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TESTING A GATEWAY TO ADDICTION: BEHAVIORAL AND NEUROPHYSIOLOGICAL EFFECTS OF EARLY EXPOSURE TO SACCHARIN, CAFFEINE, AND NICOTINE IN ADOLESCENT MICE

BETHANY LATTEN
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Reviewed and approved* by the following:

David J. Vandenbergh
Professor of Biobehavioral Health
Thesis Supervisor/Honors Adviser

Joseph Gyekis
Instructor of Biobehavioral Health
Faculty Reader

* Signatures are on file in the Schreyer Honors College.
ABSTRACT

For my honors thesis research project, I tested one facet of the gateway hypothesis of addiction to explore how nicotine consumption is altered in adolescent mice after exposure to more commonly available appetitive substances. The gateway hypothesis has been the subject of much criticism since it first became popularized several decades ago. While it was once thought that marijuana was the crucial gateway substance that compelled marijuana users to use other illicit drugs, now researchers have turned their attention to its legal yet significantly more addictive predecessor in the gateway pathway, nicotine.

The idea for studying the gateway hypothesis originated from considering how humans often progress from licit to illicit substance use. Children are continuously exposed to various substances that they learn to associate with reward. For example, sugar and caffeine containing foods and beverages lead to a pleasurable, rewarding taste (Temple, 2009). Although children are often exposed to low levels of nicotine through second-hand smoke, either in their homes or on city streets, it is unclear if this level of exposure is associated with behavioral changes. As described in this chapter, I used an animal model to study whether exposure to appetitive substances and low levels of nicotine in young mice increased nicotine consumption later in life. While the results I obtained did not demonstrate a significant association between exposure to low levels of nicotine during early adolescence and greater nicotine consumption later in life, the results do suggest that further studies should be performed to examine whether the effects of exposure to low levels of nicotine is associated with greater nicotine consumption when studied over a longer period of time.

The second component of my thesis consisted of molecular analysis of gene expression in a representative sample of the mice’s brains. Addiction has long been implicated as a form of
learning (Wikler, 1961), and recent work by Cao et al. has associated early exposure to nicotine in rodents with myelin-related gene expression changes, one of many findings that suggest the presence of a molecular component in the addiction-learning process (2013). Real-Time PCR was performed to determine if the gateway pathway described above affected expression of *Mobp*: Myelin-associated oligodendrocyte basic protein. While the expected trend of *Mobp* upregulation was observed, the results failed to reach significance. Moreover, the amount of nicotine consumed during the exposure experiment was not correlated with the amount of *Mobp* upregulation.
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Chapter 1

Nicotine’s Role in the Gateway Hypothesis of Addiction

Introduction

I. Biology of Addiction

“When you can stop you don’t want to, and when you want to stop, you can’t…”

This line describing heroin addiction in the 2006 movie Candy describes the transition from voluntary recreational drug use to uncontrollable drug dependence (Davies, 2015). The reasons for this transition, however, have been the subject of research for decades. A major breakthrough in addiction research occurred in 1961 when Abraham Wikler demonstrated that addiction is a form of learning (Wikler, 1961), and we now know that addiction results in learning by altering brain structures and communication pathways (Nestler and Aghajanian, 1997).

Addiction can interfere with any step of the normal neurotransmission process. A neuron normally functions in neurotransmission by receiving information at its dendrites, integrating the signal at the axon initial segment, and, if the excitatory signal is strong enough, transmitting an action potential down an axon toward the axon terminal. There, neurotransmitter-filled vesicles fuse with the plasma membrane and release their contents into the synaptic cleft. The neurotransmitters can then bind to receptors on a postsynaptic neuron until cleared from the synaptic cleft by enzymatic degradation, reuptake, or diffusion. The large majority of drugs have their effects toward the later stages of neurotransmission, such as neurotransmitter release, synaptic clearance, and postsynaptic receptor levels. For example, cocaine prevents dopamine
reuptake; amphetamines block degradation of monoamine neurotransmitters, and
MDMA/ecstasy promotes enhanced release of monoamines through their respective transporters. The reward pathways activated by these drugs are associated with temporary euphoria and often unpleasant withdrawal symptoms, which lead to increased drug use and ultimately drug dependence (Koob and Moal, 2006).

Other drugs, such as caffeine and nicotine, exert their stimulatory effects on downstream target postsynaptic receptors. Caffeine decreases inhibitory signals in the CNS by blocking adenosine receptors, which over time leads to down-regulation. Consequently, greater levels of caffeine are required to receive the same stimulatory effects experienced during the first use, and withdrawal symptoms of fatigue and headache are experienced when caffeine intake ceases (Leôn et al., 2002). Moreover, the degree of caffeine dependence is largely determined by an individual’s rate of caffeine metabolism, which is in part controlled by genetics (Cornelis et al., 2007).

The neurophysiological underpinnings of nicotine pharmacology are considerably more complex than caffeine and have appreciably greater implications for society. According to the National Institute on Drug Abuse, tobacco use causes nearly half a million premature deaths every year and is the leading cause of preventable deaths in the country (2014). Nicotine exerts its effects on nicotinic acetylcholine receptors (nAChRs), which are located throughout the peripheral and central nervous systems. Nicotine easily crosses the blood-brain-barrier and exerts its main rewarding effects on dopaminergic neurons in the brain’s ventral tegmental area (VTA). There, it acts as an acetylcholine agonist and enhances the release of dopamine from the nucleus accumbens. While this mesolimbic pathway is thought to be the major culprit of nicotine-induced reward and thus the deadly consequences of nicotine addiction, numerous other
signaling pathways in the CNS involve nAChRs, such as dopaminergic, serotonergic, glutamatergic, and GABAergic neurons in the prefrontal cortex and corpus striatum (Benowitz, 2009).

One of the primary focuses in the study of nicotine addiction is determining which genetic traits control the rate of nicotine metabolism. CYP2A6, the main liver enzyme responsible for the rate of nicotine metabolism, controls how likely an individual is to develop nicotine dependence, successfully cease nicotine use, and develop lung cancer (Benowitz, 2009). Those individuals who express a high-activity CYP2A6 variant are more susceptible to nicotine addiction since they require greater nicotine levels to experience a pleasurable affect (Kubota, 2006).

Despite the importance of CYP2A6 in predicting an individual’s vulnerability to developing nicotine dependence, a 2015 meta-analysis identified over 200 other genes expressed in the CNS that are also implicated in nicotine addiction (Liu et al., 2015). In their analysis, 205 genes were associated with 73 different functional classifications related to nicotine addiction, with neurodevelopment and signal transduction serving as the primary function for a large majority of the genes. Seven other functions were highly correlated with the 205 genes: synaptic transmission/neurological system processes, regulation of cell communication, channel/neurotransmitter receptor activity, transmembrane receptor/signal transducer activity, membrane fraction, response to alkaloid/nicotine; neurotransmitter or substance metabolism, and learning/memory.

