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THREE APPROACHES TO INVESTIGATING AN EPIGENETIC BASIS TO NICOTINE CONSUMPTION IN ADOLESCENT MICE: *AGOUTI VIABLE YELLOW* PROGRAMMING, METHYL DONOR SUPPLEMENTATION, AND MATERNAL CARE

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ABSTRACT

The heterogeneity of adolescent nicotine consumption of inbred mice in controlled conditions suggests a possible epigenetic etiology of individual differences in nicotine use (Chapter 1). This dissertation research approached the association between adolescent nicotine consumption and epigenetics through analysis of coat color in the *agouti viable yellow* (A^{vy}) mouse model, responses to developmental exposure to dietary methyl donor compounds, and observation of maternal care. Four major hypotheses were tested:

1) Does perinatal nicotine exposure influence expression of the environmentally sensitive epiallele, A^{vy}, as indicated by coat color? Results showed that spectrum of agouti coat color was not influenced by continuous exposure to 200 µg/ml or 50 µg/ml nicotine in parental drinking water from 2 weeks before conception to weaning (Chapter 2).

2) Are individual differences in early embryonic epigenetic programming, as indicated by extremes of coat color in A^{vy}/a mice, associated with subsequent adolescent nicotine intake? Results showed that neither extreme yellow mice nor fully mottled pseudoagouti mice were different from black or mottled littermates in their oral nicotine consumption on a three bottle choice test at age 36-42 days (Chapter 2).

3) Does dietary methyl donor availability—which can increase DNA methylation—alter adolescent nicotine consumption? Initial experiments indicated that mice born of parents fed a standard Methyl Supplemented diet during conception, gestation, and lactation showed lower adolescent nicotine consumption than offspring of control diet-fed litters. The effect included interactions with perinatal nicotine exposure (200 µg/ml free base nicotine in dams' drinking water) and test day such that methyl diet blocked the perinatal nicotine-induced increase in adolescent nicotine consumption, especially in the first days of the test. Subsequent experiments evaluated the timing of methyl diet and two concentrations of perinatal nicotine exposure (50 and

200 µg/ml). The 3-5% reduction in adolescent nicotine consumption was consistent across all groups of mice receiving methyl diet during brain development, including an additional group of C57BL/6J mice. Although the effects were not significant in each wave, the overall effect of methyl diet across studies was significant. (Chapter 3)

4) Are individual differences in maternal care, particularly licking and grooming of neonatal pups, associated with differences in adolescent nicotine consumption? Individual lick counts observed during 4 hours of sampling per day on postnatal days 1-8 were not associated with subsequent adolescent nicotine consumption in four litters of A^{vy}/a and *a/a* littermate mice (Chapter 4).

The failure to detect macroscopic factors associated with the emergence of adolescent nicotine consumption is consistent with the hypothesis that cumulative stochastic molecular events are responsible for the divergence of dizygotic-but-genetically-identical littermates in nicotine use phenotypes.

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Chapter 1

Individual Differences in Susceptibility to Nicotine Addiction and Epigenetics

The purpose of this dissertation project is to test for an association between adolescent nicotine consumption and epigenetics in a mouse model. The three approaches described, summarized in the three central chapters of this dissertation, have all focused on associations between macroscopic phenotypes and interventions that are presumed, based on previous work, to be associated with epigenetic programming at the molecular level. In this first chapter, the broad rationale for these three approaches is described, and then in each chapter the results of each test are explained. The association between adolescent nicotine consumption and prior methyl-donor supplementation during gestation and laction (described in Chapter 3), should be followed up at the molecular level in the future, especially if the finding is replicated according to the experiment proposed in Chapter 5. Additional molecular and transgenerational approaches to the epigenetics of nicotine consumption behavior should be explored, but are beyond the scope of this dissertation.

Rationale for Investigating the Role of Epigenetics in Individual Differences in Nicotine Addiction

The Question

Over 1 billion people worldwide use tobacco, and if current trends continue, tobacco use could be responsible for a billion deaths this century (Jha 2009). Even in countries that have

successfully implemented public policies to reduce this burden (Wakefield et al. 2008), therapy-resistant tobacco addiction holds millions of people in a fatal pattern of behavior (Pollock et al. 2009). The major cause of persistent tobacco use in the face of well-known harmful consequences is addiction to nicotine (USDHHS 1988). In the USA, after years of progress in reducing smoking prevalence, the 45 million remaining adult smokers tend to be more strongly addicted than the average smoker of the past—most have failed at attempts to quit due to their addiction, even those who have tried the best programs available (Shiffman et al. 2008, Pollock et al. 2009). For most of these people, addiction had a rapid onset in adolescence (DiFranza and Richmond 2008). Why do some people get so strongly addicted to smoking? Why are others able to smoke without developing dependence?

Genetic Variance in Nicotine Use Behavior

Researchers have searched for the causes of individual differences in heavy smoking and tobacco dependence for over 50 years. In this span, important effects of cost, social networks, advertising, and other environmental factors that are outside the scope of this review have been discovered, and form the basis of vital tobacco control policies that save millions of lives annually in the nations where they are implemented. However, at the same time, evidence for intrinsic biological influences on smoking behavior have also been consistently supported. Early evidence from twin studies (Friberg et al. 1959) and correlations between smoking and heritable personality traits (Eysenck et al. 1960) supported the notion that tobacco use was influenced by genetics. In the ensuing decades, increasingly well-powered and sophisticated research supported both lines of evidence in favor of strong genetic influences on tobacco use, with researchers estimating around a 50% “genetic” contribution (Pflaum 1965, Cherry and Kiernan 1976, Kaprio

et al. 1982, Pomerleau 1995, Kirk et al. 2001, Li et al. 2003, Lessov et al. 2004), which is stable across cultures (Madden et al. 1999).

Identification of nicotine as the primary addictive chemical in tobacco (reviewed by Stolerman et al. 1973) paved the way for identification of candidate genes for tobacco use. Discovery of nicotine's receptors (Changeux et al. 1970) and metabolic enzymes (Philips et al. 1985) received some initial support for association between specific variants of these genes with smoking behaviors (Lucino and Wong 2002). With 50% of the variance in smoking behaviors un explained, it seemed likely that alleles explaining 5-10% of the variance in nicotine use behavior could be discovered. However, after numerous genome wide association studies, only one locus is well replicated—the cholinergic receptor cluster on 15q25 (Caporaso et al. 2009, Vink et al. 2009, Han et al. 2009, Uhl et al. 2010, Liu et al. 2010, Tobacco and Genetics Consortium 2010, Thorgeirsson et al. 2010 Saccone et al. 2010). Individually, alleles explain at best 1-2% of the variance in smoking behavior; however by combining the effects of 11 of the most important alleles identified for main effects and 2 way epistasis with other alleles, about 10% of the population variance in cigarettes per day can be explained with existing information from SNPs (Culverhouse et al. 2011).

Role of Epigenetics

Other as yet unidentified genetic effects can still help explain the remaining 40% of the heritability of smoking behavior, but the large "missing inheritance" from twin studies could indicate major epigenetic effects that arise prior to blastocyst separation in monozygotic twins (Kaminsky et al. 2009). The clearest experimental evidence for broad phenotypic effects of early embryonic programming comes from an elegant experiment in inbred animals 30 years ago which differentiated the effects due to sharing the same zygote from sharing the same genes (Gärtner

and Baunack 1981). All same-sex mice in an inbred strain are as similar as identical twins in terms of alleles, but they generally come from different zygotes. However, it is possible to intentionally prepare monozygotic and dizygotic inbred mouse twins for use in comparative studies. In support of the epigenetic hypothesis, monozygotic mouse twins were produced by splitting the embryo in the 4 cell stage. The mouse pairs produced were more similar to one another than same sex dizygotic twins that went through the same culture procedures except embryo division. Traits measured included age of hair emerging, eye opening, ear opening; body weight from weaning to young adulthood (Gärtner and Baunack 1981). Thus, very early epigenetic factors affect timing of developmental milestones and adult body composition.

In mouse and rat models, drug self-administration phenotypes (including nicotine) have consistently shown great inter-individual variability, similar to the variability in addiction vulnerability seen in humans (Deminiere et al. 1989, Glick et al. 1996, Shoaib et al. 1997). It is a daunting task to discern whether these differences are programmed by the genetic sequence, epigenetic differences between the individual gametes, outcomes of intrauterine position and other unique gestational environs, individual differences in maternal care, or any other variable that can produce divergence in later behavior. Perhaps the easiest way to ascertain the existence of epigenetic and environmental contributions to drug use behavior is to rule out is the genetic sequence variation by using inbred animal models and seeing how much variation in drug use behavior remains in a genetically homogeneous population. The literature and public databases are replete with studies on drug use phenotypes in inbred strains (e.g. Crawley et al. 1997, Zhou et al. 2001), and variance in drug use behavior in genetically identical mice is consistently found. However, in most cases the goal is to detect between strain differences, and the within strain variance receives no further analysis.

One case where the within-inbred strain differences are not ignored is the search for phenotypic predictors of subsequent drug use. Individual differences in behavior that correlate

with self-administration are evident before the first drug exposure, as traits such as locomotor response to novelty predicted later drug use (Piazza et al. 1989). Similarly, even within inbred strains, locomotor response to novelty is correlated with subsequent nicotine consumption (Abreu-Villaça et al. 2006, Gyekis et al. 2010). Furthermore, in animals that are naturally resistant to drug self administration, environmental exposures such as repeated treatment with drugs of abuse, social isolation, or stressors can turn a resistant individual into a susceptible one (Piazza and Le Moal 1996). For example, prior chronic exposure to nicotine in utero can increase nicotine self administration in offspring female Sprague Dawley rats (Levin et al. 2006); also chronic exposure to caffeine in drinking water during the week prior to testing increases nicotine self-administration of adult male Sprague Dawley rats (Shoaib et al. 1999). In both examples, the environmental exposure appears to reduce the proportion of individuals with low self-administration profiles. As recently reviewed by Rodrigues et al. (2010), the development of the mesolimbic dopaminergic circuits involved in drug abuse appear to be sensitive to environmental stressors, especially glucocorticoid exposure, both pre- and post-natally. In reviewing potential epigenetic contributions to addiction vulnerability, Laviola et al. (2003) highlighted the prior finding that novelty seeking behavior is influenced by intrauterine position (Palanza et al. 2001), further implicating gestational steroid hormone exposure in the development of brain systems relevant to adolescent nicotine use. All of these findings support the notion that divergent environmental experiences, even very early in life, leave lasting marks on the brain and change developmental trajectories that lead to drug use. Epigenetic discoveries are waiting to be made.

Efforts to track the mechanisms linking a specific trigger to subsequent epigenetic maintenance marks like DNA methylation patterns have been successful in many cases relevant to stress, drug exposure, and subsequent drug use. Conceptually, one can consider an environmental impulse like a steroid surge to be an “epigenator signal” from outside the young brain cells that physically stimulates a specific “epigenetic initiator” signal transduction pathway

to drive into place a set of molecules that serve as “epigenetic maintainers” (Berger et al. 2009). In the interconnected network of the brain, in which nerve firing is a well-established epigenator signal in itself (e.g. Martinowich et al. 2003) and cell types are highly differentiated, diverse epigenetic outcomes can result from a single drug exposure event throughout neural circuits downstream of any intervention (Malvaez et al. 2009).

Similar complexity extends to the role of any particular epigenetic programming pathway in drug phenotypes. For example, as reviewed by McClung and Nestler (2008), cyclic-AMP response element binding (CREB) protein serves in an epigenetic initiator system that directs transcriptional machinery to alter histone tails and DNA methylation on a spectrum of genes that regulate neuroplasticity, and thus response to drugs of abuse, in certain brain regions. For example, CREB activity in the ventral tegmental area (VTA) is essential for nicotine conditioned place preference (Walters et al. 2005). However, overexpression of CREB has opposite effects on drug preference in the rostral and ventral portions of the VTA (reviewed by McClung and Nestler 2008). In addition to CREB, other transcription factors with epigenetic programming capabilities such as ΔFosB, NF-κB, and circadian clock genes are involved in dopaminergic signaling and drug use phenotypes (again reviewed by McClung and Nestler 2008). As it is becoming clear that individual differences in synaptic plasticity in response to drugs probably underlie differences in addiction vulnerability (e.g. Kasanetz et al. 2010), it seems likely that these systems controlling neuroplasticity in the reward circuit are the critical points in the epigenetic landscape where non-drug consumers and drug consumers diverge. However, future research is required to confirm which systems actually cause the individual differences in nicotine consumption we observe in inbred animals.

Alternatively, epigenetic divergence in other (though overlapping) functional systems in the brain could play the predominant role. In the case of stress, there are well established epigenetic control systems that link very early (e.g. gestational) experience to subsequent

behavior. Weaver et al. (2005) reported that being reared by high licking rat dams led to epigenetic changes in chromatin and DNA methylation to elevate hippocampal expression of the glucocorticoid receptor gene, leading to stronger negative feedback on the HPA axis, and contributing to a low anxiety phenotype. Furthermore, Murgatroyd et al. (2009) reported that periodic separation of pups from their mothers led to adult stress hyperactivity through demethylation of methyl-CpG binding protein 2 binding sites in the arginine vasopressin (AVP) gene of the hypothalamic paraventricular nucleus, which acts synergistically with CRF to produce sustained HPA axis hypersensitivity, leading to high gene expression of AVP in that brain region. Given the role of glucocorticoid signaling in dopaminergic circuits (Ambroggi et al. 2009) and the robust association of stress-related psychiatric illness with addiction (Koob 2008), it seems possible that HPA mechanisms could be sufficient to alter subsequent drug use phenotypes. As reviewed in Chapters 2 and 3, nicotine itself can serve as a epigenator signal by triggering neural firing cascades that lead to activity dependent epigenetic remodeling downstream in the neural network, or by triggering hormone releases that alter transcription and epigenetic markers.

In the past 10 years, the biotechnology needed to experimentally test for correlation between epigenetic marks on specific alleles and nicotine use has expanded greatly. Since it remains unclear which specific tissues or types of neurons are most important for determining individual differences in tobacco use and nicotine addiction, the effort required to quantify epigenetic marks on many candidate alleles in numerous candidate cell types was beyond the scope of a doctoral dissertation. Furthermore, even if the work were limited to investigating a promising allele most likely to be relevant to addiction (e.g. CREB in dopaminergic VTA neurons), it remains difficult or impossible to non-invasively measure the baseline epigenetic state that would contribute to subsequent nicotine use behaviors (without becoming confounded with nicotine exposure). To circumvent these problems, but still make a contribution to a topic of great public health and biological significance, the current dissertation sought to investigate

whether known correlates of general epigenetic programming observable in live animals were associated with nicotine use in animal models. Thus, while not specifically studying the molecular epigenetic control systems, the experiments conducted for this dissertation have been designed to be informative of the relationship between epigenetics and nicotine use in animal models.

Rationale for Evaluating A^{vy}, Methyl Diet, and Maternal Care as Potential Predictors of Nicotine Consumption Behaviors in Mice

The macroscopic phenotype approach allows for assessment of factors known to be associated with epigenetic programming processes in animal models. In order to address the notion that early embryonic epigenomic programming events were related to subsequent nicotine consumption, Chapter 2 evaluates the association between a metastable epiallele and later nicotine consumption. In order to experimentally test whether it is possible to intervene on the process of epigenetic programming to affect nicotine use behavior, Chapter 3 evaluates whether exposure to a methyl supplemented diet alters adolescent oral nicotine consumption. Finally, to investigate the potential involvement of maternal drivers of epigenetic programming after birth, Chapter 4 summarizes a pilot study on whether maternal care is associated with subsequent nicotine consumption.

The Epiallele Approach to Investigating Epigenetic Programming

Genes that are variably expressed between individuals or between different cells or tissues within an individual are called “metastable epialleles”, which are generally believed to be stochastically programmed for expression early in embryogenesis in a parent-of-origin dependent

manner (Rakyan et al. 2002). As reviewed by Richards (2006), epigenetic variation is more pronounced at certain alleles due to genetic mutations, but the variation also occurs throughout the genome—best illustrated by the increasing genome-wide divergence in epigenetic programming with age between monozygotic twins (Fraga et al. 2005). Waterland and Jirtle (2003) pioneered the use of animal models with metastable epialleles for investigating the effects of environmental exposure on epigenetics in the *Agouti viable yellow* (A^{vy}) mice, which phenotypically display a coat color indicative of their epigenetic state at the A^{vy} locus. A cumulative body of experimental evidence from this and other mouse models backs up the epidemiological finding that perinatal exposures alter people's health throughout their lifespan (Dolinoy et al. 2007a).

The primary approach has been *in utero* dietary supplementation with nutrients, such as choline, folate, and vitamin B12, that increase availability of free methyl groups in cellular metabolism, and thereby change epiallele-driven phenotypes (Wolff, et al. 1996; Cooney et al. 2002). As reviewed in Chapter 2, extensions of this work with the *Agouti viable yellow* mice have found epigenetic effects of gestational exposure to industrial chemicals commonly used in the food packaging (Dolinoy et al. 2007b), functional molecules naturally occurring in vegetables (Dolinoy et al. 2006), and a drug of abuse frequently consumed by pregnant mothers (Kaminen-Ahola et al. 2010). Thus, it was hypothesized that if nicotine exposure were influencing epigenetic programming early in embryogenesis, the *Agouti viable yellow* mouse model would manifest it. Furthermore, individual differences in epigenetic programming *in utero*, evidenced by differences in agouti coat color, might contribute to individual differences in nicotine consumption.

Involvement of Dietary Methyl Donors in Establishing Epigenetic States and Neural Phenotypes

The role of dietary availability of compounds that contribute to single carbon metabolism has emerged from studies on the mechanisms of deficiency syndromes. The interactions of choline, folate and vitamin B12 (and methionine)—which partially rescue or partially mask the deficiency of one another—indicate their converging effects on a shared and vital biochemical pathway, single carbon metabolism (Schaefer and Salmon 1950, Sauberlich 1959, Scott and Weir 1981, Niculescu and Zeisel 2002, Shin et al. 2010). In cases of extreme dietary deficiency, the differences in dietary methyl donor exposure drastically affect specific biochemical phenotypes and general health outcomes throughout the life cycle. Specific developmental periods and cellular processes are more susceptible to normal variation in dietary intake of methyl donors. The perinatal period is a time of acute sensitivity, given that dietary levels that result in no deficiency symptoms in the mother can still result in life-threatening harm to the developing child, such as spinal cord defects (Laurence et al. 1981).

Each dietary component considered a methyl donor has unique metabolic entry into the methylation pathways, but they have converging effects on neural phenotypes. Dietary folate is brought into cells by a folate receptor and then converted enzymatically to tetrahydrofolate, serving to transfer methyl groups to S-Adenosylmethionine (SAM), which in turn is a substrate for enzymes that generate methyl-group adducts such as methyl-cytosine DNA bases. Supplementation of folate beyond deficiency prevention doses can markedly alter neural development (Iskandar et al. 2010). The methylated form of vitamin B12 (methylcobalamin) is used to convert homocysteine to methionine, which then contributes to SAM stores and methylation—and supplementation produces similar neural changes as those seen from folate (Okada et al. 2010). Choline, after conversion to the metabolic product betaine, is also used to convert homocysteine to methionine and thus SAM and methylation—thus it is not surprising that

low choline intake is associated with the same neural tube defects as low folate and B12 intake and choline supplementation produces similar neuron differentiation effects as the other methyl donors (Sanders and Zeisel 2009). In animal models, perinatal choline supplementation is known to enhance neural development in the hippocampus and spatial memory phenotypes throughout adulthood (Sanders and Zeisel 2009), but further research is needed to determine whether choline exposure might affect learning and memory in humans in general or specific people with genetic susceptibility (Zeisel 2011). Regardless, the evaluation of the effects of dietary methyl donor supplementation on behavior and psychiatric disorders is a promising area that has been often promoted based on correlational studies in humans (Abou-Saleh and Coppen 1986, Bottiglieri and Hyland 1994, Bottiglieri 1996, and Coppen and Bolander-Gouaille 2005), but lacks an experimentally validated basis for inferring causation. Human clinical trials have focused on adult methyl donor supplementation and have generally been inconclusive, indicating that population supplementation expected to overcome observational differences associated with risk are not powerful enough to treat common cases of psychiatric illness if given to adults (Malouf and Grimley Evans 2008, Smith and Blumenthal 2010), but they do not address issues of supplementation *in utero* and at young ages. Further animal studies on the behavioral effects of methyl donor supplementation will translate into better informed hypotheses about the effects of methyl donors in human intervention studies.

Evidence for a Role of Maternal Care in Epigenetic Programming in the Nervous System

Decades of research have shown that early social environment can dramatically affect the development of normal behavior in mammals. When monkeys were raised by peers without their mother, Harlow found that severe learning disabilities, stereotypies, fear, and aggression resulted

(Harlow et al. 1965, Ladd et al. 2000). Primates socially isolated during the first 9 months postpartum developed with numerous alterations in neuron subtypes, including a drastic reduction in opioid peptide (leu-enkephalin) neurons in the striatum (Martin et al. 1991). In rats, a substantial body of evidence suggests that offspring of mothers that spend less time licking their pups and in arch-backed nursing are more anxious (Francis et al. 1999, Meaney 2001) and have altered brain gene expression and DNA methylation into adulthood in pathways critical to the regulation of negative feedback on the HPA axis (Fish et al. 2004, Zhang and Meaney 2010). Receiving high levels of maternal care is associated with global increases in gene expression in the hippocampus—suggesting genome-wide hypomethylation of genes in highly licked offspring (Weaver et al. 2006). Cameron et al. (2008a) reported that female rats born of high licking dams had later puberty, less sexual solicitation, increased aggression towards males, and lower plasma luteinizing hormone and progesterone as adults. These changes appeared to be functional in terms of life history and selection—the high licking rats were not only less sexually receptive, they were also less likely to get pregnant (Cameron et al. 2008b).

Meaney has proposed that the variation in maternal care in response to environmental stress represents an adaptive mechanism that allows for "programming" offspring stress response to match prevailing conditions of the day. Thus reduced maternal care could be adaptive for local environments when predation rates are high (when high anxiety and rapid reproduction generally lead to greater reproductive success), while greater maternal care could be adaptive in situations in which interspecific competition is more important (where foraging and mate selection are more important for reproductive success). Although this "Just So Story" remains to be validated in ecological contexts, it provides a scenario in which epigenetic malleability makes evolutionary sense.

Regardless of the evolutionary explanation, there are several empirical lines of evidence linking maternal care to subsequent drug consumption behaviors. Fostering of newborn DBA2/J

mice to dams of a relatively a high licking mouse strain (C3H/HeN) led to increased cocaine self-administration compared to mice fostered to dams of a lower licking strain (AKR); however, the same fostering procedure had no effect on C57BL/6J mice, experimentally verifying gene by environment interactions in drug use behaviors (van der Veen 2008).

There are several lines of evidence encouraging the hypothesis that maternal care driven epigenetic programming could contribute to individual differences in nicotine consumption. First, empirical evidence is given showing significant litter effects on nicotine consumption. Variation in maternal care is certainly one of the mechanisms that could lead to these litter effects. Second, in humans and in animal models, nicotine consumption has been linked to social behaviors and anxiety disorders (reviewed by Koob 2008), which are also influenced by maternal care. Therefore, Chapter 4 reports the results of a pilot experiment investigating whether licking and grooming received by a pup is associated with adolescent nicotine consumption.

Conclusion

Diverse lines of reasoning and sources of empirical evidence suggest that early experiences alter trajectories of development through the epigenetic landscape of potential brain functional responses to oral nicotine. In order to test whether early epigenetic programming is associated with nicotine consumption, Chapter 2 evaluates the relationship between A^{vy} coat color spectrum and nicotine consumption. In order to test whether experimental manipulation of global DNA methylation processes leads to changes in nicotine consumption, Chapter 3 reports the effects of methyl diet supplementation on adolescent nicotine consumption. In order to test whether individual differences in maternal care alter adolescent nicotine consumption, Chapter 4 tests for an association between oral nicotine consumption and maternal grooming behaviors received by the pup in the first 8 days of life. Thus, this dissertation will be the first to apply these

three prominent approaches to testing for an epigenetic basis of individual traits to the question of "what causes individual variability in adolescent nicotine consumption?"

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Chapter 2

Epigenetic programming of the A^{vy} locus is independent of nicotine exposure and nicotine consumption

Abstract

Experimental studies on perinatal nicotine exposure in animal models indicate that nicotine exposure can cause lasting changes in gene expression that may partly underlie respiratory, reproductive, metabolic, and neurobehavioral diseases induced by tobacco in humans. Of particular interest is whether epigenetic factors mediate effects of nicotine exposure on adolescent nicotine consumption.

