

Perinatal nicotine exposure upregulates ER α in the dentate gyrus of adult male rat offspring

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Table of Contents

Abstract	iv
Acknowledgments	v
Abbreviations	vi
List of figures	vii
List of tables	ix
CHAPTER 1: Introduction.....	1
Prevalence and Risks of smoking during pregnancy	1
Mechanism of nicotine addiction in the brain.....	3
Nicotine Replacement Therapy	6
<i>i. Efficacy</i>	<i>7</i>
<i>ii. Safety.....</i>	<i>8</i>
NRT use during pregnancy.....	9
<i>i. Efficacy</i>	<i>10</i>
<i>ii. Safety.....</i>	<i>11</i>
<i>iii. Safety while breastfeeding</i>	<i>12</i>
CHAPTER 2. Understanding the Brain	12
Nicotine and the brain	12
Neuroanatomy	15
Hippocampal formation.....	16
<i>i. Hippocampus.....</i>	<i>16</i>
<i>ii. Entorhinal cortex</i>	<i>17</i>
Cellular markers.....	18
<i>i. Nissl.....</i>	<i>19</i>
<i>ii. GFAP</i>	<i>20</i>
<i>iii. ER α</i>	<i>22</i>
Objectives.....	26
Hypotheses.....	26
CHAPTER 3. Method.....	27
Animals and treatments	27
Tissue collection.....	28
Nissl stain.....	28
Immunohistochemistry	29
Microscopy and cell counting.....	30
<i>i. Microscopy preparation.....</i>	<i>30</i>
<i>ii. Digital image capture.....</i>	<i>31</i>
<i>iii. Cell counting</i>	<i>31</i>
Statistical analysis	32
CHAPTER 4. Results.....	33
Body weight	33
Nissl stain in the hippocampal formation.....	33
GFAP immunoreactivity in the hippocampal formation.....	36
ER α immunoreactivity in the hippocampal formation	38

CHAPTER 5. Discussion.....	40
CHAPTER 6. Interdisciplinary Approach to NRT	49
Determinants of Health.....	49
<i>Socioeconomic Status</i>	50
<i>Degree of addiction</i>	51
<i>Social Support</i>	52
<i>Culture</i>	54
<i>Mental Health</i>	55
<i>Health Services</i>	57
Recap - Determinants.....	59
Smokeless Tobacco	60
Stress associated with smoking cessation	61
CHAPTER 7. Conclusion	63
Appendix A	66
Bibliography	74

Abstract

Cigarette smoking during pregnancy contributes to the development of neurological health problems in offspring. As a result, public health organizations are recommending NRT to pregnant women to wean them off tobacco. If nicotine itself is injurious to the developing brain, then nicotine substitution may not eliminate the deleterious health outcomes of maternal smoking.

In studies of cognitive decline, estradiol elicits a neuroprotective effect through ER activation. However, the underlying mechanism remains unclear. Evidence suggests that estrogen-mediated neuroprotection is activated through glial cell interaction, mitigating inflammation and protecting neurons critical for learning and memory. If NRT antagonizes these cellular targets, it may put individuals at risk for future cognitive impairments.

Randomly assigned nulliparous female Wistar rats were injected subcutaneously with 1 mg/kg/day of nicotine bitartrate or saline for 2 weeks before mating until weaning (PND 21). Pups (saline n=6 and nicotine, n=6) were sacrificed at 26 weeks of age and the hippocampal formation was processed for Nissl and immunohistochemical staining for GFAP and ER α .

Gestational exposure to nicotine only produced a significant increase in the expression of ER α in the DG of the hippocampus. While additional research is needed, these findings suggest that NRT might indeed interfere with proper brain development, making offspring increasingly susceptible to long-term adverse health effects.

Le tabac fumé pendant la grossesse affecte de manière importante le développement neurologique de la progéniture, y compris à long terme. C'est pourquoi, les autorités de la santé publique recommandent les substituts nicotiniques comme soutien au sevrage tabagique chez les femmes enceintes. Si le danger se situe dans la nicotine de la cigarette, alors les produits de substitution nicotiniques risquent également d'interférer avec le développement cérébral.

De nombreuses données expérimentales convergent pour attribuer un rôle protecteur à l'oestradiol sur le fonctionnement cognitif. Par contre, le mécanisme sous-jacent est inconnu. Il se peut que l'oestradiol arrive à neutraliser la réaction inflammatoire provoquée par les cellules gliales, amoindrissant la détérioration des neurones impliqués au niveau de la mémoire. Ainsi, une perturbation de ce mécanisme par la nicotine pourrait engendrer une détérioration progressive des fonctions cognitives.

Des rats femelles Wistars nullipares assignées de façon aléatoire à un groupe ont reçu soit une injection sous-cutanée de 1mg/kg/jour de nicotine bitartrate ou de saline, 2 semaines avant l'accouplement jusqu'au sevrage au jour 21 postnatal. A 26 semaines, les rats furent sacrifiés (saline n=6 et nicotine, n=6) et une analyse du Nissl et immunohistochimique de GFAP et ER α furent réalisées sur les formations hippocampiques.

L'exposition prénatale à la nicotine a seulement augmenté significativement l'expression de ER α dans le GD de l'hippocampe. Alors que des études plus approfondies sont nécessaires, ces résultats suggèrent que les substituts nicotiniques affectent le développement neurologique périnatal, ce qui risque d'entraîner des répercussions à long terme sur la santé.

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Abbreviations

CA: Cornu Ammonis;
CBT: cognitive behavioral therapy;
CNS: central nervous system;
DA: dopamine;
DG: dentate gyrus;
GD: gestational day;
ER: estrogen receptor;
GFAP: glial acidic regulatory protein;
ir: immunoreactivity;
LMIC: low- and middle-income countries;
NAcc: nucleus accumbens;
nAChR: nicotinic acetylcholine receptor;
NRT: nicotine replacement therapy;
PND: postnatal day;
SES: socioeconomic status;
VTA: ventral tegmental area

List of figures

Figure 1. Dopaminergic pathway which regulates the rewarding effects of nicotine. Dopamine synthesized in the ventral tegmental area (VTA) neurons is released at their terminals in the nucleus accumbens.