Liu explains that nicotine predominantly exerts its behavioral and neurophysiological effects via nicotinic acetylcholine receptors (nAChRs), which are encoded by a gene family of 12 nAChR subunit genes (Liu et al., 2015). Within this family, variants of CHRNA4, the gene
that codes for the crucial alpha-4 subunit of nAChR, have been demonstrated by multiple studies to have a strong genetic association with nicotine dependence (Li and Burmeister, 2009). Among numerous other SNPs, rs1044396 and rs2236196 in CHRNA4 and rs16969968 in CHRNA5, which results in expression of the risk aspartic acid residue in the alpha-5 nAChR subunit, are highly correlated with increased susceptibility of nicotine dependence (Saccone et al., 2007). In summary, these known genetic determinants of nicotine addiction provide only a brief glimpse into the complex array of molecular genetic research dedicated to studying this often fatal condition.

For a broader level of genetic analysis, twin-studies provide an effective and less-invasive method to study the role heritability plays in nicotine addiction. Twin studies have demonstrated genetics to be the leading cause of variance in nicotine dependence and smoking behavior; 75%, 80%, and 60% of the variance observed for tobacco initiation, regular tobacco use, and nicotine dependence, respectively, are accounted for by genetics (Maes et al., 2004). Twin study outcomes related to nicotine addiction have also been shown to vary throughout the lifespan due to environmental factors. Data collected from the National Longitudinal Study of Adolescent to Adult Health (Add Health) found that shared environment was a stronger predictor of concordant nicotine behavior in 16-17 year olds than additive genetic factors. Conversely, concordant nicotine behavior was found to be more significantly associated with genetics than shared environment in 18-25 year old twin pairs (Bares et al., 2015).

II. *Theories of Addiction*

This thesis will focus on three competing theories of drug addiction: the gateway hypothesis, the common liability theory, and sociological determinants of addiction. The
gateway hypothesis predicting a progression of addiction to increasingly illicit substances has been a leading theory in the field for several decades. In 1975, Denise Kandel’s research popularized a possible gateway pathway for adolescent addiction behavior, stating that initiating drug use leads to further drug experimentation. She found that almost all students in her study progressed to higher-level drugs in the same order: wine/beer, followed by cigarettes/hard liquor, followed by marijuana, followed by psychedelics, cocaine, and heroin. Of course, not all students progressed to each level, but for those who did, this sequence was followed almost without exception (Kandel, 1975).

The governing principle of the gateway hypothesis is fairly straightforward: the cause of using a new drug is the one used before it. Therefore, adolescents who try alcohol become more likely to try cigarettes and marijuana because of their experience with alcohol. Accordingly, their experience with cigarettes and marijuana makes them more likely to try other “hard” illicit drugs. In her original study, Kandel surveyed 5,468 New York State high school students, of which 62% were not using drugs when first surveyed at the beginning of the cohort study. In the next five months, 36% of these students began using legal drugs, and another 1% later started using marijuana. After another 5-6 months, 26% of the marijuana users had started using other illicit drugs (Kandel, 1975). Of the numerous ways this data could be interpreted, a major takeaway from this study was that marijuana functioned as the gateway to additional illicit drug use.

Analyzing marijuana’s significance in the supposed gateway pathway can be approached from both physiological and social perspectives. Firstly, marijuana has been shown to cause physiological changes in the brain that alter how individuals respond to other drugs. This priming process, known as cross-sensitization, was demonstrated in rats that showed an amplified behavioral response to morphine after prolonged cannabis administration, regardless of
environmental context (Cadoni et al., 2001). However, the same priming response has also been observed in rodent brains as a result of both caffeine and nicotine consumption. Caffeine exposure in rats was found to increase nicotine self-administration, possibly by increasing the sensitivity of dopaminergic neurons to low-dose nicotine and thus causing nicotine dependence to occur more quickly (Rezvani et al., 2013; Temple, 2009). In the experiment performed by Rezvani and colleagues, rats that were injected with caffeine consumed a significantly greater amount of nicotine compared to controls (2013). This finding is repeated by multiple studies discussed in Temple’s meta-analysis of current knowledge on the relationship between caffeine and nicotine, which highlights the importance of determining if children who regularly consume caffeine are more likely to have a positive first experience with nicotine (2009). Furthermore, nicotine has also been associated with cross-sensitization with other illegal drugs. Denise and Eric Kandel were involved with a 2011 study that reported chronic exposure to nicotine in mice predicted greater responsiveness to cocaine due to epigenetic mechanisms that altered synaptic plasticity in the brain dopaminergic pathway (Levine et al., 2011). This suggests that both marijuana and nicotine may play a significant role in altering responsiveness to other drugs that follow in the supposed gateway pathway.

A second way to approach drug initiation and progression is from a social perspective. Marijuana’s role in introducing individuals to illicit drug culture is undeniable. A 1999 study of adolescents of various ethnicities found that increased contact with drug users predicted greater hard drug use (Ellickson et al., 1999). Nevertheless, rather than declaring marijuana’s definitive status as the gateway to illicit drug use, this finding highlights the importance of drug availability. In such a paradigm, increased exposure to any drug increases an individual’s affinity towards experimenting with it regardless of other drugs they have tried. Moreover, it is plausible
that marijuana is the substance that most commonly introduces adolescents to the illicit drug market and functions as the gateway through which other illicit drugs can be obtained.

An alternative drug behavior theory that questions the gateway hypothesis’ emphasis on marijuana as the critical gateway drug is the common liability theory. This theory arose after a 2002 meta-analysis reported no significant association between marijuana use and hard drug initiation (Morral et al., 2002). Unlike proponents of the gateway hypothesis, other researchers believe that drug behavior varies among individuals due to a common factor, namely genetics (Vanyukov, 2012). According to this theory, an individual’s drug behavior is determined by a genotype that increases his or her likelihood of experimenting with potentially dangerous substances. This phenotype on its own cannot explain addiction, but continued drug use strengthens the addictive-prone component of the genotype, facilitating increased drug-use behavior. Morral et al. reported several other characterizations of the common liability theory. Firstly, there is a normal distribution of predisposition to drug use, and, secondly, this predisposition is associated with the risk of initiating drug use. While genetics is the driving force behind this predisposition, social opportunity for drug use also weighs heavily on how the predisposition is expressed (Morral et al., 2002).