The current study tested for a shift in the coat color spectrum of *agouti viable yellow* (A^{vy}) mice by perinatal nicotine exposure. Heterozygous male mice with the A^{vy} mutation on a C57BL/6J genetic background were mated to a/a dams. Two weeks before mating, parental mice had been randomly assigned to a standard refined 7% corn oil diet (C) or the same diet containing added folate, vitamin B12, choline, and betaine (M for methyl supplementation). On the same day, mice from both dietary groups were randomly assigned to consume either 200 µg/ml free-base nicotine or water. The diet and fluid conditions were maintained through mating, gestation, and lactation. A total of 287 agouti viable yellow pups born to 73 litters were scored for coat color at weaning.

Results showed no significant coat color shift in the litters perinatally exposed to nicotine. The expected reduction in body weight in the nicotine treated groups was equally

represented for a/a mice and A^{vy}/a mice of all phenotypes. No significant effect of MS diet exposure on probability heavy mottling/pseudoagouti phenotype was observed, possibly indicating influence of the refined diet compared to the complex diet containing ground meat and vegetable products used in previous studies on the agouti coat effects of methyl supplementation.

These results indicate that the developmental effects of nicotine are independent of the system that establishes epigenetic programming of the agouti viable yellow locus. (Funding provided by PSU SSRI L1 Grant, 2009)

Introduction

Epigenetic Effects of Nicotine and Tobacco Exposure

Tobacco smoking can induce dramatic long-term effects on physiology and behavior of humans, and some changes may be mediated by the epigenetic effects of nicotine exposure (Somm et al. 2009). Smoking has been linked to global DNA methylation and methylation of a variety of disease-specific loci in humans (see Hillemacher et al. 2008). Experimental studies have identified the effects of nicotine on DNA methylation of specific genes relevant to a variety of diseases, such as schizophrenia (Satta et al. 2008), cardiac injury (Lawrence et al. 2010), and lung cancer (Soma et al. 2006). These studies reveal that effects of nicotine on epigenetics are specific to the locus under consideration, the cell type, and the physiological effects of nicotine in the tissue of interest.

The mechanisms of nicotine effects on DNA methylation are generally downstream of nAChR-expressing cell types. Nicotine exposure downregulated DNA methyltransferase 1 and demethylated GAD67 in frontal cortex inhibitory interneurons, but not when the mice were pretreated with mecamylamine (Satta et al. 2008). The increase in methylation of the PKCε gene

promoter in the fetal heart of mice exposed to nicotine *in utero* is dependent on nicotine-induced increases in epinephrine release (Lawrence et al. 2010). A study of cultured human endothelial cells showed that nicotine altered expression of a host of genes regulated by CREB and NF-κB (Zhang et al. 2001). However, it is unclear how nicotine or nicotine metabolites induce DNMT 3 expression in cultured endothelial cells (Soma et al. 2006). The diverse effects of perinatal nicotine exposure on the development of many physiological systems have recently been reviewed at length (Somm et al. 2009, Bruin et al. 2010). The life-long effects of nicotine and their sensitivity to early developmental exposure are consistent with epigenetic alterations in many cell lineages.

Nicotine may also have transgenerational epigenetic effects on metabolic syndrome in the second generation. Holloway et al. (2007) exposed pregnant Wistar rats to 1 mg/kg/day nicotine injections throughout pregnancy and lactation. These rats that had been exposed to nicotine via their mother's blood and milk were mated, and the second generation was studied in early adulthood. The offspring of rats exposed to nicotine early in development showed hyperinsulemia, insulin resistance, higher cholesterol, and hypertension compared to the offspring of saline exposed rats. Furthermore, it has been reported that in humans, a boy's adolescent tobacco use may affect his own son's body weight (Pembrey et al. 2006). Thus, there are many lines of evidence suggesting that nicotine could be involved in epigenetic programming, and a logical next step is testing the influence of nicotine on embryonic programming of epigenetically sensitive alleles in established model systems.

Agouti Viable Yellow Mice as an "Epigenetic Biosensor"

In order to investigate whether there may be an epigenetic basis for the developmental effects of perinatal nicotine exposure, agouti viable yellow mice provide a unique opportunity to

detect whether effects are mediated by global DNA methylation at the time of conception. The *agouti viable yellow* (A^{vy}) mouse has long received attention due to the maintenance of variation in coat color of the strain even after inbreeding (Dickies 1962). In this strain of mice, the *agouti signaling peptide* gene (here abbreviated as "*agouti*", also known as "*nonagouti*") harbors a transposable element (intra-cisternal A particle) that makes expression of the agouti gene dependent upon DNA methylation within the transposon for expression, which makes the expression of the gene and the yellow coat phenotype both stochastic and sensitive to environmental exposures (Wolff et al. 1998, Waterland and Jirtle 2003, Dolinoy et al. 2010).

The wild type allele, *A*, has the classic agouti coat color phenotype—hairs have a dark base, yellow mid-shaft, and dark tip, creating a shimmery appearance. Mice homozygous for the recessive allele, *a*, generally have a black coat color, although they do have yellow hair behind the ears (Figure 1). Mice carrying the dominant A^{vy} allele display dramatic increases in agouti expression throughout the body (Waterland and Jirtle 2003) and have a coat color ranging from agouti color to pure yellow, often with a mottled pattern (Figure 1). The fully agouti coat phenotype is called "pseudoagouti" since it is agouti but not caused by the wild type Agouti allele (which causes the agouti phenotype of the C3H mouse). The degree of yellowness in the coat is inversely correlated with the amount of DNA methylation on the A^{vy} locus (Waterland and Jirtle 2003, Kaminen-Ahola et al. 2010).

Figure 2-1. Photograph of a litter of agouti viable yellow mice and black littermates.

Giving the parental A^{vy} mice a diet rich in methyl donors such as choline, betaine, folate, and vitamin B12 during gametogenesis and maintaining the mother on this diet through pregnancy leads to a higher proportion of the A^{vy}/a offspring having the agouti colored coat (Wolff et al. 1998, Waterland and Jirtle 2003, Cropley et al. 2006, Waterland et al. 2007). Similarly, gestational exposure to a soy phytoestrogen enriched diet (Dolinoy et al. 2006) or to oral ethanol consumption (Kaminen-Ahola et al. 2010) increase the probability that A^{vy}/a offspring will display a brown coat. On the other hand, exposure of pregnant dams to the plasticizer bisphenol A (Dolinoy et al. 2007) and exposure of the embryos to the process of *in vitro* fertilization (Morgan et al. 2008) both shift the coat color distribution towards yellow. The only negative finding reported thus far comes from a test for coat color shift in animals supplemented with soy protein isolate (Badger et al. 2008). Thus, coat color could potentially serve an "epigenetic biosensor" of methylation states established around the time of conception (Dolinoy 2008) that might detect whether nicotine influences DNA methylation in this critical period. Given the evidence above that nicotine can alter genetic programming in either direction

in allele specific manners, and given that the mechanism of A^{vy} programming remains unknown, a two-tailed hypothesis was appropriate.

Furthermore, the A^{vy} locus might serve as an indicator of mice with generally deregulated epigenetic programming. The brains of schizophrenics (an extremely high smoking population, de Leon and Diaz 2005) show dramatically different patterns of DNA methylation than normal subjects (Mill et al. 2008). One factor in schizophrenia may be over-expression of DNA methyltransferase 1 in inhibitory interneurons of the frontal cortex, which nAChR activation can help to control (Satta et al. 2008). We hypothesized that failure of proper epigenetic control mechanisms, either ectopic gene expression or extreme silencing, could be involved in individual differences in nicotine consumption within inbred mice, and thus tested for an association between either extreme of coat color and oral nicotine consumption.

Methods

Animal Husbandry

Experimental procedures used in this dissertation were reviewed and approved by the Pennsylvania State University's Instutitional Animal Care and Use Committee (IACUC # 28989).

Heterozygous male mice with the A^{vy} mutation on a C57BL/6J genetic background were mated to black *a/a* dams. Animals were second generation or higher descendants of progenitor animals that were a gift from Randy Jirtle at Duke University. Mice were maintained in standard "shoe-box" cages on a 12:12 light dark cycle (lights on at 8 am). All husbandry tasks and measurements were conducted 7±1 hours into the light phase (3 pm), when feeding and drinking behaviors are low (Possidente and Birnbaum 1979).

Mice were assigned to dietary and fluid consumption conditions as explained below. Sires were separated from the dams two weeks after co-housing. Dams were checked daily in the light phase to record litter date of birth. Litters were weaned on postnatal day 27 and weighed, scored for coat color (black, or, if A^{vy}, estimated percent yellow coverage), and sexed. In Wave 1, mice were photographed on a white piece of paper at weaning and the percent yellow coat (by area) on the mouse viewed from above was independently estimated by observation by three observers, resulting in a correlation of 0.9 between observers. In Waves 2-4, one of these three observers visually estimated percent yellow coat on A^{vy}/a animals at weaning. Male weanling mice were cohoused with littermates until the habituation phase of oral nicotine consumption testing (day 32).

In the first group (Wave 1), weanling A^{vy} mice were also photographed and tail lengths were measured by pulling the tail straight through a slit in a box. From photographs of A^{vy} mice, three independent observers scored percent yellow coat coverage, which was positively correlated r = 0.9 between observers.

Methyl Supplemented and Control Diets

Two weeks before mating, parental mice were randomly assigned to dietary experimental conditions. There were two control diets. The standard lab chow used in the building is LabDiet 5001, ordered from PMI Nutrition International (St Louis, MO). The control diet for comparison to the methyl diet was the AIN-93G 7% corn oil diet (C) from Harlan Teklad (Indianapolis, IN). The methyl diet (M) was the same diet containing added folate, vitamin B12, choline, and betaine (see Table 2-1). C and M diets were ordered to be supplemented in the same manner as those used in previous studies on agouti coat color shifts by methyl donors (e.g. Waterland and Jirtle 2003), except with a different dietary background. The current background diet has refined

ingredients instead of the complex ingredients (ground wheat, corn, oats, fish, soy, alfalfa, yeast, and kaolin) of the NIH-31 diet used for previous methyl donor supplementation studies. Methyl-donor and macronutrient composition of these diets is shown in Table 2-1 below.

Table 2-1. Methyl-donor nutrient composition of diets.

	Standard Chow[†]	Control Diet	Methyl Diet
Betaine	-	-	5 g/kg
Choline chloride	2250 ppm	-	7.97 g/kg
Choline bitartate	-	2.5 g/kg	2.5 g/kg
Folic acid	7.1 ppm	2.0 mg/kg	4.3 mg/kg
Vitamin B12	50 µg/kg	0.03 mg/kg	530 mg/kg of 0.1% B12
Fat	≥4.5% by weight, 13.5% of calories, from corn, soy, fish, wheat, & pork meal	7.2% by weight, 17.2% of calories, from corn oil	7.2% by weight, 17.4% of calories, from corn oil
Protein	≥23.0% by weight, 28.5% of calories	17.7% by weight, 18.8% of calories	17.7% by weight, 19.0% of calories
Carbohydrate	58.0% of calories	60.1% by weight, 63.9% of calories	59.1% by weight, 63.5% of calories
Total Calories	3.0% kcal/g	3.8 kcal/g	3.7 kcal/g
Fiber	≤6.0% by weight “crude fiber”, 15.6% “neutral detergent fiber”	50.0 g/kg cellulose (5%)	50.0 g/kg cellulose (5%)

[†] Nutrient datasheet as accessed online from PMI Nutrition International on March 4, 2011, http://labdiet.com/pdf/5001.pdf.

The schedule of dietary supplementation varied between experiments. In Wave 1, dams and sires were assigned to either C or M dietary conditions two weeks before mating and

continuously maintained on the diet until pups were weaned (about 50 days later, including conception, gestation, and lactation). Upon weaning, Wave 1 offspring were given standard chow. Wave 2 served as an identical replicate of Wave 1 except offspring were maintained on M or C diets after weaning and throughout testing. For the sake of all analyses of weaning data, Waves 1 and 2 were identical and their data are combined. Parental animals for Wave 3 were maintained on standard chow until 5 days after cohabitation and then assigned to M or C diets. Thus, Wave 3 litters were exposed to M or C conditions throughout the majority of gestation until weaning. Offspring of Wave 3 were returned to standard chow upon weaning. Parental animals in Wave 4 were given M or C diets from 14 days before mating until 5 days after cohabitation. Thus, they were exposed to M and C conditions from the period leading up to mating until up to 5 days after mating. The schedule of dietary exposures for the four Waves is shown in Figure 2 below.

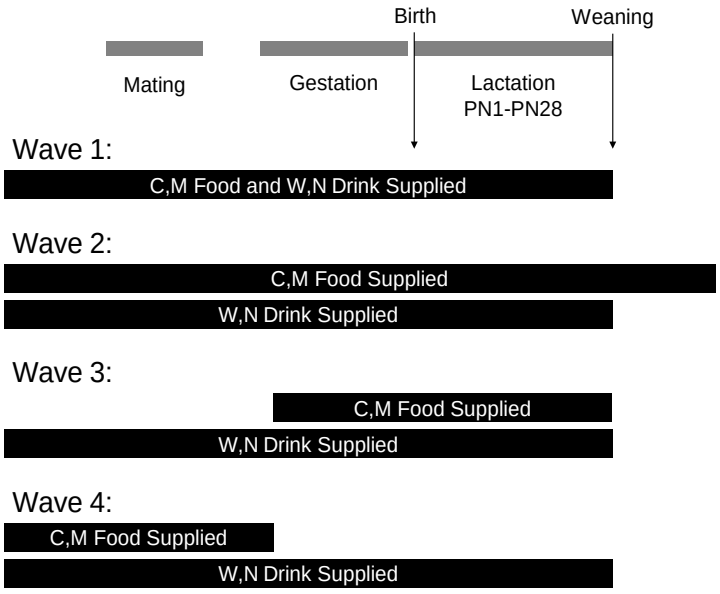

Figure 2-2. Schedule of dietary exposures for the four experimental timelines described above. PN stands for postnatal day. Both dams and sires were assigned to experimental diets at the same time in each wave.

Forced Nicotine Exposure

On the same day two weeks before mating, dams and sires from both dietary groups were randomly assigned to consume 200 µg/ml free-base nicotine (High Nicotine, HN), 50 µg/ml free-base nicotine (Low Nicotine, LN), or water (W), according to the protocol of Klein et al. (2003). The fluid conditions were maintained continuously until weaning, when mice were returned to water. In Wave 1 (see above), only W and HN conditions were assigned. In Waves 2-4, mice from each dietary condition were assigned to W, LN, or HN fluid conditions.

Adolescent Nicotine Consumption Testing

On postnatal day 32, male mice were weighed, individually housed, and offered three water bottles. On PN 35, water consumption was recorded and bottles were replaced with two bottles of nicotine solutions (200 and 50 µg/ml freebase nicotine in tap water) and one water bottle. Each day for the next 7 days, fluid consumption from each bottle was measured daily. Initially, 200 µg/ml nicotine was placed on the left (from the mouse's point of view), 50 µg/ml nicotine in the center, and water on the right. Then each day the bottles were rotated to the left, such that on the second day the order was 50 – 0 – 200 µg/ml, the third day 0 – 200 – 50 µg/ml, and so on across the seven days of testing. Nicotine solutions were replaced every 3-4 days. In Wave 1, mice were weighed on days 1, 3, 5 and 7 of the oral nicotine three bottle choice test and cages were replaced once per week. In Waves 2-4, mice were not weighed until day 7 and cages were not changed during the choice test. In Wave 1, consumption was estimated from differences in volume readings on the graduated cylinders. In Waves 2-4, consumption was estimated from

differences in bottle weights. During Wave 1, cages were changed for fresh bedding once per week, including during adolescent nicotine consumption testing, but in all subsequent experiments no cage changes were conducted during adolescent nicotine consumption testing.

Statistics

ANOVAs were calculated in SPSS version 18 (Chicago Illinois. In all analyses, two tailed tests with $p < 0.05$ was accepted as significant.

Results

Offspring Coat Color

The primary test of the hypothesis that nicotine color may shift *Agouti viable yellow* coat color came from the merged data sets of Waves 1 and 2. A total of 287 A^{vy} pups were born to 73 litters in these waves. A 2 diet × 3 drink factorial design was used to simultaneously test for effects of dietary exposures, nicotine exposures, and interactions on the average amount of yellow coat color on each A^{vy} mouse in Waves 1 and 2. No significant differences between groups were detected (all $p > 0.05$). Average yellow coat color was approximately 60% for all groups (top panel, Figure 2-3).

Smaller numbers of litters in waves 3 and 4 were inadequate for testing for effects of coat color. Still, mean percent yellow coat is also shown in Figure 2-3, and the averages are consistent with our finding that methyl diet did not shift coat color towards pseudoagouti.

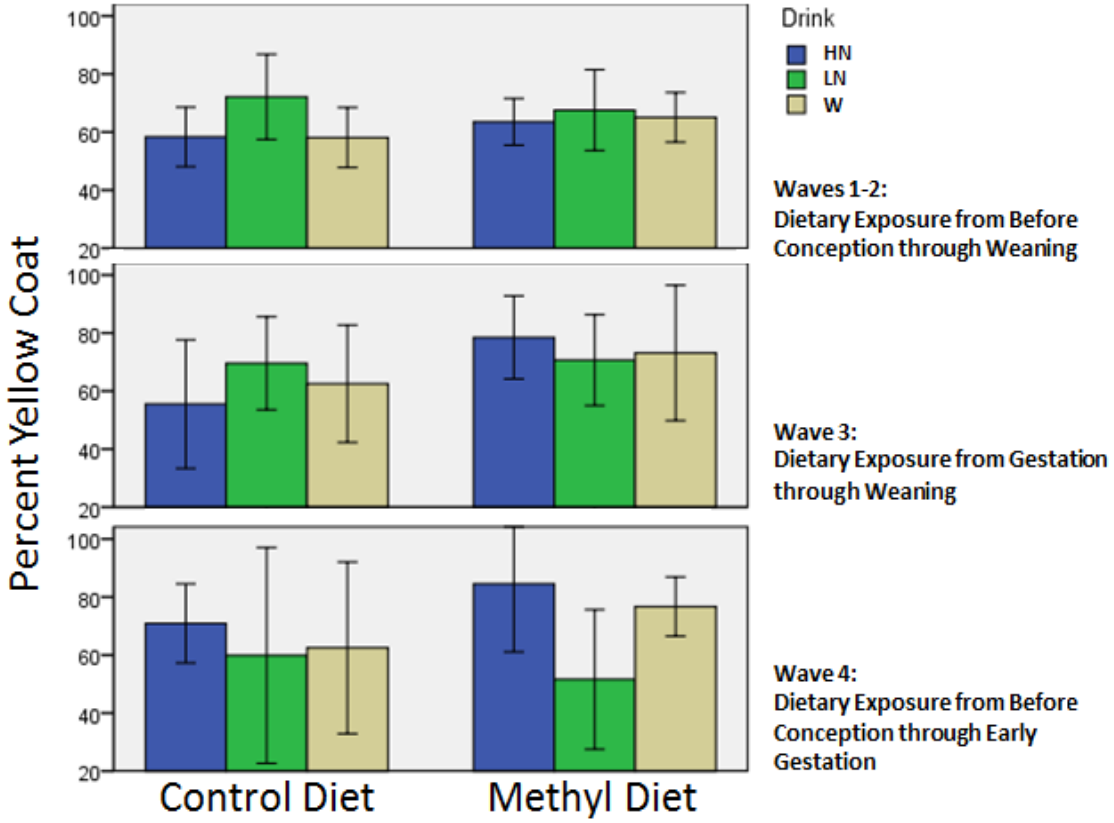

Figure 2-3. Yellow coat coloring for A^{vy} animals perinatally exposed to 200 (High Nicotine, HN), 50 (Low Nicotine, LN) or 0 (Water, W) µg/ml nicotine in their drinking water and methyl- (M) or unsupplemented (C) diets provided in Waves 2-4 (mean ± 95% confidence interval). No differences between any groups were significant (all $p > 0.05$).

For maximal power to test for effects of perinatal nicotine exposure (which had equal timing in across waves), ANOVA was used to test for differences between HN, LN, and W groups for all A^{vy} animals. No difference between groups was detected ($p > 0.05$). Histograms of coat color spectrum for each nicotine exposure group are shown in Figure 2-4. Clearly nicotine exposure had no significant effect on agouti viable yellow coat color spectrum.

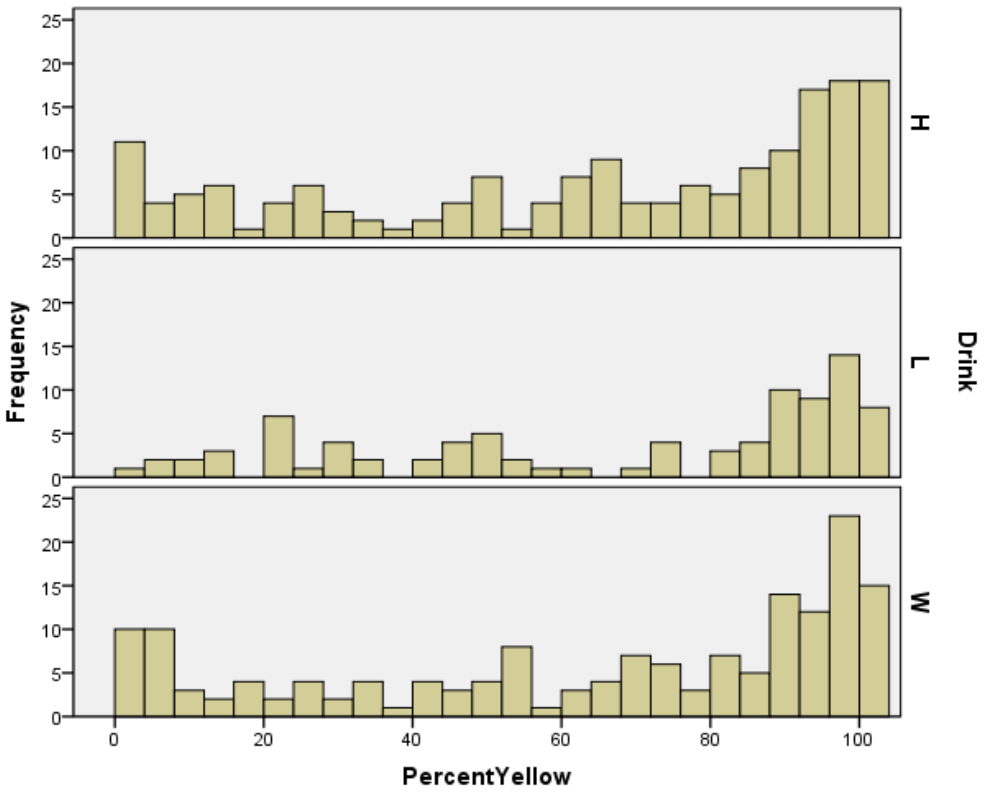

Figure 2-4. Coat color spectrum for experimental animals perinatally exposed to 200 (HN), 50 (LN), or 0 (W) µg/ml nicotine for all waves combined. Histograms show percent yellow coat on the x-axis for A^{vy} animals. No differences were significant.

No Association between Coat Color and Adolescent Oral Nicotine Consumption

Oral nicotine consumption from all adolescent male mice was analyzed to test for an association between coat color and nicotine consumption. Coat color was categorized as black for *a/a* mice and five grades of phaeomelanin coverage for A^{vy} mice (Cooney et al. 2002). Because of differences in protocol and consumption variance between Wave 1 and Waves 2-4, data were analyzed separately. ANOVA showed no significant relationship between oral nicotine

consumption and coat color in Wave 1 or in Waves 2-4 (both $p > 0.05$, clearly evident in Figures 2-5 and 2-6).