Figure 2. Nissl-stained sagittal and coronal sections/views of a rat brain with accompanying color-coded diagrams of major brain structures

Figure 3. Perforant pathway of the hippocampal formation

Figure 4. Nissl stained cell bodies showing the different cell types coloured by the Nissl procedure

Figure 5. Nissl-stained section of the rat entorhinal cortex taken with a light microscope at a 10x magnification

Figure 6. GFAP immunohistochemistry in the CA1 of the rat hippocampus taken with a light microscope at a 10x magnification

Figure 7. ER α immunohistochemistry in the CA1 and dentate gyrus of the rat hippocampus taken with a light microscope at a 10x magnification

Figure 8. Body weights represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p<0.05$).

Figure 9. Cresyl violet staining in the CA1 subregion of the hippocampus. (A) Representative photomicrographs of Cresyl violet staining in the CA1 region in controls and nicotine exposed animals taken at 10x. Counting was done at 40x. (B) Nissl body count in neurons in the CA1 represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p<0.05$).

Figure 10. Cresyl violet staining in the CA3 subregion of the hippocampus. (A) Representative photomicrographs of Cresyl violet staining in the CA3 region in controls and nicotine exposed animals taken at 20x. Counting was done at 40x. (B) Nissl body count in neurons in the CA3 represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p<0.05$).

Figure 11. Cresyl violet staining in the DG region of the hippocampus. (A) Representative photomicrographs of Cresyl violet staining in the DG region in controls and nicotine exposed animals taken at 10x. Counting was done at 40x. (B) Nissl body count in neurons in the DG represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p<0.05$).

Figure 12. Cresyl violet staining in the entorhinal cortex. (A) Representative photomicrographs of Cresyl violet staining in the entorhinal in controls and nicotine exposed

animals taken at 10x. Counting was done at 40x. (B) Nissl body count in neurons in the entorhinal represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p<0.05$).

Figure 13. GFAP-ir in the CA1 subregion of the hippocampus. (A) Representative photomicrographs of GFAP-ir in the CA1 in controls and nicotine exposed animals taken at 10x. Counting was also done at 10x. (B) GFAP count in the CA1 represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p<0.05$).

Figure 14. GFAP-ir in the CA3 subregion of the hippocampus. (A) Representative photomicrographs of GFAP-ir in the CA3 in controls and nicotine exposed animals taken at 10x. Counting was also done at 10x. (B) GFAP count in the CA3 represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p<0.05$).

Figure 15. GFAP-ir in the DG subregion of the hippocampus. (A) Representative photomicrographs of GFAP-ir in the DG in controls and nicotine exposed animals taken at 10x. Counting was also done at 10x. (B) GFAP count in the DG represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p<0.05$).

Figure 16. GFAP-ir in the entorhinal cortex. (A) Representative photomicrographs of GFAP-ir in the entorhinal cortex in controls and nicotine exposed animals at 20x. Counting was done at 20x. (B) GFAP count in the entorhinal cortex represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p<0.05$).

Figure 17. ER α -ir in the CA1 subregion of the hippocampus. (A) Representative photomicrographs of ER α -ir in the CA1 in controls and nicotine exposed animals taken at 10x. Counting was done at 20x. (B) ER α count in the CA1 represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p<0.05$).

Figure 18. ER α -ir in the CA3 subregion of the hippocampus. (A) Representative photomicrographs of ER α -ir in the CA3 in controls and nicotine exposed animals taken at 20x. Counting was done at 20x. (B) ER α count in the CA3 represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p<0.05$).

Figure 19. ER α -ir in the DG subregion of the hippocampus. (A) Representative photomicrographs of ER α -ir in the DG in controls and nicotine exposed animals taken at 10x. Counting was done at 20x. (B) ER α count in the DG represented by mean $\pm$ SEM (n=6/group). The average count of ER α + cells was greater in the nicotine vs. control group ($p<0.05$).

Figure 20. ER α -ir in the entorhinal cortex. (A) Representative photomicrographs of ER α -ir in the entorhinal cortex in controls and nicotine exposed animals taken at 20x. Counting was done at 20x. (B) ER α count in the entorhinal cortex represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p<0.05$).

List of tables

Table 1. NRT products currently licensed in Canada that are available for over-the-counter purchase

Table 2. Bregma values from the Paxinos and Watson (2007) rat brain atlas used to photograph brain areas of interest for quantification.

Table 3. Distribution of brain sections for counting of cellular markers and subsequent statistical analysis

CHAPTER 1: Introduction

The tobacco epidemic is among the greatest threats to public health the world has ever faced. It is a major contributor of the top killer diseases (cardiovascular disease, chronic obstructive lung disease and lung cancer) (WHO, 2014) making it the leading preventable cause of premature death in Canada (Public Health Agency of Canada, 2013). Worldwide, it kills one person every 6 seconds (WHO, 2014). The 20th century saw 100 million tobacco-related deaths and if current trends continue, there will be up to one billion deaths in the 21st century (WHO, n.d.).

Cigarette smoking is a prototypical addictive disorder characterized by tolerance, withdrawal and use despite high personal cost - a cost that is especially high among pregnant women who continue to smoke regardless of the risks to their unborn child or newborn. For this reason, guidelines from public health organizations worldwide have been recommending that nicotine replacement therapy (NRT) be made available to pregnant women who are unable to quit by using non-pharmacologic methods (Fiore, Htsukami & Baker, 2002; Ministry of Health, 2007; NICE, 2002; NSW Department of Health, 2006; West, McNeil & Raw, 2000; Wong, Ordean & Kahan, 2011).

In this first section, I will describe current smoking trends and the properties of nicotine that are associated with smoking dependency. I will move on to the deleterious health outcomes associated with nicotine addiction, which have brought on the prescription of NRT to aid pregnant women with weaning from tobacco use. I will finish by discussing the potential efficacy and safety concerns that have arisen since NRT has gained popularity, focusing specifically on offspring of smoking mothers.