Realizing the significance of the competing theories of drug initiation now associated with the gateway hypothesis, Denise and Eric Kandel released an updated version of the hypothesis in 2015. One of the key ideas in the 2015 article is that processes in both the gateway hypothesis and common liability theory account for the pathway through which adolescents initiate drug use. The other critical modification they made to the gateway hypothesis was to place more emphasis on nicotine as the stepping-stone substance to harder drug use by means of epigenetic modifications and synaptic plasticity. Previous studies demonstrated increased
cocaine responsiveness in mice pre-exposed to nicotine due to enhanced long-term potentiation of the signaling response. In addition, chronic nicotine pre-exposure has been shown to increase expression of genes implicated in addiction (Kandel and Kandel, 2015).

Similarly to the aforementioned genetic components of nicotine addiction, the social determinants of nicotine initiation, continued use, and addiction are multifaceted. A longitudinal study consisting of nearly 800 individuals followed throughout adolescence and early adulthood demonstrated a smoking rate of 42.8% in adolescents and 38.7% in young adults; 13.7% of individuals had quit by the end of the 10-year study while 9.5% had initiated smoking after adolescence. Social factors implicated in continued smoking from adolescence through young adulthood included low GPA, fewer years in school, being unmarried in young adulthood, having a high proportion of drug-using friends, and increased alcohol use. Later onset of smoking behavior was also associated with fewer years in school, being unmarried in young adulthood, and increased alcohol use, yet factors distinguishing early from later smoking onset remain unclear (Mendel et al., 2013). Continued use or cessation of nicotine is regulated by family norms, proportion of friends who smoke, parental supervision, religiosity, and parental education (Galea et al., 2004). Even though continued use of smoking is largely the same for adolescents of different ages, the age of smoking initiation does impact the likelihood of later drug use. According to a 1999 study, adolescents who begin smoking before age 16 have a dramatically greater risk of dependence to other drugs than those who start smoking after age 17 (Hanna & Grant). This age-dependent finding was also demonstrated by Adriani and colleagues in an animal study, which reported that mice exposed to nicotine for two hours per day in early adolescence drank significantly more nicotine than those who were exposed to nicotine in late adolescence for the same amount of time (2002). If applied to the gateway hypothesis of nicotine
exposure described in this thesis, these findings suggest that the age of first exposure to nicotine is a critical factor in predicting future drug behavior.

III. Animal Models of Nicotine Addiction

The application of the gateway hypothesis to study the progression of adolescent nicotine addiction was the purpose of the first experiment described in this thesis. Given the highly addictive nature of nicotine, it would have been unethical to recruit adolescent humans to participate in this study. Moreover, biological and environmental factors would be difficult to control in humans, and progression of nicotine addiction would take years to study. Therefore, genetically identical inbred mice were used in this study to control for all genetic variables and allow for more simple gene expression analysis in later experiments. Inbred mouse strains are created by 20 or more consecutive matings of sibling x sibling or parent x sibling. Moreover, C57BL/6J (B6) mice were used in this experiment since they are known to show affinity to alcohol and morphine, and they are often used in nicotine experiments (The Jackson Laboratory, 2015).

Since the mice used in this experiment were also housed in isolation once weaned, the only variable that differed between the experimental and control group mice was environmental exposure to appetitive substances. Such a paradigm was constructed to mimic a gateway pathway of drug exposure in developing adolescent mice. The mice were first exposed to saccharin and caffeine since many adolescent humans consume this pairing via sugary-beverages that activate the same brain pathway as nicotine and other addictive drugs (Temple 2009). The mice were later exposed to nicotine via free-choice consumption from drinking water. Although many studies that describe nicotine exposure in adolescent mice involve nicotine injection, access to
nicotine in the animal’s drinking water was essential in this study to determine if early exposure
to appetitive substances enhanced affinity to nicotine later in development without causing any
side effects associated with injection, such as infection and conditioned stress. Furthermore,
nicotine was self-administered through a neutral vehicle, water, since it would not confer
additional calories or intrude on the mice’s normal lives. Nevertheless, nicotine’s aversive taste
is a source of concern in oral self-administration experiments (Klein et al., 2003).

Methods

Male and female B6 adult mice were selected at random to be mated. All mice were
housed in a climate-controlled room on a 12:12 light:dark cycle (lights on 0800-2000 hrs) in
shoebox-style polycarbonate cages (27 cm x 15 cm x 13 cm) with corncob bedding (Bed-O’Cobs
¼®, Maumee, OH). Mice were given free access to Purina Lab Diet 5001 food. Once pups were
born, they were assigned an experimental condition. The first litter was designated as Litter 1
and the second litter as Litter 2, and so on until nine litters were obtained. Odd numbered litters
were selected to serve as the experimental group, and even numbered litters were selected to be
in the control group. Since the desired number of male and female mice was assigned to each
condition using nine litters, there was one fewer litter assigned to the experimental condition.
An overview of the exposure and two-bottle choice test experiments are summarized in Table 1, and a breakdown of each litter by size and gender is given in Table 2.
Starting between Postnatal Day (PND) 1 and 3, two bottles with 1 mg/mL (0.1%) saccharin and two tap water bottles were placed in the cages of dams that gave birth to pups in the experimental group. The mice could choose to drink water or the 0.1% saccharin solution, which was selected based on work by Shram et al. (2006). Four tap water bottles were placed in the cages of moms who gave birth to pups in the control group. The amount of water and saccharin consumed by the moms and eventually the pups was not measured. At PND 21, mice from litters 1-5 were weaned, placed in individual cages, and given two tap water bottles for one day. Due to a high mortality rate following weaning (particularly in litter 5), mice in litters 6-9 were weaned at day 23. All mice were first weighed when weaned and later after the completion
of each phase of the experiment, as follows. All PNDs presented are given in terms of litters 1-5 but occurred two days later in litters 6-9.

On PND 22, mice in the experimental group were given one tap water bottle and one bottle with 1 mg/mL (0.1%) saccharin and 300 μg/mL caffeine (Rieg et al., 2006). Again, experimental mice were allowed to choose to drink from the water or gateway-drug containing solutions. Mice in the control group were given two tap water bottles. The amount of caffeine, saccharin, and water consumed by the experimental group and water consumed by the control group was measured every other day starting on PND 24. Bottles in the experimental group were rotated after each measurement and changed once per week. This procedure continued until PND 32, upon which nicotine exposure started for the mice in the experimental group. In addition, four tap water bottles were placed in empty cages and were measured every other day throughout the experiment in order to account for the amount of liquid lost, on average, due to evaporation and dripping. These bottles were changed once per week to account for any bottles that dripped more than others.

