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METHYLATION OF THE GLIAL DERIVED NEUROTROPHIC FACTOR GENE
PROMOTER IN THE MESOLIMBIC PATHWAY

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ABSTRACT

Drug addiction and substance abuse is a major problem in the US, and it imposes major costs on society due to factors like increased mortality, loss of productive years, health costs, and criminal costs. Nicotine, a highly addictive drug, represents the single biggest preventable cause of death in adults in the US and accounts for much of the costs of drug addiction to society. There is evidence that nicotine may exert its addictive effects by altering epigenetic marks in dopamine neurons that play a role in reward and addiction. This study investigated how methylation of DNA, one type of epigenetic modification, may be measured in two regions of the mesolimbic circuit in the brain to test its role in reward and addiction in future studies. Methylation was tested in two ways. First, attempts were made to measure global methylation by using methylation of Long Interspersed Nuclear Elements (LINE) that proved unsuccessful. Second, levels of methylation were tested in the promoter of a specific gene, Glial-derived neurotrophic factor (*Gdnf*) in mice.). This gene is crucial to the maintenance and survival of dopamine neurons of the mesolimbic pathway, which has been implicated in the rewarding functions of drugs like nicotine. The current study investigated levels of methylation in the *Gdnf* promoter in two important brain regions in the mesolimbic system, the Ventral Midbrain, containing the Ventral Tegmental Area (VTA), and the Nucleus Accumbens using a qMethyl assay. Results showed that there was no significant difference in methylation of the *Gdnf* promoter between these two brain regions. Finally, methylation in the Ventral Midbrain and Nucleus Accumbens did not vary with the level of nicotine consumed by mice.

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Introduction

Drug Addiction and Substance Abuse

Drug addiction, or substance abuse, is one of the biggest societal problems faced today in the United States. A 2005 study estimated that approximately 22 million Americans over the age of 12 were dependent on alcohol or drugs (Wickizer, 2013). Substance use imposes tremendous costs on society. These costs result from premature mortality and years of productive life lost, morbidity, criminal activity, and health costs (Wickizer, 2013). In 2010, drug use overtook car accidents as the leading cause of injury and death in Americans between 25 and 60 years old (CDC, 2012). The National Institute of Drug Abuse and the National Institute of Alcohol Abuse and Alcoholism estimated the annual societal cost of alcohol and drug use at $186 billion and $181 billion dollars respectively (Wickizer, 2013).

Tobacco use and nicotine addiction represents a significant proportion of cases of substance abuse in America. It has been found that the risk of addiction to tobacco is relatively high compared to all other abused substances, when addiction is defined as the number of individuals who eventually become addicts after trying a drug (Pierce & Kumaresan, 2006). Tobacco use alone is the single most preventable cause of disease and death worldwide. Annually, over 400,000 premature deaths in the U.S. are related to smoking and second-hand smoke. Smoking also accounts for $96 billion a year in medical costs and another $97 billion in loss of productivity (CDC,2012)

Neural Basis of Addiction

Drug addiction is defined as a chronic, relapsing illness that is characterized by compulsive drug seeking and use even in the face of negative health and social consequences (Leschner, 1997). Individuals who become addicted to drugs will most likely face cravings and compulsions to use for the rest of their lives even if they quit, and most addicts will not be able to remain abstinent forever. This is because addictive drugs serve as reinforcers. Reinforcers are stimuli that act to increase the likelihood of a behavior, namely continued drug-seeking and use of a drug over time. The rewarding sensations associated with drug use serve as reinforcement that leads to continued drug use (Self & Nestler, 1995). The positive, pleasurable effects experienced when one uses a drug most often serve as positive reinforcement to sustain behavior. In cases where one becomes dependent on a drug, the cessation of negative withdrawal symptoms after taking a drug serves as negative reinforcement to increase drug –seeking and use (Koob, 1992).

The rewarding, hedonic effects of drugs are produced by the action of dopamine, a major catecholaminergic neuron in the brain. Dopamine's action in the mesolimbic system of the brain is especially important in reward. The brain regions that make up this mesolimbic system are responsible for motivational, emotional, contextual, and affective impacts on behavior (Pierce & Kumaresan, 2006). The mesolimbic system seems to function in reward learning and the prediction of future rewards. In terms of drug addiction, this mesolimbic system helps one associate drug use with reinforcing effects, and then maintains this association so that drugs continue to be associated with reward, leading to continued use (Berridge & Kringelbach, 2008). All of the classes of drugs of abuse, including nicotine, cause an increase in release of dopamine in the mesolimbic system (DiChiaro & Imperato, 1988). Two brain regions within this mesolimbic system that are of special interest in drug research are the Ventral Tegmental Area (VTA) and the Nucleus Accumbens (NAc). Dopamine neurons originate in the VTA and project

to the NAc where dopamine is released. It is this heightened dopamine release in the NAc response to rewarding stimuli is crucial to the acquisition of most learned appetitive behaviors like drug addiction (Fields *et al.,* 2007).

Research has shown that dopamine release in this mesolimbic pathway plays a role in the addictive properties of nicotine, the drug of interest in this thesis. It was found that when dopamine receptor antagonists were injected into the brains of rats trained to self-administer nicotine, "lever-pressing" to administer the drug was immediately and significantly reduced (Balfour, 1994). Furthermore, when the NAc was selectively lesioned, nicotine self-administration was markedly reduced (Corrigall *et al,.* 1992). This evidence substantiates the importance of the mesolimbic pathway in establishing the reward seeking behaviors associated with nicotine addiction. Further, the administration of nicotine was found to increase accumbal dopamine levels by nearly 100% (DiChiaro & Imperato, 1988). Such rapid and substantial changes in the amount of dopamine may induce changes in both the neurons and glia in these two brain regions that maintain the addicted behavioral state. Understanding the molecular mechanisms that generate this state and maintain it are important to understand for progress in treatment of drug abuse.

Epigenetics and Addiction

Drug use can have long lasting effects on reward pathways in the brain that they activate, which may account for the chronicity of addiction (Leschner, 1997). These effects may be molecular, cellular, structural, or functional changes to specific brain regions and neurons that are involved in the neural response to drugs, such as the VTA and NAc (Nestler & Aghajanian,1997). It is hypothesized that these changes lead to changes in the reinforcement mechanisms and motivational states associated with the drug (Nestler, 2002). Regulation of gene expression is a

common example of a molecular alteration that leads to stable changes in the neurons that affect their functioning in the response to drugs (Nestler, 2002) Regulation of gene expression can occur at many levels. Transcription may be regulated through the alteration of signal transduction pathways such as the cAMP and CREB paths. Alternately, gene expression may be regulated post-translationally, which may include changes in mRNA translation or protein degradation (Nestler, 2002). There has been more research into the alteration of gene expression through transcriptional changes. Epigenetic modification to DNA and chromatin may be one potential mechanism of transcriptional alteration that occurs as a result of chronic drug use and leads to alterations in neural signaling (Robinson & Nestler, 2011). These epigenetic mechanisms affect the expression of genes without changing the nucleotide sequence of DNA. Epigenetic mechanisms include chromatin remodeling through histone modification, translation repression through the binding of microRNAs, and altered transcription resulting from DNA methylation (Robinson & Nestler, 2011). Epigenetic processes lead to stable and, in some cases, heritable phenotypes. These changes can occur in response to developmental timing and also environmental factors (Suzuki & Bird, 2008). Epigenetic patterns, including DNA methylation patterns that have been established during development or from exposure to different environmental factors, can continue to change during the life of an organism in an experience dependent manner, making them important in learning, adaptive behavior responses, and synaptic plasticity, all of which are related to the development of addiction (Day *et al.,* 2013).

DNA methylation is a common epigenetic mechanism that involves the addition of a methyl group to a cytosine residue, converting it to 5-methylcytosine (5mC). Usually this occurs at cytosine residues that are followed by a guanine nucleotide, or a CpG site. In fact, 60-90% of all CpG sites are methylated, which accounts for almost all of the 5-methylcytosines found in the genome (Bird, 1986). These CpG dinucleotides are relatively rare in the genome as a whole and are mostly found clustered together as CpG islands in the promoter regions of genes, specifically

those genes that are differentially expressed at different times in development or in different tissues. Methylation of promoter regions allows for altered expression and repression of genes in response to various factors including developmental timing, environmental exposures to drugs or different diets, or disease processes in the body like cancer or chronic stress (Bird, 2002). Methylation of promoter regions is normally associated with repression of gene expression or gene silencing (Suzuki & Bird, 2008). Methylation affects transcription by altering the interactions of transcriptional proteins with DNA, usually by changing chromatin structure (Jones & Takai, 2001). Methylation of CpG sites can also recruit methylated CpG binding proteins and other transcriptional repressors that block transcriptional machinery from accessing the gene promoter, resulting in gene silencing (Jones & Takai, 2001). It is possible to assess methylation on a global level throughout the entire genome, or at specific gene sites. Changes in either of these measures of methylation could be important to understanding how methylation may affect gene expression, contributing to the underlying neural mechanisms that contribute to reinforcement, reward, and addiction.

Global Methylation and LINEs

Global methylation refers to the total level of 5-mC in the genome, and it can provide a broad measure of general patterns of methylation in the genome like hyper- or hypo-methylation. (Yang, *et al, 2004)*. Recently, there has been interest in associating global levels of DNA methylation with various clinical outcomes related to factors like illness, diet, and drug use (Weisenberger *et al.*, 2005) For example, global hypomethylation has been found to be characteristic of many cancerous tumors and even pre-cancerous lesions (Suter, Martin, & Ward, 2004). Further, fetal choline deficiency was associated with global hypomethylation and altered neural development (Niculescu, Craciunescu, & Zeisel, 2006). Repetitive elements in the genome

have been targeted for this global methylation analysis because they account for nearly half of the mammalian genome sequence, and contain many CpG sites that are targets for methylation (Weisenberger *et al.*, 2005). Long Interspersed Nuclear Elements (LINEs), a specific type of repetitive element, represent an especially good target for global methylation measures for a variety of reasons. First, at 19% of the genome they represent the single largest fraction of interspersed repeats in the mouse, which is the model of choice in this thesis (Martens *et al.,* 2005). Second, LINEs have a highly conserved sequence, which makes them an easier and more accurate target to measure using techniques that assess multiple LINEs in one assay (Nelson *et al.*, 2011). Finally, LINE element methylation has been shown to vary with environmental exposures to substances like air pollutants, metals, and alcohol (Nelson *et al.*, 2011). In the case of alcohol, an addictive drug, LINE hypomethylation was enhanced in a dose-dependent manner (Thapar, *et al.*, 2010). While there is no published evidence that levels of methylation vary in LINE elements with nicotine exposure, it is a hypothesis worth testing given the effects that other substances have on LINE methylation.