Prevalence and Risks of smoking during pregnancy

Studies show that approximately 13 to 27% of women in industrialized countries will smoke while pregnant (Colman & Joyce, 2003; Connor & McIntyre, 1999; Penn & Owen, 2002; Schneider, Huy, Schutz & Diehl, 2010; Schneider & Schutz, 2008). However, it is important to keep in mind that this is not an accurate representation of the actual proportion of women who smoke while pregnant. Due to the sensitive nature of this information, it is

not uncommon for women to underreport their smoking status to avoid the social stigma and feelings of guilt surrounding smoking in pregnancy. Reliance on self-reporting to identify pregnant smokers has been shown to underestimate smoking by approximately 25%, causing thousands of smokers each year to go undetected (Shipton et al., 2009).

The 2005 Canadian Community Health Survey reported that 13.4% of women who had given birth in the previous 5 years had smoked while pregnant (Heaman, Lindsay & Kaczorowski, 2009; Lindsay, Royle & Heaman, 2008), similar to recent statistics revealing that 15% of Ontarian women aged 15-49 years smoked while pregnant (Schwartz et al., 2010). These figures are slightly below the 17.7% reported in 2001 (Millar & Hill, 2004), a trend also observed in the United States whereby the rate of maternal smoking has seen a decline from 15.2% in 2000 to 13.8% in 2005 (Tong et al., 2009). Despite what these promising data would have us believe, health interventions over the past three decades, designed to diminish smoking in pregnancy, have not been especially effective (Greaves et al., p.4). It appears that the drop in the proportion of pregnant women who smoke is due to an overall decline in smoking rates among women of childbearing age, not by increased rates of smoking cessation related to pregnancy (Ebrahim et al., 2000; Husten, Chrismon & Reddy, 1996)

For those women who manage to quit smoking, it is often short-lived; we see an approximate 25% relapse rate during pregnancy (Klesges, Johnson, Ward & Barnard, 2001). A systematic review of smoking cessation in pregnant women living in developed countries revealed that 4.0% to 69.7% for population-based studies, and 26.5% to 47.0% for clinic-based studies, did not manage to quit smoking completely during their pregnancy (Schneider, Huy, Schutz & Diehl, 2010). Predictors of smoking in pregnancy included: a smoking partner, a large number of children, a high rate of tobacco consumption and poor prenatal care.

Meanwhile, tobacco use is increasing in many low- and middle-income countries (LMICs) in an effort to replace lost consumers from wealthier countries. Historically, few women living in LMICs have smoked tobacco. Because of women's lower social and economic status, smoking rates among pregnant women remained low (9%) (Bloch 2008) but there is strong evidence to suggest a change in this trend. This number is expected to rise to 20% by 2025, shifting the tobacco epidemic from high-income countries to LMICs

(Samet 2001; Richmond 2003). This increase has been attributed in part to aggressive marketing from the tobacco industry, which is predicting high profits from sales (Kaufman 2001), along with increased tobacco production (FAO 2003). In order to make up for the declining sales in industrialised nations, the tobacco industry has been employing clever advertising ploys in LMICs to capitalize on women's desire for equal rights by associating smoking with social desirability, success and freedom (Amos, Greaves, Nichter & Bloch, 2012).

In addition to targeting this largely untouched population of new tobacco users, weak regulation of tobacco marketing has led to a rapid increase in smoking among women, especially those considered vulnerable (Kaufman, 2001; Gilmore & McKee, 2005; Graham, 2009). A survey conducted among pregnant women in two African countries is just one example that goes to show this particular group is not only exposed to advertising for tobacco products but their knowledge of the risks associated with smoking and second hand smoke is extremely limited (Chomba 2010), making them particularly vulnerable to tobacco marketing. The surge in smoking rates among women in the developing world will worsen pregnancy outcomes and potentially undermine or reverse hard-won gains in maternal and fetal health. If predictions are correct, 80% of deaths caused by tobacco will occur in LMICs by the year 2030 (Nichter et al., 2010).

Tobacco use is an important preventable cause of poor pregnancy and infant outcomes. Exposure of the fetoplacental environment to cigarette smoke has been associated with an array of obstetrical, fetal and developmental complications. Researchers estimate that eliminating cigarette smoking in pregnancy could prevent up to 5% of perinatal deaths, 20 to 30% of low birth weight deliveries, and 15% of preterm deliveries (Crawford et al., 2008). A more recent study in Alberta looking at data from 2001 to 2005 revealed that successful smoking cessation could indeed reduce neonatal morbidity by 10 to 15% (Burstyn, Kapur & Cherry, 2010).

Mechanism of nicotine addiction in the brain

A proper understanding of the physiological basis of nicotine addiction and smoking behavior provides the necessary foundation that precedes the conception of therapeutic

advances in smoking cessation therapy. Hence, it is important to describe the steps involved when nicotine dependence develops, particularly as it pertains to the central nervous system.

When cigarette smoke is inhaled, nicotine is absorbed through the pulmonary venous system, reaching the brain within 10 to 20 seconds (Le Houezec, 2003). Similarly to other drugs of abuse such as cocaine and amphetamines, nicotine stimulates dopamine (DA) release in the brain, a key chemical involved in the reward response and the development of dependencies (Court et al., 1998; Di Chiara & Imperato, 1988; Imperato et al., 1986). It does so by altering the activity of the mesolimbic dopaminergic pathway (Figure 1), whereby ventral tegmental area (VTA) neurons enhance DA release in the nucleus accumbens (NAcc) (Pidoplichko et al., 1997).

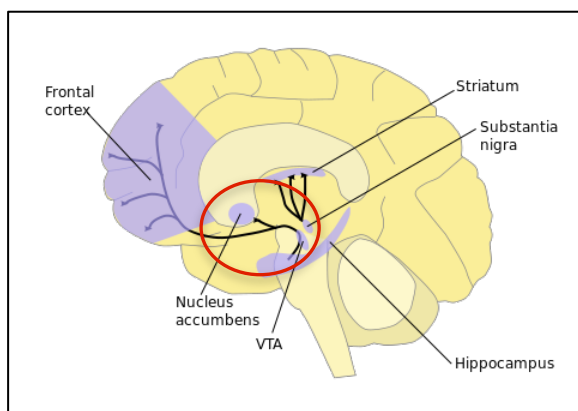


Figure 1. Dopaminergic pathway which regulates the rewarding effects of nicotine. DA synthesized in the ventral tegmental area (VTA) neurons is released at their terminals in the NAcc (National Institute on Drug Abuse, 2008).