Starting on PND 32, mice in the experimental group were given a water bottle with 1 mg/mL (0.1%) saccharin, 300 μg/mL caffeine, and 25 μg/mL nicotine in addition to their tap water bottle. This concentration of nicotine was shown to elicit preference in a previous mice study (Klein et al., 2004). As before, these bottles were rotated after every measurement. Mice in the control group continued to be given two tap water bottles, and measurements continued to be taken every other day.

After measurements were taken on PND 50, the final phase of the experiment began: the nicotine two-bottle choice test. All bottles were removed from the mice’s cages, and all mice were given two new bottles; one bottle contained tap water, and the other bottle contained a 50
μg/mL nicotine solution. The amount of water and nicotine consumed was measured once per day, and bottles were rotated after each measurement. After 10 measurements were recorded for each mouse, they were given only tap water for 24 hours. The data for Litter 1 mice was lost on the seventh and eighth day of their two-bottle choice test due to computer error.

On the day following the end of the two-bottle choice test, the female and male mice from each litter with the lowest identification number were sacrificed, and brain samples from the ventral midbrain and cerebellum were obtained. Dr. David Vandenbergh performed all dissections. Cerebellum and ventral midbrains dissections took 1.5 and 2.75 minutes, on average, respectively to complete once the mouse was deceased. All brain tissues were immediately put into RNA Later solution and refrigerated for 24 hours, after which the RNA Later was removed, and the samples were stored at -70°C.

These experiments were conducted with approval from the Penn State Use of Vertebrate Animals (IACUC) protocol 29898.

Statistical Analysis

With the data obtained from the bottle-readings, t-tests were performed to test for significance in the percent and dosage nicotine consumed during the two-bottle choice test between the experimental and control group mice. Next, since the obtained bottle reading results showed an interesting pattern of change over time between the two groups, a repeated measures ANOVA test was performed to determine if there was a difference between the percent nicotine consumed by the experimental and control group mice over the course of the two-bottle choice test. However, since the ANOVA could not generate accurate results without the two days of
missing data for litter 1, repeated measures analysis with maximum likelihood estimation was performed using PROC mixed in SAS, which included a full interaction model with experimental condition, sex, and the polynomial effects of time. Then, higher order non-significant terms were deleted one by one until reaching a simplified, statistically significant model between the variables in question. This PROC mixed analysis was carried out with the assistance of Dr. Joseph Gyekis.

Results

I. Nicotine Consumption

The growth rates of all mice are illustrated in Figure 1. Although mice were only weighed on PNDs 21, 32, 50, and 60, weights on all other days were interpolated so that dosages of drugs consumed by the mice could be determined for each bottle reading.

![Growth of Mice After Weaning Through End of Experiment](image)

**Figure 1.** Weight of all mice throughout course of experiment
As demonstrated by Figure 1, the control group and experimental group mice had similar weights throughout the majority of the experiment. The slightly greater weights observed in the experimental group at the beginning and end of the experiment were not significant.

Results from the saccharin, caffeine, and nicotine exposure period are as follows. Milligrams caffeine and nicotine consumed by mice in the experimental group during the exposure period are illustrated in Figure 2.

![Caffeine and Nicotine Consumed by Experimental Group During Exposure Period](image)

**Figure 2: Average caffeine and nicotine consumed during the exposure period by males and females in the experimental group**

In Figure 2, the primary axis (Caffeine Consumed) demonstrates that males and females consumed comparable amounts of caffeine throughout all phases that included caffeine exposure. In general, the average amounts of caffeine consumed by both genders combined indicate an upward trend, which parallel the increase in body weight that occurred as the mice aged.
Figure 3 illustrates the trends observed in nicotine and caffeine dosages consumed by mice in the experimental group during the exposure period.

**Dosage Caffeine and Nicotine During Experimental Group Exposure**

![Graph showing caffeine and nicotine dosages](image)

**Figure 3: Caffeine and nicotine dosages consumed by mice in the experimental group during the exposure period**

As illustrated by Figure 3, the dosage of caffeine consumed stayed relatively constant throughout the entire exposure period in both genders when body weight was accounted for.

Figures 4-6 present the findings observed during the next phase of the experiment, the two-bottle choice test. First, Figure 4 illustrates the percent of nicotine-containing water consumed by both groups during the two-bottle choice test.
Mice from both groups consumed a high amount of nicotine on the first day and much less on the second day of the two-bottle choice test. Nevertheless, there was no overall difference in total nicotine consumed between experimental and control mice ($p < 0.62$). With PROC mixed in SAS, a simplified model that only included the linear effect of time, effect of condition, time by condition interaction, and the quadratic effect of time was obtained. The time by condition interaction was found to be significant, thus indicating a steeper decline over time in the control group than the experimental group ($F_{1,43} = 7.93$, $p < 0.008$).

Next, Figure 5 illustrates the average milligrams of nicotine consumed per kilogram body weight over the course of the two-bottle choice test for both groups of mice.
As demonstrated by Figure 5, the control group consumed a fairly steady amount of nicotine on days 5-8, and then showed a decrease between days 9 and 10. The overall difference in nicotine consumption between the two groups was insignificant, yet the experimental group demonstrated an upward trend in nicotine dosage between days 2 and 10. Noting these trends, the same SAS procedure as previously described was used and, again, a time by condition interaction was significant ($F_{1,43} = 7.86, p < 0.008$). For additional SAS output on percent nicotine and dosage nicotine consumed over time, please refer to Appendix I.

Another research question studied in this experiment was whether early caffeine exposure predicted greater nicotine consumption during the two-bottle choice test. These variables are plotted in Figure 6.
Figure 6. Caffeine consumed during the exposure period and percent nicotine consumed during the two-bottle choice test

With $R^2=0.058$, $r=0.24$, little to no association was found between percent nicotine consumed during the choice test and caffeine consumed during phase 3 of the exposure period. After regression analysis, the p-value obtained for the independent variable caffeine consumed during phase 3 was 0.16 (not significant).

Discussion

The major finding of this experiment was the observation of a complicated pattern of nicotine consumption, which suggested that mice pre-exposed to appetitive substances and nicotine experienced increased nicotine affinity over time compared to mice that were not pre-exposed to nicotine. Moreover, the diverging trends observed in Figure 5 suggest that the mice may have learned to respond to nicotine and develop preference. While the non pre-exposed mice appear to have responded to nicotine by developing aversion, the pre-exposed mice may
have responded more favorably to nicotine’s positive effects by developing preference. A two-bottle choice test of greater length may have led to more insightful findings, such as those achieved by Renda and Nashmi in 2014. Over the course of a 20-day two-bottle choice experiment, mice that were pre-exposed to nicotine consumed a greater amount of nicotine than those that were not exposed to nicotine before the two-bottle choice test (Renda & Nashmi, 2014). Therefore, instead of drawing any clinically applicable conclusions from the results of this experiment, it can be suggested that further research should be performed to study the effects of previous nicotine exposure of low concentrations on nicotine consumption later in life.