One goal of this thesis was to relate nicotine exposure to levels of global methylation in a mouse model by quantifying methylation in LINEs. Previous research validated using LINE methylation as a marker of global methylation. A high correlation was shown between methylation levels measured in LINE elements and total genome methylation measured using High Performance Liquid Chromatography, which has proven a reliable method to quantify total 5mC content in the genome (Weisenberger *et al.*, 2005). Also, as mentioned previously, LINE methylation specifically had been shown to vary with exposure to various factors such as air pollution, metals, organopollutants, and alcohol suggesting that LINES might be more likely than other repetitive DNA elements to vary with nicotine exposure (Nelson *et al,* 2011).This study aimed to quantify methylation levels using a methyl-sensitive restriction enzyme digest combined with quantitative real time PCR. The target region chosen for analysis was a part of the LINE

open reading frame-2 and contained a single restriction enzyme site sensitive to the action of MspI/HpaII. The open reading frame was chosen because previous experiments had been able to efficiently amplify this region using Real-Time PCR (Martens *et al.,* 2005). Also, this region remains intact in most LINE elements because it contains the genes for retrotransposon activity, as opposed to the 5' promoter sequence, which can often be deleted in many of the repeat sequences (Nelson *et al.*, 2011). This conservation of ORF2 sequence means that many LINEs, scattered throughout the genome, will be assessed in each PCR sample.
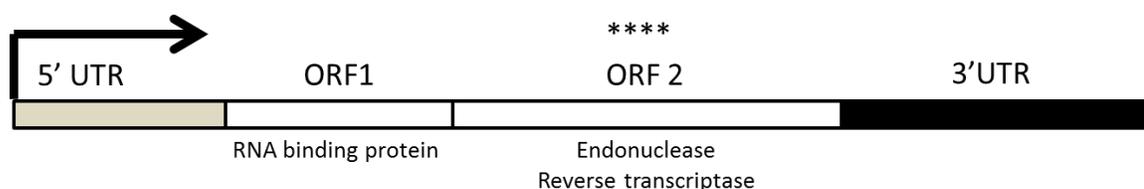
Figure 1-1: Structure of a mouse LINE element. LINE elements are the most abundant class of repeat elements and make up 19% of the mouse genome. They are about 5,000 bp in length and contain a 5'untranslated region, 2 open reading frames, and a 3' untranslated region. The open reading frames are highly conserved and carry genes for retrotransposon activity. Primers were designed to target the open reading frame 2. The bold arrow indicates the direction of transcription. * Figure adapted from Martens *et al* 2005 and Ergun *et al* 2004.

The methyl sensitive restriction enzyme pair MspI/ HpaII was to be used to analyze methylation. These enzymes differentially cut DNA depending on the methylation state of the target CCGG sequence. MspI is methylation insensitive and will cut the DNA whether a methyl group is present on cytosine or not. HpaII is methylation sensitive and will not cut if there is a methyl group on the cytosine in the CCGG sequence (Fernandez-Peralta *et al.,* 1994). After the DNA is digested, it is amplified using Real-Time PCR. It is important to note that the restriction enzyme digestion must occur before the PCR because PCR does not maintain the pattern of methylated DNA. The assay is therefore based on the presence of intact DNA *post*-digestion. Then, a comparison can be made between the cycle threshold values obtained from the PCR of the MspI digested samples and the HpaII digested samples to determine the percent methylation

in the sample. If a sample is not highly methylated, HpaII will digest the DNA more fully, and Ct values will be similar to those seen in the MspI reactions. If there is high levels of methylation, the DNA will remain largely uncut by HpaII and the Ct values of these reactions will be much earlier than the MspI digested samples because there will be more intact template DNA. Use of this system would assess whether nicotine consumption in mice could be related to differences in LINE methylation, which could represent levels of global methylation.

Site Specific Methylation and the Glial-Derived Neurotrophic Factor Gene

DNA methylation has been implicated in changing gene expression in brain regions associated with reward and addiction like the VTA and NAc (Renthal & Nestler, 2008). These methylation changes may be occurring in neuronal cells, or in glia. Glia are non-neuronal cells that play an important structural role in the nervous system. Both of these cell types are targets of methylation and show distinct patterns that correlate to neuron-specific and glia-specific gene expression (Kozlenkov *et al.*, 2013). One possible outcome of altered methylation relevant to addiction is that dopamine neurons in the VTA are able to show plasticity and change during the formation of reward-related memories as a result of altered gene expression (Day *et al.,* 2013). Specific genes that are important for memory formation in other brain regions, such as *Egr1* and *Fos,* were found to be upregulated during the process of stimulus-reward learning that occurs in the mesolimbic system. The upregulation of these genes was associated with changes in the methylation of CpG sites in their promoters in the VTA (Day *et al.,* 2013) Methylation was necessary for the formation of the stimulus-reward associations that are an important part of the formation of addiction (Day *et al.,* 2013). Although only a small sample of genes were tested in this study, it may be possible that other genes that are important to the function of the mesolimbic dopamine pathway may be regulated by methylation. This study tested whether methylation may

play a role in the expression of another gene that plays an important role in this reward system, *Gdnf.*

Glial Derived Neurotrophic Factor (*Gdnf*) is a growth factor that has been shown to be crucial for the development and maintenance of dopamine neurons in the mesolimbic system. *Gdnf* is essential in adult dopaminergic neurons, given that other neurotrophic factors cannot compensate for loss of *Gdnf* (Pascual *et al.,* 2008). A reduction of *Gdnf* in the VTA leads to a decrease in mRNA levels of tyrosine hydroxylase, a key enzyme in biochemical formation of dopamine, and subsequent wide-scale cell death of dopaminergic neurons (Pascual *et al.,* 2008). Receptors for *Gdnf* are found in the neurons of all brain regions of the mesolimbic system, including those of the VTA and NAc, and the factor acts on all of these cells (Trupp *et al.,* 1997). However *Gdnf* mRNA levels are not consistent throughout the system, indicating *Gdnf* is not synthesized in all the mesolimbic regions (Wang *et al.,* 2010). *Gdnf* mRNA levels are low in the VTA, the source of dopaminergic cell bodies in the mesolimbic system; however mRNA levels are higher in VTA target regions, most notably the NAc (Trupp *et al.,* 1997). It appears that *Gdnf* is synthesized in VTA target regions, and then is delivered to the dopamine neurons of the VTA through retrograde transport (Pascual *et al.,* 2008).

Gdnf expression in the NAc is upregulated when VTA neurons fire and cause a release of dopamine in this target region. This *Gdnf* was originally identified as being secreted by glial cells in the mesolimbic system, where it exerts its neurotrophic effects on midbrain dopaminergic neurons (Lin 1993). However, evidence shows that in the NAc it is medium sized neurons that produce *Gdnf* and not glial cells (Trupp *et al.,* 1997). The *Gdnf* produced by these neurons in response to dopamine that is transported back to the VTA leads to enhanced firing of the dopamine neurons in the VTA, which further heightens the levels of dopamine in the NAc and in turn increases *Gdnf* expression. In this way, *Gdnf* appears to be able to auto-regulate its own activity and also enhance mesolimbic dopamine activity overall (Wang *et al.,* 2010).

Recent evidence shows that the activity of *Gdnf* in the mesolimbic system, specifically the pathway between the VTA and NAc, may play a role in addiction. As mentioned previously all drugs of abuse cause an increase in dopamine in the NAc due to activation of the dopamine neurons in the VTA. Concomitantly, a rise in the level of *Gdnf* is seen in the NAc following exposure to various drug classes including opioids, psychostimulants, and ethanol (Carnicella & Ron, 2009). It appears that this rise in *Gdnf* may actually inhibit drug seeking and self-administration in rodents. Action of *Gdnf* in the VTA inhibited alcohol consumption and self-administration of various drugs including cocaine and methamphetamines (Carnicella & Ron, 2009). Increased *Gdnf* in the VTA also inhibited the development of alcohol induced condition-place preference in mice, which is considered a measure of drug seeking (Barak *et al.*, 2011). Further, animals that were deficient in *Gdnf* showed increased sensitivity to the rewarding effects of drugs (Carnicella & Ron, 2009).

The suppressive effects of *Gdnf* on drug intake can be long-lasting, due to the ability of *Gdnf* to upregulate and maintain its own expression, as described above (Barak & Ahmadiantehrani, 2011). Although there are many proposed mechanisms for how *Gdnf* leads to long-lasting suppression of drug intake, one line of evidence is that *Gdnf* reduces the negative effects associated with withdrawal of a drug (Barak *et al.*, 2011). As mentioned, all classes of drugs lead to an increase in dopamine in the NAc. Comparatively, drug withdrawal is associated with a decrease in accumbal dopamine (Barak *et al.,* 2011). Infusion of *Gdnf* into the VTA helped to alleviate this deficiency of dopamine in the NAc following withdrawal of a drug, specifically alcohol. This may help to reduce the drive to consume more of a drug (Barak *et al.,* 2011).

The body of literature surrounding the relationship between *Gdnf* and nicotine is not as extensive as that surrounding the relationship between *Gdnf* and alcohol, or other drugs like cocaine. However, nicotine is a highly addictive drug that increases dopaminergic activity in the mesolimbic pathways of the brain, suggesting that nicotine may also cause the same upregulation

of *Gdnf* seen after administration of other drugs (Pierce & Kumaresan, 2006). Also methylation in the VTA has been shown to be necessary for the formation of stimulus-reward associations. This methylation is associated with upregulation of certain genes involved in plasticity (Day *et al.,* 2013). While *Gdnf* methylation has not been investigated in drug addiction, methylation of the *Gdnf* promoter has been shown to vary in response to stress in rats (Uchida, *et al.*2011). This study also tested whether methylation may also play a role in regulation of *Gdnf* in response to drug use, specifically nicotine. It was hypothesized that nicotine consumption would lead to decreased levels of methylation of the *Gdnf* promoter in the NAc, the mesolimbic region where *Gdnf* is expressed. This hypomethylation could allow for the increased *Gdnf* transcription seen in the NAc in response to drug intake.

To test whether Gdnf promoter sequences are methylated, the OneStep qMethyl^{TM} Kit (Catalog No. D5310, Zymo Research Corporation) that utilized the action of various Methyl Sensitive Restriction Enzymes was used in combination with Real-Time Quantitative PCR. Levels of methylation were analyzed in the Ventral Midbrain (VMB), which contains the VTA, and the Nucleus Accumbens (NAc) of mice exposed to nicotine. It was hypothesized that methylation levels may be lower in the NAc because this is where *Gdnf* is synthesized in response to dopamine release. This *Gdnf* is then transported back to the VTA neurons, where it increases dopamine release. This project also attempted to determine if there was any relationship between the level of promoter methylation in these tissues and the amount of nicotine that was consumed by the mice.

Materials and Methods

Mice and Treatment

All animal procedures were performed within the standards and specifications of the Pennsylvania State University Institutional Review Board and Institutional Animal Care and Use Committee (IACUC #'s 37244 and 38357). Adult C57BL6/J (B6) female mice received nicotine starting at 14 weeks of age. Each mouse was given 4 bottles of nicotine (25 µg/ml) and one bottle of water. These bottles were provided to the mice for 10 consecutive days. Readings were taken every other day to determine the amount of nicotine and total fluid consumed. Nicotine consumed (mg/kg/day) was calculated using the volume consumed and the body weights of the mice for subsequent analysis. Due to limitation of resources, it was not possible to obtain control mice that had not been exposed to nicotine at the time of the experiment.