More specifically, nicotine interacts with specific membrane receptors called neuronal nicotinic acetylcholine receptors (nAChRs) (Mansvelder & McGehee, 2002). Although acetylcholine is the natural ligand for these receptors, nicotine has a higher affinity for certain nAChRs subtypes (Albuquerque, Pereira, Alkondon & Rogers, 2009).

Tobacco dependency is mediated by nAChRs on neurons of the VTA, which project their axon terminals to the NAcc (Champtiaux et al., 2003). Although nAChRs are expressed throughout the brain in areas such as the NAcc, hippocampus and cortex, it is the nAChRs within the VTA that mediate the rewarding effects of nicotine (Corrigall, Coen & Adamson, 1994; Nisell, Nomikos & Svensson, 1994), as part of the mesolimbic reward pathway. Their activation transmits the signal down the axon and stimulate the release of DA in the NAcc

(Mansvelder & McGehee, 2002), thus, generating short-lived feelings of wellbeing, improved mood and increased attention (Benowitz et al., 2008).

A single exposure to nicotine increases DA release in NAcc from VTA DA neurons for more than an hour *in vivo* (Di Chiara and Imperato, 1988; Imperato et al., 1986; Schilstrom et al., 1998). However, the nAChRs on the DA neurons desensitize in seconds to minutes in the presence of physiologically relevant nicotine concentrations (Pidoplichko et al., 1997; Dani, Radcliffe & Pidoplichko, 2000). Between cigarettes, chronic smokers maintain a high enough concentration of nicotine to deactivate the receptors and slow down their recovery (the ability to go from the non-functional desensitized state back to a functional resting state where it has the potential to once again be activated), causing nAChRs to become temporarily unresponsive to their endogenous neurotransmitters or any other agents (Benowitz, 2008). This creates a state of desensitization that is artificially prolonged by continual exposure to nicotine (Quick & Lester, 2002). Generally, arterial blood and brain nicotine concentrations will increase sharply following exposure, then decline after a 20 to 30 minute period as nicotine redistributes to other body tissues (Le Houezec, 2003), with a steady-state volume of distribution of about 2.6L/kg (Hukkanen, Jacob & Benowitz, 2005). The slow release of nicotine from these tissues will lead to a similar pattern in cotinine levels (nicotine's main metabolite), gradually rising during the day, peaking at the end of smoking and maintaining high concentrations overnight (Benowitz et al., 1983).

In humans, approximately 70-80% of nicotine is converted to cotinine by the liver, via the cytochrome p450 2A6 enzyme (Benowitz & Jacob, 1994). In non-pregnant adults, the average half-life of nicotine is 1.85 hours and that of cotinine is approximately 16.6 hours (using urinary excretion), whereas during pregnancy they drop to 1.62 and 8.8 hours (Dempsey, Jacob & Benowitz, 2002). As nicotine is eliminated, DA levels decline which results in a craving for more nicotine (Benowitz, 2008). After a brief period without nicotine exposure, (such as a night's sleep), the baseline concentration of nicotine drops and some of the receptors regain their sensitivity (Dani & Heinemann, 1996). Once these receptors are functional, cholinergic neurotransmission is raised to an unusually high level that affects all the cholinergic pathways in the brain. This causes smokers to experience withdrawal symptoms such as irritability, depressed mood, restlessness, anxiety, difficulty

concentrating, increased appetite, insomnia and tobacco cravings that leads them to smoke another cigarette (Government of Canada, 2013). Over time, nAChRs undergo adaptive changes such as up-regulation and desensitization that lead to a stronger need for stimulation in order to achieve the reward of smoking – thus leading to nicotine tolerance and in most individuals, dependence (Benowitz, 2008).

Cigarette exposure can increase nAChRs up to 4-fold in chronic smokers (Perry et al., 1999). This neurophysiological anomaly is not exclusive to the smoker; it will also produce a 20-50% fetal nAChR up-regulation, depending on the brain region examined, in offspring of smoking mothers (Nachmanoff et al., 1998).

Nicotine Replacement Therapy

Nicotine replacement therapy is a smoking cessation aid that delivers nicotine to reduce cravings and alleviate withdrawal symptoms, thereby easing the transition from cigarette smoking to complete cessation (West & Shiffman, 2001). As evidence condemning tobacco products as a major cause of premature death worldwide continues to mount, so does NRT accessibility and prescription by medical professionals.

Various formulations of these "quit smoking aids", such as nicotine gum, transdermal patch, nasal spray, inhaler, sublingual tablets, and lozenges (see Table 1), are buffered to alkaline pH to facilitate absorption of nicotine through cell membranes (Benowitz, Hukkanen & Jacob, 2009). But unlike inhalation (as in smoking cigarettes), the oral, nasal and transdermal routes of nicotine absorption are much slower and result in a slower gradual increase of nicotine concentrations in the blood and brain.

These different types of NRT also differ in terms of their dosage and rate of nicotine delivery depending on the needs of the smoker. The chewing gum, sublingual tablet, lozenge, inhaler and nasal spray provide fast-acting, intermittent doses of nicotine whereas transdermal patches deliver nicotine slowly and continuously to the brain (Perera et al., 2008). Table 1 summarizes the products currently licensed in Canada that are available for over-the-counter purchase.

Type	Available dose	Plasma nicotine
Transdermal patch	5mg, 10mg, 15mg worn over 16 hours 7mg, 14mg, 21mg worn over 24 hours	10-20 ng/ml*
Chewing gum	2mg, 4mg	5-15ng/ml*
Sublingual tablet	2mg	
Lozenge	1mg, 2mg, 4mg	
Inhalation cartridge plus mouthpiece	10mg	
Nasal spray	0.5mg/spray	

Table 1. NRT products currently licensed in Canada that are available for over-the-counter purchase

*Benowitz et al., 1987, 1995; Schneider, Olmstead, Franzon & Lunell, 2001)

In comparison, an average cigarette contains 10-14 mg of nicotine (Kozlowski et al., 1998), of which approximately 1-1.5 mg is absorbed systematically during smoking (Benowitz & Jacob, 1984). Therefore, the typical pack-per-day smoker will absorb 20 to 40 mg of nicotine each day (Henningfield, 2005) and plasma concentrations from daily smoking will vary between 10-37 ng/ml (Schneider, Olmstead, Franzon & Lunell, 2001).