Additional features of the experimental design that may account for the insignificant results were allowing the mice continuous access to nicotine and not pairing nicotine self-administration with a reinforced behavior. Fowler and Kenny reported that significantly greater nicotine self-administration occurred when mice were only allowed to consume nicotine after pressing a lever that turned on a light (2011). The results of the experiment may also have been improved by designing and implementing a protocol that was known to promote nicotine addiction via self-administration in mice before beginning the two-bottle choice test. Consequently, nicotine addiction could have been achieved in most or all mice, and then different variables could have been manipulated to increase or decrease addictive behaviors.

Comparing this experiment to others performed under similar conditions also brings forth the idea of publication bias. There is evidence that publication of positive results in animal research occurs more frequently than publication of negative results, which has resulted in biased reporting of animal research studies (ter Riet et al., 2012). With little exception, publications report significant findings, and insignificant findings are rarely made available to the public’s perusal. This is especially relevant to mouse research due to its ease and low cost; experiments
can often be repeated or modified until the desired significance is achieved. Nevertheless, many unpublished findings still hold value to scientific research and can shape the direction of future studies (Foster & Putos, 2014).
Chapter 2

Gene Expression Analysis of Myelin-Associated Oligodendrocyte Basic Protein ($Mobp$)

Introduction

The neurophysiological events leading to addiction are thought to occur predominantly in neurons of the midbrain. Neurons originating in the ventral tegmental area (VTA) of the midbrain produce large amounts of dopamine, a neurotransmitter implicated in reward, pleasure, attention, memory, and learning (Koob, 1992). More specifically, the dopaminergic pathway that originates in the VTA and projects to the nucleus accumbens is implicated as the essential pathway through which drug reinforced behaviors and addiction is formed (Kelly, 2004).

This critical dopaminergic pathway, often called the mesolimbic dopamine system, affects both cellular and behavioral memory (Nestler, 2013). At the cellular level, drug exposure most notably causes changes in gene expression and the levels of underlying transcription factors. One of these key transcription factors is CREB, cAMP response element binding protein, which is highly expressed in the nucleus accumbens. When CREB is activated by a drug, sensitivity to the rewarding effects of the substance is decreased, thereby increasing the amount of the drug needed to produce a reward response equivalent to the original experience (Nestler, 2013).

Another family of transcription factors that is strongly associated with addiction-related behaviors is related to the protein known as Fos. After initiation of any addictive drug, ΔFosB becomes the dominant Fos transcription factor in dopaminergic neurons of the nucleus accumbens, through which it increases sensitivity to the reward properties of drugs and promotes
continued self-administration of the drug. Moreover, these ΔFosB-mediated behavioral effects were found to be especially pronounced in adolescents and may play a role in altering sensitization to other addictive substances.

These variations in transcription factor levels become especially relevant to the previously discussed gateway pathway of addiction when considered from the perspective that certain substances may prime the brain for increased sensitivity to subsequently used drugs. For example, children are often exposed simultaneously to sugar and caffeine in caffeinated beverages. Sugar has been shown not only to activate a similar dopaminergic reward pathway as addictive drugs, but also to promote addiction-related behaviors. Similarly, caffeine is known to activate the dopaminergic mesolimbic system pathway, which may enhance the rewarding properties of sugar and caffeine when both are consumed simultaneously (Temple, 2010).

The effect of caffeine on the body’s response to nicotine is also a topic of significant research. A question that influenced the hypothesis of this experiment is if children who have been repeatedly exposed to caffeine have a more rewarding initial experience with nicotine. Since caffeine and nicotine are often consumed together by humans, numerous animal studies have been designed to determine if sensitization to one drug affects behavior related to the other. Such an association was demonstrated by Shoaib et al. in 1999, who reported that rats self-administered 90% more nicotine when caffeine was present in their drinking water. Although caffeine and nicotine affect different neurotransmitter pathways, the blockade of adenosine A₁ and A₂A receptors that results from chronic caffeine exposure is thought to prime the body’s response to nicotine, thus decreasing the amount of nicotine an individual must consume to discern the substance’s rewarding stimulatory effects. These ideas may seem contradictory since caffeine is thought to lower the threshold amount of nicotine required to elicit a rewarding
experience, yet it is plausible that tolerance to nicotine accumulates over time and that greater amounts of nicotine must be consumed to achieve a similarly rewarding response (Justinova, 2008).

**Oligodendrocyte Study**

In addition to neurons, support cells in the brain offer another layer of complexity in the study of the neurophysiological process of addiction. These cells, termed glia, outnumber neurons in the human brain by at least 10 to 1 and occupy as much volume as neurons (Herculano-Houzel, 2009). A major sub-type of glial cells is oligodendrocytes, which function in forming the myelin sheath to enhance the efficiency of signal transmission along axons. Each oligodendrocyte can myelinate 30-40 axons, thus resulting in a complex organization of support cells surrounding neurons. Due to myelin’s essential role in rapid signal transmission in the brain, any change in myelination patterns can have a significant impact on proper neuron communication and axon stability (Jessen, 2004). This idea of altered neuron communication pathways is particularly relevant to the mechanisms of addictive drugs. For example, a study by Cao et al. demonstrated that prenatal exposure to nicotine affected expression of proteins essential to myelin formation, such as myelin basic protein, proteolipid protein, and myelin-associated oligodendrocyte basic protein (Mobp) [2013].

*Mobp* was chosen as the target gene of interest in this study due to its virtually exclusive expression in the postnatal Central Nervous System. The *Mobp* gene is expressed during a late stage of myelination, myelin sheath compaction, and is transcribed from Chromosome 9 (Holz and Scwab, 1997; NCBI, 2015). As illustrated in Figure 7, which was adapted from the Allen Brain Atlas of the mouse brain, *Mobp* expression varies by brain region. The dark fuchsia
midbrain illustrated in Figure 7a is demonstrated in Figure 7b to express the highest amount of *Mobp* relative to other brain regions (Allen Institute for Brain Science, 2015).