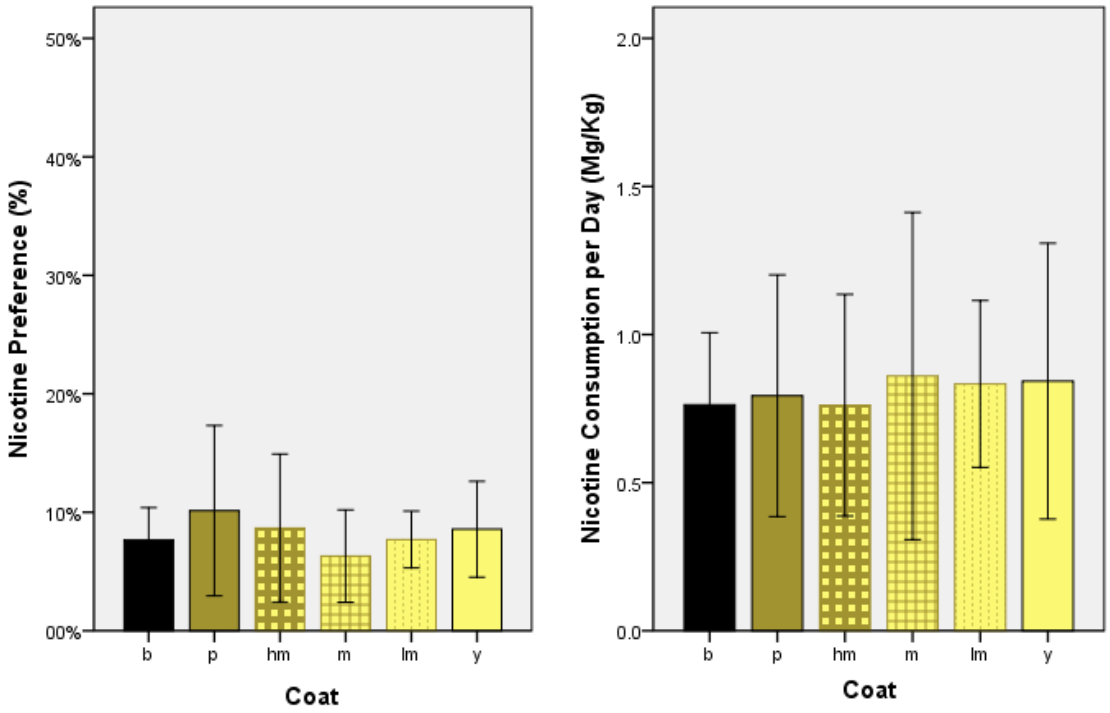

Figure 2-5. Nicotine consumption as a percent of total fluid consumption and as nicotine exposure in mg/kg across the choice test from postnatal day 36-42 of mice of each coat color classification (mean, 95% confidence interval) in Wave 1.

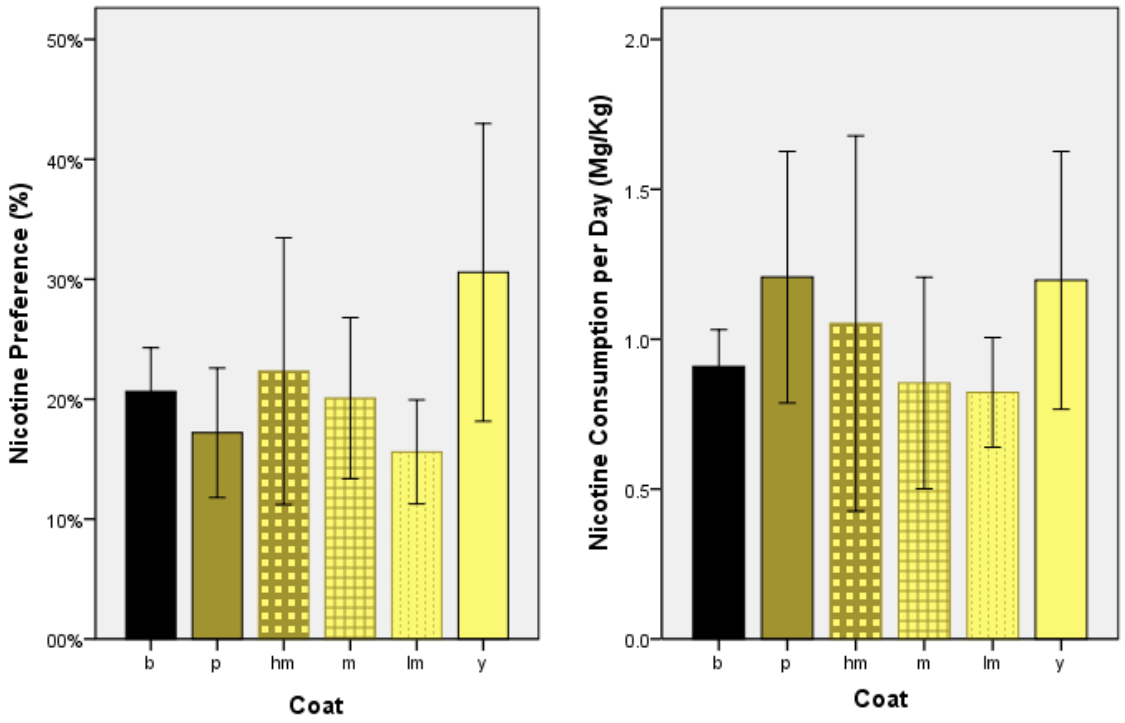

Figure 2-6. Nicotine consumption as a percent of total fluid consumption and as nicotine exposure in mg/kg across the choice test from postnatal day 36-42 of mice of each coat color classification (mean, 95% confidence interval) in Waves 2-4.

Discussion

Maternal Oral Nicotine Consumption Did Not Influence Developmental Programming of A^{vy}

The A^{vy} locus is still "labile" to nutritional exposures in mid-gestation (Cropley et al. 2006). A variety of nAChRs are expressed throughout the skin and hair follicles, on neuronal and non-neuronal cell types, including melanocytes, although it is unclear when the majority of this expression begins (Kurzen et al. 2004, Misery 2004, Wessler and Kirkpatrick 2009). In the brain, functional nicotinic receptors can be found in brain tissue as early as after embryonic day 10

(Atluri et al. 2001). Mechanisms of changes in DNA methylation induced by nicotine exposure in previous studies are generally mediated by nicotinic receptors, which then precipitate either changes in signaling within the same cell (Soma et al. 2006, Satta et al. 2008) or endocrine signals that later alter DNA methylation elsewhere (Lawrence et al. 2010). Thus, the early embryo may be protected from epigenetic effects of nicotine on imprinted loci due to lack of receptor expression, particularly in the melanocyte cells, but this remains to be validated. These results indicate that the developmental effects of nicotine are independent of the system that establishes epigenetic programming of the agouti viable yellow locus in this strain.

Emerging work indicates that these results have only limited value for interpretation beyond this particular mouse line. Work in Skinner's lab has shown that the epigenetic response signatures across the genome for embryonic exposure due to endocrine disruptors are profoundly different for inbred vs. outbred strains, and the loci that respond to a chemical exposure in mice are completely non-overlapping with the loci found in rats (Skinner 2011). Thus, interpretation from inbred to outbred animals, or across species of rodents, is not likely to be merited. Furthermore, there is a recent preliminary report from Murphy et al. (2011) that maternal smoking was associated with increased DNA methylation at the IGF2 imprinted region of infant cord blood DNA; the hypermethylation of this growth factor in the smoke-exposed children was significantly associated with low birth weight. If confirmed and found to be due to nicotine, it would indicate that nicotine might indeed regulate DNA methylation on imprinted genes to alter human health.

Methyl Donors Supplemented in a Refined Diet Did not Shift A^{vy} Coat Color Spectrum

The failure to detect a shift of coat color towards pseudoagouti in groups exposed to methyl diet in any wave of the trial merits further discussion. The work of Waterland and Jirtle (2003) indicated that a sample size of 10 litters per group would be adequate to detect an increase in eumelanin this period of diet exposure and this strain of mice (the mice used in this study were direct descendants of the colony at Duke University). The current data did not even show a trend towards more pseudo-agouti coat in our combined data from Waves 1-2, which had nearly double sample size of Waterland and Jirtle (2003) in the water-only groups and 3-fold larger sample size across all fluid exposures. Thus, the lack of a methyl diet effect is unlikely to be a statistical anomaly.

Compared to the NIH-31 diet used in previous studies of methyl diet shift of A^{vy} coat color, the refined AIN-93G Corn Oil diet is different on many levels. The NIH-31 diet contains ground wheat, corn, oats, fish, soy, alfalfa, yeast, and kaolin in addition to various supplements. In contrast, the AIN-93G diet is composed almost entirely of purified ingredients. In addition to lacking a vast array of complex biomolecules not specifically added to the diet or found in corn oil, the refined diet has a very different mineral content. It contains half as much calcium, copper and selenium; one quarter of the magnesium, one seventh of the iron, and an order of magnitude less manganese and iodine. Perhaps the most critical mineral, zinc, is found at 24% lower concentration in the refined diet. Furthermore, because the AIN diet is more energy dense (3.8 vs 3.0 kcal/g), mice in the current study consumed even less of these minerals. The diet appeared to be nutritionally adequate for breeding, since litter sizes on the AIN diet were similar to studies using the NIH-31 diet (data not shown). However, the refined diet still may have caused a functional change in the metabolism of dietary methyl donor compounds—leading to an amelioration or reversal of the effect of the methyl supplemented diet on A^{vy} coat color spectrum.

Conclusion

The current project showed no differences in nicotine consumption between pseudoagouti individuals (with hypermethylated A^{vy}) and yellow individuals (with hypomethylated A^{vy}). *In utero* variability in epigenetic programming at this locus is thus independent of the hypothetical systems that might program individual differences in nicotine use behaviors within inbred animal populations. Furthermore, no change in coat color spectrum of the agouti viable yellow mice was seen due to nicotine exposure or methyl diet supplementation on this refined diet.

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Chapter 3

Effects of Perinatal Nicotine and Methyl Diet Exposure on Adolescent Nicotine Consumption in Agouti Viable Yellow Mice

Abstract

Nicotine exposure during early development alters adolescent and adult behavior, and methyl-donor compounds have been shown to block the behavioral effects of perinatal ethanol exposure. The *Agouti viable yellow* mouse model was used to test the hypothesis that chronic exposure to nicotine during conception, gestation and lactation (Wave 1) will increase adolescent oral nicotine consumption, while concurrently testing whether methyl diet could block this effect. In the first round of experiments (Wave 1), this hypothesis was supported. A replication experiment tested whether the effect would be altered by continuation of the methyl diet into adolescence (Wave 2) or limiting the dietary exposure to only gestation and lactation (Wave 3). In both Wave 2 and 3, the main effect of methyl diet in reducing adolescent nicotine consumption was in the same direction and magnitude as Wave 1, however, the p values were not significant due to increased variance. Furthermore, in Waves 2 and 3, there were no main effects of perinatal nicotine exposure on adolescent nicotine consumption, making it impossible to test if methyl diet blocks that effect. Meta-analysis indicated that the main effect of methyl diet was significant across studies, and merits further investigation. Additional tests indicated that methyl diet reduced ethanol consumption in a bottle choice test and in a “drinking-in-the-dark” limited access protocol, but did not change place conditioning response to injections of nicotine or cocaine. Methyl diet treated mice showed no marked changes in bitter taste response in a bottle choice test

for quinine consumption, but the trend was towards reduction. Overall, the results support the hypothesis that methyl diet induces a modest change in adolescent nicotine consumption and ethanol consumption, but it remains unclear whether these effects are due to specific changes in response to drugs of abuse or are generally true for novel tastants.

Introduction

Similar to the case in humans, perinatal nicotine exposure may cause increased adolescent nicotine consumption in male C57BL/6J mice (Klein et al. 2003). The experiments described in this chapter investigate whether the effect of perinatal nicotine exposure can be replicated in a much larger sample and a slightly different strain, and to determine whether the effect can be altered by perinatal exposure to a methyl supplemented diet. Any effects of methyl supplementation on adolescent behavior could act via epigenetic programming around the time of conception or during brain development later in gestation and after birth. Literature on methyl donor diets indicates that effects of supplementation on behavior occur during brain development, and suggest that supplementation during this period is sufficient to generate effects similar to those produced by supplementation throughout conception and gestation. Therefore, mice were given methyl supplementation either throughout conception, gestation, and lactation or during only a subset of this period (see Figure 2-2). In order to determine whether the voluntary nicotine consumption changes induced by methyl diet are specific to this drug, groups of mice were tested for patterns of consumption of ethanol and conditioned place preference responses to cocaine. In addition, to determine whether nicotine choice differences may be due to variations in response to bitter taste, a subset of mice were also tested for patterns of consumption of quinine.

The Problem: Behavioral Teratogenicity of Tobacco Smoke

Nicotine is a highly addictive drug, on par with heroin and cocaine (U.S. Department of Health and Human Services 1988, Stolerman and Jarvis 1995). Despite decades of public health warnings and legal action to reduce tobacco use, around one quarter of American children are still exposed to nicotine *in utero* or after birth, mainly due to exposure via parents' nicotine intake (Goodwin et al. 2007). Prenatal nicotine exposure appears more harmful than cocaine and certainly induces a far greater public health burden (Slotkin 1998). Eliminating addiction prior to pregnancy is most desirable, but until that is consistently achieved, it is important to investigate the actions of nicotine and the methods of preventing harm to the developing child.

The main effects of maternal smoking on pregnancy that have been identified and replicated through epidemiological studies include pregnancy complications, premature birth, low-birth-weight for gestational age, stillbirth, and sudden infant death syndrome (CDC 2004). Smoking is associated with higher risk of birth defects such as cleft palate (Viera 2008, Shaw et al. 2009), undescended testes (Barthold 2008), enamel defects in the teeth (Velló et al. 2009), renal hypoplasia (Slickers et al. 2008), and others. Long-term alterations in respiratory, renal, cardiovascular, and metabolic systems have also been observed (Pattenden et al. 2006, Doherty et al. 2009, Al Mamun et al. 2006, Somm et al. 2009), and the nervous system is not spared (Cornelius and Day 2009, Button et al. 2007, Ernst et al. 2001). Harm to brain development that leads to altered behavioral patterns can be called "behavioral teratogenicity" (Anandam et al. 1980, Paz et al. 2007).

One of the first strong pieces of evidence that parental smoking around the time of gestation influenced child behavior came from a study by Weitzman et al. (1992). With data on over 2,000 children aged 4-11 from the National Longitudinal Survey of Youth, the authors were able to control for many covariates of smoking, including family structure, income, education,

maternal psychological traits, home environment, and so on. Analyses showed independent effects of maternal smoking during pregnancy on child behavioral problems, with a dose response relationship based on a breakpoint of mothers' smoking more or less than a pack per day. Al Mamun et al. (2006) reported that adolescent smoking rates among children born to mothers who were smokers, but who had temporarily quit during pregnancy, were similar to children of non-smokers, while those whose mothers continued smoking during pregnancy had elevated smoking rates. A study by Neuman et al. (2007) reported that the effect of smoking on the risk for ADHD interacted with dopaminergic genotypes, with the greatest increase in risk of ADHD occurring among children with high risk DAT1 and DRD4 genotypes as well as smoke exposure. The literature on these topics is vast, and different studies have different strengths of design and magnitudes of outcomes. Overall, reviews continue to confirm the association between smoking tobacco and adverse behavioral outcomes in children, with modest but consistently measurable effects independent of known confounders. Among the more clearly supported phenotypes of children exposed to tobacco prenatally include increased risk of irritability and poor language responses in infants; increased risk of conduct disorder and inattention in early childhood; and attention deficit hyperactivity disorder and nicotine addiction in adolescents (see reviews by Cornelius and Day 2009, Button et al. 2007, Ernst et al. 2001). A substantial literature exists to suggest that second hand smoke exposure, often driven by paternal smoking, has similar effects (Herrmann et al. 2008).

A variety of hypotheses have been raised to account for the effects of tobacco smoke on the developing brain. Smoking causes low birth weight, which is a risk factor for poorer brain development. The carbon monoxide of tobacco smoke causes hypoxia, which causes brain damage and suppresses brain growth. The nicotine in tobacco influences receptors in the brain that guide brain development and could be responsible for altered cellular development. Hundreds of other potentially toxic chemicals are present in tobacco smoke that can also harm the

brain. Clearly, the effects of smoking observed in epidemiological studies are not necessarily the effects of tobacco smoke. There could be unmeasured confounding variables, such as maternal genes that increase the risk both for her own smoking and the adverse behavioral outcomes in her child. Furthermore, all of these genetic risk factors, harm mechanisms, and other environmental factors may interact. Facing this complexity, investigation of the specific biochemical effects of nicotine certainly requires research in controlled experiments (see reviews by Ernst et al. 2001, Thapar et al. 2009, D'Onofrio et al. 2008, Maughan et al. 2006).

Dissecting the precise role of nicotine in the harm that tobacco smoke does to the developing brain will help to guide preventative medicine for the unborn and treatments for babies and children who are exposed to tobacco smoke. Experiments specifically on nicotine are also important in identifying the risks of mothers who expose their children to pure nicotine. Pregnant women with strong tobacco addiction are offered nicotine replacement therapy during pregnancy to avoid the added burden of tobacco smoke if they fail to quit smoking by any other means (Benowitz and Dempsey 2003). In addition, some research supports using nicotine as a drug to prevent even more severe harm induced by other drugs during pregnancy (e.g. Yanai et al. 2010, Billauer-Haimovitch et al. 2009). Although rates of nicotine addiction have declined in many demographic groups, there are high rates of tobacco use in the home in many developing countries and in some regions smoking has increased among women after advertising campaigns by international companies (Graham 2009). Thus, the need to discover effective interventions against the harms of tobacco exposure during pregnancy is greater than ever.

Animal Experiments to Investigate Specific Effects of Perinatal Nicotine on Behavior

Controlled research on teratogenicity has been carried out in animals, usually rodents, and has helped to identify chemicals that increase of prenatal harm and guide policies to reduce human exposure. It has also empowered the investigation of treatments for pervasive teratogens, again providing the potential to improve the lives of children unfortunately conceived in and born into harmful environments. The effects of perinatal exposure on animals can be evaluated by examining the underlying neural processes that we share, even if our overt behaviors are different. In order to develop and validate a treatment for the behavioral teratogenicity of nicotine, the first step is to identify instances in which nicotine induces harmful changes in the brains of developing animals, and then provide treatments to those groups of animals in order to demonstrate that harm can be prevented, or at least ameliorated. Future studies can evaluate whether the prevention translates to humans.

Nicotine is a natural alkaloid of the tobacco plant (*Nicotiana* sp.). It apparently evolved as a chemical defense molecule against herbivory (Ohnmeiss and Baldwin 2000), but in mammals, it has attractive qualities that lead many humans to intentionally consume it regularly throughout their lives. The small molecule is soluble in water and lipids and passes freely across all membranes in the body. About 100 years ago, nicotine was found to have nerve-stimulating actions similar to acetylcholine (ACh), but only acts as an agonist on a subset of the ACh-sensitive nerves, while the remaining nerves could be stimulated by muscarine (Dale 1914). This original work had been done with plant extracts, and it was not known whether ACh was the natural ligand. A decade later, Loewi discovered that the vagus nerve released a substance that was capable of slowing the rate of contraction of an isolated frog heart (Loewi and Navratil 1926). Dale discovered that the active ingredient in the vagus secretion was the same ACh that he

had extracted from ergot fungi years before, and they earned the Nobel Prize for the discovery of neurotransmitter signaling.

The receptor molecules, although hypothesized nearly 100 years earlier, were not biochemically identified until they could be isolated via strong binding to α-bungarotoxin (Changeux et al. 1970), a lethal toxin from the venom of a Taiwanese snake. Subsequent work showed that nicotine binds to nicotinic cholinergic receptors (nAChRs) embedded in a cell membrane and opens a channel through the protein, allowing sodium, potassium, and occasionally other ions to flow into the cell (Changeux 1990, Lindstrom 2007). Thus, nicotine causes neural firing via specific receptors.

The functions of nAChRs are regulated by developmental control of receptor subtype. Each receptor is a ligand-gated ion channel constructed from five peptide subunits. At the neuromuscular junction, nicotinic receptors composed of α1, β1, δ, γ, and ε subunits are responsible for neuronal control of muscle twitching, but the effect of nicotine on these receptor types is relatively weak. The majority of the effects of nicotine of interest to this dissertation come from the other subtypes of nAChRs that are present on neurons throughout the body. They are generally composed of one or more α2-α10 and β2-β4 subunits. Nicotine is most effective at the lowest dose (as measured by dosage at which 50% of ligand opening occurs, the EC50 value) on the α4β2 receptors (5 μM), followed by α7 (40 μM), α3β4 (80 μM), and α3β2 (132 μM) (data from human receptors, Royal College of Physicians of London 2000).

The effects of nicotine on cholinergic signaling over the long term are complex and remain poorly understood because of the multiple adaptations that occur following initial binding. For example, neurons expressing α4β2 receptor subtypes experience rapid neural firing upon first exposure to nicotine, but they also begin to desensitize immediately, causing a subsequent decrease in natural ACh signaling on those receptors, which then can lead to compensatory changes in gene and receptor expression (Watkins et al. 2000).

Nicotine treatment prenatally and postnatally interferes with the normal role of acetylcholine in brain growth and development. Cholinergic neurons begin to express nAChRs in the brain around gestational day 12 in rodents and around the time of brain formation in gestational week 5 in humans (Naef et al. 1992, Dwyer et al. 2009). Once present on the neural membranes, the ligand-gated channels receive information from the neurotransmitter milieu outside the cell and stimulate cellular depolarization accordingly. These depolarization events mediated by nAChRs influence molecular signaling cascades and developmental trajectory of the neuron cell expressing the receptor and the post-synapic cells it innervates.

Depending on the intensity, depolarization can be necessary for cell survival and differentiation or a direct cause of cell death. ACh signaling through alpha-7 receptors tends to prevent the elongation of neurites (Pugh and Berg 1994, Lipton et al. 1988). Thus, as soon as ACh can guide the flow of cations, the fates of the cells expressing the receptor (and cells they innervate) are sensitive to cholinergic signaling. Nicotine's effects are primarily apoptotic early in development (Roy et al. 1998) and in adolescence (Abreu-Villaca et al. 2003), but in the adult hippocampus reports on the effects of nicotine have been mixed. Some found nicotine administration to be associated with increased neurogenesis, including studies with nicotine administration during the early postnatal period (Son and Winzer-Serhan 2009), adulthood (Mudò et al. 2007), and aged animals (Belluardo et al. 2008). However, others reported decreased neurogenesis, hypotrophy, and increased apoptosis after nicotine treatment (Abrous et al. 2002, Shingo and Kito 2005, Scerri et al. 2006, Huang et al. 2007).

During the equivalent of the first and second trimesters of human pregnancy (the prenatal period in rodents), nAChRs play a critical role in the development of catecholaminergic neurons in the ventral tegmental area (VTA), substantia nigra (SN), and the brain stem regions that control respiration and heart beat (Dwyer et al. 2009). For example, neurons in the VTA and SN express mRNA for α4 and β2 subunits and functional receptors within days of formation (Azam et al.

2007), and point mutations in the α4 cause failure of development of the substantia nigra (Labarca et al. 2001). These effects on dopaminergic cells are of primary interest for studies on drug use because nicotine's reinforcing properties are believed to be largely mediated by this system, like those of all addictive drugs (Koob and LeMoal 2005).

During the equivalent of the third trimester of human pregnancy (shortly after birth in rodents), transient upregulation of nAChRs occur together with the development of the cortex, hippocampus, and cerebellum (Dwyer et al. 2009). During this period, α7 receptor activity causes increased rates of cortical cell pruning during neuronal maturation. Increasing α7 receptor signaling by a genetic point mutation increases the rates of apoptosis (Orr-Urtreger et al. 2000). Loss of the β2 subunit in the cortex specifically during this period leads to learning deficits in adulthood (King et al. 2003). In addition, expression of nAChRs in the hippocampus govern GABA's switch from excitatory to inhibitory signaling that leads to electrical maturation (Dwyer et al. 2009). The effects of nicotine on brain development are likely to be mediated by the effects of nicotine on cholinergic signaling during these critical periods (Dwyer et al. 2009).

Ernst et al. (2001) reviewed behavioral changes induced by perinatal nicotine exposure in controlled animal studies and found that many, but not all, reported increased spontaneous locomotor activity. For example, Fung (1988) found that prenatal nicotine exposure caused an increase in locomotor activity by 14 day old rat offspring. However, initial locomotion during the first 5 minutes of exploration in a novel open field apparatus was reduced by nicotine in 19 day old rats; at the same time, the gestational nicotine group also showed reduced activity in the center of the apparatus, consistent with an anxiety-like trait (LeSage et al. 2006). A variety of studies report modest attention and memory impairments on various mazes, but nicotine has been found to increase fear learning (Paz et al. 2007) and to delay fear extinction learning (Eppolito et al. 2010). In order to address the confounding effects of forced nicotine treatment in most experimental treatments, which contrast with the voluntary intake by mothers in naturalistic

settings, Paz et al. (2007) studied non-forced nicotine exposure in the prenatal period of C57BL/6J mice. They found that voluntary nicotine intake caused hyperactivity, improved fear learning, increased preference for a cocaine-associated place, and lengthened the time to escape from learned helplessness tests. Vaglenova et al. (2004) found that even after cross fostering of prenatally nicotine treated mice to non-nicotine treated mothers, offspring still showed cognitive deficits and increased anxiety responses. These studies further support the hypothesis that nicotine exposure is a direct cause of the increased rates of hyperactivity disorders, major depression, and substance abuse in people exposed to tobacco early in life.