Tissue Extraction

Immediately following treatment, mice were sacrificed using CO_2 euthanasia. Brains were dissected by Dr. David Vandenbergh of the Pennsylvania State University. The ventral midbrain, and nucleus accumbens were collected from all mice.

DNA Isolation

Following dissection, brain tissue samples were immediately homogenized in 1.5 ml tubes and 700 µl of lysis buffer containing 673 µl STE (100 mM NaCl, 10mM Tris (pH 8), 10mM EDTA),

17.5 μl 20% SDS, 1.5 μl RNase A (10 mg/ml) and 8 μl Proteinase K (20 mg/ml) was added to each sample. The samples were incubated overnight at 37°C with gentle shaking. DNA extraction was carried out using a phenol-chloroform extraction. A single volume of 24:24:1 phenol/cholorform/isoamyl alcohol mixture was added to each tube. Samples were shaken vigorously for 2 minutes and then centrifuged at 13,000 rpm for 5 minutes. The upper aqueous phase was removed to a clean tube and a second extraction was performed, this time using 1 volume of 24:1 chloroform/ isoamyl alcohol. A 1/50 volume of 5M NaCl was added to each sample and DNA was precipitated with 2 volumes of 100% ethanol. Samples were stored at 4°C overnight. DNA was pelleted by centrifuging samples at 13,000 rpm for 15 minutes. Pellets were washed once using cold 70% ethanol and 5 μl of H_2O was added before incubating at 42°C with the top open to allow remaining ethanol to evaporate. The DNA was then dissolved in 100 μl of 1/10 TE (1mM Tris, 0.1 mM EDTA, pH 8.0) and stored at 4°C. Sufficient DNA was collected for further analysis from 8 mice for the VMB and 9 for the NAc.

DNA Purity and Quality Assessment

A Nanodrop ND-1000 spectrophotometer was used to assess DNA concentration and also its purity. DNA maximally absorbs at 260 nm, while protein and RNA absorbs at 280 nm. The A_{260}/A_{280} ratio can be used as a measure of DNA purity, with a ratio of ~1.8 representing pure DNA.

Gel electrophoresis was also used to further assess DNA quality by running approximately 150-250 ng of DNA from each sample on a 1% agarose gel (49ml ddH_2O, 1 ml 50x TAE, 0.5 g agarose plus 2 μg Ethidium Bromide (EtBr)). After 15 min of electrophoresis at set 100 V with modulating amperage, the gel was imaged under UV illumination. Bands were analyzed for any evidence of smearing that would indicate degradation.

LINE Global Methylation Study

Mouse LINE Primer Design

The region of interest in the mouse LINE retrotransposon open reading frame 2 region (ORF2)

was identified using existing primers previously described in mouse embryonic stem cells

(Martens *et al.,* 2005). The primers' specificity for the murine LINE retrotransposon was

determined using NCBI blast. This indicated that the primers were specific to the known LINE

sequences in the NCBI data base. It is important to note that this may not be representative of all

LINE elements in the genome, but measures many sites across the genome (Martens *et al*., 2005).

Nucleotide sequence from this region of interest was aligned and consensus sequence was

inserted into the New England Biolabs' NEBcutter (http://tools.neb.com/NEBcutter2/) to look for

methyl sensitive restriction enzyme (MSRE) sensitive sites. Specifically, target of amplification

was a CCGG site that would be cut by the MspI/HpaII pair of enzymes. Primers were designed

using Primer3 (http://primer3.ut.ee/) to amplify a 153 base pair region containing a single

MspI/HpaII restriction enzyme site in the ORF2 reading frame of the LINE element (Figure 2-1).

The oligonucleotide PCR primers (sequences written 5' to 3') chosen to be synthesized at the

PSU Genomics core were M1For1: AAAGCCAGAAGAGCCTGGAC, and M1Rev1;
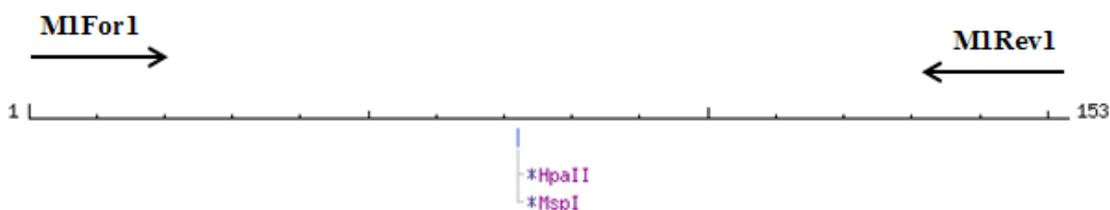
CGTGGAGAGATAATGCGTGA

Figure 2-1: Map of MspI/HpaII cut site in ORF2 region of mouse LINE element. M1For1 and
M1Rev1 primers were designed to contain a single CCGG site that is sensitive to the MSRE isoschizomer
pair MspI/HpaII. Reproduced from the NEBcutter website (not copyrighted).

Real Time qPCR

Before testing whether there were differences in methylation status in the genomic DNA, it was

necessary to ensure that the primers for the LINE sequences would amplify the target LINE DNA

successfully. PCR was performed using a reaction mixture prepared in house to test the

specificity and efficiency of the primers designed to target the mouse LINE element. First, 3 µl of

RockStart^{TM} Buffer (500mM Tris base, 160mM $(NH_4)_2SO_4$, 1% Tween 20, 50 mM H_3PO_4, at

final pH 8.8) was combined with 3 µl of 35 mM $MgCl_2$ in each reaction well, and incubated at

room temperature for 15 minutes at room temperature to allow formation of a white precipitate

removing the magnesium as a phosphate salt (Barnes & Rowlyk, 2002). Following precipitate

formation, a reaction mixture containing 0.6µl 10mM dNTPs, 1.5 µl 20xSYBR, 1.5 µl Taq

Polymerase, 1.2 µl of 5 µM forward primer, 1.2 µl of 5 µM reverse primer and 13 µl ddH$_2$O was

added to each tube. 5 µl of genomic DNA was added to bring the final reaction volume in each

tube to 30 µl. The final amount of the DNA in reactions of the standard curve experiment ranged

from 2.5ng – 40 ng. Reaction tubes were centrifuged at 1500 rpm for 1 minute to remove any air

bubbles.

The PCR reaction was run on an ABI StepOne Real-Time PCR system. PCR cycle

parameters were as follows: 95°C x 5 min; 40 cycles of 95°C x 30 sec, 63°C x 1 min; 72°C x 7

min. The magnesium needed to allow DNA polymerization dissolves during the early cycles of

the PCR. Thus there is no amplification in the first cycle, which is the source of much of the

background product generated in a PCR, but by the third or fourth cycle, sufficient magnesium

has dissolved from the precipitate to allow amplification (Barnes & Rowlyk, 2002). A melt curve

of the amplification products was then generated with the following parameters 95°C x 15 sec;

60°C x1 min; 95°C x 15 sec.

Verification of Product

Melt Curve analysis was used to determine if PCR reactions had formed a single product. PCR products were run out on a 5% acrylamide gel stained with EtBr to check whether the product was the correct size. The 5% acrylamide gel contained 5 ml of 40% acrylamide, 0.8 ml 50xTAE, and 32.8 ml ddH$_2$O. 10 µl of PCR sample was combined with 2 µl loading dye and 10µl ddH$_2$O. Phi X ladder was used for comparison of product size. After electrophoresis for 90 min at 150 V with modulating amperage, the gel was stained with EtBr and imaged under UV illumination.

Mouse LINE Primer Efficiency

Primers were tested for efficiency using a standard curve. A two-fold serial dilution series of the following concentrations was tested: 40 ng, 20 ng, 10 ng, 5 ng, 2.5 ng. The Log[DNA] was graphed against the average cycle threshold value for each concentration. A line was fit to the data and the slope of this line was used to calculate an estimate of efficiency. Efficiency was calculated using the equation $E = 10^{(-1/slope)}$. A value of 2 represents perfect efficiency, meaning that the amount of DNA doubled with every cycle.

GDNF **Methylation Study**

Gdnf Primer Design

A region of interest in the *Gdnf* promoter region was identified using existing primers previously described in the rat (Uchida, *et al.*2011) for use in the OneStep qMethyl assay kit from Zymo Research Corporation. The choice of primers was done with assistance from Dr. Jill Petrisko, Scientist with Zymo Research Inc. The primers' specificity for the mouse *Gdnf* promoter was

determined using NCBI blast. This result indicated that the primer sequence generated from the

mouse genome database (genome.ucsc.edu) corresponded to the *Gdnf* promoter, which is a single

copy gene. It is important to note that the primers will not detect all known CpG sites in this

region. Nucleotide sequence from this region of interest was inserted into NEB cutter

(http://tools.neb.com/NEBcutter2/) to look for restriction enzyme cut sites. Primers were designed

to amplify a 132 base pair region containing two Methyl Sensitive Restriction Enzyme (MSRE)

cut sites. One site was sensitive to AccII (CGCG), and the other for HpaII. (CCGG) (Figure 2-2).

Both of these enzymes were contained in the MSRE reaction mix used for methylation analysis.

The oligonucleotide PCR primers (sequences written 5' to 3') chosen to be synthesized at the

PSU Genomics core were GdnfF2: CTCTTCCGCGCTTCTTCTT and GdnfR2:

TTCGAAGGCTGTCCTTCCT.
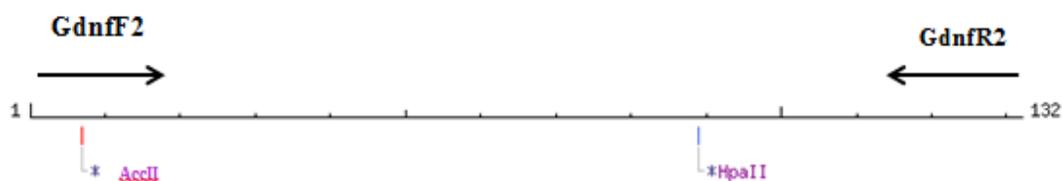
Figure 2-2: Map of restriction enzyme cut sites in *Gdnf* promoter region. Primers were designed to amplify a region containing 2 MSRE cut sites. The first site (CGCG) was within the forward primer and sensitive to the enzyme AccII. The second was downstream and sensitive to HpaII digestion

Gdnf Primer Concentration Optimization

PCR reactions were performed using a Zymo Taq reaction premix from Zymo Research

Corporation. Preliminary analysis compared the effectiveness of the following *Gdnf* primer

concentrations: 750 nM, 1000 nM, and 2000 nM.