![Figure 7a: Illustration of mouse brain location of featured regions](image)

![Figure 7b: Relative gene expression of *Mobp* in adult mouse brain](image)

**Figure 7: *Mobp* gene expression measures from Allen Institute for Brain Science**

In the Cao et al. experiment, rats were injected with a nicotine dose equivalent to what humans would achieve by smoking 1.5 packs of cigarettes per day (2013). Their study found that significantly altered expression of *Mobp* occurred in the frontal cortex (downregulation), caudate putamen (upregulation), and nucleus accumbens (upregulation). Although not always the case,
these animal model findings are supported by results of a human study; Mobp expression was found to be altered in brains of cocaine abusers, resulting in dysregulated myelination patterns (Albertson et al., 2004).

Regulators of Mobp expression, such as transcription factors, may also be affected by nicotine exposure. For example, Methyl CpG binding protein 2 (MeCP2) is a transcription factor that can activate or repress genes found in neural or glial cells in the brain, including Mobp. The literature surrounding nicotine exposure and MeCP2 expression is limited, yet a 2015 article by Gong et al. suggested that MeCP2 interactions with other genomic markers in the presence of nicotine may result in decreased methylation of MeCP2, and thus greater expression of this transcription factor.

Given the complex array of factors potentially involved in myelination changes, this experiment was designed to test the most likely end-protein of the nicotine exposure-induced pathway described above. Therefore, to test the hypothesis that Mobp expression increases in the midbrain of mice exposed to nicotine throughout adolescence, the effects of nicotine exposure on Mobp expression levels were compared between mice that were first exposed to nicotine in adolescence and those first exposed to nicotine in adulthood.

Methods

Several months after the animal study of Chapter 1 was completed, molecular analysis of the collected brain tissues began. DNA and RNA were extracted using the Zymo Research ZR-Duet DNA/RNA MiniPrep kit (Zymo Research Corp., Irvine, CA) and following the provided protocol. Approximately 20mg of each tissue sample were lysed using the provided lysis buffer,
transferred to a provided spin column held in a collection tube, and centrifuged at 12,000g for 1 minute. The spin column was then placed into a new collection tube, DNA Prep Buffer was added, and the system was again centrifuged. The original collection tube was not discarded but rather stored until later for RNA extraction. This pattern of adding solution followed by centrifugation was repeated with DNA Pre-Wash Buffer and g-DNA Wash Buffer. The resulting contents in the spin column were then centrifuged in a new collection tube and placed in a sterile microcentrifuge tube. DNase/RNase-Free Water was added to the column matrix to elute the DNA and after 5 minutes, the system was centrifuged to collect the samples. The eluted DNA was then stored at -20°C. Next, ethanol was added to the collection tube containing the RNA, and the contents of this collection tube were transferred to an RNA spin column, placed in a collection tube, and centrifuged. The flow-through was then discarded, RNA Prep Buffer was added, and the system was again centrifuged. This pattern of adding a solution followed by centrifugation was repeated with RNA Wash Buffer of 700μL for 30 seconds and 400μL for 2 minutes. The spin column was then placed in a new collection tube, centrifuged, and placed in a sterile microfuge tube. DNase/RNase-Free Water was added to the spin column, the system was centrifuged, and the eluted RNA was stored at -70°C until cDNA conversion.

To convert the extracted RNA into cDNA, the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Life Technologies, a part of Thermo Fisher) was used. The RNA was removed from storage and allowed to thaw on ice. Once thawed, the RNA samples were diluted to 25ng/μl, mastermix of 10XRT Buffer, 25X dNTP Mix (100mM), 10X Random Primers, MultiScribe Reverse Transcriptase, RNase Inhibitor, and Nuclease-free Water was added to each sample, and the samples were placed in a thermal cycler programmed at the
recommended conditions (Table 3). Once the thermal cycling program was complete, the samples were refrigerated at 4°C (Applied Biosystems, 2006).

<table>
<thead>
<tr>
<th>Table 3. Thermal cycling program used to convert RNA to cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature (°C)</strong></td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Time (min)</strong></td>
</tr>
</tbody>
</table>

Real-Time PCR was performed to determine whether differential gene expression in Myelin-associated oligodendrocytic basic protein (Mobp) occurred in experimental and control group mice. Beta-actin (Bact) was chosen as the endogenous control reference gene due to its abundant expression throughout the rodent brain (Chatchaisak et al., 2013) and frequent use as a reference in gene expression studies. To best match the cDNA and prevent genomic DNA from being amplified, primers were designed to span gaps between one exon in either the forward or reverse primer, which was accomplished by finding the messenger RNA sequence of each gene in NCBI GenBank FASTA. The sequences of the exon-spanning primers were determined using NCBI’s Primer Blast tool, and the primers were ultimately made in the Genomics Core Facility in the Penn State University, Chandlee Building.

Each of the 36 samples was tested in triplicate for both targets, Mobp and Bact, for a total of 216 samples. The following table, adapted from Power SYBR Green PCR Master Mix, describes the content of each well (Thermo Fisher, 2011):

<table>
<thead>
<tr>
<th>Table 4. Reaction mixture of each experimental Mobp and Bact sample</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Component</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
</tbody>
</table>

The SYBR Green program on a ThermoFisher Scientific StepOne instrument was selected for Real-Time PCR to quantify gene expression levels of *Mobp* and *Bact*, with melting temperature set to 60°C (Table 5).

**Table 5. SYBR Green PCR program used to amplify *Mobp* and *Bact* samples**

<table>
<thead>
<tr>
<th></th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Melt Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of cycles</strong></td>
<td>1</td>
<td>40</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Step 1</td>
<td>Step 2</td>
<td>Step 1</td>
</tr>
<tr>
<td><strong>Temperature (°C)</strong></td>
<td>95</td>
<td>95</td>
<td>85</td>
</tr>
<tr>
<td><strong>Time (min)</strong></td>
<td>10:00</td>
<td>0:15</td>
<td>0:15</td>
</tr>
</tbody>
</table>

*Statistical Analysis*

Gene expression levels were quantified using the C\(_{T}\) values reported at the end of each PCR experiment, which is summarized in Figure 8. By definition, the C\(_{T}\) value is the number of cycles that occur before signal fluorescence exceeds background fluorescence; thus, low C\(_{T}\) values correspond to samples containing greater cDNA. The next step in analysis, calculating \(\Delta C_{T}\) values, was performed by subtracting the average *Mobp* C\(_{T}\) value from the average B-Actin C\(_{T}\) value, thus resulting in the difference in expression between the experimental and control brain regions. The \(\Delta \Delta C_{T}\) value was then found by subtracting the average \(\Delta C_{T}\) of the control (non pre-exposed) mice from the average \(\Delta C_{T}\) of experimental (pre-exposed) mice for both genes.
Then, an R-value was found for each brain region to determine the ratio of gene expression change in pre-exposed mice relative to the control mice, and a t-test was performed to determine the overall difference in expression between the two groups (Integrated DNA Technologies, 2012).