Thus, clear evidence from controlled animal research demonstrates that nicotine is a biologically active component in tobacco that alters brain development and nervous system function. Several of the effects of nicotine described from animal model experiments are consistent with the epidemiological findings in humans for risk of tobacco exposure early in development. Of particular interest to this dissertation is literature relevant to the hypothesis that perinatal nicotine can affect the responses to subsequent nicotine use, which is reviewed in the following section.

Effects of Perinatal Nicotine Exposure on Adolescent Nicotine Use

Many studies have reported that perinatal nicotine exposure increases use of nicotine and other addictive drugs. These outcomes have been found in a variety of measurement paradigms. For example, Klein et al. (2003) found that male (but not female) C57BL/6J mice born of dams given 50 µg/ml nicotine in their drinking water from the ninth day of gestation through weaning on postnatal day 21 showed higher nicotine consumption on a two bottle choice test. Adolescent male rats show higher levels of cocaine induced locomotor sensitization (Franke et al. 2007) and higher self administration of high dose cocaine in an operant responding paradigm (Franke et al.

2008) if they have been exposed to nicotine perinatally. Studies on adult female rats exposed to nicotine both *in utero* and during adolescence showed that prenatal exposure caused increased intravenous self administration of nicotine (Levin et al. 2006). Sobrian et al. (2008) found that 450 day old male rats that had been exposed to high dose nicotine during gestation (GD8-20) showed locomotor sensitization to nicotine that was not present in rats exposed to low doses of nicotine or saline. As mentioned above, Paz et al. (2007) reported increased conditioned place preference response from cocaine injections. One negative report used a very limited time of exposure (embryonic day 15 to birth) which might account for a lack of effect on nicotine consumption (Maehler et al. 2000). Combined, these results indicated that long-term nicotine exposure can cause stable changes in drug use phenotypes. Of greatest relevance to this dissertation are the effects of perinatal nicotine exposure on adolescent nicotine consumption in male mice.

The majority of adult smokers were exposed to nicotine during their youth. Initiation of smoking is more likely to lead to an enduring habit among adolescents; and animal studies show this may be due in part to increased susceptibility of adolescent neural systems to addiction (Laviola et al. 2003). Studies on rats suggest that adolescent animals acquire self-administration behavior faster and attain higher levels of nicotine self-administration (Chen et al. 2007). Adolescent mice have also been reported to prefer nicotine more than adult mice in a conditioned place preference test (Kota et al. 2007). One study found that early adolescents appear to be at a particularly high risk for high oral consumption (Adriani et al. 2002). Furthermore, Adriani et al. (2004) showed that nicotine injections in adolescence cause more long-term changes in certain addiction-relevant AMPA receptors than nicotine injections adulthood; and Placzek et al. (2009) found that adolescent but not adult animals showed glutamatergic AMPA receptor changes in ventral midbrain neurons in response to a single nicotine injection consistent with long-term

potentiation of excitatory dopamine firing. Thus, adolescence is the key widow to assess drug use outcomes that arise following perinatal exposure to nicotine.

In adolescence any gestational impacts of nicotine on the brain's developmental trajectories are still emerging as the brain matures. A variety of changes at the cellular and molecular levels could be involved in a predisposition to drug use. The most obvious possibility for perinatal nicotine exposure is alteration in cholinergic receptors that in turn influence dopaminergic activity in the reward circuit. nAChRs are present on dopaminergic neurons in the VTA, and stimulation of the VTA with a potent nAChR agonist increases nicotine self administration (Corrigal et al. 1994), probably via enhancing the response of VTA dopamine neurons to nicotine, and thereby enhancing the effect of nicotine on dopamine release to the NAcc. Even *in utero*, acute nicotine injections to the dam were found to cause increased forebrain dopamine release in the pups within half an hour (Ribary and Lichtensteiger 1989). In adolescence, basal activity in parts of the prefrontal cortex and nucleus accumbens is still altered after prenatal nicotine exposure (Park et al. 1996). As reviewed by Koob and LeMoal (2005), there are several neural routes by which nicotine can cause changes in the reward circuit, and it remains unclear which receptor locations and subtypes are most important for determining reward and dependence.

Several effects of perinatal nicotine exposure on cholinergic receptors in the reward circuit have been reported in adolescent animals. Van de Kamp and Collins (1994) found that in C3H mice treated with nicotine prenatally there was almost a two-fold increase in nAChR binding in the striatum, but in B6 mice nAChR binding was about one fourth lower in the prenatal exposure group. Most other findings from other rodent models are consistent with the downregulation of nAChRs in adolescence (similar to B6 mice). Abreu-Villaca et al. (2004) found a reduced number of cells in the midbrain of adolescent rats that had been exposed to nicotine prenatally. Chen et al. (2005) reported that after gestational treatment with nicotine,

nAChR binding in the reward circuit (PFC, NAcc, VTA) of adolescent rats was reduced by one fourth to one third, and the total number of VTA neurons was reduced by 15%.

Not surprisingly, these changes in nAChR levels are correlated with changes in response to nicotine in adolescence. Kane et al. (2004) found that in adolescent rats that had been exposed to nicotine during gestation, acute nicotine treatment induced a smaller increase in dopamine release to the nucleus accumbens shell (attenuated initial response). Interestingly, Gold et al. (2009) reported that in control animals, repeated nicotine exposure led to a decline in the amount of dopamine that was released to the striatum after nicotine exposures in adolescent male rats; however, in animals that had been exposed to nicotine prenatally, the dopamine release did not decrease. This failure to habituate might be a mechanism for sustained reward after nicotine exposure in adolescent animals.

Abreu-Villaca et al. (2004) studied the effects of prenatal nicotine exposure on the expression of nAChRs in response to nicotine in adolescent rats. Control rats showed the normal upregulation of nAChRs after exposure to nicotine, but this response was attenuated in the rats that had been exposed to nicotine prenatally. Control rats also showed a depression of cholinergic neuronal firing following nicotine withdrawal, and this response was actually exacerbated in the prenatal nicotine group. This exacerbated withdrawal phenotype could indicate more severe nicotine dependence in adolescent rodents exposed to prenatal nicotine.

Thus, a variety of differences in the function of reward pathways and cholinergic signaling have been reported in adolescence between rodents exposed to nicotine before birth. These may be responsible for the increases in drug use phenotypes observed between nicotine exposed animals and unexposed controls. However, it remains unclear which (if any) of these neural factors explain the large differences between individuals in nicotine consumption within treatment groups (Dadmarz and Vogel 2003), even between inbred mice (Gyekis et al. 2010). By replicating Klein et al.'s finding that perinatal nicotine consumption increases adolescent nicotine

consumption (2003), a model will be in place to test methods of blocking the behavioral teratogenicity of nicotine. Given the consistency of nicotine effects on drug consumption behaviors between animal and human studies, any positive findings have the potential for making important contributions to the prevention of substance abuse.

The Role of Perinatal Methyl-Donor Supplementation in Nervous System Development

A classic method of intervention in health is nutrient supplementation, which remains important today. Even among people who can afford to eat a highly hedonic diet, sub-optimal nutrition is still commonplace, especially during pregnancy, and especially for compounds that contribute to single carbon metabolism like choline, folic acid (folate), and vitamin B12. For example, spina bifida is a neural tube defect that can be almost completely prevented with dietary supplementation, with significant effects from each of the three methyl-donor compounds mentioned above (Shaw et al. 2004, Pitkin 2007, Kirke et al. 1993). Only in 1998 was choline declared an essential nutrient in the United States; in the same year the U.S. mandated supplementation of grain products with folic acid in order to reduce risk of birth defects and cardiovascular disease in the general population (Lucock 2000). Recent research indicates that these compounds may also be beneficial for brain development (Fernstrom 2000), but despite the known benefits of supplementation, concerns remain about potential harms of chronic exposure to high doses of folate and related compounds (McNulty and Scott 2008, Lucock and Yates 2009).

As carbon based life forms, it is not surprising that methyl donor compounds are required for survival, or that dietary sources of methyl donors are vital. The biochemical actions of choline, folate, and vitamin B12 all converge in their influence on the formation of S-

adenosylmethionine (SAM). SAM serves as the methyl donor cofactor for methyltransferase enzymes that synthesize nucleic acids, neurotransmitters, and protein functional groups. Knocking out enzymes required for single carbon metabolism are usually embryonic lethal, and alleles that alter the function of these enzymes are associated with increased risk of birth defects, higher dietary requirements for methyl donor compounds, and increased risk for psychiatric disorders (Zeisel 2006a, Lucock 2000). SAM also facilitates the methylation of homocysteine back to methionine. Because systemic high levels of homocysteine are toxic to a variety of cell types, maintenance of adequate levels of SAM may slow rates of neurodegeneration with aging (Mattson and Shea 2003).

In addition to the methylation effects it shares with other methyl donors, choline can also alter cholinergic neuronal activity and membrane properties. Cholinergic effects can be directly mediated by direct effects of the dietary choline or they can be mediated by increased production of ACh, for which choline is the direct precursor (Blusztajn 1998). Choline itself is a selective α7 agonist that causes upregulation of α7 receptors, but not other types of nAChRs, in adolescent rats (Guseva et al. 2006). The majority of dietary choline serves as the charged moiety of the abundant double chained lipids that form cell membranes in the brain and throughout the body, both sphyngomyelin and phosphatidylcholine (Blusztajn 1998). It is difficult to separate the effects of membrane choline from methyl donor choline and neurotransmitter choline, because choline can be converted back and forth to each form. However, knockout mouse studies have shown that knocking out enzymes that govern phospholipid metabolism are often embryonic lethal and CTP:phosphocholine cytidylytransferase β knockout mice show defective axon branching (Vance and Vance 2009). Thus, choline could be an especially important contributor to methyl diet effects on neural development, because it influences methylation, cholinergic activity, and neural membranes.

A wide variety of animal studies show the learning and memory benefits of diets supplemented with more choline than standard chow (see review by Zeisel 2006b). These effects have been seen on many apparatuses and in both rats and mice, and appear to be mediated by choline effects on survival of developing hippocampal neurons and later improvement in LTP in adulthood (Zeisel 2006b). Of particular interest are the findings that choline supplementation in early postnatal days can modulate some of the negative consequences of perinatal exposure to alcohol that are also seen with perinatal exposure to nicotine. For example, choline injections on postnatal days 4-30 can block the adolescent hyperactivity and learning deficits caused ethanol exposure on days 4-9 (Thomas et al. 2004). In further experiments by the same group, choline injections on days 11-30 were found to only partially reduce the spatial learning deficits, indicating a broad therapeutic window of choline supplementation from the third trimester equivalent throughout childhood (Ryan et al. 2008). Thus, there is already evidence that methyl donor supplementation can block the behavioral teratogenicity of a drug of abuse.

One key mechanism of action for choline is its role in neurotrophic factor expression. Studies have long shown that perinatal choline supplementation increases hippocampal neuronal survival, LTP, and learning (Blusztajn 1998); blocks excitatory neurotoxicity in adolescent rats (Guo-Ross et al. 2002, Holmes et al. 2002); and prevents the learning deficits induced by early maternal separation (Tönjes et al. 1986). Concurrent with these functional changes, choline induces a long-term sustained upregulation of brain derived neurotrophic factor (BDNF) in the hippocampus of prenatally exposed animals (Glenn et al. 2007). Neurogenesis may critically influence susceptibility to addiction, because whole brain irradiation, which impairs neurogenesis, increases cocaine self administration in rats (Noonan et al. 2010). As the authors reviewed, drug taking behaviors are reduced in a variety of experimental conditions that increase hippocampal neurogenesis, and vice versa. Further experiments are needed to identify effects of

pro-neurotrophic environmental conditions and pharmacological exposures on nicotine consumption.

In sum, perinatal methyl donor supplementation can increase cholinergic receptor levels, upregulate neurotrophic factors and neurogenesis, and raise cognitive abilities. Each of these are opposite of the traits found in perinatal nicotine exposure. Therefore, dietary supplementation might block some of the harms of gestational nicotine treatment summarized in the section described above. The hypothesis follows that the increased adolescent nicotine consumption of male offspring exposed to nicotine could be blocked by concurrent methyl donor treatment in the perinatal period. The studies on fetal alcohol syndrome indicate that dietary supplementation throughout the rodent brain developmental period are likely to be important.

An Animal Model to Test the Effects of Methyl Supplements on Nicotine Consumption

The genetic background of the Agouti viable yellow mice is ideal for study of nicotine consumption phenotypes and the role of methyl supplements. The A^{vy} allele, which arose on a C3H background, has been largely bred onto the C57BL/6J genomic background, but between 1/16 to 1/4 of C3H background remains (Yen et al. 1994). The B6 background is ideal for studying drug addiction phenotypes because these mice have a strong preference for many drugs compared to other mouse strains (McClearn and Rodgers 1959, Horowitz et al. 1977, Morse et al. 1993, Robinson et al. 1996). The relatively high drug consumption is observed across experimental paradigms and in nicotine oral consumption the high nicotine consumption is not due to high initial acceptance of bitter taste, but rather differential response to the reinforcement after ingestion (Glatt et al. 2009). Since C3H mice consume less nicotine than B6 mice (Robinson et al. 1996) and specific C3H alleles are known to be associated with lower oral nicotine

consumption than B6 strains (Li et al. 2007), Xiaofan Li (no relation) and David Vandenbergh genotyped A^{vy}/a mice at the four alleles with the greatest contribution to variance in nicotine consumption in Li et al. (2007) and found that all loci had B6 alleles, confirming that the agouti model has the B6 genetic background needed for studying substantial nicotine consumption at these 4 loci. In addition, a variety of studies suggest that changes in the agouti gene might also affect response to drugs of abuse such as cocaine and opiates (Alvaro et al. 1996, Alvaro et al. 2003, Bronstein et al. 1990, Hsu et al. 2001, Hsu et al. 2005, Lindblomb et al. 2002, Rapaka et al. 2008, Starowicz et al. 2003). Therefore, we tested for effects of A^{vy} genotype on nicotine consumption.

To test for perinatal nicotine effects on adolescent nicotine consumption, nicotine is administered through maternal drinking water, as described previously (Sorenson et al. 1991, Sparks and Pauly 1999, Klein et al. 2003). The route of administration can dramatically affect perinatal outcomes (see Muneoka 1997), and it is particularly important to avoid stressing the dams given the effects of stress on behavior and that these effects overlap with perinatal nicotine effects (Dwyer et al. 2009). Oral nicotine does not involve handling, injections, or surgery, making it the least invasive method available, but it leads to variation in exposure between dams depending upon to their total fluid intake, which is a variable measured in the current study.

These mice have also served an important role in assessing the consequences of methyl diet supplementation. The dietary exposure is a standard methyl supplemented diet previously found to alter coat color spectrum of A^{vy} mice when supplemented around the time of conception and early gestation (Waterland and Jirtle 2003, Cooney et al. 2002). The recipe is the Standard Harlan Teklad AIN-93G 7% corn oil diet (which already includes 2.5 g/kg choline bitartate) further supplemented with 7.97 g/kg choline chloride, 4.3 mg/kg folic acid, 530 mg/kg of 0.1% vitamin B12, and 5 g/kg betaine. Mice were exposed to the diet during breeding. In the first experiment, Wave 1, both parental animals were given the test diets beginning two weeks before

mating and the diets continued until weaning. In Wave 2, the diet also started 2 weeks before weaning but continued throughout offspring phenotyping. In Wave 3, the test diets only began 5 days after cohabitation of the male and female for mating and ended at weaning. In Wave 4, the test diets began 2 weeks before mating but were discontinued at 5 days after cohabitation of the male and female for mating. In the B6 cohort, the diet began 2 weeks before mating and continued throughout offspring phenotying.

The effects of perinatal condition on adolescent nicotine consumption of male mice are evaluated in a three-bottle choice test in adolescence from postnatal (PN) days 35-42 (similar to Klein et al. 2003). The modification to include three bottles (0, 50 and 200 µg/ml nicotine) is expected to increase overall nicotine consumption on account of the increased number of nicotine bottles available (Tordoff and Bachmanov 2003). The bottle choice method is a high throughput, minimally invasive approach to drug consumption phenotyping with reasonable face validity and good correspondence with addiction-relevant phenotypes in other drugs of abuse, but additional work needs to be done to validate the relevance of voluntary oral nicotine consumption to addiction (Dingman et al., in preparation).

Overall, this model allows for the ready detection of differences in consumption according to perinatal nicotine exposure and methyl diet with minimal stress to the mice.

Summary: Testing the Hypothesis that Methyl Diet Can Block the Behavioral Teratogenicity of Perinatal Nicotine Exposure

Exposure to tobacco smoke in humans is associated with a broad array of physiological and behavioral health problems. Some of these problems, including increased propensity for substance abuse, have been linked to perinatal nicotine exposure in controlled experimental studies of rodents. Methyl supplemented diets have the potential to block this behavioral

teratogenicity, as it blocks some similar behavioral harms of perinatal alcohol exposure and has the potential to alter acetylcholinergic development, the initial target of perinatal nicotine. Therefore, tests of adolescent nicotine consumption in male A^{vy}/a and *a/a* mice after exposure to nicotine and/or methyl supplementation evaluate interactive effects of these exposures on substance use.

The essence of the proposed dissertation rests on six main hypotheses that build upon each other. First, it is hypothesized that nicotine exposure of male agouti mice via drinking water with 200 μg/ml nicotine will produce an increase in adolescent nicotine consumption of offspring similar to the effect seen in C57BL/6J dams given 50 μg/ml nicotine (Klein et al. 2003). Second, it is hypothesized that treatment of the parental animals with the methyl supplemented diet as described above can prevent the increase in adolescent nicotine consumption induced by perinatal nicotine exposure. The first and second hypotheses were tested synchronously in each wave of the study.

Third, it is hypothesized that the effects are mediated by methyl diet treatment during brain development from around embryonic day 5 to weaning. Thus, supplementation during this time period will generate the same effect as supplementation of both dam and sire diet starting two weeks before conception. A fourth hypothesis (either alternative or complementary to the third) is that dietary supplementation prior to mating through conception also influences adolescence nicotine consumption. By changing the timing of food delivery to the parental animals in Waves 3 and 4 (Figure 2), it is possible to separately test the effects of dietary timing and thereby gain insight into critical periods of dietary treatment. If true, the fourth hypothesis would predict that higher levels of early embryonic methylation activity would reduce nicotine consumption in adolescence due to prenatal effects.

The fifth hypothesis is that changes in adolescent nicotine consumption by methyl diet could translate to changes in voluntary consumption of other drugs of abuse. Therefore, mice will

be tested for differences in ethanol consumption induced by each perinatal condition in Wave 2. The hypothesis that methyl diet proffers protection against other drugs will be supported if methyl diet supplemented groups show reduced alcohol consumption in a three-bottle preference test similar to the nicotine test described above. Similarly, a group of C57Bl/6J mice in the B6 cohort will test the effects of methyl diet on limited access ethanol consumption and cocaine conditioned place preference. Lower ethanol consumption and reduced cocaine place preference would support the hypothesis of lower nicotine consumption being part of a more generalized change in response to drugs of abuse.

Finally, it is possible that differences in oral drug consumption on the three bottle choice tests could be mediated by changes in ability to learn bottle discrimination (Blizard et al. 2008) or effects of diet on taste preference. Therefore, the sixth hypothesis is that methyl diet may influence preference for quinine-flavored water. By giving some mice from each prenatal treatment group access to a quinine three-bottle choice test, it will become evident if general bitter taste avoidance is altered by perinatal nicotine and/or methyl diet exposure.

From these hypotheses, we should be able to confirm whether perinatal nicotine exposure influences adolescent nicotine consumption in male mice, and determine whether this effect of perinatal nicotine can be blocked by methyl diet supplementation.

Methods

Animal Husbandry

In Waves 1-4, heterozygous male mice with the agouti viable yellow (A^{vy}) mutation on a C57BL/6J genetic background were mated to *a/a* dams. Mice were maintained in standard “shoe-

box" cages and assigned to dietary and fluid consumption conditions as explained below. Sires were separated from the dams two weeks after co-housing. Dams were checked daily in the light phase to record litter date of birth. Litters were weaned on postnatal day 27 and weighed, scored for coat color (black, or, if A^{vy}, estimated percent yellow coverage), and sexed. Coat colors were categorized into six groups following the method of Cooney et al. (2002). Male weanling mice were cohoused with littermates until the initiation of oral nicotine consumption testing.

In the first group (Wave 1), weanling A^{vy} mice were also photographed. From photographs of A^{vy} mice, three independent observers scored percent yellow coat coverage, which was correlated r = 0.9 between observers.

In addition, six litters of C57BL/6J mice derived from the colony in the Penn State barrier facility were subsequently tested with breeding and weaning procedures the same as for the agoutis. These are referred to below as the "C57BL/6J Cohort" or simply "B6".

Methyl Supplemented and Control Diets

Two weeks before mating, parental mice were randomly assigned to dietary experimental conditions. There were two control diets. The standard lab chow used in the building is LabDiet 5001, ordered from PMI Nutrition International (St Louis, MO), with 4.5% fat and 3.0 kcal/g. The control diet (C) for comparison to the methyl diet was the AIN-93G diet, modified to use 7% corn oil instead of soybean oil (Harlan Teklad), 3.8 kcal/g (the same control diet as Dolinoy et al. 2006). The methyl diet (M) was the the same diet containing added folate, vitamin B12, choline, and betaine. C and M diets were ordered from Harlan Teklad to be supplemented the same as in previous studies (Waterland and Jirtle 2003—though note slight differences). Methyl donor and macronutrient composition of these diets is shown in Table 2-1.

The schedule of dietary supplementation varied between experiments. In Wave 1, dams and sires were assigned to either C or M dietary conditions two weeks before mating and continuously maintained on the diet until pups were weaned (about 50 days later, including conception, gestation, and lactation). Upon weaning, Wave 1 offspring were given standard chow. Wave 2 served as an identical replicate of Wave 1 except offspring were maintained on M or C diets after weaning and throughout testing. For the sake of all analyses of weaning data, Waves 1 and 2 were identical and their data are combined. Parental animals for Wave 3 were maintained on standard chow until 5 days after cohabitation and then assigned to M or C diets. Thus, Wave 3 litters were exposed to M or C conditions throughout the majority of gestation until weaning. Offspring of Wave 3 were returned to standard chow upon weaning. Parental animals in Wave 4 were given M or C diets from 14 days before mating until 5 days after cohabitation. Thus, they were exposed to M and C conditions from the period leading up to mating until up to 5 days after mating, the same as described in Chapter 2 and Figure 2-2.

In addition, three litters of C57BL/6J mice were given the same control diet and three litters were given the same methyl diet. The schedule of treatment was continuously through conception, weaning, and phenotyping (akin to wave 2).

Forced Perinatal Nicotine Exposure through Maternal Drinking Water

Two weeks before mating dietary conditions were imposed and mice from both dietary groups were randomly assigned to consume 200 µg/ml free-base nicotine (High Nicotine, HN), 50 µg/ml free-base nicotine (Low Nicotine, LN), or water (W). Fluid conditions were maintained continuously until weaning, when mice were returned to water. In Wave 1 (see above), only W

and HN conditions were assigned. Water was administered through standard cage bottles, while nicotine was administered through graduated cylinders positioned on the opposite side of the cage. In Waves 2-4, mice from each dietary condition were assigned to W, LN, or HN fluid conditions, and all fluids were administered through graduated cylinders.

The number of successful litters delivered in each experimental dietary by nicotine exposure treatment condition is summarized in Table 3-1. The low number of matings in Wave 4 Control Diet group was due to fewer matings established, not low breeding success.

There was no perinatal nicotine exposure for the C57BL/6J cohort.

Table 3-1. The number of offspring born (and number of successful litters delivered in parentheses) in each experimental dietary by nicotine exposure treatment condition.

	Water	**Low Nicotine**	**High Nicotine**
Wave 1 Control Diet	73 (11)	-	75 (11)
Wave 1 Methyl Diet	81 (10)	-	85 (11)
Wave 2 Control Diet	71 (9)	47 (6)	44 (7)
Wave 2 Methyl Diet	50 (7)	43 (7)	44 (7)
Wave 3 Control Diet	40 (5)	37 (5)	67 (4)
Wave 3 Methyl Diet	23 (4)	34 (4)	30 (4)
Wave 4 Control Diet	9 (1)	23 (3)	36 (4)
Wave 4 Methyl Diet	37 (4)	30 (4)	14 (2)

Adolescent Nicotine Consumption Testing

On postnatal day (PN) 32, male mice were weighed, individually housed, and offered three water bottles. On PN 35, water consumption was recorded and bottles were replaced with two bottles of nicotine solutions (200 and 50 µg/ml freebase nicotine in tap water) and one water bottle. Each day for the next 7 days, fluid consumption from each bottle was measured daily. Initially, 200 µg/ml nicotine was placed on the left (from the mouse's point of view), 50 µg/ml nicotine in the center, and water on the right. Then each day the bottles were rotated to the left, such that on the second day the order was 50 – 0 – 200 µg/ml, the third day 0 – 200 – 50 µg/ml, and so on across the seven days of testing. Nicotine solutions were replaced every 3-4 days.