Every reaction contained 10 μl of 2x Zymo Taq Premix, 1 μl of 50 μM SYTO 9 (Life

Technologies). There was variation in the concentration and amount of primers, and the amount

of water used in the different reaction concentrations. The 750 nM reactions also contained 1.5 µl

of 10 µM forward and reverse primers, and 1 µl of DNase/RNase free H_2O. The 1000 nM

reaction contained 2 µl of 10 µM forward and reverse primers. The 2000 nM reaction contained 2

µl of 20µM forward and reverse primers. The total volume of master mix in each reaction was

15 µl. 5µl of DNA at 4 ng/µl were added to each reaction to a total volume of 20 µl. Total input

DNA was 20 ng in all samples.

Reactions were run on an ABI StepOne Real-Time PCR system. PCR cycle parameters

were as follows: 95°C x 10 min; 40 cycles of 95°C x 30 sec, 63°C x 1 min, 72°C x 1 min; 72°C x

7 min. A melt curve of the amplification products was then generated with the following

parameters 95°C x 15 sec; 60°C x1 min; 95°C x 15 sec.

Verification of Product

Melt Curve analysis was used to determine if PCR reactions had formed a single product. PCR

products were run out on a 8% acrylamide gel stained with EtBr to check for correct product size

as described above. The 8% acrylamide gel contained 8 ml of 40% acrylamide, 0.8 ml 50xTAE,

and 30.76 ml ddH_2O. 10 µl of PCR sample was combined with 2 µl loading dye and 10µl ddH_2O.

Phi X ladder was used for comparison of product size. After electrophoresis for 90 min at 150 V

with modulating amperage, the gel was stained with EtBr and imaged under UV illumination.

Gdnf Primer Efficiency

Primer efficiency for 2000 nM concentrations of *Gdnf*F2 and *Gdnf*R2 primers was tested using a

standard curve of the following DNA dilutions: 50 ng, 25 ng, 12.5 ng, 6.25 ng, and 3.125 ng.

Efficiency was determined with the equation E= $10^{(/1/\text{slope})}$ as described above.

Determining effective reaction volume of OneStep qMethyl Kit

The OneStep qMethyl assay from Zymo Research Corporation was used to determine levels of methylation in the *Gdnf* promoter across multiple samples with replicates. This kit uses comparisons between test reactions containing methyl-sensitive restriction enzymes (MSREs) and reference reactions to determine the amount of methylation in each tissue sample. The first step in this process is a digestion of DNA using MSREs followed by amplification of the digested DNA using real-time PCR.Before methylation analysis was completed, a test was done to determine effective reaction volume to use the smallest volume that would amplify consistently. Test and reference reactions were set up in triplicate in the following volumes: 20 μl, 15 μl, and 10 μl. The test reaction mix for the 20 μl reaction included 10 μl of 2x Test Reaction PreMix with MSRE's, 2 μl of 20 μM forward primer, 2 μl of 20 μM reverse primer. The reference reaction mix contained 10 μl of 2x Reference Reaction PreMix with MSRE's, 2 μl of 20μM forward primer, 2 μl of 20μM reverse primer. 15 μl of the appropriate reaction mix was added to each reaction tube, and then 5 μl of 4 ng/μl DNA was added to bring the final reaction volume to 20 μl and the final DNA concentration to 20 ng. The volumes used in the 15 μl reaction were 75% of the 20 μl reaction, and the volumes in the 10 μl reaction were 50% of the volumes in the 20 μl reaction respectively.

The MSRE digestion stage of the process was performed by incubating the samples at 37°C for 2 hours. RT-PCR was performed immediately following digestion using the following parameters: 95°C x10 min; 45 cycles of 95°C x 30 sec, 63°C x 1 min, 72°C x 1 min; 72°C x 10 minutes. Products were then held at 4°C.

Methylation Analysis using OneStep qMethyl kit

Comparison of levels of methylation in the *Gdnf* promoter between the NAc and VMB were analyzed using the OneStep qMethyl kit from Zymo Research Corporation. For each sample, a test reaction and a reference reaction was set up. The test reaction mix included 7 μl of 2x Test Reaction PreMix with MSRE's, 1.4 μl of 20 μM forward primer, 1.4 μl of 20 μM reverse primer, and 0.7 μl of RNase/DNase free H_2O. The reference reaction mix contained 7 μl of 2x Reference Reaction PreMix with MSRE's, 1.4 μl of 20μM forward primer, 1.4 μl of 20μM reverse primer, and 0.7 μl of RNase/DNase free H_2O. 10.5 μl of the appropriate reaction mix was added to each reaction tube, and then 3.5 μl of 4 ng/μl DNA was added to bring the final reaction volume to 14 μl and the final DNA concentration to 1 ng/ μl for optimal enzyme digestion. All samples were run in triplicate.

The MSRE digestion stage of the process was performed by incubating the samples at 37°C for 2 hours. RT-PCR was performed immediately following digestion using the following parameters: 95°C x10 min; 45 cycles of 95°C x 30 sec, 63°C x 1 min, 72°C x 1 min; 72°C x 10 minutes. Products were then held at 4°C.

Percent methylation was determined for all samples using the equation below, in which Ct is cycle threshold at which the sample fluorescence is reliably detected and in the early phase of the amplification, and ΔCt = Ct value of Test Reaction – Ct value of Reference Reaction.

$$\textbf{Percent Methylation = 100 x } 2^{\textbf{-}\Delta\textbf{Ct}}$$

Statistical Analysis

All statistical analysis was carried out in Microsoft Excel 2010 with the Data Analysis Tool Kit installed. A two-sample F-test was conducted to determine if there was a significant

difference in the variability of methylation levels seen in the NAc and VMB samples respectively. Differences in methylation of the *Gdnf* promoter between the NAc and VMB were analyzed using a two-tailed t-test, assuming samples had unequal variance. A t-test of the same data was also run in the statistics program R (**cran**.us.**r**-project.org) to corroborate the findings from Excel and to generate t-values. Linear regressions were used to determine if a relationship existed between the amount of nicotine that mice consumed and the levels of *Gdnf* promoter methylation in the NAc and VMB respectively. Differences in the means for the two tissues was considered significant if the observed F or t statistic had a probability ≤ 0.05.

Results

Global Methylation Study

Mouse LINE Primer Specificity

M1For1 and M1Rev1 primers were designed to target the mouse LINE element open reading frame 2. The specificity of these primers was tested using Real-Time PCR and a homemade master mix. Primers showed high specificity and amplified a single product. Figure 3-1 shows melt curve analysis which revealed a single major peak at a melting temperature (Tm) of 77.52°C corresponding to a single product.
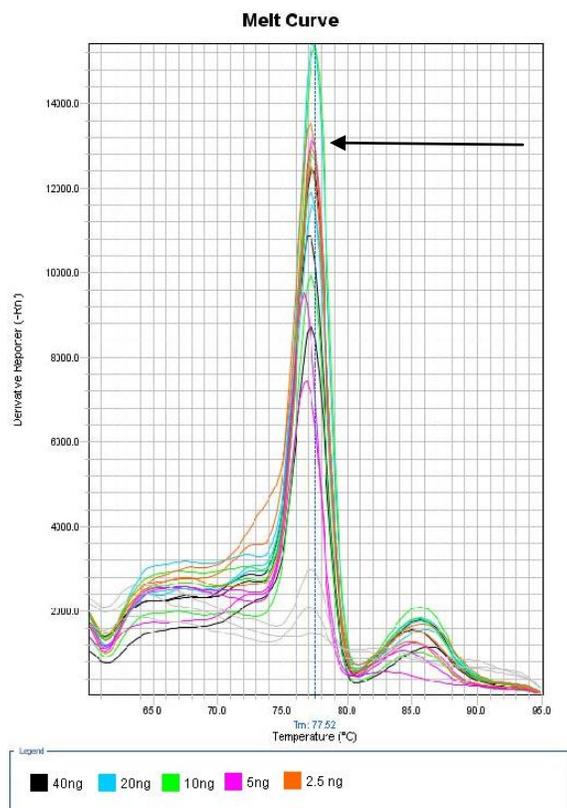
Figure 3-1: Melt curve analysis of products of LINE amplification. One major peak was observed at a Tm of 77.52°C

Electrophoresis of the PCR products out on a 5% acrylamide gel revealed that a single product of the expected 153 base pairs was formed. There was minimal background fluorescence and no secondary bands that could indicate other products or primer dimers (Figure 3-2).
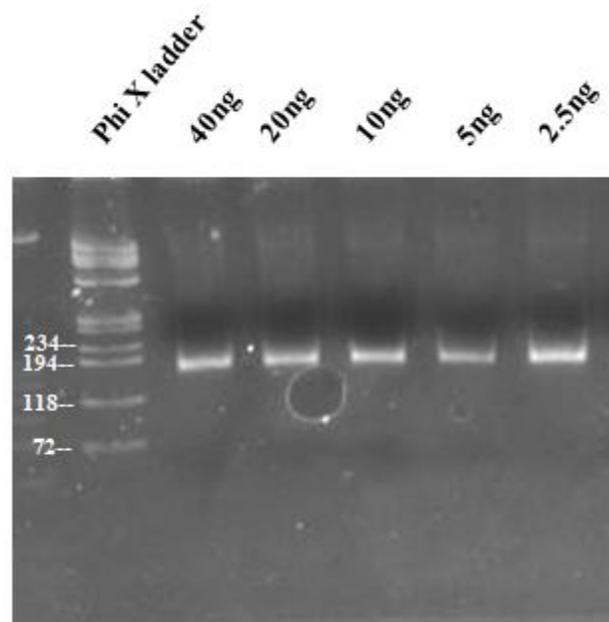
Figure 3-2. Verification of primer specificity and product formation of a region in the mouse LINE with 5% acrylamide gel. Comparing the product to the Phi X ladder reveals that it migrates approximately as expected for a 153 bp product.

Mouse LINE Primer Efficiency

The M1For1 and M1Rev1 primers did not amplify the product efficiently, as revealed by a standard curve experiment. Figure 3-3 shows an amplification plot of a DNA dilution series in which Ct values were not inversely associated with input DNA concentration, and replicates were highly variable. Multiple attempts were made to assess the causes of the poor quality PCR, one example of which is shown in this figure.
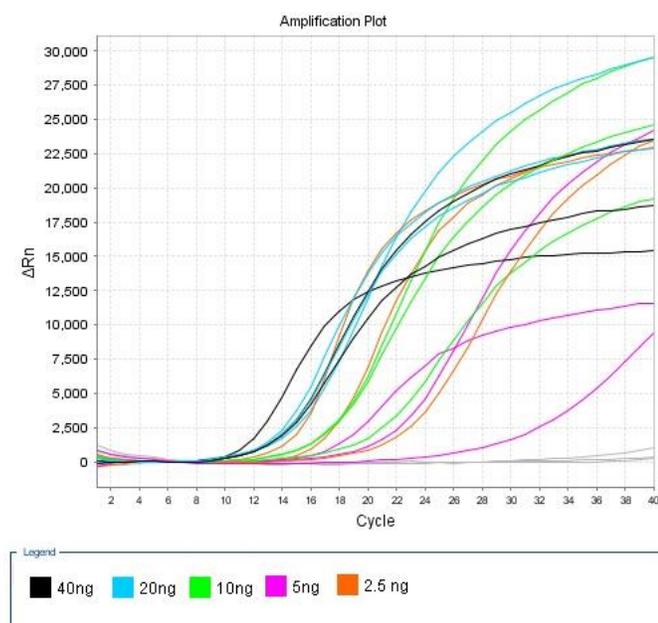
Figure 3-3: Amplification plot of five-fold dilution series using LINE primers. Results from a two fold dilution series beginning at 40 ng and decreasing to 2.5 ng was prepared run in triplicate. High variability among the triplicates and a lack of relationship between template DNA concentration and Ct values can be seen.