Nicotine, the main alkaloid found in tobacco products, is considered one of the components most harmful to fetal development (Yildiz, 2004). It crosses the placenta and can be detected in the fetal circulation at levels that exceed maternal plasma levels by 15% and amniotic fluid levels by 88% (Luck, Nau & Hanesen, 1985).

i. Efficacy

The reasoning behind NRT is that it delivers nicotine, the key ingredient involved in tobacco dependency. As such, medicinal nicotine products also stimulate NACHRs in the VTA of the brain, thereby releasing DA in the NAcc and lessening nicotine withdrawal symptoms in regular smokers who abstain from smoking.

The primary downfall of NRT products is their varying kinetic profiles, which lead to dosing and compliance problems. Nicotine pharmacotherapy relies on systemic venous absorption, which results in lower levels of nicotine over a period of minutes (for nasal spray or oral products such as gum, inhaler, sublingual tablet, or lozenge) and hours (for transdermal patches) (Molyneux, 2004). On the contrary, when cigarette smoke is inhaled, it is absorbed via the pulmonary circulation allowing nicotine to reach the brain in a matter of seconds. This inability to reproduce the rapid and high levels of arterial nicotine compromises NRT's ability to eliminate withdrawal symptoms entirely.

For example, absorption from the nicotine gum is slow and persists even after chewing stops; therefore it is very difficult to adjust the dose (Benowitz, 1988). Similarly, the transdermal patch's gradual release (3-8 hour peak) (Fant et al., 2000) negates its ability to satisfy a craving. Though the 24-hour patch was developed to solve this problem (as an alternative to the 16-hour patch), the wearer is subjected to a lengthy exposure period that far surpasses that of an actual smoker (Le Houezec, 2003). Finally, while the nasal spray has a more comparable pharmacokinetic profile to smoke inhalation (5-10 min peak) that allows for immediate self-administration, it is difficult to control for the dose administered and treatment regimen compliance (Schneider et al., 1995).

When NRT is taken in the prescribed manner, it increases the likelihood of quitting smoking. According to a meta-analysis of 117 randomized control trials with over 50 000 participants, NRT increases the long-term cessation rate by 50 to 70% in smokers who are motivated to quit and have high levels of nicotine dependence (Stead et al., 2012). The authors do make it a point to highlight the lack of evidence to support these conclusions in individuals smoking fewer than 10 to 15 cigarettes a day.

ii. Safety

In comparison to tobacco products, NRT's primary benefits are twofold. Firstly, it is composed of a sole chemical rather than the thousands found in cigarettes/tobacco. Secondly, it delivers a smaller dose of nicotine than an habitual smoker would be exposed to, allowing for gradual weaning from nicotine altogether. When nicotine is provided via NRT, the user avoids approximately 4000 other chemicals that are inhaled with nicotine in tobacco smoke (Stedman, 1968). Because NRT provides a clean nicotine delivery system without the other dangerous chemicals found in tobacco, it is associated with fewer side effects than cigarette smoking.

A first avoidable side effect is that its potential for abuse is low (West et al., 2000; West & Shiffman, 2001). The potential for abuse of a substance largely depends on the dose and the speed of delivery to the brain. Since administration and central reinforcement occur closely together in the case of tobacco smoking, it can easily become addictive (De Wit, Bodker & Ambre, 1992; Henningfield & Keenan, 1993; Jaffe, 1990). On the contrary, the slow increase in arterial plasma and brain nicotine concentrations produced from NRT mean

that users are not at risk for developing a physiological addiction (West & Shiffman, 2001; OMA, 2008). Additionally, cigarettes contain additives that enhance nicotine delivery and absorption such as ammonia (which speeds the delivery of nicotine by raising the pH of tobacco) and theobromine (which dilates the airways facilitating inhalation) (Le Houezec, 2003).

Another common misconception is that NRT will exert similar damaging effects on the respiratory and cardiovascular systems as cigarette smoking; in fact NRT has not been shown to increase the risk of lung cancer, chronic respiratory disorders and heart disease (Russell, 1991; OMA, 2008; Perera, 2008). Nicotine is not responsible for such diseases but rather the tar, carbon monoxide and other harmful gases found in cigarette smoke (Le Houezec, 2003).

NRT use during pregnancy

When women smoke during pregnancy, nicotine readily crosses the placental barrier and is detected in the amniotic fluid and umbilical cord of newborns (Luck, Nau, Hansen, Steldinger, 1985). Fortunately, data support improved pregnancy outcomes with early smoking cessation. If smoking is discontinued prior to the first prenatal visit, the risk of complications is diminished to that of a non-smoker (Greaves et al., 2011, p.3). Mid- to late-gestation appears to be the period most vulnerable to the harmful effects of nicotine for the developing fetus (Prabhu et al., 2010). Thus, the objective is to be nicotine free especially during the third trimester when the fetus responds most adversely to nicotine administration (Bergsjö, Bakketeig & Lindmark, 2007; Bernstein et al., 2000; Lampl, Kuzawa & Jeanty, 2003; SOGC, n.d.; Zaren, Lindmark & Bakketeig, 2000). If NRT use is required, it is crucial that treatment begins early in the pregnancy in order to be discontinued as soon as possible, thus, minimizing the risks to the fetus associated with perinatal exposure.

NRT use is cautioned rather than contraindicated in numerous developed countries. In the US (Fiore, Htsukami & Baker, 2002), Australia (NSW Department of Health, 2006), New Zealand (Ministry of Health, 2007), United Kingdom (NICE, 2002; West, McNeil & Raw, 2000) and Canada (Wong, Ordean & Kahan, 2011) guidelines recommend that with some qualifications, NRT be used by pregnant women. The Society of Obstetricians and Gynaecologists of Canada advocate for NRT use in women for whom behavioural therapy

alone for smoking cessation has failed and only after an informed discussion of the benefits and risks of therapy. They also suggest using the lowest dose of NRT, which, in the case of the transdermal patch, means removing it at night (Wong, Ordean & Kahan, 2011).