Methodology differed between study periods. In the first study period (Wave 1), 126 adolescent male mice were weighed on days 1, 3, 5 and 7 of the oral nicotine three bottle choice test and cages were changed once during the test. During Wave 1, cages were changed for fresh bedding once per week (according to normal caretaking schedules), including during adolescent nicotine consumption testing, but in all subsequent experiments no cage changes were conducted during adolescent nicotine consumption testing (to reduce disturbance during the test). In Wave 1, consumption was estimated from differences in volume readings on the graduated cylinders. Apparent errors in bottle readings led to occasional extremely high or negative fluid consumption values found during data analysis, which were deleted according to outlier status.

In the second study period (Waves 2-4), 287 adolescent male mice were weighed on day 1 and again on day 7 and cages were not changed during the choice test. In Waves 2-4, consumption was estimated from differences in bottle weights. Data were only deleted if bottles were observed as leaking or vapor locked at the time of bottle handling.

Daily measurements of fluid loss from bottles on empty cages were on average 0.3 ml, which was subtracted from bottle readings. Testing on cages without mice but experimenters

drawing water from the sippers by touching the tips with paper towels showed that consumption readings based on weighing the bottles and by reading volume of the graduated cylindars were well correlated (R^2 = 89.7.8%). In this test, change in mass of the paper towels was considered the “true consumption”. “True consumption” explained 93.8% of the variance in volumetric readings and 98.8% of the variance in readings from bottle weights. The reduced error in measures using bottle weights justified the transition in methodology from Wave 2 forward.

Comparison of weight and volume measurements indicated excellent agreement between the methods, but bottle weights had a lower variance than volume readings, apparently due to occasional gross errors in reading the bottle volumes (data not shown).

Note that percent nicotine measures have equal contributions of variance from both nicotine bottles, while nicotine consumption outcomes in mg/kg diminish the effect of variance in the 50 µg/ml bottle by 4-fold relative to the 200 µg/ml bottle. Thus, preference focuses on bottle discrimination irrespective of concentration, while mg/kg consumption focuses on physiological exposure to the drug. If an experimental effect is more significant in percent consumption and less significant in dosage consumed, this may be due to greater effects of that treatment on consumption from the low nicotine bottle.

The oral nicotine consumption phenotyping of the B6 cohort offspring followed different methodologies and schedules. 42 mice of both sexes were tested. The choice tests began in the juvenile period, postnatal day 29, and continued throughout adolescence. For the first 9 days, the bottle choice method used a three bottle system that was symmetric (no side preference effects) and only evaluated one concentration of nicotine at a time (Tordoff and Bachmanov 2003). Mice were randomly assigned to begin nicotine choice testing with either 50, 200 or 400 ug/ml nicotine solutions, and randomly assigned to have either 2 water bottles and 1 nicotine bottle (W-N-W) or 1 water and 2 nicotine bottles (N-W-N). After 3 days, consumption was measured. Then, bottles were reset, and concentration of nicotine moved from 50 to 200, 200 to 400, or 400 to 50 ug/ml,

while number of nicotine bottles switched from 1 to 2 or 2 to 1. This process was repeated for three rounds, so that each mouse experienced each nicotine concentration for three days. By this time mice were 38 days of age. Then mice were assigned to a nicotine choice system similar to the method described in Chapters 2-4 (three bottles, containing 0, 50 and 200 ug/ml nicotine solution), but bottles were only measured and rotated every three days. This continued for another 9 days, until mice were 47 days old. Access to nicotine was continued in the second three bottle choice procedure until conditioned place preference testing began between 7-8 weeks of age.

Additional Wave 2 Fluid Consumption Testing: Ethanol and Quinine

In Wave 2, on PN 35, mice were randomly assigned to initially consume nicotine, ethanol, or quinine (see Table 2). Ethanol and quinine consumption tests were three bottle choice tests identical to nicotine consumption tests except for bottle contents. For ethanol consumption testing, the high ethanol bottle (HE) contained 10% ethanol and the low ethanol bottle (LE) contained 2.5% ethanol. These concentrations of ethanol have long been used to discern strain differences in ethanol choice testing (e.g. Kakihana and McClearn 1963) and preferences have predictive validity for other ethanol-related phenotypes (e.g. Metten et al. 1998). For quinine consumption testing, the high quinine (HQ) bottle contained 0.4 mM quinine and the low quinine (LQ) bottle contained 0.1 mM quinine. The LQ condition is considered “mildly unpalatable” (Blizard and Adams 2002), and higher concentrations are increasingly aversive. For both quinine and ethanol, individual variability in consumption phenotypes were expected at these concentrations.

After this first week of testing, Wave 2 mice that had first been assigned to nicotine consumption tests were then assigned to a second week of testing (by the same protocol) with either ethanol or quinine (as described above). The Wave 2 mice tested for quinine or ethanol

consumption in the first week were tested for nicotine consumption in the second week (age 42-50 days), see Table 3-2.

Table 3-2. Bottle choice sequences for mice in Wave 2.

	35-42 Days	**43-50 Days**
Test Group 1	Nicotine 3 Bottle Choice	Ethanol 3 Bottle Choice
Test Group 2	Nicotine 3 Bottle Choice	Quinine 3 Bottle Choice
Test Group 3	Quinine 3 Bottle Choice	Nicotine 3 Bottle Choice
Test Group 4	Ethanol 3 Bottle Choice	Nicotine 3 Bottle Choice

Wave 1 Hole Board Activity Testing

On three consecutive nights during baseline water consumption observations (PN 32-34), at 3-4 hours into the dark cycle, Wave 1 mice were placed in a 16” by 16” square hole board apparatus under low light conditions for 3 minutes (red lights and computer screen). During this test, infrared beam breaks recorded nose pokes in the 4 holes, rears, and ambulation throughout the apparatus.

Conditioned Place Preference Testing: Waves 2 and 3

About one week after the end of fluid consumption tests, 39 mice from Waves 2 and 3 were included in a nicotine conditioned place preference test based on Grabus et al. (2006) and Brunzell et al. (2009). The conditioned place preference testing chamber was a 16×16 inch square box with a divider down the center and white floor/walls on one side and black floor/walls on the

other. The divider could be open to allow access to both sides of the apparatus in the baseline and retention trials, or it could be closed for conditioning trials. Mice were randomly assigned for conditioning in the black or white chambers, and for first entry into the black or white chamber for baseline and retention trials. Testing order was randomly assigned but remained the same throughout the baseline testing, conditioning, and retention trials. All testing was conducted under red light. Between mice, the testing apparatus was simply wiped with a wet paper towel (with water) then wiped dry.

On testing day 1, mice were placed into the testing chamber for 15 minutes about 2 hours into the light phase. Time spent in each side of the chamber was recorded with Anymaze (Stoelting, Wood Dale, Illinois). Baseline tests were conducted 2-4 hours into the light phase.

On testing days 2, 3, and 4, each mouse experienced two procedures on the same day. In the first procedure, about 2 hours into the light phase, mice were weighed. Then, 2-4 hours into the light phase, mice were given a subcutaneous injection of saline (0.9% NaCl) of about 0.1 ml in volume. Then, after 5 minutes, mice were placed in the non-drug paired side of the apparatus for 30 minutes. In the second procedure, about 5-7 hours into the light cycle, mice were given a 0.5 mg/kg subcutaneous injection of nicotine solution of about 0.1 ml. Then, after 5 minutes, mice were placed in the drug-paired side of the testing chamber for 30 minutes.

On testing day 5, mice were again placed into the testing chamber for 15 minutes with access to each side of the apparatus, and time spent on each side was recorded. Baseline tests were conducted 2-4 hours into the light phase.

Preference scores were calculated as time (seconds) spent on the drug-paired side of the chamber during the retention trial minus time spent on the same side during the baseline test.

Conditioned Place Preference Testing: C57BL/6J Cohort

Five litters (3 control diet, 2 methyl diet), with 35 individuals of both sexes, were tested for nicotine or cocaine conditioned place preference. The place conditioning protocol varied between litters as the lab explored different factors that might influence whether nicotine preference would emerge. The protocol variants are all designed to test the same underlying construct, so data are merged for analysis. Since protocol differences were each in different litters, when the mixed model analysis controls for litter effects it also controls for main effects of protocol. In all cases, mice were randomly assigned to drug condition; doses were 10 mg/kg for cocaine and 0.3 mg/kg for nicotine injected 5 minutes prior to entry into the conditioning chamber. Testing order was randomly assigned but remained the same throughout the baseline testing, conditioning, and retention trials.

The conditioning apparatus was a box with a small central neutral chamber and two side chambers each 8 inches square. For baseline and follow-up preference testing mice were intitially placed in the neutral chamber for each test. The side chambers were distinguished by differences in flooring (mesh vs smooth) or either wall coloring (black vs. white or vertical vs. horizontal black and white stripes).

In litter 1, the baseline test was the first exposure to the apparatus. Baseline side preference testing was 15 minutes. Then 3 conditioning sessions for both saline and drug were conducted with saline conditioning in the morning and drug conditioning in the afternoon. Each conditioning session was 30 minutes. Follow-up side preference testing was 15 minutes.

In litter 2, the protocol was the same as litter 1 except for the following changes: animals had one day of habituation to the apparatus and on each day of conditioning; animals had at least 30 minutes of habituation to the testing room before injection; and the baseline and follow-up tests lasted for 20 mintues each.

In litters 3-5, the protocol was the same as litter 2 except for the following change: conditioning sessions to saline and drug were conducted on alternate days, for a total of 4 saline and 4 drug sessions (8 days of conditioning).

Preference scores were calculated as time (seconds) spent on the drug-paired side of the chamber during the retention trial minus time spent on the same side during the baseline test. By linear transfer, these can be converted into a change in percentage of time spent on the drug side, which does not affect inferential statistical tests (merely dividing by total number of seconds).

Conditioned place preference protocols were developed and carried out by Marc Dingman based on previous work (Grabus et al. 2006; Brunzell et al. 2009) in order to test the hypothesis by the author (Joseph Gyekis) that methyl diet might reduce place preference for these two potentially rewarding or aversive drugs.

Limited Access Ethanol Consumption: C57BL/6J Cohort

After place preference testing, C57BL/6J mice were given limited access ethanol exposure. Mice were randomly assigned to either ethanol only or ethanol with nicotine conditions for the “drinking in the dark” procedure. For the ethanol only group, the procedure essentially followed the standard “drinking in the dark” procedure (Rhodes et al. 2005). On day 0 of testing, mice were randomly assigned to go through drinking in the dark during a nicotine choice test or not. If assigned to the nicotine group, on day 0, mice were given one bottle of 50 ug/ml nicotine and one water bottle. If assigned to the water group, mice were given two water bottles. Some mice were moved to a novel home cage, with metal floor plates, for lickometer measurements of nicotine consumption. During the 10 pm to midnight period on days 1-4, mice from the nicotine condition were given a 20% ethanol bottle to replace their water bottle, and continued to receive

the 50 ug/ml nicotine solution. During this dark phase, both water bottles for mice in the non-nicotine group were replaced with 20% ethanol. The rest of the day on days 1-4, mice continued to receive either one water bottle and one 50 ug/ml nicotine bottle or two water bottles.

Statistics

Statistical testing was completed with SAS version 9.2 (Cary, North Carolina). The Proc Mixed procedure was used for multilevel restricted maximum likelihood regression models (Littell 2006). In short, nicotine consumption outcomes were evaluated with a repeated measures design accounting for the nesting of individual mice within litters (in repeated statement). The degrees of freedom were calculated according to the method developed by Kenward and Roger (1997). After estimating the base model with compound symmetry covariance structure and accepting covariates based on significance ($p < 0.05$), models were run again with unstructured, one step autoregressive, and Toeplitz error structures and the final model was accepted based on the lowest Bayesian Information Criterion (BIC) score among these covariance structures. Covariates tested in nicotine consumption regressions included day of testing, current body weight, and water position (left, right, or center). Between subjects effects included perinatal exposure conditions (diet, nicotine), study period (wave 1 vs. waves 2-3), and week of testing (only relevant to wave 2). The main output of the regression is two tables: “solutions for fixed effects” and “type 3 test of fixed effects”. The solutions for fixed effects table provides the average difference between each specific group vs. a reference condition for class variables and beta coefficients for quantitative variables after accounting for each other predictor in the model (with t-values). The type 3 fixed effects reports the overall effect of a variable jointly testing all of the conditions within the class, akin to normal ANOVA (with F values).

This method of regression depends upon the assumption of normally distributed data. Distributions were evaluated with Kolmogorov-Smirnov (KS) tests for deviance from normality in SAS. Exponential transformations were attempted to normalize data, and if data were normal after transformation, the transformed data were used in the regression models. The significance threshold of $p < 0.05$ was accepted as significant.

Results

Wave 1

In this experiment, 126 male heterozygous Agouti viable yellow mice (A^{vy}/a) and black littermates (a/a) from 33 litters were treated with the control vs. methyl diet and exposed to either tap water or 200 µg/ml nicotine continuously from conception through weaning.

Wave 1 Body Weight

Perinatal exposure to either methyl diet and/or nicotine was associated with reduced body weight at weaning on day 28, and the two exposure conditions interacted such that the body weight reduction effect of nicotine exposure was greater in mice on the control diet (Figure 3-1A). These main effects of diet and fluid intake remained significant after controlling for significant covariates—litter size, agouti genotype, and sex. Specifically, methyl diet mice were found to be 1.82±0.28 g lighter ($t = -6.51$, $p < .0001$). Nicotine exposed mice were 1.12±0.25 g lighter ($t = -4.41$, $p < .0001$). Interaction of these perinatal conditions showed that control diet and high nicotine exposed mice were 1.11±0.38 g lighter than expected from the main effects ($t = -2.96$, $p = 0.0034$). Meanwhile, as expected, litter size reduced body weight (by 0.17±0.07 g for each additional littermate, $t = -2.29$, $p = 0.0230$), A^{vy} mutation increased body weight (by

1.10±0.19 g, t = 5.89, p <.0001), and female sex was associated with lower body weight (1.06±0.19 g lighter, t -5.62, p <.0001).

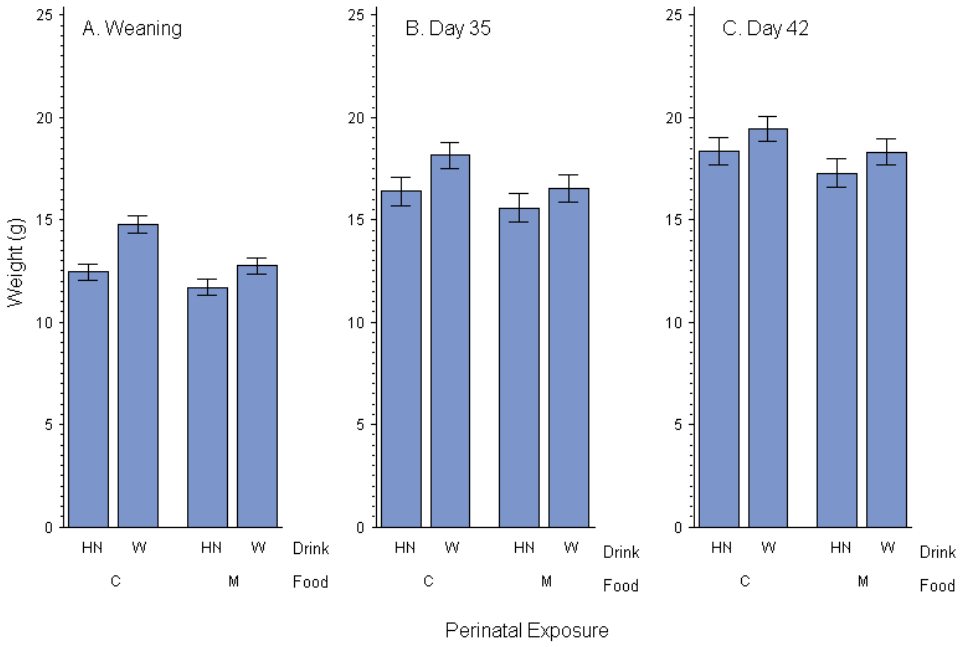

Figure 3-1. Body weight in mice from the four perinatal exposure conditions (mean ± standard error), at A) weaning, B) nicotine consumption testing day 1, and C) testing day 7.

The main effects of perinatal diet and nicotine exposures on body weight were persistent (Figures 3-1B, 1C). At the end of adolescent nicotine choice testing at 42 days of age, methyl diet mice were 1.01±0.32 g lighter (t = -3.12, p = 0.0022) and nicotine exposed mice were 0.97±0.32 g lighter (t = -3.01, p =0.0032; interaction no longer significant), even after controlling for litter size (for each additional littermate mice were 0.32±0.12 g lighter, t = -2.73, p = 0.0072) and agouti genotype (A^{vy} mice 1.31±0.31 grams heavier, t = 4.19, p <.0001).

Wave 1: Fluid Consumption During Habituation

Baseline fluid consumption during the 3 days prior to adolescent nicotine choice tests was measured in bottles on the left, center, and right side of the cage (from the mouse's point of view). Baseline side preference and baseline total fluid consumption per day were derived metrics. In terms of side preference, mice exposed to water (instead of nicotine) before weaning showed a preference for the water bottle on the right hand side of the cage (closer to the position where water was administered before weaning (6.51± 2.32% higher fluid consumption from the right bottle than nicotine exposed mice, t = 2.81, p = 0.0058). Perinatal exposure conditions also influenced total fluid consumption. Even after adjusting for body weight (0.32±0.03 additional mls per day for each gram of body weight, t = 10.86, p <.0001), fluid consumption was affected by perinatal diet (1.03±0.29 higher ml per day in methyl group, t = 3.54, p = 0.0006) and nicotine (0.74± 0.28 fewer ml/day, t = -2.63, p = 0.0098), with a significant interaction (if mice were exposed to control diet and nicotine in the perinatal period, this was associated with an additional 1.01±0.37 ml/day, t = 2.72, p = 0.0076). The interaction is illustrated in Figure 2.

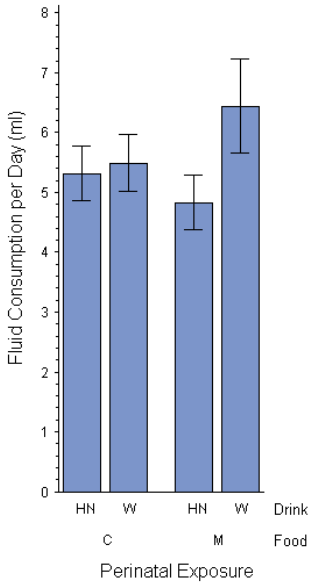

Figure 3-2. Total fluid consumption during habituation in mice from the four perinatal exposure conditions (mean ± standard error).

Wave 1: File Box Activity

Activity on an automated scoring hole board apparatus was monitored on 3 consecutive nights in 32-34 day old mice (testing conducted 1-3 hours after lights off). Number of nose pokes in the four holes, rears, and ambulation for each 3 minute test was tallied.

No significant differences in nose pokes were seen between dietary and nicotine exposure groups, but there was a quadratic effect of test day ($t = 6.40$, $p < 0.0001$, Figure 3-3A). Rearing behavior was lower in mice exposed to nicotine in the perinatal period (0.19± 0.08 fewer rears, $t = -2.43$, $p = 0.0165$, Figure 3-3B). Total ambulation showed a linear trend by day which interacted with diet and nicotine exposure conditions ($t = 2.39$, $p = 0.0174$)—in essence, ambulation tended to increase over time in all groups except the group exposed to methyl diet with water in the perinatal period (see Figure 3-3C). Proportion of activity in the center vs. the periphery of the File Box was also measured. Center time decreased across nights, but decreased faster in *a/a* mice than A^{vy}/a mice ($t = 2.34$, $p = 0.0197$, see Figure 3-3D).

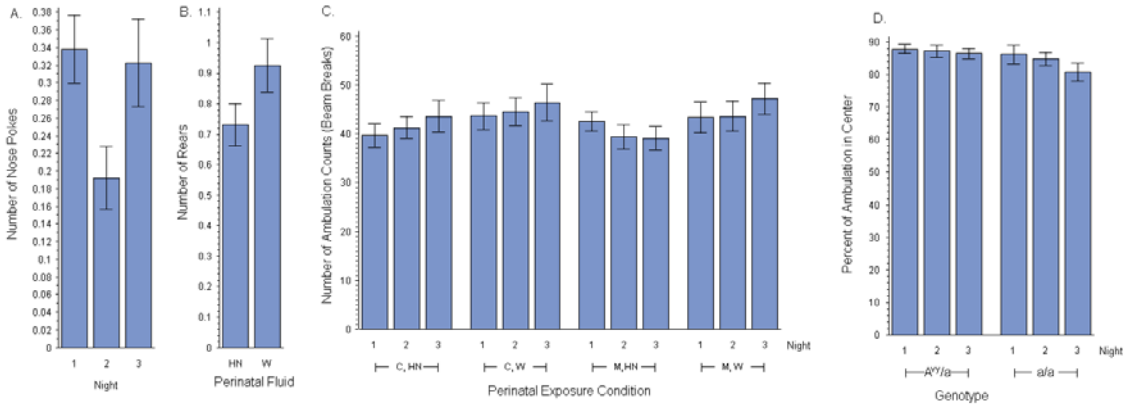

Figure 3-3. Locomotor activity. A) Nose poke behaviors vs. test day. B) Perinatal nicotine exposure vs rearing behavior. C) Interaction of perinatal exposure conditions with change in ambulation over time. D) Genotypic effects on center vs. peripheral ambulation. All graphs show mean ± standard error for raw values in each condition indicated.

Wave 1: Adolescent Nicotine Consumption: "Preference"

Adolescent mice were given access to solutions of tap water with 0, 50, and 200 μg/ml of free base nicotine daily for 7 days. The primary outcome variable analyzed was percent nicotine consumption, sometimes referred to as "preference".

Perinatal nicotine exposure and methyl diet both interacted with testing day ($F = 12.55$, $p = 0.0004$, see Figure 3-4A) when controlling for bottle position effects, which is another significant covariate ($F = 11.50$, $p < 0.0001$). In the broadest sense, the mice exposed to control diet and nicotine before weaning started with higher nicotine consumption in the first half of the week and consumption gradually declined while in other exposure conditions there was little change.

To estimate the main effects of diet and drug exposures, the interactions of perinatal exposure conditions were not included in a second model. Methyl diet reduced nicotine consumption by 4.4±1.5% ($t = -2.85$, $p = 0.0051$), and preweaning exposure to 200 μg/ml nicotine reduced nicotine consumption by 3.4±1.6% ($t = 2.14$, $p = 0.0340$). The model controlled for the main effect of test day (0.7±0.2% decline per day, $t = -3.60$, $p = 0.0003$) and bottle placement (compared to placing water on the right, when water was on the left consumption was no different but when water was in the center, nicotine consumption as 4.3±1.0% lower, $t = -4.46$, $p < 0.0001$)

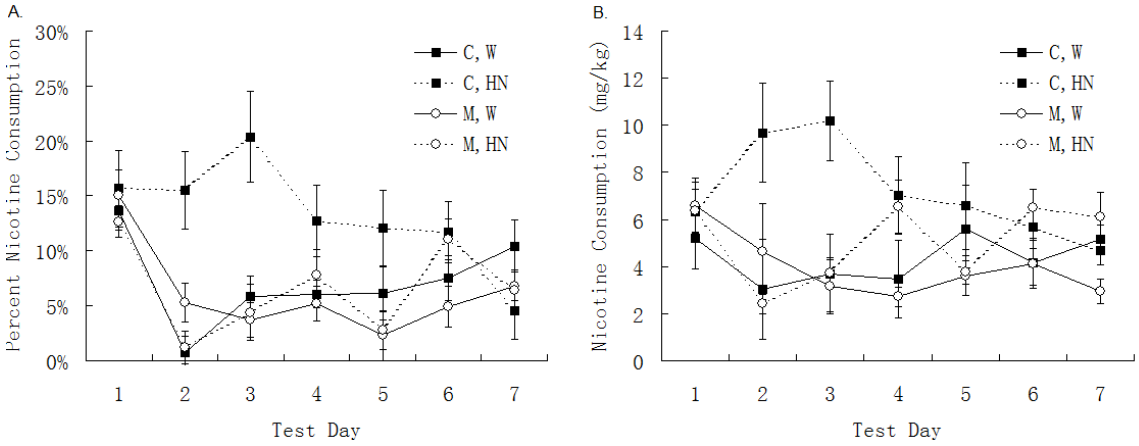

Figure 3-4. Adolescent nicotine consumption in Wave 1 A) Percent nicotine consumption by day, and B) Nicotine consumption (mg/kg) by day for the 4 perinatal exposure conditions. All graphs show mean ± standard error for raw values in each condition indicated.