Following amplification, the average Ct value for each DNA dilution was graphed against the log of the concentration of DNA. Primer efficiency was estimated using the equation E= $10^{(/1/slope)}$. This experiment resulted in an E of 1.45, which corresponds to an efficiency of 45.16%. This was not within the desired E range of 1.8 – 2.2 (Figure 3-4). Because of the low reliability and efficiency of this reaction, it was determined that trying to measure the global methylation levels of mouse LINE elements would not be feasible using this assay.

Figure 3-4: Changes in cycle threshold as a function of the concentration of DNA for M1For1 and M1Rev1 primer analysis . Mean Ct values for each concentration of DNA used in the standard curve were graphed against the Log[DNA]. The slope of this line was used in the equation E= $10^{(-1/\text{slope})}$ to determine efficiency.
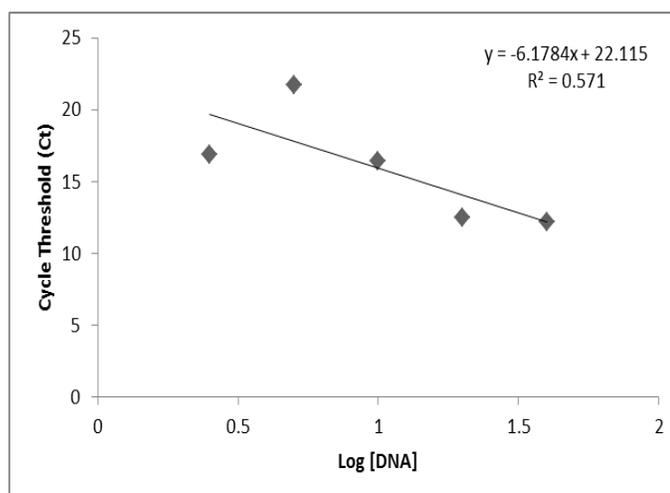
GDNF **Methylation study**

Gdnf Primer Optimization Preliminary Analysis

A second goal of this thesis was to analyze methylation levels in the promoter region of a single gene, in contrast to the attempt to measure global methylation with the test of LINEs. Glial-derived neurotrophic factor was chosen as described above, to test for methylation changes across two brain regions in the mesolimbic dopamine pathway. The first step in this process was to optimize reaction conditions for the *Gdnf*F2 and *Gdnf*R2 primers that had been designed to amplify a region containing two MSRE sites in the promoter of the gene. At the suggestion of Dr. Jill Petrisko, a three-concentration analysis comparing primers at 750 nM, 1000 nM and 2000 nM was completed. The amplification plot seen in Figure 3-5 showed that the 2000 nM primer concentration gave the tightest replicates. Also, the cycle threshold (Ct) values obtained from the 2000 nM samples showed the least variance within the triplicates.
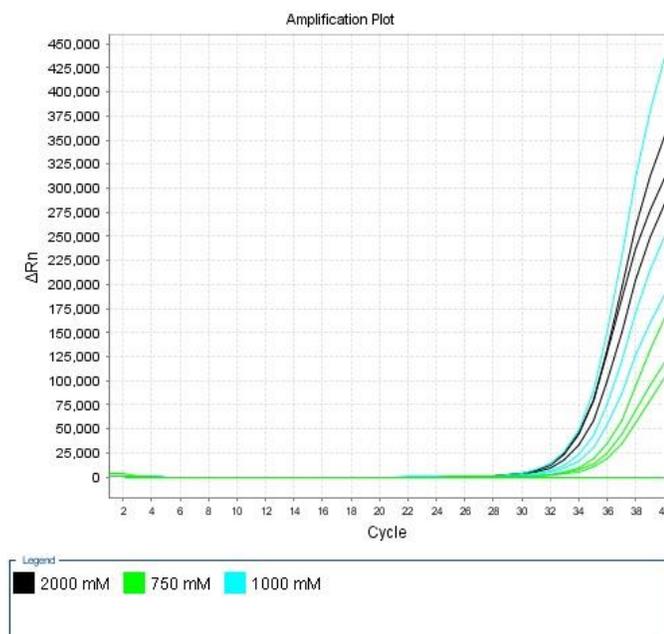
Figure 3-5: Amplification plot of various GdnfF2 and GdnfR2 primer concentrations. Primers were tested at concentrations of 750 nM, 1000 nM, and 2000 nM. Every concentration was run in triplicate. 20 ng of input DNA was used in each reaction. 2000nM concentration showed the least variability between replicates and showed robust amplification.

Melt curve analysis indicated that *Gdnf*F2 and *Gdnf*R2 primers showed high specificity.

Every concentration of primer formed a single, unique product, indicated by a single peak in the

melt curve at a Tm of 84.97°C (Figure 3-6).
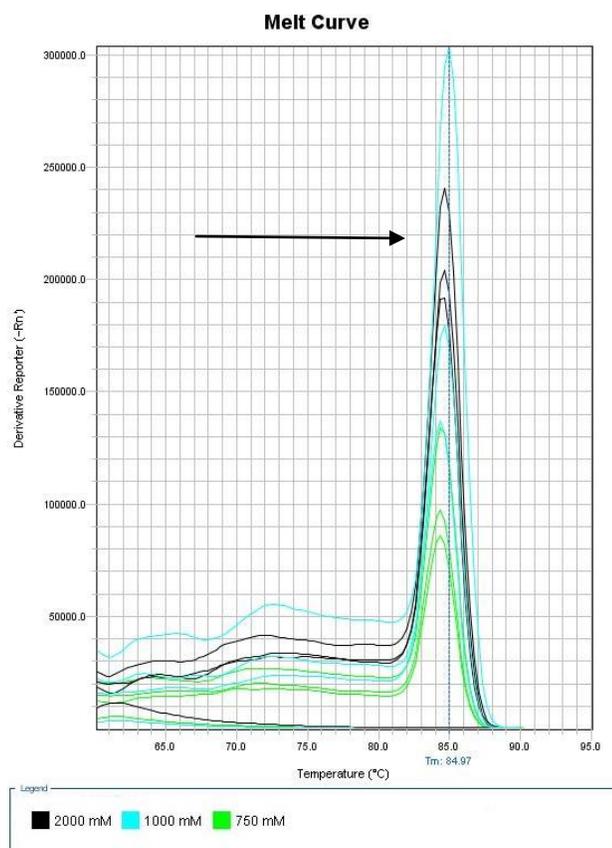
Figure 3-6: Melt curve analysis of various primer GdnfF2 and GdnfR2 concentrations. A single major peak was observed at 84.97°C. All primer concentrations led to the formation of this product. Minimal background products were observed.

Gdnf Primer Efficiency

After preliminary analysis showed that 2000 nM was working well, a standard curve experiment was performed to determine primer efficiency. A two-fold dilution series of the following concentrations was used: 50 ng, 25 ng, 12.5 ng, 6.25 ng. 3.125 ng. The amplification plot seen in Figure 3-7 shows that triplicates of the various concentrations all showed tight amplification. Also, samples with lower concentrations of input DNA amplified at later cycles than samples with higher concentrations of input DNA, as was expected.
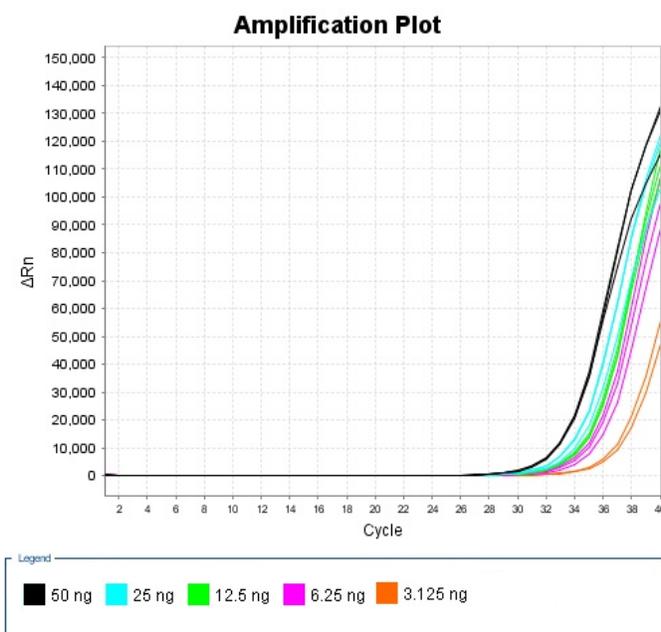
Figure 3-7: Amplification plot of five-fold dilution series using GdnfF2 and GdnfR2. A primer concentration of 2000 nM was used in these experiments. All DNA concentrations were run in triplicate.

*Gdnf*F2/*Gdnf*R2 primers produced an efficiency (E) of 2.21 in a 5-fold dilution series experiment (Figure 3-8). This represents an efficiency of 121.37%. An E value of 2.0 represents perfect efficiency. It was decided that this some what elevated efficiency was adequate to use for future experiments to determine the levels of methylation of the *Gdnf* promoter in the NAc and the VMB.
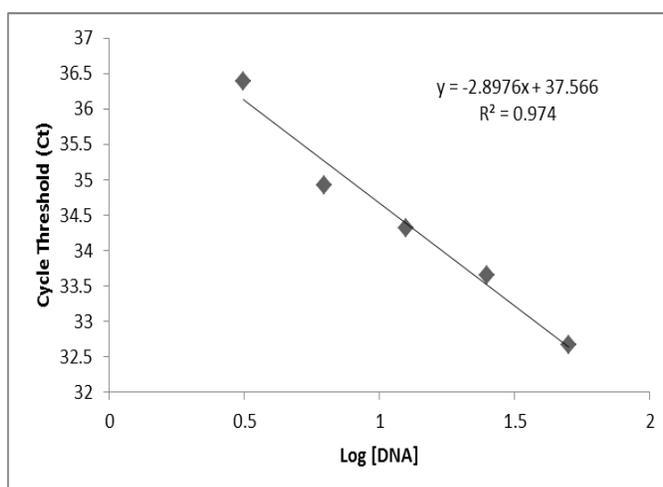
Figure 3-8: Changes in cycle threshold as a function of the concentration of DNA in *Gdnf*F2/*Gdnf*R2 primer analysis. Mean Ct values were graphed against the Log [DNA]. The slope of this line was used in the equation E= $10^{(-1/slope)}$ to determine efficiency.

