, there appears to be no difference between groups in this analysis. Behavior tests conducted with the SHR strain can help determine whether there are withdrawal effects in the adult phase from adolescent nicotine exposure.
DISCUSSION

In this study, the Sprague Dawley (SD) and Spontaneously Hypertensive (SHR) strains showed changes due to nicotine exposure, but the changes varied within the two strains. Previous human studies state that there are beneficial effects of nicotine on ADHD individuals (Potter, 2007). Thus, it was hypothesized that the SHR strain would not only exhibit an effect on behavior and brain integrity but also exhibit an improvement in cognition due to nicotine exposure. As shown by the MRS and DTI, the SD strain showed an effect of drug in brain integrity where the nicotine group exhibited a decrease in the level of metabolites and an increase in the FA value. However, the SHR strain showed little sensitivity to the drug. As shown by locomotion, the nicotine group of the SD strain exhibited an effect of drug over time shown by the increase in activity, while the SHR strain showed an effect of drug but not over time. In the adult phase, the SD strain showed no effect of withdrawal from the drug.

The MRS data shown is that of an adolescent SD rat (figure 7). The levels of metabolites shown by the spectrum are the levels of a healthy animal. Any major shift in the parts per million (ppm) of the metabolites would mean that there had been a change in the brain integrity due to drug. However, no such shifts were observed. Since this is an MRS of an SD rat in the saline group, it was expected that no significant change would occur.

In figure 8, changes in brain metabolites are shown in both strains but more in the SD strain. It seems that the SD strain showed an effect of drug much more significantly than the SHR strain. However, it is important to consider the difference in sample size of both strains. The sample size of each group of the SD strain is 3 while the sample size of each group in the SHR strain is 8 or 9. Thus, due to the small sample size in the SD strain, there may have been an outlier that contributed significantly to the results of the SD strain seen in figure 8.
In the analysis of the hippocampal FA value (figure 9) where the sample size was 4 for each group of both strains, the SD strain showed an effect of drug whereas the SHR strain did not. Previous studies have shown that smokers exhibited significantly elevated FA compared with nonsmokers in the whole corpus callosum (Paul, et al., 2008). That may explain why the nicotine group of the SD strain showed an increase in FA value. However, the FA value did not change within the drug groups of the SHR strain, suggesting that the SHR strain may not be sensitive to nicotine exposure.

Similarly, the SD strain exhibited an effect of drug over time in the locomotion behavior test while the SHR strain showed an effect of drug but not an effect of drug over time (figure 10). The nicotine group showed an effect of drug over time as it became sensitized to the drug and became more active throughout the week of nicotine exposure. However, the nicotine group of the SHR strain became more active due to nicotine exposure but did not become sensitized to the drug over time.

In addition to behavior, the effect of nicotine on memory was assessed. There is significance in the frequency of exploration (figure 11) in that the SHR strain shows much less object exploration than the SD strain. The hyperactivity of the SHR rats can explain their lack of focus toward each of the objects. Data from the percentage of exploration (figures 12-15) showed no statistical significance. Further testing is necessary to test the effects of nicotine on memory in adolescence. Additionally, the adult testing of the SD strain (figures 16-20) showed no significance. However, future testing involving the SHR adult strain can help determine any significant changes within both strains from nicotine withdrawal.

It was hypothesized that nicotine would improve the cognitive or behavioral deficits present in individuals with ADHD. However, we were unable to reject the null hypothesis of no
difference between SHR rats on nicotine vs. saline. Thus, there is no evidence from this study supporting the idea that nicotine improves cognition in SHR rats. However, the data presented is consistent in showing that the SHR rats are not very sensitive to nicotine. This may suggest that this a lowered sensitivity to the drug is why cigarette smoking is more common in adolescents with ADHD (Gray, et al., 2011). In order to test this further, future experiments will be beneficial for the purpose of acquiring more data that will allow for deeper analysis into the effect of nicotine exposure on behavior, cognition, and brain integrity in adolescence.
CONCLUSION

The SD strain exhibits functional changes in many of the metabolites as seen in the MRS data but the SHR strain shows very little effect of nicotine exposure. Additionally, only the SD strain shows an effect of drug in brain integrity as shown by the DTI data, in which there is an increase in the FA value in the nicotine group, and an effect of drug over time in the locomotion, in which the nicotine group shows higher activity than the saline group. However, the SHR strain shows no effect of drug in brain integrity and only shows an effect of drug in locomotion in which the nicotine group shows higher activity than the saline group. Further behavior tests need to be conducted to assess whether there are any effects of nicotine on locomotion and memory during adolescence. Furthermore, additional testing for the SHR strain in the adult phase will allow for better analysis of withdrawal effects due to adolescent nicotine exposure.
FUTURE DIRECTIONS

There are many factors to consider for the future in order to gain more accurate results. One consideration is the possible variability within the SHR strain. Some of the rats seem to be more hyperactive than the rest of the rats just within the SHR strain. Additionally, it is important to consider the time delay after the familiarization phase.

For behavior testing, the time of injection may be important. It may make a difference in data acquisition if the rat is placed in the open field for testing right after the injection or a few minutes after the injection.

During NOR testing, a forty-five minute delay after the familiarization phase may be too long of a delay. Another important factor that could affect data acquisition during NOR testing is the quality of objects. Some of the objects used during this project proved to be very easy for the rats to climb onto, which would increase their preference for the object and is irrelevant to their memory of it. Finally, it would be ideal to keep the time of testing fairly consistent (only in the morning, only afternoon, etc.)
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