Wave 1: Adolescent Nicotine Consumption: "Dosage"

Dosage was calculated by (ml of consumption from the high nicotine bottle times 200 µg/ml) divided by body weight on that day of testing (inferred by interpolation on days not weighed) plus (ml of consumption from the low nicotine bottle times 50 µg/ml) divided by body weight. The resulting value in microgram nicotine per gram body weight is equal to the more commonly used units of milligram nicotine per kilogram body weight).

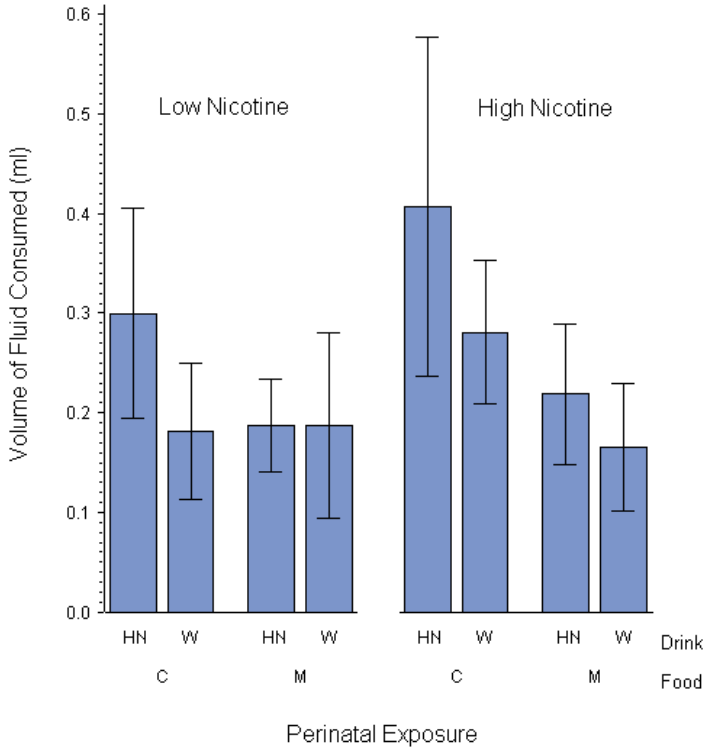

Figure 3-5. Average daily adolescent nicotine consumption in Wave 1 from the low (50 µg/ml) nicotine bottle and the high (200 µg/ml) nicotine bottle (both in mls). Graphs show mean ± standard error for raw values in each condition indicated.

Nicotine dosage consumed was similarly influenced by time and perinatal exposure conditions. The interaction of perinatal diet × perinatal nicotine × test day showed a trend that was near significant ($F = 3.38$, $p = 0.0669$, Figure 3-4B). In a model testing only for main effects of perinatal exposure conditions (without interactions), methyl diet did not reduce nicotine consumption ($p > 0.05$), while perinatal nicotine exposure continued to have a significant effect to increase adolescent nicotine consumption (by 1.02±0.50 mg nicotine per kg body weight per day, $t = 2.05$, $p = 0.0419$). The diminished significance of methyl diet effects on nicotine consumption measured in mg/kg is explained by the less consistent effect of methyl diet exposure on consumption from the 200 µg/ml nicotine bottle than the 50 µg/ml bottle (Figure 3-5).

Replication Experiment: Waves 2 - 4

Waves 2-4 were designed to a) replicate the effects of methyl diet on nicotine consumption in adolescence, b) investigate critical time points of diet administration, and c) further investigate the interaction effects of perinatal nicotine and methyl diet exposure on adolescent nicotine consumption. Wave 2 also had additional groups of mice for investigating whether individual differences in adolescent nicotine consumption correlate with ethanol or quinine consumption.

Due to less efficient breeding than expected (data not shown) and budgetary restrictions, a shortage of breeder dams led to inadequate sample size in most conditions of Wave 4. Some Wave 4 conditions only had 1-2 litters. Regression models that account for the nested structure of data essentially use litters as the unit of analysis, and n of 1 or 2 is inadequate for hypothesis testing on perinatal diet × nicotine exposure subgroups in Wave 4. Therefore, Wave 4 animals were excluded from analyses except where mentioned explicitly.

Replication Experiment: Body Weight

Combined analysis of wave 2 and 3 data showed that exposure conditions again influenced weaning body weight while controlling for covariates (female sex was associated with 0.94±0.18 g lighter $t = -5.18$, $p < 0.0001$; A^{vy} genotype was associated with a 0.41±0.18 g increase in body weight, $t = 2.23$, $p = 0.0261$; litter size was associated with a 0.42±0.06 g decrease in body weight for every additional sibling, $t = -6.41$, $p < 0.0001$). Perinatal exposure to nicotine reduced weaning body weight in a dose-dependent manner (compared to mice exposed to water, 50 μg/ml exposure led to 1.42±0.32 g reduction in body weight, $t = -4.39$, $p < 0.0001$; 200μg/ml exposure led to 2.50±0.32 g reduction in body weight, $t = -7.83$, $p < 0.0001$). Furthermore, the Wave 2 condition was associated with heavier body weight, apparently due to the parental

animals' exposure to high fat methyl or control 7% corn oil diet before conception (Wave 3 parental animals were maintained on the ~4.5% fat standard chow until 5 days after matings were set up). Methyl diet exposure showed a significant interaction with perinatal nicotine exposure ($F = 3.17$, $p = 0.0429$), but the pattern was different from Wave 1 (see Figure 3-6). The interaction between methyl diet and nicotine exposure did not persist through adolescent nicotine consumption testing (data not shown, $p > 0.05$), and only the effect of nicotine exposure remained significant ($p < 0.05$).

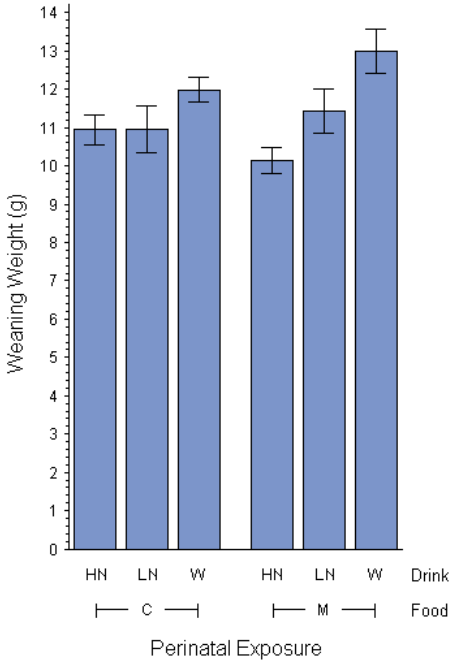

Figure 3-6. Effect of methyl diet on weaning body weight in combined data from Waves 2-3.

Replication Experiment: Baseline Fluid Consumption

Baseline fluid consumption during the 3 days prior to adolescent nicotine choice tests was measured in bottles on the left, center, and right side of the cage (from the mouse's point of view). In this cohort, there was no side bias in pre-weaning bottle placement between exposure

conditions and, as expected, there were no significant effects of exposure condition on side preference.

Total fluid consumption was influenced by Wave (0.84±0.12 fewer ml/day in Wave 2 than Wave 3, $t = -4.30$, $p < 0.0001$) in a model accounting for differences in body weight (0.32±0.03 additional mls per day for each gram of body weight, $t = 10.86$, $p < .0001$). Presumably the reduced fluid consumption in wave 2 is due to the higher fat and lower fiber diets administered during phenotyping than the standard chow fed to mice during phenotyping in Wave 3 mice.

Replication Experiment: Adolescent Nicotine Consumption

A replication study has specific directional predictions and therefore probabilities are based on one-tailed hypotheses. From findings in Wave 1, percent nicotine consumption was the nicotine consumption outcome variable. Methyl diet was expected to reduce adolescent nicotine consumption and perinatal nicotine exposure was expected to increase adolescent nicotine consumption.

In addition to the replication component, this portion of the study had a two-tailed hypothesis about the potential interaction between methyl diet and exposure duration (Wave). Methyl diet might have a stronger effect on adolescent nicotine consumption in wave 2 if ongoing methyl diet exposure after weaning increased the methyl diet effect. Alternatively, methyl diet might have a stronger effect in wave 3 if the effect of methyl diet were actually related to a withdrawal syndrome after switching from the methyl supplemented diet to standard chow at weaning. The null hypothesis, that wave would not interact with methyl diet, is best supported by the findings in the literature that methyl donor ingredients in the test diet have their greatest effects on behavior when administered during early brain development (i.e. perinatal periods).

Results showed that in Wave 2 and 3 mice, percent nicotine consumption was influenced by methyl diet (4.6±2.4% lower in methyl diet groups, t = -1.91, $p_{1\text{-tailed}} = 0.0289$) but not perinatal nicotine exposure. At the same time, several covariates had significant effects on consumption: body weight (1.7±0.4% increase in consumption for each g of body weight, t = 4.26, p < 0.0001), bottle setup position (compared to when water was on the right, 3.2±1.1% less nicotine consumption with water bottle on left, t = -2.91, p = 0.0038 and 4.6±1.1% less when water bottle is in the middle, t = -4.11, p <0.0001), and test day (consumption declined 1.8±0.5% per day, t = -3.98, p <0.0001).

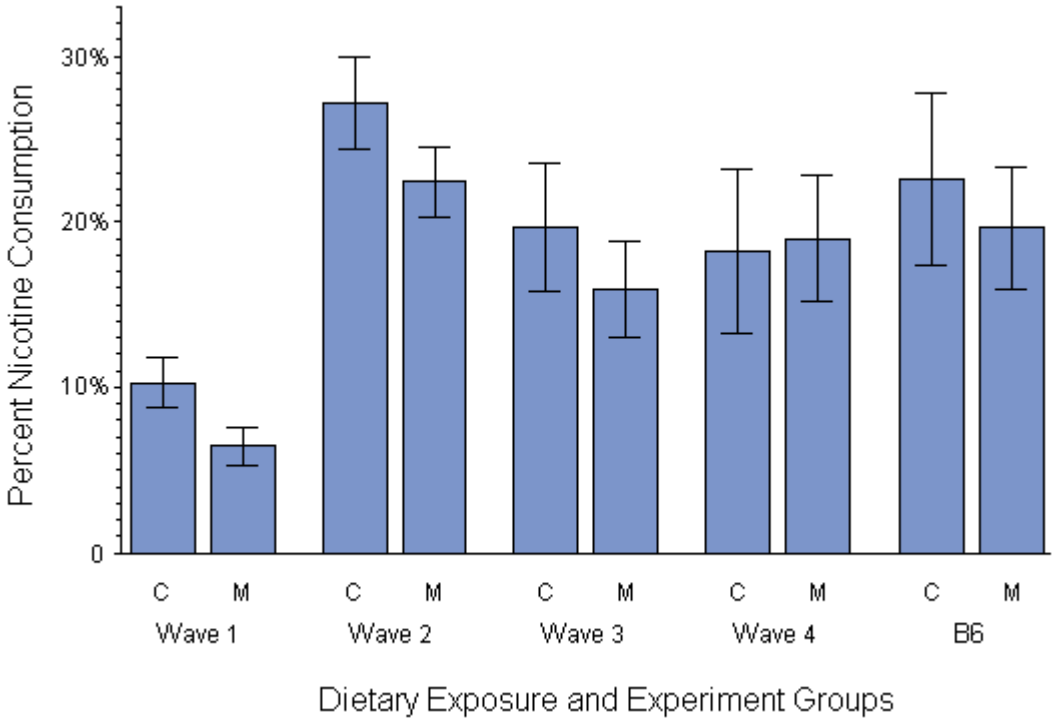

Figure 3-7. Main effect of methyl diet on percent nicotine consumption across studies. Note that all groups except wave 4 show roughly the same trend towards lower nicotine consumption in the methyl diet group. The commonality between these groups is methyl supplementation in the dams' food during gestation and lactation.

In order to investigate whether outliers could account for the effects of methyl diet in this part of the study, the distribution of consumption was analyzed. Percent nicotine consumption in Waves 2 and 3 were significantly different from a normal distribution (in Kolmogorov-Smirnov test, $D = 0.220$, $p < 0.01$). Instead, the data fit a lognormal distribution (see histogram in Figure 3-8), indicating need for log data transformations to bring the data towards a normal distribution (KS test $D = 0.033$, $p = 0.01$). A secondary minor peak at the highest consumption values was responsible for the remaining deviation from normality. Re-analysis of the log transformed percent nicotine consumption increased the F statistic for the same significant covariates, but not the methyl diet effect, which was insignificant ($t = -0.77$, $p_{1\text{-tailed}} = 0.2220$).

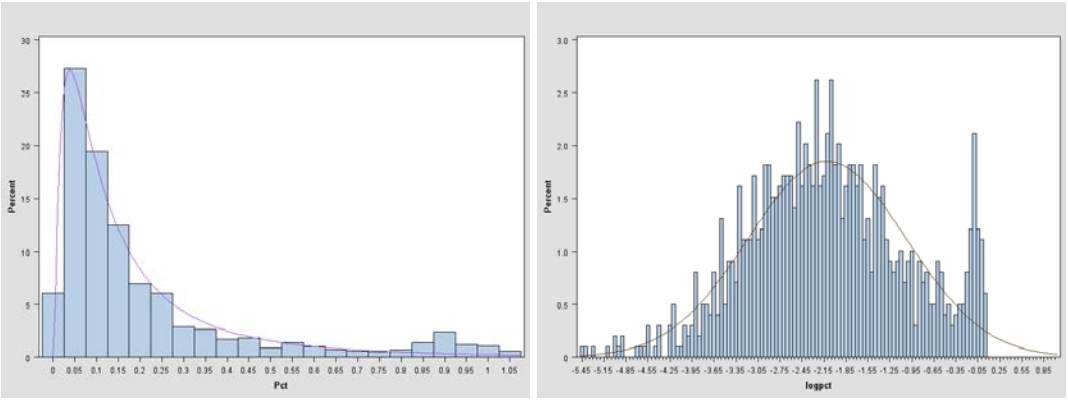

Figure 3-8. Distribution of percent nicotine consumption in waves 2-4. Raw data, fit with a lognormal distribution curve, are shown at left. The data after log transformation are shown at right, fit with a normal distribution curve.

The analysis of data across waves is described in the meta-analysis section below. First, the additional Wave 2 experiments are described.

Replication Experiment: Quinine Consumption

47 male mice from 24 litters in Wave 2 were given access to a three bottle choice test with quinine instead of nicotine during week 1 (36-42 days) or week 2 (43-50 days). Percent quinine consumption was not different between perinatal exposure groups. The only significant predictor of percent quinine consumption was test day (3.9±0.8% decline per day, $t = -5.0$, $p < 0.0001$). Similar to nicotine consumption, quinine consumption was lognormally distributed, and thus data were re-analyzed after log transformation. Perinatal diet and nicotine exposure conditions remained insignificant. Log-transformed quinine consumption declined with test day ($p < 0.0001$), increased with body weight ($p = 0.0228$), and showed a side preference similar to that of nicotine as described above ($p = 0.0384$).

Quinine consumption was correlated with nicotine consumption. Most mice tested for both quinine and nicotine consumption were tested for nicotine in week 1 and quinine in week 2. Because data were not normally distributed, non-parametric correlations were conducted. Average percent nicotine consumption in week 1 was positively correlated with average percent quinine consumption in week 2 ($\rho = 0.452$, $p = 0.0018$, $n = 45$).

Data were split based on diet for an exploratory analysis of whether dietary exposure moderated the relationship between nicotine and quinine consumption. The correlation coefficient was higher and p value was lower in mice on the control diet ($\rho = 0.590$, $p = 0.0049$, $n = 21$) than the methyl diet ($\rho = 0.394$, $p = 0.0568$, $n = 24$).

Data were also split based on perinatal nicotine exposure conditions for an exploratory analysis of whether nicotine exposure moderated the relationship between nicotine and quinine consumption. Correlation magnitude and significance appeared different for mice from litters given water ($\rho = 0.382$, $p = 0.0598$, $n = 25$), 50µg/ml nicotine ($\rho = 0.771$, $p = 0.0724$, $n = 6$), or 200 µg/ml nicotine ($\rho = 0.332$, $p = 0.2464$, $p = 14$).

In these two sets of subgroup analyses, the groups with lower nicotine consumption estimates in the regression analyses above had a lower correlation between nicotine consumption and quinine consumption.

Replication Experiment: Ethanol Consumption

69 individuals from 31 litters of Wave 2 mice were given access to a three bottle choice test with ethanol during week 1 or week 2. Percent ethanol consumption was lower in mice in the methyl diet group than control mice (9.3±4.4% lower, $t = -2.11$, $p = 0.0397$). Perinatal nicotine exposure groups affected ethanol consumption and interacted with test week. The bulk of the effect was due to higher ethanol consumption in week 2 for mice given 200µg/ml nicotine in the perinatal period (36.6±13.3% higher, $t = 2.76$, $p = 0.0078$). The only significant covariate was test day (ethanol consumption declined by 2.8±0.6% per day, $t = -4.46$, $p < 0.0001$). Ethanol consumption was not normally distributed (skewed left, outliers had low ethanol preference), but due to a large ceiling effect, exponential and polynomial transformations did not appreciably improve the distribution.

Spearman's correlations were used to test for a relationship between ethanol consumption and nicotine consumption. Data were available to check for a correlation between ethanol consumption before nicotine, and nicotine before ethanol. Week 1 ethanol consumption was significantly correlated with week 2 nicotine consumption ($\rho = 0.622$, $p = 0.0020$, $n = 22$). Week 1 nicotine consumption, however, was not significantly associated with week 2 ethanol consumption ($\rho = 0.005$, $p = 0.9708$, $n = 55$). No significant differences in the association between nicotine consumption and other tastants was observed depending on perinatal condition (nicotine consumption in week 2 was correlated with nicotine consumption in week 1 no matter if mice were in the methyl diet or control diet groups).

Replication Experiment: Perinatal Nicotine Exposure Effects on Oral Nicotine Consumption in the Second Week of Testing

The sample of mice from Wave 2 given nicotine in the second week of choice testing had adequate power to test the hypothesis that perinatal exposure to 50 µg/ml nicotine would increase nicotine consumption in offspring. Results showed that exposure to 50 µg/ml nicotine was associated with a 27.3±5.6% increase in nicotine consumption during the choice test ($t = 4.85$, $p < 0.0001$).

Replication Experiment: Nicotine Conditioned Place Preference

Nicotine conditioned place preference tests were conducted on 39 mice from Waves 3 and 4. Baseline data from the black/white box showed that mice had a significant preference for the black side (614.8±11.1 seconds the black side, $t = 14.82$, $p < 0.0001$ for t-test with null hypothesis of 450 seconds), indicating a biased CPP procedure with a 68% baseline preference for black. but there was no influence of prior exposure conditions or nicotine preference on baseline black-white preference.

Preference scores were normally distributed. CPP was not predicted by prior nicotine consumption during the choice test, wave, perinatal methyl diet, or perinatal nicotine exposure. The only significant predictor of preference score was the nicotine conditioning chamber. Mice conditioned on the black side had an 88.5±27.3 second lower score than the mice conditioned on the white side ($t = -3.24$, $p = 0.0025$, Figure 3-9). Based on post-hoc t-tests, there was a significant conditioned place aversion for the mice in the black side ($t = -3.87$, $p = 0.0011$), but no net effect of conditioning in mice exposed to nicotine in the white chamber ($t = 0.70$, $p = 0.4937$). Thus, methyl dietary supplementation did not influence conditioned responses to nicotine injections at these doses.

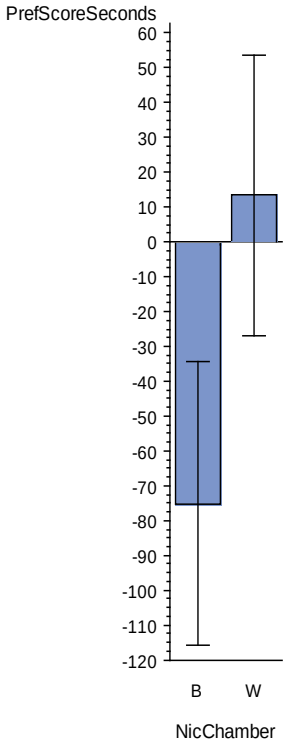

Figure 3-9. Effect of testing chamber on nicotine conditioned place preference. B = black side, W = white side. The y-axis represents the number of seconds change in time spent on the drug paired side of the apparatus in the retention test vs. the baseline test (15 minute tests).

C57BL/6J Cohort: Oral Nicotine Consumption

In the first 9 day period, using the Tordoff and Bachmanov three bottle setup, percent nicotine consumption was significantly higher when mice were given access to two bottles ($F_{1,72.5}$ = 33.97, $p < 0.0001$), and the degree of increase was dependent on the concentration of nicotine provided ($F_{2,81.9}$ = 3.30, $p = 0.0419$, see Figure 3-10A). Nicotine consumption in mg/kg per day was also dependent on number of bottles ($F_{1,86}$ = 21.73, $p < 0.0001$), which again interacted with nicotine concentration (Figure 3-10B). By either measure, sex and dietary conditions did not have significant effects on nicotine consumption.

A. B.

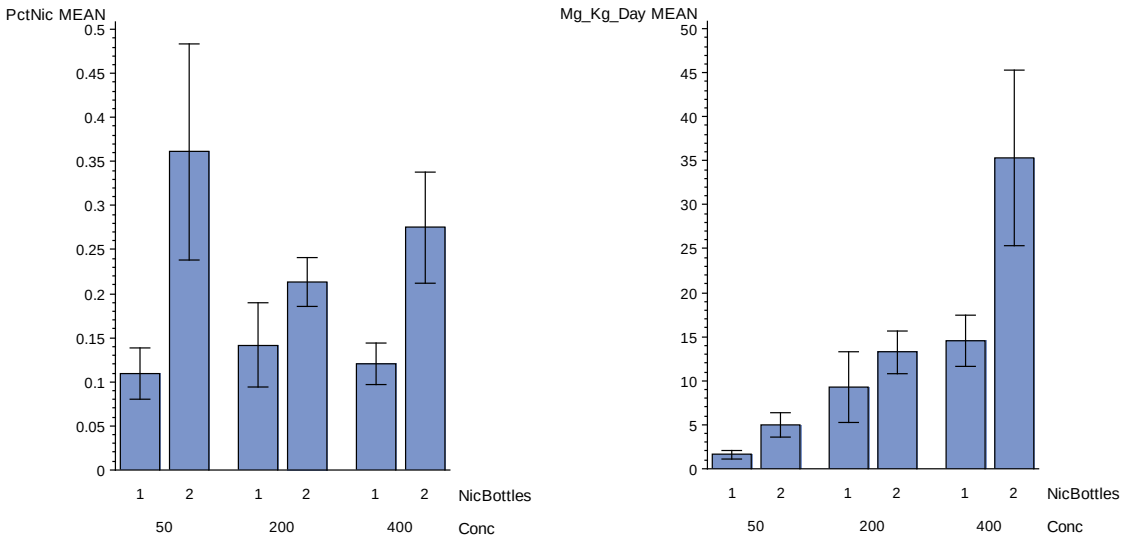

Figure 3-10. Left, A: Percent nicotine fluid consumption (need to adjust y-axis from ratio to pct), showing significant effects of bottle number and bottle number by nicotine concentration interaction. Right, B: Nicotine dosage showing significant effects of bottle number and bottle number by nicotine concentration interaction.

In the second 9 day period, bottle number and nicotine concentration were the same from day to day. No significant effects of sex or methyl diet were observed on either percent nicotine or consumed dosage. Thus, no test variables were significantly associated with nicotine consumption.

In both time periods, diet was not significantly associated with nicotine consumption, but the trend was towards lower consumption in the methyl diet group (Figure 3-11).

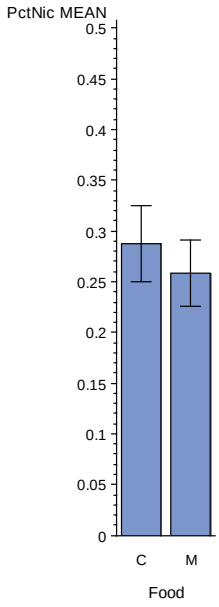

Figure 3-11. Trend towards lower percent nicotine consumption in the methyl diet group across the choice period (18 days). Y-axis is in fraction rather than percent units.