Gdnf Primer Product Verification

Melt curve analysis of PCR products revealed a single, defined peak at a Tm of 84.38 ° C which

demonstrates a single unique product (Figure 3-9)
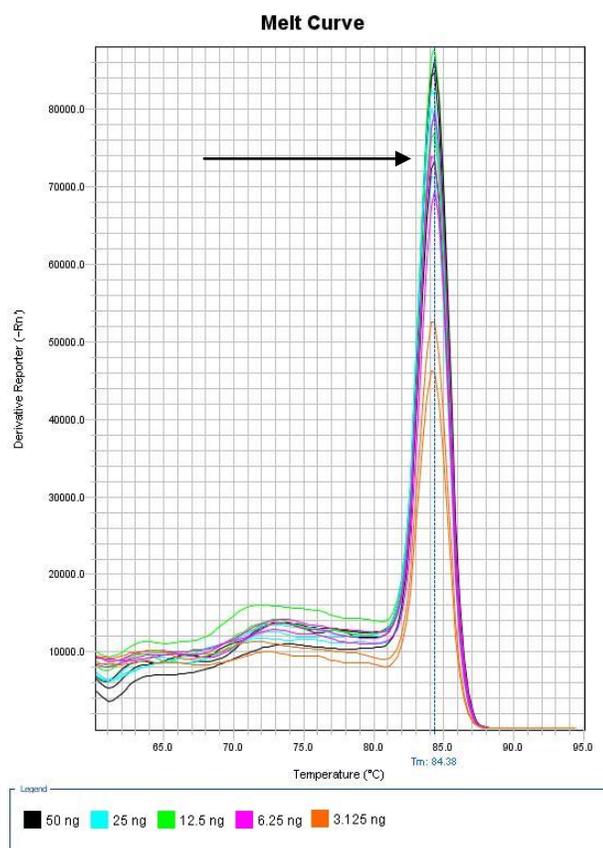
Figure 3-9: Melt curve analysis of various primer concentrations. A single major peak was observed at a Tm of 84.387°C, indicative of a single unique product.

PCR products were also run out on an 8% acrylamide gel. A single band of the expected product

size (132 bp) was formed. There was no streaking or secondary band formation, which could

represent primer dimers or secondary products (Figure 3-10).
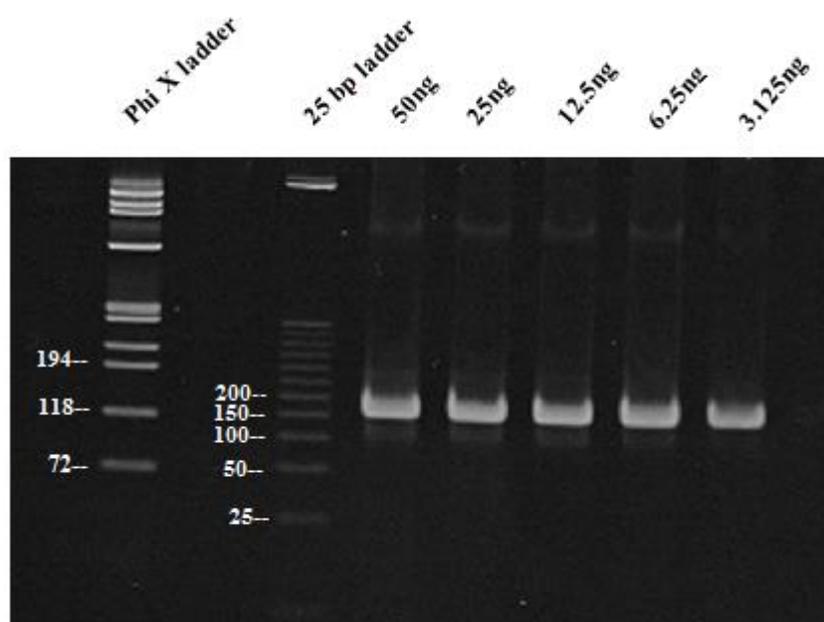
Figure 3-10: Acrylamide gel of *Gdnf*F2 and *Gdnf*R2 primer products. PCR products were run out on an 8% agarose gel. One sample from each tested concentration in the serial dilution was used. Expected product size was 132 bp. The band falls just behind the 118 bp marker on the Phi X ladder, and between the 100 and 150 bp markers on the 25 bp ladder. No streaking or secondary bands were observed. The gel confirms that a single unique product was formed.

Determining effective reaction volume of OneStep qMethyl Kit

Before running the final experiments with the qMethyl assay, experiments were performed to determine if the reaction could be performed effectively using lower volumes than the 20 μl. This was done for practical purposes, being that the reagents were limited and we wanted to be able to test as many samples as possible. Also, if this assay were to be used in the future it would be useful to know that the reaction volumes could be reduced to conserve reagents. A small experiment was run testing reaction volumes of 20 μl, 15 μl, and 10 μl. Relative reagent concentrations remained the same, and the amount of input DNA was also reduced proportionally with reaction volume. This would assure that the MSRE digestion would run to completion and result in complete digestion. After the reaction was run, the percent methylation was estimated as described above and the values between the reaction volumes were

compared. Analysis of the amplification plot did not show any visible differences among the

reaction volumes (Figure 3-11). There was some variability in triplicates across the test and

reference reactions for each volume. However, the reference reaction did amplify at earlier Ct

values on average than the test reactions did, which is expected. The reference reactions contain

no MSREs, so there was no digestion of DNA and more intact template DNA available for

amplification, which resulted in an earlier Ct value for those samples. Test reactions contain

MSREs and non-methylated DNA would be digested. This reduces the amount of available

template DNA and results if amplification at later Ct values.
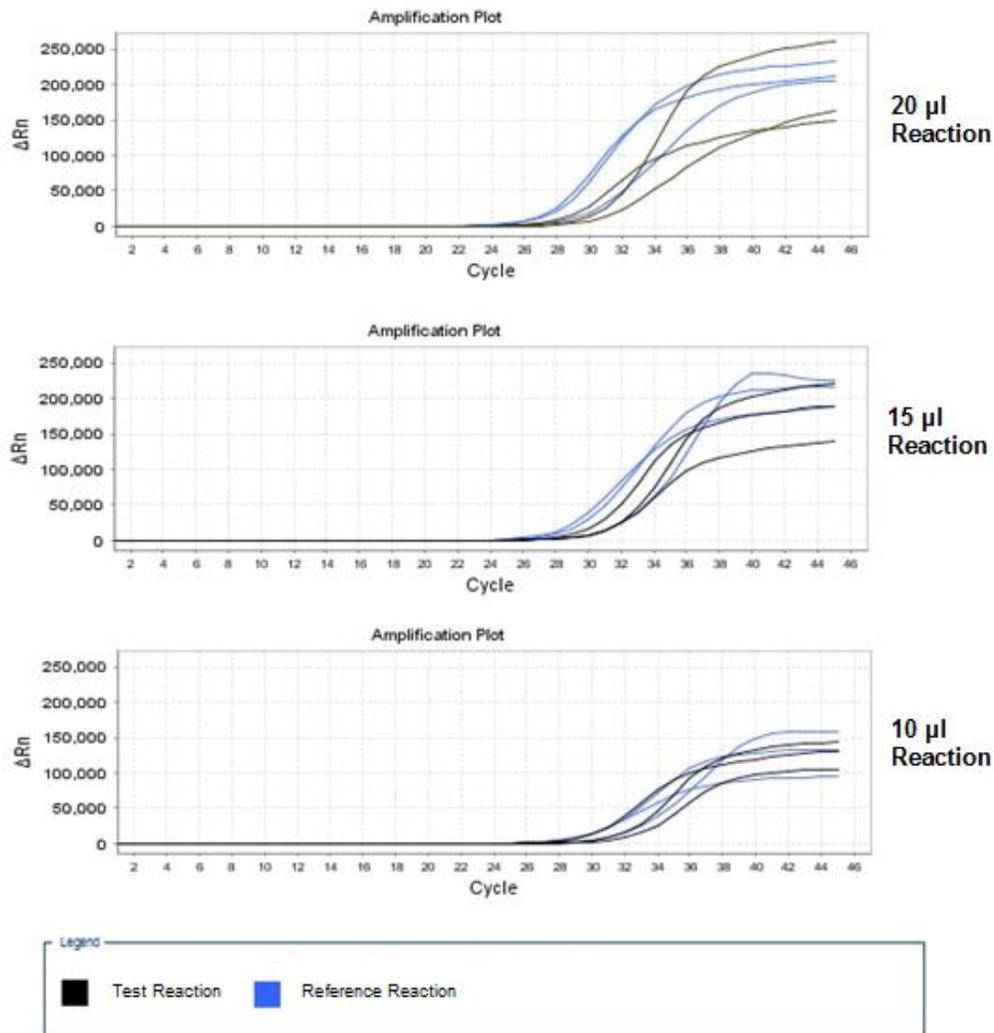
Figure 3-11: Amplification plot of qMethyl assay test and reference reactions at different volumes.
The qMethyl Reaction was tested at 3 reaction volumes to determine if a lower reaction volume using less of the reagents could be used effectively. 20 μl, 15 μl, and 10 μl reaction volumes were used in test reactions with MSREs and reference reactions with no enzymes. All reactions were run in triplicate.

Gdnf Promoter Methylation in the NAc and VMB

After the primer concentration and reaction volume had been optimized, the final qMethyl assay was used to measure levels of methylation in the promoter of *Gdnf* in the VMB (N=8) (containing the VTA) and the NAc (N=9) of mice that had been exposed to nicotine. For each tissue sample, a test reaction containing MSREs was compared to a reference reaction containing no MSREs. By comparing the Ct values of the two reactions, percent of methylation could be determined. The reference reaction should always amplify at an earlier Ct value than the test reaction because the reference contains intact DNA and the test reaction contains DNA that is digested to some degree. When a specific sample contains high levels of methylation, there is not a large difference in the Ct values between the test and the reference reaction (Figure 3-12). This is because when there are high levels of methylation, the MSREs cannot cut the DNA at the restriction sites. Therefore there is a high level of undigested DNA that is close in amount to the completely undigested DNA in the reference reaction.

Figure 3-12: Amplification plot of sample with high methylation. This sample had 55.26% methylation of restriction sites in the *Gdnf* promoter. The reference and test reactions had Ct values that were closer together because high methylation prevented extensive MSRE digestion in the test sample.