Despite NRT's popularity, a systematic review of randomized control trials in which NRT was used, concluded that information on the safety and efficacy of NRT during pregnancy is lacking (Coleman et al., 2011).

i. Efficacy

Since pregnant women are largely excluded from drug trials (Rayburn, Bogenschutz, 2004), little information is available about efficacy or safety in that group. What's more, the metabolic clearance of nicotine and cotinine is markedly faster during pregnancy (60% and 140% respectively) (Dempsey et al., 2002), increasing steadily between the first and third trimester (Koren, Blanchette, Lubetzky & Kramer, 2008). Thus, higher doses of NRT may be needed to attain an effect for cessation.

In order to control for this potential underdosing of nicotine stemming from increased nicotine metabolism, a group of researchers individually adjusted the NRT dose to match levels attained by smoking. They found that NRT did not improve relapse rates compared with placebo controls. Of the 402 pregnant women, only 5.5% (n=11) in the nicotine patch group achieved abstinence compared to 5.1% (n=10) in the placebo patch group (Berlin, Grange, Jacob & Tanguy, 2014).

This coincides with a large randomized control trial (n=1050) conducted by Coleman et al., (2012) the results of which suggested that smoking cessation treatment failure may stem from high participant dropout rate (Coleman et al., 2012). Only 7.2% of women (35 of 485) who received 15 mg patches and 2.8% (14 of 496) assigned to the placebo group reported using trial medications for more than 1 month. In addition to these two large studies, numerous smaller studies have experienced poor NRT compliance rates, rarely exceeding one month of use (Wisborg et al., 2000; Pollack et al., 2007; Coleman, Chamberlain, Cooper & Leonardi-Bee, 2011).

Overall, controlled trials have failed to substantiate claims that NRT increases smoking cessation rates (Oncken et al., 2008; Kapur et al., 2001). Nevertheless, a link between NRT use and a reduction in the number of cigarettes smoked has been established. A randomized

trial (n=181) found that adding NRT to cognitive behavioral therapy (CBT) resulted in higher cessation rates than counseling alone (Pollack et al., 2007). Unfortunately, this trial was stopped early because of increased incidence of serious adverse events in the CBT+NRT arm compared with the CBT-only group. It was later reported that the rate of negative birth outcomes was partially confounded by the fact that a larger proportion of women in the CBT+NRT arm had had a history of preterm births than women in the CBT-only arm.

It is possible that NRT efficacy might vary based on individual differences such as the level of addiction. Two reviews of NRT during pregnancy agree that pregnant women smoking fewer than 5 cigarettes per day would benefit more from behavioral support than NRT, while moderate and heavy smokers should use NRT under medical supervision (Forinash et al., 2010; Osadchy, Kazmin & Koren 2009).

ii. Safety

The link between NRT and congenital anomalies and poor perinatal outcomes remains ambiguous (Oncken et al., 2008; Pollack et al., 2007; Dwyer, Broide & Leskie, 2008; Slotkin, 2008; Gaither, 2008; Morales-Suárez-Valera, Bille, Christensen & Olsen, 2006). A systematic review and meta-analysis in which NRT was used with or without behavioral support revealed that birth outcomes such as low birth weight, preterm birth, perinatal mortality and neonatal intensive care admissions were less frequent among infants born from mothers who used NRT than those who continued to smoke or received placebo (N=695 pregnancies) (Coleman et al., 2011). However, none of these differences reached statistical significance.

Nevertheless, women may be offered NRT if counseling is not successful and after an informed discussion about the benefits and risks during pregnancy (Rore, Brace, Danielian & Williams, 2008; Crawford, Tolosa, Goldenberg, 2008; Pauly & Slotkin, 2008; Coleman, 2007). Moreover, nicotine coupled with the thousands of chemicals present in cigarette smoke is likely far more harmful than NRT. When used properly, the level of nicotine to which the fetus is exposed with the transdermal patch or gum is lower than that of cigarettes (Benowitz et al., 2000; Oncken et al., 2008).

The Society of Obstetricians and Gynaecologists of Canada advises using the lowest

effective dose of NRT such as nicotine gum or nasal spray, which provide an intermittent dose of nicotine instead of the transdermal patch, which offers a continuous dose. On the chance that the transdermal patch is used, guidelines advise women to remove it at night to avoid any unnecessary nicotine exposure. Finally, NRT should be discontinued if the patient continues to smoke at the same pre-NRT rate (Wong, Ordean & Kahan, 2011).

iii. Safety while breastfeeding

Nicotine is excreted into breast milk in small quantities, but the concentrations are much lower than in adults who smoke or use NRT (Dempsey & Benowitz, 2001). It is possible that the NRT formulation plays a role in the level of exposure to the infant. Again, transdermal patches provide a steady dose of nicotine in maternal plasma and thus in breast milk. NRT products that are taken intermittently might be preferable to prolong nicotine-free intervals for breastfeeding. While the risk associated with such small doses of nicotine is minimal (Dempsey & Benowitz, 2011), women are recommended to breastfeed before using NRT to minimize infants' exposure (Action on Smoking and Health, 2007).

Complete avoidance of all nicotine should therefore be the objective in pregnancy and breastfeeding. The majority of women will do so without formal intervention (Anderka et al., 2010; Crawford, et al., 2008; DiFranza, et al., 2004; Salmasi, et al., 2010) but in cases where women failed to quit, NRT use is justifiable in relation to the risk of continued smoking.

CHAPTER 2. Understanding the Brain

Nicotine and the brain

Evidence from animal models suggests that *in utero* nicotine exposure during critical periods of brain development may be harmful to cognitive functioning of offspring. Nicotine is a toxicant with the ability to alter cellular cascades that modify long-term synaptic patterning, increasing vulnerability for behavioral and learning disabilities later in life (Blutstein, 2013). While deleterious health outcomes might not be noticeable immediately, nicotine remains a teratogen with the ability to interfere with neurodevelopment in certain

brain areas such that functional consequences of exposure may only be noticeable later in life (Slotkin, 2008).

One such brain area is the hippocampus, a key brain region involved in cognition, learning and memory. In the rodent, differentiation of the hippocampal formation (composed of the hippocampal proper and entorhinal cortex, see the neuroanatomy section below) occurs during the late prenatal and early postnatal periods (Avishai-Eliner et al., 2002). Thus, exposure to teratogens such as nicotine during this stage of development has the potential to elicit detrimental effects on the maturing hippocampus. These cellular and molecular changes may result in neurobehavioral changes later in life (Levin et al., 1996; Lichtensteiger et al., 1988) such as hastening the onset of neurodegenerative disorders or other cognitive deficits in adulthood and senescence.