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><strong>Average CT of each triplicate</strong></td>
</tr>
<tr>
<td>2.</td>
<td>( \Delta C_T = \text{average B-actin } C_T - \text{average } Mobp \ C_T )</td>
</tr>
<tr>
<td>3.</td>
<td>( \Delta \Delta C_T = \text{average } \Delta C_T \text{ experimental group} - \text{average } \Delta C_T \text{ control group for both genes} )</td>
</tr>
<tr>
<td>4.</td>
<td>( R = 2^{\Delta \Delta C_T} \text{ for each gene} )</td>
</tr>
<tr>
<td>5.</td>
<td><strong>t-test</strong></td>
</tr>
</tbody>
</table>

Figure 8. Statistical analysis scheme to find difference in *Mobp* and positive control gene expression levels between experimental and control group mice in both brain regions tested.

**Results**

*Primer Design*

Using UCSC Genome Browser and NCBI’s Primer Blast, the primer set shown in Table 6 was demonstrated to successfully amplify and quantify both *Mobp* and *Bact* gene expression levels.

**Table 6: Primer sequences selected to study differential gene expression in *Mobp* and *Bact* genes**

<table>
<thead>
<tr>
<th>Primer sequence (5’→3’)</th>
<th><em>Mobp</em></th>
<th><em>Bact</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer and melting temperature (°C)</td>
<td>CACAACAGCCATTACTCGCA</td>
<td>TGTTACCAACTGGGACGACA</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As illustrated in Figure 9, the *Mobp* primers were designed to span across introns spliced during mRNA processing so that only cDNA was amplified. The intron-spanning primer sequences for *Bact* were obtained from the results of a previous experiment (Hair, 2015).

**Figure 9. Design of *Mobp* primers to span introns in order to amplify exons present in cDNA**

The desired sequence length of the amplified *Mobp* and *Bact* fragments were 130 and 164 base pairs, respectively. After facing difficulty in amplifying the correct fragments, the lengths of the PCR products finally obtained by using a Power SYBR Green PCR Master Mix were determined using polyacrylamide gel electrophoresis (Figure 10).
Figure 10. Polyacrylamide gel electrophoresis of Real-Time PCR Products

Using Quick-Load Purple 2-Log DNA Ladder (0.1 – 10.0 kb) from New England BioLabs Inc. (Ipswitch, MA), the observed bands for Mobp and B-actin amplification were confirmed to correspond to the correct fragment lengths.

Quantification of Gene Expression

Once Real-Time PCR CT values were reported and averaged for each of the 72 triplicates, ΔCT, ΔΔCT, and R values were found. For reactions that tested differential expression of Mobp in the cerebellum between pre-exposed and non pre-exposed mice, R was found to equal 0.765, p < 0.666, and the ratio of Mobp gene expression between experimental and control group mice midbrains was found to equal 1.44, p < 0.06.
Finally, Figure 11 presents the comparison of nicotine exposure values from Chapter 1 with gene expression data from Chapter 2 to determine if nicotine exposure in early adolescence was correlated with up-regulation of \textit{Mobp} in experimental group mice.

![Total Nicotine Exposure vs. \Delta\Delta C_T](image)

**Figure 11. The effect of adolescent nicotine pre-exposure on gene expression in experimental group adult mice**

Figure 11 suggests that there was not an association between the level of nicotine exposure in adolescence and midbrain \textit{Mobp} expression.

**Discussion**

The results of this gene expression experiment support the hypothesis that pre-exposed mice tend to express more \textit{Mobp} in their midbrain, with an effect reaching near-significance. In comparison with the findings of Cao et al. that early nicotine exposure in rats causes up-regulation of \textit{Mobp} in the nucleus accumbens ($p < 0.001$), it seems plausible that \textit{Mobp} alters different components of the mesolimbic pathway (e.g. nucleus accumbens and ventral midbrain) of addiction via similar mechanisms. Moreover, the insignificant difference in \textit{Mobp} expression
in the cerebellum indicates that the positive control brain region did not vary in gene expression levels between experimental and control group mice and that technical errors did not account for the findings in the midbrain.

While Cao et al. did achieve significant results of varied *Mobp* expression in a mesolimbic pathway structure, their results were obtained by delivering the same amount of nicotine each day via injection (2013). Unlike the mice in this experiment, Cao’s rats experienced a steady dose that may have transcended to a more consistent level of up-regulation across the sample. As explained in Chapter 1, free choice consumption of nicotine was essential to this experiment because it attempted to mimic the voluntary choice that adolescent humans have when using nicotine. However, this method of nicotine delivery eliminated the possibility of all animals receiving an equivalent amount of baseline nicotine exposure.

Another important consideration related to these findings is that exposure to nicotine changes has the potential to alter postnatal brain development of glial cells. Similarly to mice, myelination continues until early adulthood in humans (Stiles and Jernigan, 2010). Therefore, nicotine exposure in humans at any point in adolescence may have the potential to alter brain development, thus warranting additional exploration of how nicotine alters neurodevelopmental processes in humans and how these changes may alter drug-use related behavior later in life. Furthermore, since altered gene expression is known to be involved in these phenotypic variations, a subsequent step is to study the underlying transcription factors that control the expression of these genes and how nicotine-induced changes in transcription affect sensitivity to subsequently used drugs.
Chapter 3

General Summary

These experiments were designed to test if exposure to caffeine and nicotine in adolescent mice leads to behavioral and neurophysiological changes. In Chapter 1, a paradigm based on the gateway hypothesis of addiction was applied to mice that were sequentially exposed to saccharin, caffeine, and nicotine before their nicotine consumption patterns were compared to mice that had not been previously exposed to the appetitive substances. Although a similarly designed 2014 experiment by Renda and Nashmi demonstrated significantly different amounts of nicotine consumption between the pre-exposed and non pre-exposed mice, the results of this experiment did not indicate that the mice’s nicotine consumption varied as a result of previous exposure to sweet taste, caffeine, and nicotine.