When there are low levels of methylation in a sample, the MSREs are not inhibited and they cut the DNA. This results in far less input DNA and the test reaction amplifies at Ct values that may be much later than the reference reaction (Figure 3-13).
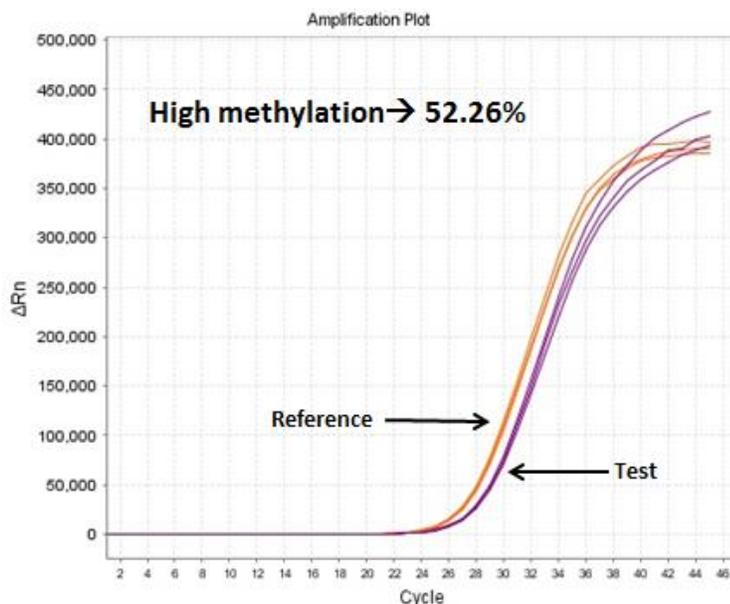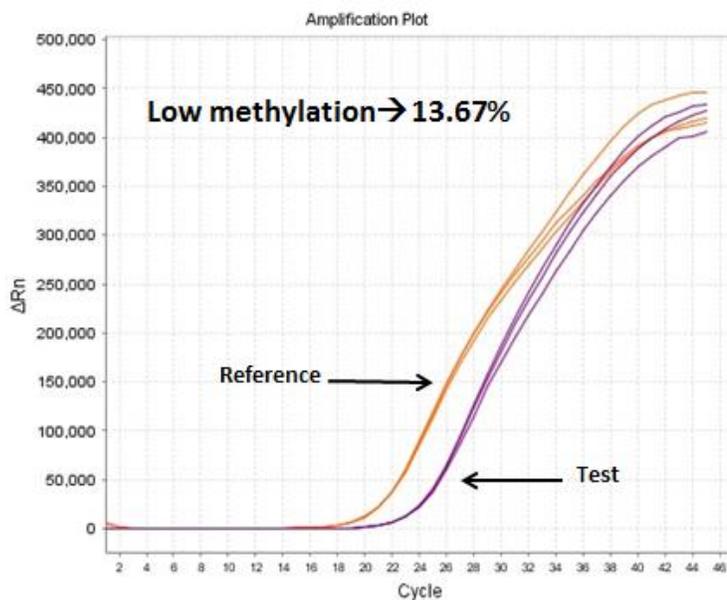
Figure 3-13: Amplification plot of sample with low methylation. This sample had 13.67% methylation of restriction sites in the *Gdnf* promoter. The reference and test reactions had Ct values that were far apart because low methylation allowed extensive MSRE digestion in the test sample, resulting in less input DNA and later amplification.

To determine the amount of methylation in the NAc and VMB of all the mouse samples, the average Ct values across triplicates of the test and reference reactions were calculated for both brain regions (see Appendix A). Then the equation Percent Methylation = 100 x 2-ΔCt, where ΔCt = Ct value of Test Reaction – Ct value of Reference Reaction, was used to calculate the methylation of both the NAc and VMB for each mouse. Average amount of nicotine consumed (mg/kg/day) was also calculated for each mouse (Table 3-1).

Mouse Sample ID	% Methylation of Nucleus Accumbens	% Methylation of Ventral Midbrain	Nicotine Consumed (mg/kg/day)
96.1	28.70	23.18	7.59
96.2	27.95	53.77	4.36
96.3	29.98	45.10	3.26
96.4	14.40	23.65	4.65
96.5	32.10	48.60	2.68
96.6	25.34	38.73	3.67
77.1	39.33	52.26	2.20
72.1	31.83	13.67	1.83
77.3	33.02	N/A	4.35

Table 3-1: Descriptive data of all mouse samples from qMethyl assay. The percent methylation of NAc and VMB and amount of nicotine consumed for each mouse in the sample are displayed in this table. See Appendix A for the mean Ct values of the test and reference reactions and standard error for each PCR. These mean Ct values were used to calculate a single percent methylation value from each DNA sample.

A comparison of the variance between the two samples was conducted using an F-test. Results (two-sample for variance) showed that the variance within the NAc samples differed significantly from the VMB samples ($F_{(8,7)}$= 0.1981, p=0.0183).

A two-tailed T-test assuming unequal variance was used to determine if there was any difference between levels of *Gdnf* promoter methylation in the NAc (M= 29.18%, SE=$\pm$ 2.26%) and VMB (M= 37.37%, SE= $\pm$ 5.046074) across samples. No significant difference was found between the levels of methylation in the NAc and VMB (t(9.425)= -1.3999, p=0.1936). The statistical analysis was run in both Microsoft Excel and the program R, both yielding the same results.

Linear regressions were used to compare the amount of nicotine consumed by each mouse to the levels of methylation in the NAc and VMB respectively (Figures 3-14 and 3-15). No significant relationship was found between the amount of nicotine consumed and the amount of *Gdnf* promoter methylation in the NAc (F(6,9)= 4.635, p= 0.075) although there is a trend for reduced methylation with higher nicotine consumption.
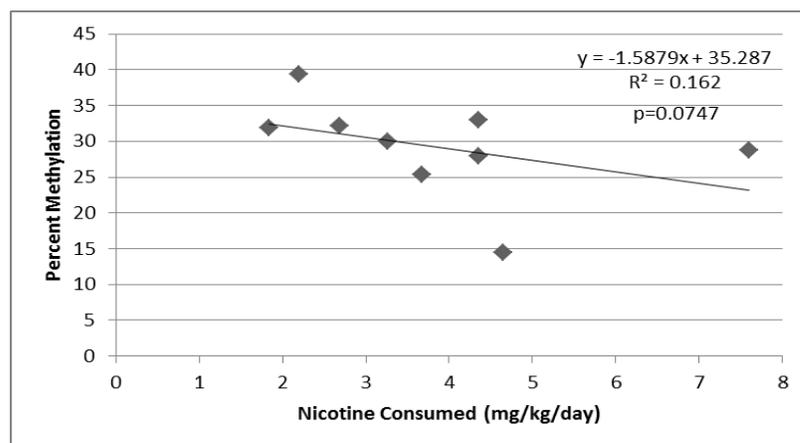
Figure 3-14: Linear Regression comparing nicotine consumed to *Gdnf* methylation in NAc. Linear regression analysis was performed in excel. Equation of the line along with R^2 and p value are provided. There was no significant relationship between nicotine consumption and the level of methylation in the NAc.

Similarly, no significant relationship was found between amount of nicotine consumed and levels of *Gdnf* promoter methylation in the VMB (F(5,8)= 0.0683, p=0.804).
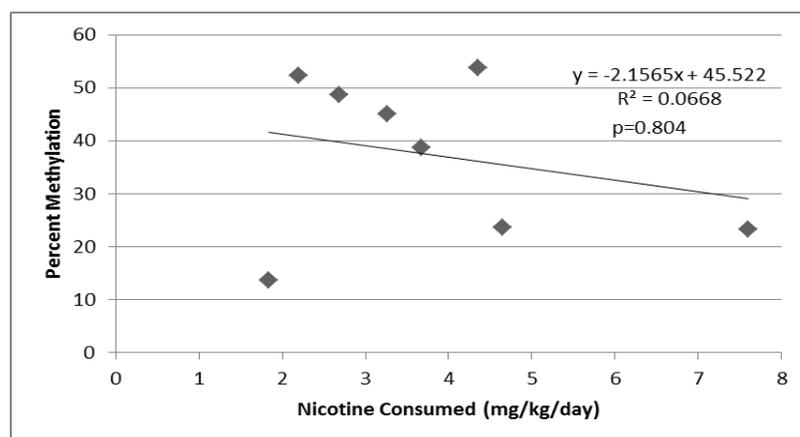
Figure 3-15: Linear Regression comparing nicotine consumed to *Gdnf* methylation in VMB. Linear regression analysis was performed in excel. Equation of the line along with R^2 and p value are provided. There was no significant relationship between nicotine consumption and the level of methylation in the VMB.

There was one mouse that consumed much higher levels of nicotine on average than the rest of the mice (see Table 3-1, sample # 96.1). To determine if the drinking data from this mouse represented an outlier, the mean and standard deviation of amount of nicotine consumed was calculated. To determine if the data point was an outlier, the convention that any data point that is three or more standard deviations from the mean can be dropped was tested. Using this criterion, the data point did not qualify as an outlier (it was 2.17 deviations from the mean). However, this is just convention and that the amount of nicotine consumed by this mouse may have been abberent, especially if analysis of a bigger sample had been possible. Dropping this mouse from the analysis yielded a higher *p*-value in the NAc analysis.

Discussion

Drug addiction and substance abuse are major problems in the US today and impose major costs on our society (Wickizer, 2013). Use of nicotine, a highly addictive drug, is the number one preventable cause of death in the US (CDC,2012). Drugs like nicotine exert their rewarding effects through the action of the mesolimbic dopamine pathway. These drugs induce dopamine neurons in the VTA to fire, leading to increase levels of dopamine in key regions like the NAc(Pierce & Kumaresan, 2006). Evidence has shown that DNA methylation may be involved in the regulation of dopamine transmission in the mesolimbic pathway, and therefore may play an important role in the development and maintenance to addiction (Renthal & Nestler, 2008). This study attempted to investigate this link between methylation and addiction through two experiments involving mice that had been exposed to nicotine. In the first experiment, attempts were made to measure global changes in methylation as indicated by methylation of LINE repetitive elements that were unsuccessful. Following this attempt at global analysis, the methylation of the promoter of a specific gene, *Gdnf*, was analyzed in the NAc and VMB, two brain regions in the mesolimbic dopamine pathway.

LINE Global Methylation Study

The first goal of this thesis was to use methylation levels at LINE repetitive elements in the mouse genome as a measure of global methylation. Previous research had shown that this was a valid measure of global methylation by comparing it to known measures of global methylation such as high performance liquid chromatography (Weisenberger *et al.*, 2005). Also, methylation of LINE elements had been shown to vary with environmental exposure, which was of interest

because the aim of this project was to study the effects of nicotine exposure on global methylation (Nelson). A methyl sensitive assay utilizing the methyl sensitive restriction enzyme pair MspI/HpaII, combined with Real-Time PCR was to be used to detect methylation.