To date, studies that have evaluated the safety of NRT use during pregnancy have focused mainly on the acute risks of nicotine exposure on the developing fetus (Kolas, Nakling & Salvensen, 2000; Morales-Suarez-Varela, Bille, Christensen & Olsen, 2006; Oncken et al., 2008; Pollack, Oncken et al., 2007; Strandberg-Larsen, Tinggaard, Nybo, Olsen & Gronback, 2008; Williams, Evans & Newnham, 1997; Wisborg, Henriksen, Jespersen & Secher, 2000). What's more is that the study designs have made results difficult to interpret, especially when ascertaining the role of nicotine alone. Such flaws include important confounding variables not accounted for. Because self-reported smoking status is not accurately validated by measurement of cotinine, researchers cannot ensure that participants use NRT products correctly or use them in conjunction with cigarettes. There is also a risk of recall bias and underreporting of nicotine consumption. These may lead to skewed results and fail to properly assess the safety and efficacy of NRT in pregnant women.

As for the long-term consequences of NRT, there are no prospective epidemiological studies that have examined its use during pregnancy. Nevertheless, the consensus seems to be that impaired neurobehavioral and cognitive function is a chief outcome associated with prenatal nicotine exposure, seen in both animals and humans (Levin, Briggs & Rose, 1993). Therefore, while cigarette smoke is composed of numerous toxicants, the literature strongly points to nicotine as a key chemical involved in mediating the adult-onset neurological outcomes of cigarette smoke exposure. For example, epidemiological studies determined

that maternal smoking leads to deficits on tasks that require learning, memory, and problem solving skills in children up to age 10 (Cornelius et al., 2001; DiFranza, Aligne & Weitzman, 2004). It has also been strongly linked to attention-deficit hyperactivity disorder (Braun, Kahn, Froehlich, Auinger & Lanphear, 2006; Button, Maughan & McGuffin, 2007; Jacobsen et al., 2007; Obel et al., 2008; Schmitz et al., 2006; Thapar et al., 2003; Tong & McMichael, 1992).

This is comparable to animal studies in which rodents have been prenatally exposed to nicotine. The offspring exhibit hyperactivity, cognitive impairment, increased anxiety and persistent neurochemical alterations (Dwyer, McQuown & Leslie, 2009; Pauly & Slotkin, 2008; Winzer-Serhan, 2008). Attentional and spatial memory deficits have also been observed in the Morris water maze and radial-arm maze tasks in prepubertal, adolescent, and adult rats exposed to nicotine *in utero* (Cutler, Wilkerson, Gingras & Levin, 1996; Levin, Briggs & Rose, 1993; Sorenson, Raskin & Sub, 1991). Nevertheless, the long-term outcomes and mechanisms underlying such deficits remain largely unknown.

One hypothesis as to the underlying mechanism involves neuroimmunomodulation, a process involving the communication between cells of the nervous, immune and endocrine systems (Arevalo et al., 2011). Central nervous system (CNS) damage, which occurs under pathological conditions, such as exposure to environmental toxicants, disrupts homeostasis in these systems and can trigger a number of deleterious health outcomes. Neural cell response to injury is referred to as neuroinflammation (Streit, Mrak & Griffin, 2004) and can be either acute or chronic (Lucas, Rothwell & Gibson, 2006). An acute response is associated with an abnormal increase in the number of cerebral astrocytes; this is caused by CNS trauma and accompanied by the release of pro-inflammatory molecules that aim to protect the neural tissue. The latter consists of a prolonged activation of some glial cell types, which can promote neural damage if not properly controlled. Estradiol can play a modulatory role in this reaction; when acting through its receptors, this hormone serves to regulate the inflammatory response by modulating glial cell activation and the release of these inflammatory molecules (Arevalo et al., 2011).

Though scarce, recent work has begun to address the contribution of perinatal nicotine exposure on long-term changes to neuronal and glial populations underlying cognitive and behavioral changes (Abdel-Rahman et al. 2004, 2005; Abou-Donia et al. 2006;

Blutstein et al., 2013; Roy et al. 2002). Nevertheless, the emphasis tends to be on animals whose developmental stage is equivalent to childhood or adolescence in humans. More research is needed to examine if the aforementioned changes affecting learning and memory persist in adults. Particular attention should be given to neuronal and glial elements in the hippocampal formation as well as estrogen receptors that play a role in modulating the chronic inflammatory response in brain tissue.

Neuroanatomy

The rat brain is composed of 3 parts: the forebrain, the midbrain and the hindbrain (Paxinos & Watson, 2013). The forebrain is divided into the telencephalon, the hypothalamus and the diencephalon (Figure 2). The frontal part of the forebrain is the olfactory bulb that receives nerve fibers from the roof of the nose. The midbrain connects the forebrain and the hindbrain. The latter comprises the isthmus, the cerebellum and the rhombomeres, which is connected with the spinal cord.

The mammalian brain is surrounded by a smooth structure termed the cerebral cortex (Paxinos & Watson, 2013). It's the cerebrum's outermost layer composed of gray matter consisting mainly of neuronal cell bodies accompanied with an underlying layer of white matter composed of myelinated axons (Zhang & Sejnowski, 2000). Different cortical areas are utilized in the performance of motor, visual, auditory, olfactory and somatosensory functions (Paxinos & Watson, 2013).

Sub-cortically, we find the corpus callosum, the major commissure of the forebrain connecting the right and left sides of the cerebrum (Paxinos & Watson, 2013). Deeper in the brain we find a series of structures that form the limbic system. They include regions necessary for species survival, specifically arousal, emotion, motivation and memory (Morgane, Galler & Mokler (2005). Some of the more prominent areas composing the limbic system include the hypothalamus, amygdala and hippocampus. The hippocampus is between the corpus callosum and thalamus and occupies a large portion of the forebrain in the rat.