C57BL/6J Cohort: Place Conditioning to Nicotine and Cocaine

There was no significant effect of dietary condition on place conditioning. There was also no significant effect of sex. Mice had significantly greater place preference for cocaine than nicotine conditioning (significant main effect of drug : $F_{1,33} = 9.26$, p 0.0046), cocaine generated a place preference, while nicotine generated a marginal place aversion.

"Drinking in the Dark"

The current study successfully detected the expected escalation of volume of ethanol consumed across the first four days of standard drinking in the dark testing in the control group. This increase interacted significantly with dietary exposure ($F_{1,29.1} = 5.79$, $p = 0.0227$), such that methyl diet group did not increase, while the control diet group did increase consumption (Figure

3-12A). In the group of mice with concurrent nicotine access and only one ethanol bottle during the drinking in the dark testing, a similar pattern emerged but was not significant (Figure 3-12B). Once again, the expected trend towards reduced nicotine consumption in the methyl diet group was observed, but was not significant (Figure 3-13).

A. B.

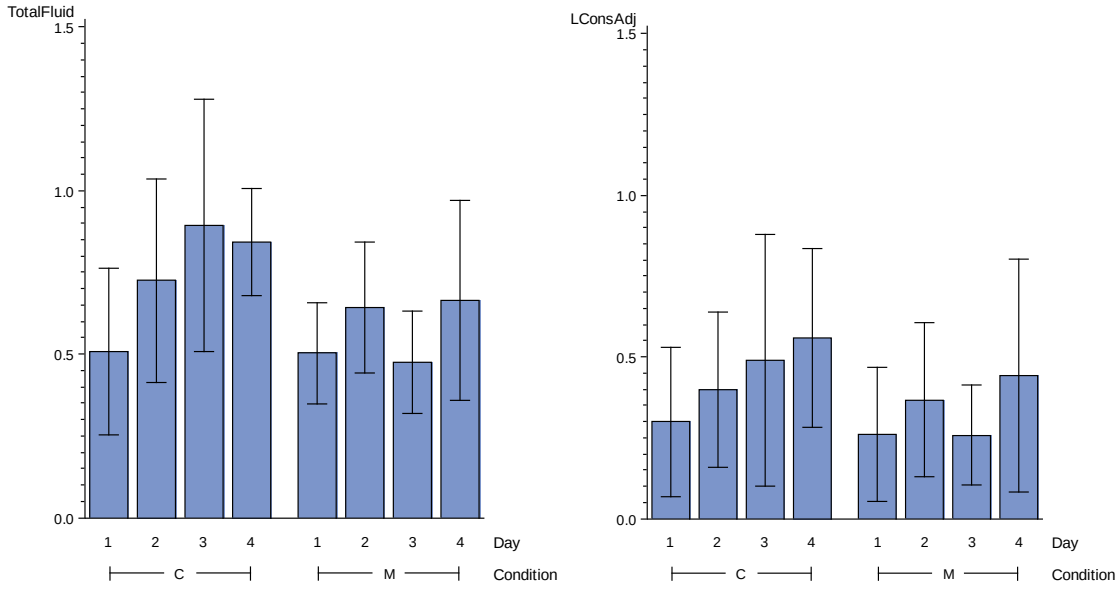

Figure 3-12. Left, A. Ethanol drinking in the dark escalates in control diet condition but not in methyl diet mice. Right, B. Ethanol drinking in the dark with concurrent nicotine access shows a similar, but insignificant trend. Y-axes indicate total 20% ethanol consumption during the 2 hour limited access period in milliliters.

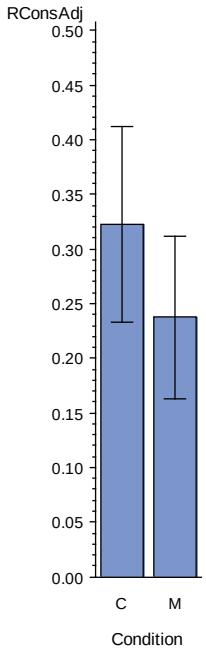

Figure 3-13. Trend towards lower volume of nicotine consumed (milliliters) during the drinking in the dark procedure in methyl diet mice.

Meta-Analysis: Perinatal Methyl Diet Exposure Effects on Adolescent Nicotine Consumption

A simple meta-analysis was conducted on all studies of mice exposed to methyl diet in the perinatal period and phenotyped for nicotine consumption in adolescence. Percent nicotine consumption across the adolescent week was averaged for each animal, and then the natural log of this value was taken as the scale of measurement for the phenotype. Then, descriptive statistics of this "ln consumption" variable were summarized (Figure 3-14) and entered into a meta-analysis calculator for continuous data developed by Basu (accessed March 15, 2011). The weighted average differences between groups indicated a significant main effect of methyl diet reducing adolescent nicotine consumption across studis, with a 95% confidence interval that did not overlap with zero (Figure 3-14). Thus, even after accounting for the tendency to skew in the percent nicotine consumption distribution, the methyl diet effect remains significant.

Descriptive Statistics for the Natural Log of Percent Nicotine				
Wave	**Diet**	**N**	**Mean**	**StdDev**
1	**C**	61	-2.68	1.00
	M	58	-2.88	0.76
2	**C**	80	-1.47	1.00
	M	81	-1.53	0.75
3	**C**	44	-1.85	0.99
	M	47	-2.27	0.79
4	**C**	30	-2.10	0.95
	M	39	-2.28	1.05
B6	**C**	20	-1.55	0.43
	M	21	-1.67	0.24
All	**C**	235		
	M	246		

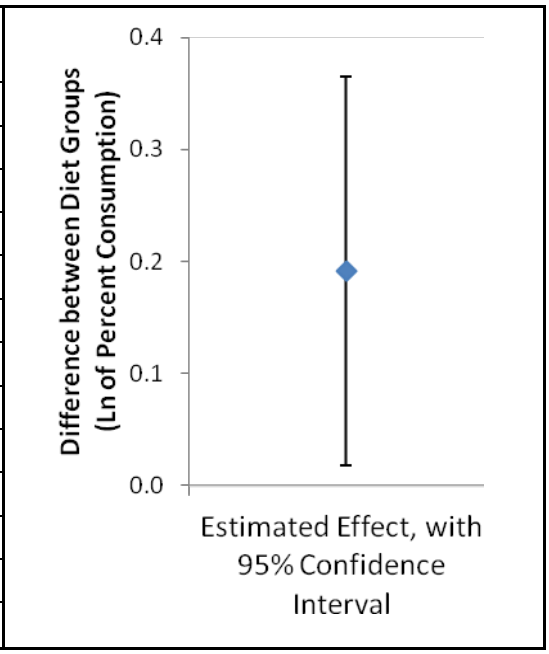

Figure 3-14. Meta-analysis of log-transformed percent adolescent nicotine consumption in all mice exposed to methyl diet in the perinatal period.

Discussion

Limited Ability to Test whether Methyl Diet Blocks the Effects of Perinatal Nicotine Exposure on Nicotine Consumption

The effects of perinatal nicotine exposure on adolescent nicotine consumption were less robust than expected, resulting in limited ability to test the major proposed hypotheses. Perinatal nicotine exposure was expected to increase adolescent nicotine consumption. In wave 1, this was true for 200 µg/ml nicotine administered through the dams' drinking water continuously from before conception until pups were weaned. Furthermore, perinatal methyl donor supplementation blocked the effect of perinatal nicotine exposure. However, the main effect of perinatal nicotine exposure on adolescent nicotine consumption was not observed in Waves 2-4. This was

unexpected, because in Waves 2-4, mice were exposed to 50 µg/ml nicotine in the perinatal period, which was reported by Klien et al. (2003) to have large effects on the consumption of nicotine by adolescent males. Furthermore, the effect of 200 µg/ml detected in Wave 1 was not apparent in data from Waves 2-4. Thus, in the section of the study that was powered to test for methyl diet effects, there was no way to investigate whether methyl diet was blocking the behavioral effects of nicotine's perinatal exposure in the replication experiment, because there were no main effects of nicotine detected under these conditions.

Interestingly, the effect of perinatal exposure to 50 µg/ml nicotine on subsequent nicotine intake was detected in the group of mice tested for nicotine consumption in week 2. Why would the effect of perinatal exposure be so strong in this group, comparable to Klien et al. (2003), but completely absent in littermate mice tested a week earlier? The age of testing, around adolescence, is a time of critical neurological development. The timing of the pubertal transition presumably determines this functional age. Differences in pubertal transition due to mouse genetics are not expected to be responsible for differences between studies, since the genetic background of C3H mice are expected to reduce pubertal age (Nelson et al. 1990), while the current study, with mice containing some C3H genetic background, showed the sensitive period one week later. Pubertal transition can also be affected by a variety of environmental variables (Ebling 2005), some of which may be relevant to the current study. This work differed from Klein et al. (2003) in several ways, including later weaning of the mice (4 weeks of age in the current study compared to 3 weeks of age in Klein et al.), longer forced nicotine exposure in the perinatal period, no shipping of the parental mice (see Laroche et al. 2009), and less frequent handling of the mice (especially in the juvenile period). With later weaning, it is possible that the crowding as the litter gets larger may lead to the dam secreting signals in her urine to delay puberty in the offspring (Massey and Vandenbergh 1980, Jemiolo and Novotny 1994). Thus it is conceivable

that nicotine exposed mice in the current study reached a critical period of sensitivity at a later age due to environmental variables.

Main Effect of Methyl Diet on Adolescent Nicotine Consumption?

Despite the inability to test for the hypothesized interaction of perinatal exposure to nicotine and methyl diet, an independent main effect of methyl diet was suggested by all waves of the study that supplemented during brain development (gestation and lactation).

In Wave 1, the main effect of methyl diet was significant and robust. In Waves 2 and 3, the main effect of methyl diet was significant in a model adjusting for the nested structure of the data and all covariates, but not after adjusting for deviation from normality. Thus, the differences in nicotine consumption between dietary exposure groups seen in this wave are fairly likely to be due to non-random distribution of high nicotine consumers between groups. Perhaps methyl diet prevents some mice that would have become relatively heavy nicotine consumers from developing that behavior.

Due to the discovery of the same mean difference in nicotine consumption in all 4 separate experimental groups exposed to methyl diet during brain development, the results appear to be small but reliable. The meta-analysis on exponentially shifted data confirms this interpretation. Furthermore, the observation that methyl diet reduces oral ethanol consumption in both a bottle choice and a drinking in the dark protocol support the notion that methyl diet is changing responses to orally consumed drugs of abuse.

In terms of relevance to the human condition, a small effect on a health outcome with high incidence is of interest to public health if it can be readily translated into cost-effective interventions. In contrast to the relatively light nicotine use in these mice, heavy nicotine use is common in humans and causes a huge a financial and social burden on society. Concurrent

supplementation with the 4 methyl donor ingredients in this test diet is readily achievable, and would be a cost effective intervention if it could prevent even 3% of smoking behavior. Thus, future experiments should investigate methyl diet supplementation to determine whether the effect is real and robust, or spurious.

The first step towards replication should be to test the effects of methyl diet in a population of experimental subjects with a greater incidence of high nicotine use. If the effects of methyl diet are manifested by prevention of heavy nicotine use, then the effects should be stronger in a population of high nicotine consuming animals. A second step should be to investigate existing epidemiological data to evaluate whether normal dietary folate, choline, and B12 exposure during gestation is associated with lower nicotine use behavior in human adolescents. Research in humans should proceed with caution and pay attention to nutrient interactions because prior research has indicated that prenatal supplementation with folate alone may be linked to increased risk of asthma (Witrow et al. 2009), and some experimental studies have suggested involvement of excess folate supplements in development of certain cancers (Ly et al. 2010).

A Mechanism Linking Methyl-Diet Supplementation to the Rewarding Properties of Nicotine

Further evidence is needed to confirm or refute the effects of methyl diet exposure on adolescent nicotine consumption seen in Wave 1 in the study, since the evidence from Waves 2-4 and a group of B6 mice were suggestive but not confirmatory. Conditioned place preference testing did not show direct changes in reward to injections of specific doses of nicotine but do not test for the degree of reward induced by self-administed doses of the drug by the oral route. It remains possible, though certainly not confirmed, that methyl diet reduces the reward response to

voluntary oral nicotine consumption. At least two lines of evidence from the literature suggest that methyl diet could influence the rewarding properties of nicotine.

Nicotine microinjections directly into the VTA are reinforcing and the reinforcement is dependent on both cholinergic and dopaminergic receptors (David et al. 2006). Unlike other drugs of abuse, the effect of nicotine on the activity of dopaminergic VTA neurons is uniquely dependent on cannabinoid signaling pathways acting via perioxisome proliferator-activated receptor alpha (PPAR-α) (Luchicchi et al. 2010). PPAR-α downregulates β2 nAChR subunits in VTA dopamine neurons and PPAR-α activation down-regulates basal VTA dopaminergic neuron firing (Melis et al. 2010), which is believed to lead to blockade of nicotine self-administration (Mascia et al. 2011). This essential modulator of nicotine addiction has a longer history of study for its role in regulation of lipid homeostasis (Reddy and Mannaerts 1994), especially in the liver (Reddy and Hashimoto 2001). Research indicates that, throughout life, PPAR-α is epigenetically regulated and sensitive to perinatal environment, such as protein restriction (Burdge et al. 2004). The effect of prenatal protein restriction on PPAR-α promotor methylation and expression in liver and adipose tissue can be blocked by maternal dietary folate supplementation during the same period (Lillycrop et al. 2005, Lillycrop et al. 2008, Burdge et al. 2009), indicating that early folate exposure can upregulate PPAR-α. If folate had this effect in the brain, reduced nicotine addiction would be an expected outcome, and thus could mediate the effects of methyl diet observed in the current study. This hypothesis can be tested directly by evaluating effects of methyl diet supplementation on PPAR-α expression and activity in the midbrain.

Alternatively or additionally, the effects of choline supplementation on brain development, especially in the hippocampus, could play a role in the prevention of nicotine use behavior. Perinatal choline supplementation above standard levels increases hippocampal neurogenesis in the fetal period and improves memory throughout life (see reviews by Meck and Williams 2003, Zeisel 2009). Hippocampal neurogenesis has been experimentally linked to

addiction liability in a recent study by Noonan et al. (2010) which used cranial radiation to depress hippocampal neurogenesis—this treatment was associated with increased cocaine intravenous self administration. The involvement of hippocampal function in nicotine addiction is especially prominent in the process of learning of drug associated cues and withdrawal (see discussion section of Davis and Gould 2009 and Caguilla et al. 2002). Given that perinatal choline supplementation leads to stable improvements in hippocampal function throughout the lifespan, it seems possible that it could influence addiction liability and thus adolescent nicotine consumption in the current mouse model.

As reviewed in the introduction of this chapter, the chronic treatment with a weak nAChR agonist, choline, can alter cholinergic receptor function and thereby affect how brain responds to nicotine. Experiments are planned to test whether choline alone is sufficient to alter nicotine consumption phenotypes, and if these experiments are carried out in specific knockout strains that prevent the entry of choline into specific metabolic pathways, it is possible to differentiate the effects of choline on methylation, lipids, and cholinergic receptors.

Alternative Mediators of the Effect of Methyl-Diet Supplementation on Nicotine Consumption

The benefits of perinatal choline supplementation for learning suggest that the benefit seen in Wave 1 could be mediated by choline-induced improvements in nicotine avoidance learning. Perhaps some of the variance in nicotine consumption seen in the current study is due to unintentional consumption by mice that failed to remember which bottle served water and which bottles served nicotine. Mice with stronger memory would naturally have fewer incidents of such bottle discrimination error.

The potential effects of methyl diet exposure on bitter taste sensitivity is also still a viable hypothesis. The sample of mice that consumed quinine before nicotine was inadequate to test for a methyl diet effect. The data on quinine consumption in mice on the second week seems to indicate no differences in bitter taste preference, but there is a possible confounder. The correlation of quinine consumption in week 2 to nicotine consumption in week 1 could indicate a conditioned taste preference for mice undergoing nicotine withdrawal. Blizard et al. (personal communication) tested for nicotine conditioned taste aversion to a variety of substances and found that C57BL/6J mice who had been previously exposed to LiCl after nicotine consumption subsequently consumed less quinine than control animals. On the other hand, the correlation between nicotine and quinine consumption could indicate individual differences in general foraging activity. Mice tend to consume more of a substance if more bottles of that substance are provided, irrespective of whether the tastant is preferred or avoided (Tordoff and Bachmanov 2003a, Tordoff and Bachmanov 2003b). Future studies should use a design better suited to dissecting the possible interactions between nicotine consumption and the other tastants evaluated as a comparison.

Conclusion

Tests of the proposed hypotheses yield a complex picture. Only partial support was found for the first hypothesis, that perinatal nicotine exposure would induce changes in adolescent male nicotine consumption—the mice with perinatal exposure to 200 µg/ml nicotine in Wave 1 showed a statistically significant but very small increase in adolescent nicotine consumption, while the mice in Wave 2 showed no effect of either 50 µg/ml or 200 µg/ml perintal exposure on adolescent nicotine consumption. The second hypothesis, that the perinatal nicotine–induced increase in consumption could be blocked by methyl diet supplementation received support in

Wave 1 but not in subsequent experiments. Instead, all groups treated with methyl diet from gestation through weaning showed slightly lower adolescent nicotine consumption irrespective of perinatal nicotine treatment. The third hypothesis that methyl diet's effects on nicotine consumption depends upon exposure during brain development throughout gestation and weaning was confirmed. The fourth hypothesis, that methyl diet exposure in the parental animals prior to and shortly after mating would also be able to change adolescent nicotine consumption was not supported. The fifth hypothesis, that methyl diet would also change consumption of other drugs of abuse, was supported by reduced consumption of ethanol across a week of three-bottle choice and also reduced consumption of ethanol in a drinking in the dark procedure by the methyl diet mice. However, no changes in conditioned place preference for nicotine or cocaine injections were observed. The sixth hypothesis, that methyl diet might influence bitter taste response, was neither strongly supported nor convincingly ruled out, and requires testing with larger samples of nicotine-naïve animals. The balance of evidence supports the conclusion that methyl diet supplementation during brain development in this model reduced oral consumption of nicotine and ethanol, but it remains unclear if this modest change is due to drug-specific changes in reward circuitry or due to non-specific changes in the way that mice respond to tastant presentation. Further work is needed to determine whether specific compounds in the methyl diet (e.g. choline) are sufficient to alter nicotine consumption and to more carefully test for non-specific effects on response to novel tastants. Perhaps most importantly, additional work is also needed to investigate whether the main effect of methyl diet in reducing adolescent nicotine consumption extends to reducing of long-term addiction-like behavior. If so, the finding would merit epidemiological work in humans to investigate relationships between maternal methyl-donor consumption and subsequent adolescent drug use behaviors.

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Chapter 4

No Effect of Nest Behaviors on Adolescent Nicotine Consumption in Adolescent Male *Agouti Viable Yellow* Mice

Introduction

Epidemiological research in humans indicates that early childhood experiences may influence subsequent risk of many psychiatric illnesses and health risk factors, including smoking. Analysis of the Childhood Experiences study data showed strong association between the degree of abuse or traumatic environment experienced by a child and smoking behavior from adolescence through adulthood, even after adjusting for age, sex, race, and education in the family (Felitti et al. 1998, Anda et al. 1999). Various efforts have been made to disentangle the association between stressors, other associated environmental variables, genetics, and smoking across the lifespan. Epidemiological studies have frequently shown an association between tobacco smoking and anxiety disorders in adults (Lawrence et al. 2009, Lawrence et al. 2010) and adolescents (Dudas et al. 2005, Boys et al. 2003). Investigations of longitudinal data in both adolescents and adults have been used to support the idea that smoking increases subsequent risk for anxiety disorders/depression (Ismail et al. 2000, Johnson et al. 2000, Klungsøyr et al. 2006). Others have shown that various measures of depression (Boden et al. 2010, Patton et al. 1996) anxiety (Patton et al. 1996), neuroticism (Cherry and Kiernan 1976, Byrne et al. 1995), social fears (Sonntag et al. 2000) and stress (Byrne et al. 1995, Byrne and Mazanov 2003) raise the risk for subsequent increases in smoking. Thus, there is ample reason to believe that individual life

experiences related to stress and social environment do alter nicotine consumption. Still, experimental research is needed to confirm this, uncover the mechanisms, and determine how early in life the links between nicotine and social environment emerge.

Prominent in the field of biobehavioral health is the theory that stressful life events alter the neurophysiological systems that govern the hypothalamic-pituitary-adrenal axis, leading to differential responses to subsequent social environmental cues. The biological—and to a modest extent the psychological and social—components of a biopsychosocial model can be applied and tested in animal experiments. A model for the effects of early social environment on behavioral development focuses on the effects of maternal care by rodent mothers on their offspring. Fostering pups to dams that give lower maternal care leads to animals becoming more anxious than pups fostered to high licking dams (Francis et al. 1999), paralleling the human condition after childhood neglect (see review by Meaney 2001). Such effects of maternal care appear to mediated by the influences of social experience on epigenetic programming in the developing brain (Weaver et al. 2006, Zhang and Meaney 2010, McGowan et al. 2011), especially via regulation of glucocorticoid receptor expression in the brain, which affects negative feedback on the HPA axis and responses to stress (Weaver et al. 2004, McGowan et al. 2009). Few studies have evaluated the effects of maternal care on drug use behaviors, and limited information from one study suggests that the effects may exist, but may be strain specific—and perhaps non-existent in the C57BL6/J mouse strain (van der Veen 2008).

Recent work has begun to investigate the association between individual differences in maternal care received within a litter (Cavigelli et al. 2010). This model allows for analysis of the effects of individual pup solicitation behaviors on maternal licking. In order to test whether individual differences in each pups' behavior and maternal care received by each pup influence adolescent nicotine consumption, four litters of mice were analyzed.

The first hypothesis was that increased maternal care would be associated with reduced nicotine consumption. Previous research suggests that at least between litters, increased maternal care leads to reductions in anxiety-like behavior. This in turn is linked to nicotine use behaviors. Thus, anxiety-like behavior might mediate changes in nicotine consumption. The second hypothesis was that increased solicitation behaviors might be linked to increased nicotine consumption. This is based the possibility that associations between juvenile hyperactivity and subsequent nicotine use might extend to associations of higher activity in the early postnatal period and adolescent nicotine consumption.

Methods

Heterozygote Agouti viable yellow mice were a gift the Jirtle Lab at Duke University and bred for two generations under standard conditions at Pennsylvania State University under conditions approved by IACUC # 29898. Mice were maintained in shoe box cages on a 12/12 hour light dark cycle, with lights on from 08:00 hrs. Mice were observed in the evening under red light. The colony room was maintained at ~75ºF and 50% humidity throughout.

Four litters born to A^{vy}/a dams impregnated by *a/a* sires were observed by a protocol developed previously (Cavigelli et al. 2010). In brief, pups and dam were observed at 4 time points per day for 60 minutes per time point from postnatal days 2-8 with the day of birth being postnatal day 0. Three tests were conducted in the light phase (starting 1 hours, 5.5 hours, and 9 hours into the light phase) and one test was conducted in the dark phase (starting 1 hour into the dark phase). After the final observation each day (2 hours into dark phase), pups were removed from mother for 15 minutes and then each pup was weighed and marked with a non-toxic Sharpie marker on the belly, back, and back of head. Pup handling lasted less than 1 minute per pup. The number of maternal licking bouts per pup was recorded during each nest behavior observation

session. Each pup's maternally-directed behaviors were recorded. These included frequency of making snout contact with the mother, frequency of ventral probing (seeking a nipple to nurse), and pup location in relation to mother's snout (near or far). Pup location was recorded at 3-minute intervals, and ventral probing and snout contact were recorded as they occurred during the 60-minute observations. Each metric (licks, snout contact, ventral probing, and pup location as "easy licking distance") was summed across days 2-8 to test for their association with nicotine consumption outcomes.

Pups were weaned at postnatal day 28 and housed in groups of 2-4 by sex. Mice were also exposed to two 5 minute sessions of behavioral testing with exposure to novel objects and novel social animals between weaning and the initiation of nicotine consumption testing. Hypotheses about these data, while interesting (Gyekis et al. 2010, Ragan et al. 2010) are beyond the scope of this dissertation.

At postnatal day 36, mice were individually housed for fluid consumption tests. Each mouse was offered three bottles: tap water, 50 μg/ml nicotine, and 200 μg/ml nicotine in graduated cylinders (free base nicotine acquired from Sigma-Aldrich, St Louis). Bottles were rotated daily and evaporation rates measured from bottles on empty cages were subtracted from raw measures of daily change in volume from each bottle. The standard protocol of changing cages for fresh bedding once per week continued during adolescent nicotine consumption testing. In addition to daily fluid consumption, body weight and food weight were also recorded daily until completion of the three bottle choice test on postnatal day 49. Percent of fluid consumed from the nicotine bottles was the measure chosen for analysis of effects of nest behaviors on nicotine consumption.