Before this methyl sensitive assay could be performed, primers had to be tested to determine if they could effectively target the ORF2 of the LINE element, which was chosen because this region is highly conserved in the repeats and had also been successfully amplified by PCR in previous experiments (Martens 2005). The primers that were designed appeared to be selective for the target region. Melt curve and acrylamide gel analysis revealed a single specific product. Also, all samples in the LINE amplification appeared at very early cycle threshold values (between 10 and 20) when compared to what is usually seen when amplifying a single site in the genome. This result is consistent with finding that repeat elements had Ct values up to 15 cycles earlier than a single genome site amplified on the same reaction plate (Weisenberger *et al.*, 2005). Unfortunately, although the primers were specific, they showed poor efficiency. Normally in standard curve experiments, one expects samples with less input DNA to show up at later cycle threshold values, but no consistent pattern was observed here. Also, there was high variability within triplicates, which should in theory yield the same Ct values because all triplicates contained the same amount of input DNA. This inconsistency may be due to the low efficiency with which the primers performed. Polymorphisms and truncations that accumulate in the LINEs may make it difficult to attain reproducibility among samples (Nelson *et al.*, 2011). It is also possible that better results may have been obtained using a commercial assay rather than reagents that were prepared in house. There had been previous efficiency problems due to the age of the Taq polymerase enzyme used in this study. The study of global methylation in LINE elements was not continued after these early primer optimization experiments.

Methylation of the *Gdnf* Promoter

The second goal of this thesis was to determine if the methylation status of the *Gdnf* promoter varied in the NAc and the VMB, brain regions involved in the mesolimbic dopamine reward circuit. *Gdnf* is a neurotrophic factor that is crucial for the development and maintenance of the dopaminergic neurons in these regions (Pascual *et al.,* 2008). Although *Gdnf* is utilized by neurons in all these brain regions, its expression is not uniform throughout the mesolimbic pathway. *Gdnf* is synthesized in the NAc, a target region of VTA dopamine neurons, and retrogradely transported back to the VTA in the VMB (Wang *et al.,* 2010). Since *Gdnf* is synthesized at higher levels in the NAc, it was hypothesized that there may be a difference in methylation between this region and the VMB. Since methylation is most often associated with repression of transcription, it was possible that hypomethylation would be observed in the NAc in comparison to the VMB, which would allow for heightened expression in this region.

Analysis of the methylation levels between the NAc and the VMB did not show any significant differences. One possible explanation for this is that there are numerous other factors that play a role in gene expression besides methylation. Epigenetic modifications such as chromatin restructuring through histone remodeling, and silencing of translation through the binding of microRNAs are just some factors that may also influence gene expression (Robinson & Nestler, 2011). It is also important to remember that methylation, although more frequently involved in transcriptional repression, may sometimes lead to enhanced expression of a gene. This can occur if the methylation sites are within the binding site of transcriptional silencers or insulators (Robinson & Nestler, 2011). Methylation prevents the binding of these proteins that normally inhibit expression, leading to increases in gene expression.

In analyzing methylation of these two tissues, it is also worth noting that there was a significant difference in the variance of methylation levels between the NAc and the VMB. The

variance in the VMB was much greater across all samples than it was in the NAc. This was not due to any obvious technical error as VMB and NAc samples were run together using the same reaction mixtures on the same reaction plate. Although no further means were available to analyze the cause of this variance in the present study, it may be possible that the smaller amount of variance in *Gdnf* promoter methylation in the NAc may be due to stricter regulation of expression in this tissue.

After comparing methylation between the NAc and VMB, I asked whether levels of methylation in the mesolimbic system were related to nicotine consumption in mice. Expression of *Gdnf* has previously been implicated in the response of the mesolimbic pathway to drugs, making it an appealing target for analysis. *Gdnf* is upregulated in the NAc in response to drug-induced dopamine. This has been documented after exposure to alcohol, cocaine, amphetamines, and ethanol (Carnicella & Ron, 2009). It was important to test if this upregulation after drug exposure may be due to changes in methylation of the gene in mesolimbic regions. In addition, it will be of interest to explore *Gdnf* regulation in relation to nicotine exposure, an area of research that has not been studied as extensively as the relationship between *Gdnf* and other drugs.

Levels of *Gdnf* promoter methylation in both the NAc and VMB were individually compared to the amount of nicotine consumed by mice using linear regression. There was no significant relationship between the amount of nicotine consumed by mice and methylation levels of *Gdnf* in the VMB. There were no prior expectations for a relationship to exist in this case because *Gdnf* regulation and transcription is not known to be altered in the VTA (contained in the VMB) following exposure to a drug. However, *Gdnf* expression is altered in the NAc following exposure to another drug of abuse, ethanol. Therefore, it was expected that there may be some relationship between the amount of nicotine consumed by the mice and levels of *Gdnf* promoter methylation in the NAc. Analysis revealed that there was no significant relationship in this case either. However, the relationship did approach significance (p=0.0747). It is possible that with a

larger sample size, and possibly more sensitive methods, a relationship between the amount of nicotine consumed and methylation levels of the *Gdnf* promoter in the NAc may have been established.

There are a number of technical concerns with the assay that may have affected the outcome of these experiments. For one thing, the CpG sites that were used in this assay were only two out of a large number of CpG sites in the *Gdnf* promoter region. It is possible that other CpG sites in this region may be differentially methylated to control expression of *Gdnf*. If these other sites had been measured, differences between the methylation levels in the NAc and the VMB may have become apparent, and there may have been a relationship between the methylation of other sites and consumption of nicotine. Unfortunately, none of the other methylation sites provided acceptable primer binding sites to allow analysis by PCR. Another technical issue with this assay is that due to limits in primer design imposed by the sequence being used, it was necessary for us to include two CpG sites in the product. These sights could be differentially methylated, but there was no way of determining if there was any difference between these two sites. If methylation of one site was increased while methylation at the other site was decreased, the fraction of undigested DNA would remain the same. Again, this could affect all of the analyses run in this study. In the future, it may be beneficial to design primers around a single methyl-sensitive site in the promoter of *Gdnf*, and then to test a number of different sites in the promoter region individually to determine if methylation plays a role in the differential expression of *Gdnf* in the NAc and VMB. One final explanation as to why no significant relationships were observed may be specific to this assay. This assay is not extremely sensitive to small changes in methylation. While it may be very accurate at picking up large differences, for example between 75% and 25%, it may not be able to pick up subtle differences in the levels of methylation that could still be exerting an effect on gene expression (Dr. Jill Petrisko, personal communication).

It is also important to consider the accuracy of estimating the amount of nicotine consumed by the mice in this analysis, which was based on average amount of consumption per day. The mice showed consistency across the days, and did not show much variation in the amount of nicotine consumed across days, so the average amount of nicotine in g/kg/day is probably an accurate measure of consumption to use. Also, a cage was set up with control bottles so that the amount of evaporation could be taken into account in the analysis of the drinking. The estimated average volume of evaporation/leakage was subtracted from the volumes measured in the experiment. In addition, bottles were checked for air locks that would prevent the mice from drinking, due to the smaller diameter glass tubes used and the small volume of fluid in the tube. (The large plastic bottles standard in a mouse cage are often squeezed when placed in the cage to ensure that there is no air lock.). Data from locked nicotine bottles was thrown out, and if there was a lock on a water bottle that would force mice to consume more nicotine to reach adequate fluid intake this was thrown out as well. There were no air lock issues detected in this experiment.

Despite not finding any significant results in the current analyses, it may be valuable to pursue analysis of levels of methylation in the *Gdnf* promoter, especially in the NAc. As mentioned before, a near significant relationship between methylation in this brain region and nicotine consumption in mice was discovered. The present study was limited by the availability of mice to use in the experiment, and expanding the sample size could give a more accurate picture of this relationship. Also, this study showed that the qMethyl assay may be a useful tool in continuing to analyze differences in methylation of specific sites. However, if used in the future, it would be useful to include standards that could be used to accurately quantify methylation. One limit in the current study was that a relative comparison of methylation across samples could not be made, but there were no known standards of methylation with which to compare these values to in order to determine exact amounts of methylation in samples. Zymogen has prepared this type of reference methylated DNA for tests of human samples but not mouse.

In the future, investigation of how nicotine affects methylation of DNA in LINE elements and specific gene sites could also be analyzed using more quantitative methods such as bisulfite sequencing, which can test the methylation status of a number of CpG sites independently. There is potential for many lines of inquiry expanding on topics that were addressed in this thesis. Further research is needed to more clearly characterize the relationship between nicotine exposure and global or gene-specific changes in methylation that may play a role in the formation and maintenance of nicotine addiction.

Appendix A: Average Ct Values and Standard Error for All Reaction Triplicates in qMethyl Assay

Mouse Sample ID	Average Ct Values $\pm$ SE Nucleus Accumbens		Average Ct Values and SE Ventral Midbrain	
	Test Reaction	Reference Reaction	Test Reaction	Reference Reaction
96.1	32.60$\pm$ 0.22	30.81$\pm$ 0.04	30.83$\pm$ 0.04	28.72$\pm$ 0.10
96.2	32.60$\pm$ 0.10	30.76$\pm$ 0.02	31.55$\pm$ 0.05	30.66$\pm$ 0.04
96.3	32.64$\pm$ 0.33	30.90$\pm$ 0.08	31.94$\pm$ 0.09	30.79$\pm$ 0.08
96.4	32.70$\pm$ 1.12	29.90$\pm$ 0.08	31.72$\pm$ 0.11	29.64$\pm$0.94
96.5	32.34$\pm$ 0.11	30.66$\pm$ 0.04	31.55$\pm$ 0.04	30.51$\pm$ 0.01
96.6	28.81$\pm$ 0.02	26.83$\pm$ 0.41	28.53$\pm$ 0.02	27.15$\pm$ 0.04
77.1	28.61$\pm$ 0.09	27.27$\pm$ 0.12	24.87$\pm$ 0.04	27.59 $\pm$ 0.06
77.2	28.84$\pm$ 0.09	27.19$\pm$ 0.15	28.48$\pm$ 0.04	22.00$\pm$ 0.01
77.3	28.73$\pm$ 0.09	27.14 $\pm$ 0.21	NA	NA

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Education

The Pennsylvania State University, Schreyer Honors College
Eberly College of Science: B.S in Biology, Neuroscience Option
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Research Experience

The Vandenbergh Lab (Molecular Genetics Lab),   **University Park, PA**
Mentor: David J. Vandenbergh, Ph. D.
Summer 2011- Present
- Conducted research in mouse models to investigate the molecular basis of addiction, specifically how methylation patterns are effected by and related to nicotine use
- Used wet-lab techniques including DNA extraction, PCR amplification, agarose gel and polyacrylamide gel imaging
- Participated in mouse work which included basic care and handling, weighing animals, breeding, mice cessarian sections, and brain dissections

Center for Neuroscience at University of Pittsburgh   **Pittsburgh, PA**
Mentor: Robert A. Sweet, M.D.
Summer 2012
- Conducted research on Alzheimer's Disease, specifically its effect on synapses and spines
- Developed techniques that would allow for the use of Array Tomography, a fluorescent imaging technique, for use in the Sweet Lab
- Worked on an independent project and presented work at final conference

Berenbaum Cognitive Psychology Lab   **University Park, PA**
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Spring 2010- Fall 2010
- Conduct research on the variations in development, behavior, and cognition from a neurological perspective. Focus is on neuro-endocrine and genetic influences.
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Honors and Awards

Dean's List, Member of the Golden Key Honor Society, National Collegiate Scholar

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Global Medical Brigades, Springfield THON Organization, OPP Committee THON 2011