After testing the behavioral effects of a gateway hypothesis of appetitive substance consumption, additional experiments were performed to determine if one measure of mouse brain physiology was affected by early caffeine and nicotine exposure. Chapter 2 presents the results from a gene expression study of Myelin-associated oligodendrocyte basic protein (Mobp), which demonstrated insignificant upregulation of Mobp in pre-exposed mice. While the results of these experiments did not support the hypothesis that sweet taste, caffeine, and nicotine exposure in adolescent mice leads to greater nicotine consumption later in life and pronounced changes in brain gene expression, the experiments suggest that any changes, given the limits of the experimental design, will be subtle. However, these negative results should not undermine the urgency of understanding how early substance exposure affects drug-related behavior in adulthood.
Appendix A

SAS Output for Chapter 1 Results

The following equation for the control group were constructed as follows:

Estimate of Intercept +/- Estimate of Day (d) +/- Estimate of Day*Day (d2)

The following equation for the experimental group were constructed as follows:

(Estimate of Intercept +/- Estimate of Condition) +/- (Estimate of Day*Condition +/-
Estimate of Day)(d) +/- Estimate of Day*Day (d2)

SAS Output for Figure 4 data (percent nicotine consumed during two-bottle choice test over time):

Solution for Fixed Effects

| Effect      | Condition | Estimate | Standard Error | DF | t Value | Pr > |t| |
|-------------|-----------|----------|----------------|----|---------|------|---|
| Intercept   |           | 39.37094  | 4.7511         | 43 | 8.29    | <.0001|   |
| Day         |           | -4.7768   | 1.1733         | 43 | -4.07   | 0.0002|   |
| Condition e |           | -4.0153   | 5.8643         | 43 | -0.68   | 0.4972|   |
| Condition w |           | 0         | .              | .  | .       | .    |   |
| Day*Condition e |   | 1.7057    | 0.6057         | 43 | 2.82    | 0.0073|   |
| Day*Condition w |   | 0         | .              | .  | .       | .    |   |
| Day*Day     |           | 0.3145    | 0.0899443      | 3.50 | 0.0011 |     |   |
Type 3 Tests of Fixed Effects

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>1</td>
<td>43</td>
<td>12.19</td>
<td>0.0011</td>
</tr>
<tr>
<td>Condition</td>
<td>1</td>
<td>43</td>
<td>0.47</td>
<td>0.4972</td>
</tr>
<tr>
<td>Day*Condition</td>
<td>1</td>
<td>43</td>
<td>7.93</td>
<td>0.0073</td>
</tr>
<tr>
<td>Day*Day</td>
<td>1</td>
<td>43</td>
<td>12.22</td>
<td>0.0011</td>
</tr>
</tbody>
</table>

The percent nicotine consumed by the control group mice is represented by:

$$39.4 - 4.77d + .31d^2$$

The percent nicotine consumed by the experimental group mice is represented by:

$$35.4 - 3.06d + 0.31d^2$$

The difference between the two equations above was significant, with a greater decline over time in the experimental group mice.

SAS Output for Figure 5 data (dosage nicotine during two-bottle choice test over time):

Solution for Fixed Effects

<p>| Effect     | Condition | Estimate | Standard Error | DF | t Value | Pr &gt; |t| |
|------------|-----------|----------|----------------|----|---------|-------|---|
| Intercept  | 3.6995    | 0.4974   | 43             | 7.44 | &lt;.0001  |
| Day        | -0.2847   | 0.1384   | 43             | -2.06 | 0.0458  |
| Condition  | -1.8472   | 0.6430   | 43             | -2.87 | 0.0063  |
| Condition  | 0         | .        | .              | .    | .       |       |   |
| Day<em>Condition | 0.2316   | 0.0826243| 2.80           | 0.0076 |
| Day</em>Condition | 0      | .        | .              | .    | .       |       |   |
| Day*Day    | 0.02685   | 0.0108243| 2.48           | 0.0171 |</p>
<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
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<tbody>
<tr>
<td>Day</td>
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<td>43</td>
<td>1.70</td>
<td>0.1993</td>
</tr>
<tr>
<td>Condition</td>
<td>1</td>
<td>43</td>
<td>8.25</td>
<td>0.0063</td>
</tr>
<tr>
<td>Day*Condition</td>
<td>1</td>
<td>43</td>
<td>7.86</td>
<td>0.0076</td>
</tr>
<tr>
<td>Day*Day</td>
<td>1</td>
<td>43</td>
<td>6.15</td>
<td>0.0171</td>
</tr>
</tbody>
</table>

With day = d, the equation used to represent the dosage of nicotine consumed by the control group during the two-bottle choice test is:

\[ 3.7 - 0.3d + 0.03d^2 \]

The equation used to represent the dosage of nicotine consumed by the experimental group is:

\[ 1.9 - 0.5d + 0.03d^2 \]
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Academic Vita of Bethany J. Latten

EDUCATION
- Bachelor of Science in Biobehavioral Health with minors in Biology and Spanish
- The Pennsylvania State University, University Park, PA

HONORS AND AWARDS
- Schreyer Honors College Scholar
- Member of the Honor Society of Phi Kappa Phi
- Evan Pugh Scholar Award recipient for being in top 0.5% of Penn State University Class of 2016
- Evan G. and Helen G. Pattishall Undergraduate Research Endowment in Biobehavioral Health Recipient

UNIVERSITY INVOLVEMENT
- Schreyer Honors College Career Development Program (Spring 2014-Spring 2016)
- College of Health and Human Development student marshal for spring 2016 commencement ceremony
- Teaching Assistant- Biobehavioral Health 101 (Summer 2013)
- University Choir (Fall 2012-Fall 2013)
- Biobehavioral Health Society (Fall 2012-Spring 2013)

RESEARCH EXPERIENCE
- Laboratory of Molecular Genetics (Spring 2013-Spring 2016)
- Thesis title: Testing a Gateway to Addition: Behavioral and Neurophysiological Effects of Early Exposure to Saccharin, Caffeine, and Nicotine in Adolescent Mice
- Thesis Supervisor: David J. Vandenbergh

WORK EXPERIENCE
- Kaplan Faculty Instructor (September 2015-present)
- Grader for Chemistry 212 (Spring 2016)
- CNY Medical Professionals (Summer 2015)
- Schreyer Honors College Scholar Assistant (Fall 2013-Spring 2015)

COMMUNITY INVOLVEMENT
- State College Pregnancy Resource Center- student volunteer
- Westminster Presbyterian Fellowship- campus ministry with State College Presbyterian Church
- Shadowed Physicians in Pediatrics, Family Medicine, Anesthesiology, Otolaryngology, Psychiatry, and the Emergency Department
- Volunteered in Cortland Regional Medical Center Emergency Department

INTERNATIONAL TRAVEL
- Seville, Spain- studied abroad for 8 weeks to complete Spanish minor (Summer 2014)
- Costa Rica- toured hospitals and clinics with Health Policy and Administration class (December 2014)