Statistical tests for effects of nest behaviors (independent variables) on percent nicotine consumption (dependent variable) were calculated in SAS 9.2 with the Mixed procedure. Repeated measures of nicotine consumption (accounting for the nested structure of the data given

that siblings came from the same litters) were tested for effects of nest behaviors that were total numbers of observations from day 2 to day 8 (28 observation sessions, 1 hour each). The analysis did not control for between-dam effects on total licking (litters differed significantly), because here we were interested in whether absolute levels of licking received was associated with nicotine consumption. $P < 0.05$ was accepted as significant.

Results

Descriptive Statistics

The four litters of mice ranged from 6 to 11 offspring. In 7 days with 4 hours per day of observation, offspring were frequently observed ventral probing the dam, infrequently observed making snout contact with the dam, and frequently located near the dams snout ("easy licking distance"). Descriptive statistics of nest behaviors are shown in Table 4-1. Note that the differences in observed sample size for weaning weight are due to offspring mortality between birth and weaning. In each case, the pup that died began losing weight 1-3 days before death, and died in the first week post partum. Data from these pups were included in the descriptive statistics for nest behaviors, but obviously not in comparison of adolescent behaviors.

Table 4-1. Descriptive statistics on litter size, weaning weight, and nest behaviors of the four litters studied in this chapter. Means and standard errors are the averages for each offspring within the litter.

Dam ID	Litter Size	Variable	N	Mean	StdErr
55	9	Weaning Weight	8	15.01	0.69
		Total Maternal Licking	9	37.22	2.41
		Percent Near Maternal Snout	9	14.67	2.33
		Total Snout Contact	9	5.89	0.84
		Total Ventral Probing	9	56.78	3.29
63	6	Weaning Weight	6	16.50	0.42
		Total Maternal Licking	6	52.17	3.17
		Percent Near Maternal Snout	6	45.17	2.18
		Total Snout Contact	6	3.83	1.35
		Total Ventral Probing	6	80.83	5.79
87	10	Weaning Weight	10	12.09	0.47
		Total Maternal Licking	10	24.40	1.48
		Percent Near Maternal Snout	10	21.40	2.37
		Total Snout Contact	10	4.90	0.57
		Total Ventral Probing	10	84.10	4.16
91	11	Weaning Weight	9	11.85	0.28
		Total Maternal Licking	11	50.09	3.74
		Percent Near Maternal Snout	11	21.82	2.59
		Total Snout Contact	11	3.36	0.74
		Total Ventral Probing	11	67.18	3.19

Dams differed varied in their rate of licking across the observation period. Dam #55 licked 12.0 times per hour, #63 licked 11.2 times per hour, #87 licked 8.6 times per hour, and #91licked 19.7 times per hour. Substantial variation was observed in the number of licks received per pup, both within and across litters (Figure 4-1).

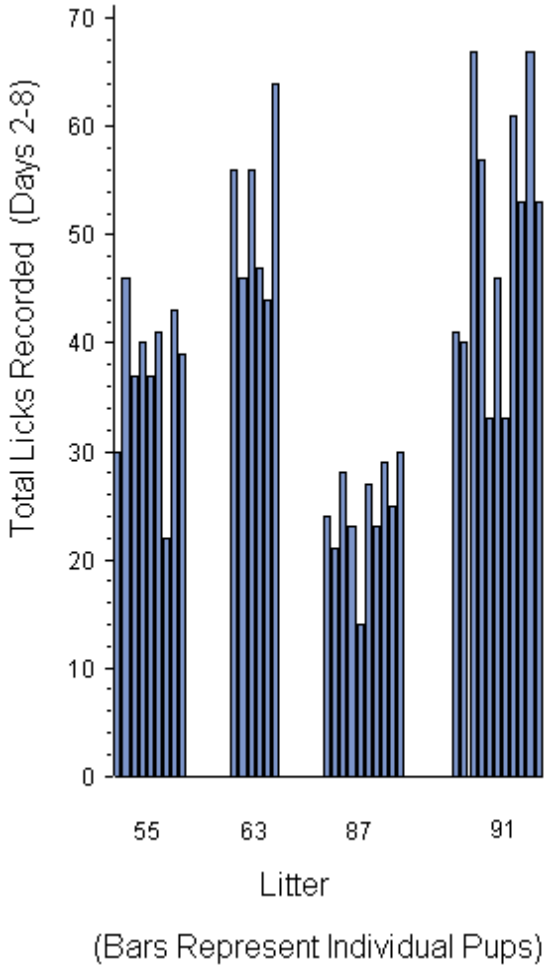

Figure 4-1. Illustration in the variability in total number of observed licks per pup days 2-8.

Offspring sex and genotype were unrelated to nest behaviors, but, as expected, nest behaviors were interrelated with one another (generally positively correlated), showed diurnal variation (more pup-dam interaction during the light phase), and changed across the week of observation (most pup-dam interaction in the first days after birth and declining thereafter; analyses not shown).

Tests for Nest Behaviors as Predictors of Nicotine Consumption

Linear regression models were used to test for effects of nest behaviors on nicotine consumption from adolescence to very early adulthood. Nest behaviors were totaled from days 2-8. Percent nicotine consumption was not associated with genotype, litter, neonatal maternally-directed behaviors (ventral probing, snout contact, “easy licking distance”) or maternal licking (all $p > 0.05$). The only significant predictor of nicotine consumption was an interaction between sex and test day (see Table 4-2), with females showing more of a decline in nicotine consumption across the two weeks of nicotine choice testing (Figure 4-2). Thus, no support was found for the hypothesis that high maternal licking reduced nicotine consumption. The hypothesis that higher rate of solicitation behavior would be associated with increased nicotine consumption was also not supported.

Table 4-2. Final model predicting nicotine consumption. No nest behaviors were significantly associated with nicotine consumption.

Effect	Sex	Estimate	Std Error	DF	t Value	Pr > |t|
Intercept		0.1701	0.01654	143	10.28	<.0001
Day		0.000060	0.001943	156	0.03	0.9754
Sex	f	0.04315	0.02362	144	1.83	0.0698
Sex	m	0	.	.	.	.
day*Sex	f	-0.00677	0.002783	157	-2.43	0.0162
day*Sex	m	0	.	.	.	.

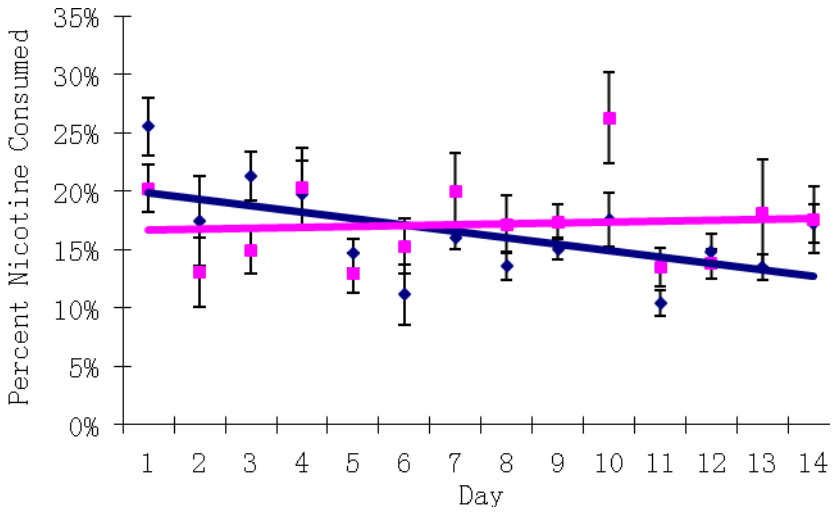

Figure 4-2. Illustration of the effect of sex on nicotine consumption across time. Day 1 of nicotine consumption testing corresponds to an age of 36 days. Consumption declined significantly for females (diamonds, blue line) but not males (squares, pink line).

Discussion and Conclusion

Previous studies have found that the nest behaviors measured in this study are significant predictors of young adult behavior in rats—increased maternal licking was generally associated with *slower* approach novel objects and *increased* anxiety in young adulthood (Ragan et al., *in press*). These same trends held in these agouti viable yellow litters (Ragan et al. 2010). Thus, this protocol for detecting individual differences in licking received within a litter appears to be relevant to subsequent behavior and, likely, neurological development. Despite adequate power to detect associations between nest behavior and subsequent phenotypes, no association was observed between variation in the amount of maternal care received by a pup in the first week of life and subsequent nicotine consumption behavior in adolescence.

At the same time that this study was initiated, van der Veen (2008) reported that fostering C57B6/J pups to either high or low licking dams had no effect on subsequent cocaine addiction

behaviors in an intravenous self-administration paradigm. Thus it is possible that the B6 genetic background is not sensitive to epigenetic programming by maternal licking behavior, or that such programming occurs in a fashion that does not influence drug use phenotypes. Alternatively, variation in maternal care between siblings could contribute to nicotine addiction liability, but in a manner that only emerges over the long term, as it may take a matter of months for rodents to develop addiction to oral nicotine (Galli and Wolffgramm 2011).

Additional work is also needed to confirm the presence of litter effects on adolescent nicotine consumption within inbred animals. Significant sibling correlations would indicate a shared environmental effect on nicotine consumption that could be due to intrauterine environment or nest behaviors of the dam. The lack of observed association with nest behaviors in this study suggests that intrauterine factors could be more important for determining litter effects on nicotine consumption.

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Chapter 5

Future Directions: What Determines Individual Differences in Adolescent Nicotine Consumption?

The current work indicates that gross differences in adolescent nicotine consumption are largely independent of imprinting (at least at one allele) and maternal care in the first week of life (at least the domains observed). Exposure to different dietary methyl-donors appear to slightly alter nicotine consumption, but only accounts for a small portion of the variance. Future research should continue to investigate the sources of individual differences in addiction susceptibility, especially for nicotine, but take different approaches. Two major lines of work appear particularly important:

- **Identifying epigenomic risk factors for nicotine addiction**: Are germ line gene expression control systems associated with nicotine use behaviors in adolescence and into adulthood? For example, do differences in DNA methylation or histone code on neurodevelopmental genes in the embryo predispose to subsequent drug use? Although exceedingly expensive now, technology is making it possible to divide embryos, use half of the cells for genome-wide sequencing of epigenetic marks (even including histones and RNAs), and use the other half of the embryonic stem cells for growth of the mouse. Thus, quantitative trait loci for phenotypes like nicotine consumption can be examined at the epigenetic level within inbred animals. This would provide a strong yes or no answer to the question that prompted the current dissertation—are epigenetic factors responsible for individual differences in nicotine consumption in inbred mice?

- **Development of a high throughput model of nicotine addiction**: Is it possible for mice to develop addiction like behavior in an efficient experimental paradigm? In the context of animal studies on drugs of abuse, "addiction" normally refers specifically to substance dependence evidenced by compulsive use, loss of control in limiting intake (for example loss of normal avoidance of secondary consequences in order to take the drug), and evidence of side effects of withdrawal (particularly dysphoria) when the drug is not available (Koob et al. 1998). While not all researchers use dependence and addiction as synonyms in the context of animal research, within the context of the current dissertation, dependence is considered a central manifestation of addiction that can be studied in animal models. By these standards, oral nicotine consumption has been shown to be addictive over the long term in some rodent studies, at least in the sense of nicotine-induced loss of control of bitter taste aversion (Galli and Wolffgramm 2011). However, the model developed by Galli and Wolffgramm (2011) requires year-long experiments and yields only a small fraction of "addicted" animals. This is inadequate for screening potential treatments for nicotine dependence. This concern has led to the proposal (Gyekis and Vandenbergh lab) to develop a line of mice selected for high nicotine consumption. Furthermore, it is important to evaluate patterns of consumption. One of the most widely used quantitative indicators of human tobacco addiction is time between getting up in the morning and first use (DSM-V). Can similar metrics be used in animals? Also important to the process of nicotine addiction in humans is the effect of nicotine-related cues on the brain (e.g. Goudrain et al. 2010, Franklin et al. 2011, Rubinstein et al. 2011), which can be modeled in animals (e.g. Scott and Hiroi 2011, de Bruin et al. 2011). Will addition of additional peripheral reinforcers—believed to be important for human nicotine addiction—result in more rapid acquisition of addiction-like behavior in an affordable oral consumption model?

Identifying Epigenomic Risk Factors for Nicotine Addiction

As conceptually predicted by Waddington (1942), the experience and conditions of parent cells can affect the phenotypes of the following generation of cells; this canalization turns out to be mediated by the patterns of DNA methylation, chromatin structure, and chromosome packing that are established during cell division (see Jablonka and Raz 2009 for a broad perspective). Variability in these epigenetic programs can strongly influence transcription throughout the genome and disease risk in subsequent generations (Skinner 2011). Not only do different individuals transmit different DNA methylation profiles in their gametes, but within an individual different gametes carry different DNA methylation profiles (Flanagan et al. 2006). Furthermore, recent research indicates that a spectrum of gene regulatory proteins, small RNAs, and mRNAs are inherited along with the "traditional" epigenetic molecules (DNA methylation and histones), indicating a broad spectrum of molecular mechanisms for transgenerational epigenetic programming (Bourc'his and Voinnet 2010).

Skinner (2011) has shown that for a variety of endocrine disrupting chemicals, intense exposures during a critical period of embryogenesis can cause changes in DNA methylation that alter transcription in a tissue specific manner and that these epigenomic changes are passed down through the male gametes for many generations after a single round of exposure. These have consequences for the brain and behavior—including expression of nicotine consumption related genes like DRD2 (Crews et al. 2007, Skinner et al. 2008), providing proof of concept for transgenerational epialleles affecting behavior. Thus, it appears quite possible that epigenetic programs could play a role in individual differences in nicotine consumption behaviors.

Whether or not heritable differential methylation profiles exist within inbred animals remains to be established—Skinner reports that that inbred animals did not show the transgenerational epigenetic responses seen under the same protocols with outbred animals in his

lab. Furthermore, selection based on behavioral traits within inbred strains showing variability in a trait does not result in a selection effect. However, just because an effect is not replicated with affinity in the population mean of the next generation, it does not rule out epigenetics. The stochastic variance in epigenetic programming, perhaps manifest in greater susceptibility to "environmental" effects in genetic models, can still be the determinant of future behavioral states.

To test the hypothesis that DNA methylation in the early embryo is associated with subsequent nicotine use behaviors, in the future, an embryo separation experiment can be attempted in either inbred or outbred animals. Using *in vitro* fertilization, the early embryo can be separated, one half isolated for evaluation of DNA methylation marks, histone marks, or regulatory RNAs, while the other half is allowed to develop into a mouse. These can be tested for association with adult behavior in the same manner as genome-wide association studies or microarray studies are conducted today. Ideally, such a study would be carried out with the collaboration of a great number of researchers who would collect a myriad of developmental and adult phenotypes for comparison to embryonic state.

Skinner's work also suggests an interesting avenue to follow up the behavioral effects of nicotine exposure. Nicotine can act as an endocrine disruptor, including reducing anogenital distance (Gyekis et al. 2010) and altering disease phenotypes at least into the F2 generation (Holloway et al. 2007). Based on Skinner's work, a multi- study on nicotine injection in an outbred strain in the critical window of fetal sex determination followed up into the subsequent three generations would provide the opportunity to evaluate the effects of direct whole-fetus nicotine exposure on behavior (F1), gamete exposure to nicotine (F2), and transgenerational effects (F3). Of particular interest to this dissertation is the possibility that dietary factors, including methyl donor supplementation, might moderate some of the transgenerational exposures

Development of a High Throughput Model of Nicotine Dependence

Oral nicotine consumption, if shown to have addiction-like properties, could be an ideal approach for developing a high-throughput animal model of nicotine addiction. Unlike intravenous self administration procedures, oral methods do not require surgery or such an expensive dose delivery apparatus. When the goal is to process a large number of animals in order to screen a large number of potential treatments, low cost and simplicity become priorities. However, oral consumption's strengths in terms of low cost per day and simplicity in terms of animal handling and apparatus are counterbalanced by the low proportion of of mice and the long period of time it takes to demonstrate oral nicotine addiction (Galli and Wolffgramm 2011). Thus, in order to explore whether the advantages of oral nicotine consumption can be realized in an addiction model, the following section proposes several changes to existing oral nicotine consumption tests that might lead to a model for nicotine addiction.

The first priority is to increase the proportion of mice that show a relatively high consumption of nicotine. By selectively breeding mice for high three-bottle choice nicotine consumption from a population of B6 x D2 advanced intercross mice, an increase in the proportion of mice consuming significant quantities of nicotine is expected.

However, even among high nicotine consuming mice, it remains unknown whether nicotine is addictive. Addiction is defined differently depending upon the intellectual tradition and the context of the research. In the current context, the neurophysiological dependence is of critical importance, especially insofar as the dependence involves evolutionarily conserved mechanisms shared between mice and men. As humans, other primates, and rodents become addicted to the drugs of abuse that they share, they show preference for use, willingness to suffer negative consequences for use, and withdrawal symptoms when use is terminated or delayed.

In rodent models of oral nicotine consumption, research rarely shows preference for consumption of nicotine solutions over water. The 50% threshold is useful for determining overt preference, but even if nicotine solution consumption is consistently less than half of fluid intake, it does not mean that the low nicotine consumption is not reinforcing at those levels. Clearly, the bottle choice method used for tastant preference is inadequate for evaluating the reinforcing properties of oral nicotine. Thus far, little work has been done to develop conditioned association studies for oral nicotine. A recent proposal (brainstormed by Gyekis, planned and in progress by Dingman and Vandenbergh) to test for oral nicotine conditioned place preference, conditioned odorant preference, and conditioned visual stimulus preference is underway to address this question.

The increased willingness of a subset of rats to consume nicotine-adulterated quinine after a long period of access to low concentrations of oral nicotine has been interpreted as a loss of control over nicotine consumption due to addiction, leading to consumption of quinine despite the undesirable bitter taste (Galli and Wolffgramm 2011). It remains unclear whether oral nicotine consumption is reinforcing under an operant setting. If nicotine is shown to support traditional operant reinforcement schedules, that would be the strongest approach to demonstrating that nicotine is addictive. To achieve this, it is likely that nicotine will be more reinforcing if paired with conditioned stimuli like light flashes, and it is possible that nicotine exposure will make these conditioned reinforcers more reinforcing even if they are not synchronous with nicotine doses (Cagguila et al. 2002). Careful planning of yoked control conditions, should be conducted. If mice are willing to work for nicotine, whether by licking a dry sipper, nose poking a hole, lever pressing, or running a maze, this would provide a strong corrolary to the willingness of people to work for tobacco products, while also avoiding the potential confounds of interactions between nicotine taste and quinine (for example, in Galli and Wolffgramm 2011 nicotine might simply reduce the bitter taste of quinine for some animals, and

not be an example of loss of control, but actually be a willing consumption of a reasonably palatable taste).

Evaluation of withdrawal symptoms upon loss of access to nicotine or upon exposure to nicotinic signaling antagonist drugs is one approach to demonstrate withdrawal. Mice that are identified as potentially addicted by other methods should be evaluated for overt somatic withdrawal signs (e.g. Kwilasz et al. 2009, Hamilton et al. 2010) to determine whether their addiction results in overt behavioral signs of withdrawal. However, from humans, people do not show stereotypies in response to lack of access to cigarettes still experience withdrawal, but do have a broad spectrum of other symptoms of withdrawal that can be modeled in animal models (reviewed by Malin and Goyarzu 2009). These include depressed intracranial self-stimulation thresholds (Johnson et al. 2008), impaired fear learning (André et al. 2008), weight gain (Fornari et al. 2007), increased anxiety-like behavior and conditioned place aversion (Jackson et al. 2008). Another approach to testing whether or not mice experience withdrawal is to evaluate the pattern and timing of voluntary comsumption. This is the logic behind one of the most commonly used questions about nicotine dependence (including the DSM-V): how long after waking do you smoke your first cigarette? To test the hypothesis that mice become addicted to oral nicotine, locomotor video monitoring will be established in the home cage and compared to nicotine use behavior. Relatively long periods of inactivity (stationary position) are strongly associated with EEG-verified sleep (Pack et al. 2007). Is the time between long bouts of immobility and first nicotine consumption shorter in mice that also show other indicators of addiction-like behavior? Is nicotine consumption behavior preferred over water, eating, or other activities immediately upon waking? If so, this could be an indication of preference presumably due to withdrawal.

Irrespective of the relationship to sleep, it is important to evaluate the schedule of use throughout the day. It is very possible that nicotine use behavior in the oral consumption test is simply part of a feeding and foraging behavioral habit. Under this hypothesis, it is predicted that

nicotine consumption will consistently co-occur with oral fluid consumption of water and eating. Alternatively, if nicotine consumption is a separate phenomenon, nicotine consumption is expected to follow a schedule of use significantly different from water drinking and feeding.

A final approach to addiction is the evaluation of a correlated endophenotype. Hyperactivity in response to psychostimulant drugs is believed to be an indicator of addiction. After repeated exposure to nicotine, cocaine, and other addictive drugs to the point of addiction, hyperactivity response to the drug becomes stronger and stronger—this is called locomotor sensitization (e.g. Philips and Di Ciano 1996). To test whether voluntarily consumed nicotine is associated with subsequent increased locomotor activity in mice, home cage activity will be monitored 24 hours per day over the course of one or more weeks in animals with access to nicotine, water, and alternative tastant (e.g. quinine) solutions. Locomotor speed in the ~15 minutes post nicotine fluid consumption will be evaluated in comparison to average waking locomotor activity (adjusted for time of day), activity following consumption of the control tastant, and activity following water consumption. If transient increases in locomotor speed following nicotine consumption are observed in a manner that is similar to the pharmacokinetics of oral nicotine, then this will be interpreted as a locomotor activating effect of voluntary nicotine consumption. If this locomotor activation increases over time, especially during the early stages of nicotine access, then this will be interpreted as a locomotor sensitization to nicotine and an indicator of the emergence of neurological processes that underlie addiction.

Because these techniques allow measurements on mice dozens or hundreds of times with an automatic electronic setup, the stress and work burden of laboratory technicians is reduced, while the number of repeated measures increases enough to allow for estimation of mouse-specific variance parameters (Molenaar et al. 2009). If successful, this project will not only allow for testing of addiction treatments, but will further the study of individual differences in

spontaneous behavior, instead of group differences in responses to forced experimental procedures.

Conclusion

The development of increasingly low cost, high throughput methods for both evaluating epigenetic marks and nicotine addiction behavior will be necessary to truly evaluate whether or not embryonic and early developmental programming plays a role in nicotine addiction. Work in the Vandengergh laboratory will continue to address many of the questions raised above about whether or not mice given access to voluntary oral nicotine starting in adolescence show signs of addiction by young adulthood. If successful, it will be possible to conduct stronger tests of hypotheses about the effects of perinatal exposure conditions on subsequent addictions, effects of epigenetic factors on perinatal conditions, and screen for potential treatments of nicotine addiction.

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Publications

Gyekis J, Anthony K, Foreman JE, Klein LC, Vandenbergh DJ. Perinatal nicotine exposure delays genital development in mice. *Reproductive Toxicology*. 2010 Jun;29(3):378-80.

Gyekis J, Foreman JE, Anthony K, Klein LC, Vandenbergh DJ. Activity-related behaviors in the hole-board predict nicotine consumption in C57B6 mice perinatally exposed to nicotine. *Behavioral Brain Research.* 2010 Jan 5;206(1):139-42.

Foreman JE, Lionikas A, Lang DH, Gyekis JP, Krishnan M, Sharkey NA, Gerhard GS, Grant MD, Vogler GP, Mack HA, Stout JT, Griffith JW, Lakoski JM, Hofer SM, McClearn GE, Vandenbergh DJ, Blizard DA. Genetic architecture for hole-board behaviors across substantial time intervals in young, middle-aged and old mice. *Genes Brain and Behavior*. 2009 Oct;8(7):714-27.

Support and Experience

Kligman Graduate Fellowship, August 2010-August 2011

Hintz Graduate Enhancement Scholarship, July 2009-July 2011

Teaching Assistant and Instructor in Biobehavioral Health, May 2007-August 2010

Graham Research Fellowship, August 2006-May 2007

Research Assistant, Penn State Cooperative Wetlands Center, June 2005-August 2007

Fully funded every summer, oral and poster presentations at conferences

Teaching Assistant in Geography, August 2006-May 2007

Science Writing Intern, *Research Penn State* Magazine, January 2004-Spring 2005

9 articles published in print and/or online

Schreyer Honors Scholarship, Fall 2002-Spring 2006

Hauser Prize for Best Presentation of Undergraduate Research 2004