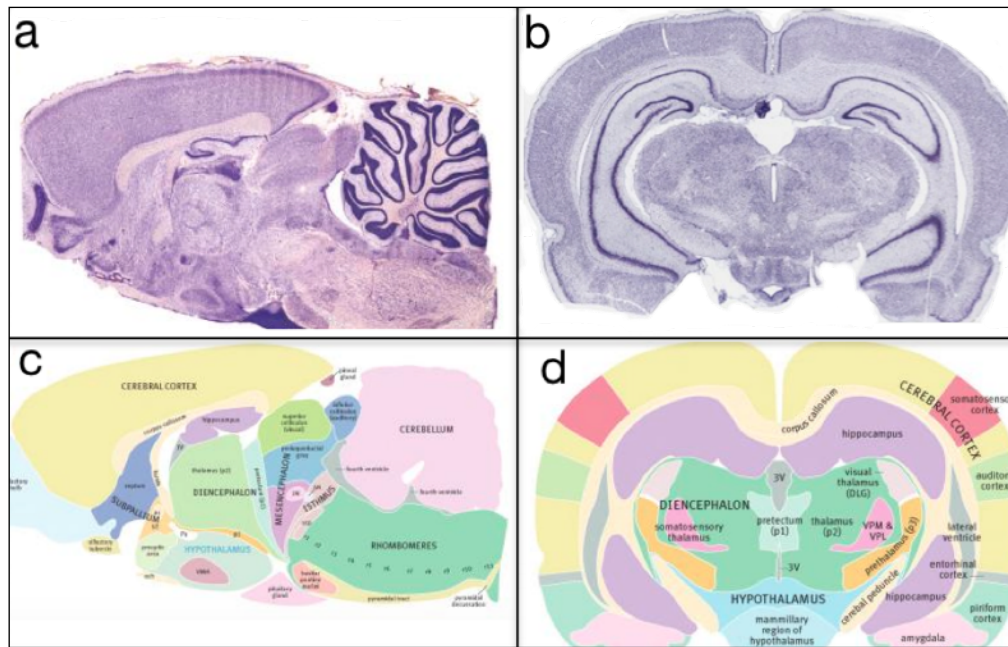


Figure 2. Nissl-stained sagittal (a) (Paxinos & Watson, 2013) and coronal (b) (Jones, E., 2007) sections/views of a rat brain with accompanying color-coded diagrams of major brain structures (c, d) (Paxinos & Watson, 2013)

Hippocampal formation

i. Hippocampus

The hippocampus has long been a major focus of neuroscience research in large part because of its involvement in cognitive function. An extensive body of evidence suggests that the hippocampus is essential for fast encoding and storage of new memories (O'Reilly & Norman, 2002) and studies in humans, monkeys and rodents have shown that damage to this region impairs performance on tasks of learning and memory (Broadbent, Squire & Clark, 2004; Squire, 1992; Suzuki & Eichenbaum, 2000).

In the mammalian brain, the hippocampus is divided into two halves located in each hemisphere of the medial temporal lobe and meeting near the center of brain (Isaacson & Larry, 2004). It encompasses the dentate gyrus (DG) and Ammon's horn (*cornu ammonis*), which is divided into four subfields, CA1 to CA4 (Thompson & Lein, 2009), in humans; in rodents, the existence of the CA4 is still debated. The DG is primarily composed of small densely packed granule neurons while triangular-shaped pyramidal neurons comprise the major neuronal population in the CA regions, varying in morphology from one subfield to

the next. Pyramidal neurons and granule neurons also serve different functions in the hippocampus. CA1 and CA3 pyramidal neurons process sensory and motor cues to form a cognitive representation of spatial, contextual and emotional information, which they transmit throughout the brain (Graves et al., 2012), while dentate gyrus granule cells appear to be involved in the formation of spatial memories (Colicos & Dash, 1996). In addition to neuronal cells, there are prominent non-neuronal populations, including astrocytes, which are uniformly distributed throughout the hippocampus.

The presence of ERs in the hippocampus is indicative of its importance as a target for estradiol activity in the brain (Prange-Kiel, Wehrenberg & Rune, 2003; Rune et al., 2002). Estradiol can be synthesized *de novo* in the hippocampus from endogenous cholesterol by a series of enzymes. Differing enzyme levels in hippocampal tissue sections suggest that steroid expression varies across the subfields of the hippocampus. According to Rune et al. (2002), estradiol is highest in the CA3 region of adult rats, which, in turn, upregulates ER α . This is consistent with the stronger expression of ER α in CA3 pyramidal neurons compared to CA1 neurons.

Estradiol-mediated neuroprotection was first observed in the brain of rodents following excitotoxic injury and stab wound injuries in the hippocampus (Garcia-Segura et al., 1999). In both cases, there was an increase in aromatase activity coupled with *de novo* expression of aromatase in astrocytes; note that aromatase is the enzyme necessary for converting androgens to estrogens. More recently, antisense oligonucleotides were used to inhibit aromatase expression and estradiol synthesis in the rodent hippocampus (Zhang et al., 2014). This increased the detrimental effects of global cerebral ischemia, enhancing neuronal loss and reactive gliosis in the CA1 of the hippocampus. Taken together, these data suggest the regulation of anti-inflammatory and neuroprotective actions of estradiol following brain injury occur in the hippocampus.

ii. Entorhinal cortex

Information flows to and from the hippocampus. Afferent activity originates from all major sensory regions of the cerebral cortex (Isaacson, 2004). This is referred to as the perforant pathway (Figure 3). Input signals follow a ‘neural chain’, arising from the cerebral

cortex, transmitted to the entorhinal cortex, the major gateway between the cerebral cortex and hippocampus.

Memory consolidation in the cerebral cortex is slow and gradual, requiring repeated interactions with the hippocampus (Squire & Alvarez, 1995; McClelland, McNaughton & O'Reilly, 1995). Inputs arise from olfactory, visual and auditory cortical fibers that make synaptic connections with the entorhinal cortex. The passage of activity runs through to the lateral or medial entorhinal areas and reaches the dendrites of the DG (Isaacson, 2004). Axons from DG granule cells, in turn, run through the proximal dendrites of neurons in the CA3. Efferent signals are then forwarded to the CA areas before reaching the subiculum, the most inferior component of the hippocampal formation.

At the interface between the hippocampus and cerebral cortex, the entorhinal cortex is a crucial structure involved in the formation and expression of memory (Izquierdo et al., 1997; Fyhn et al., 2004; Steffenach, Witter, Moser & Moser, 2005).

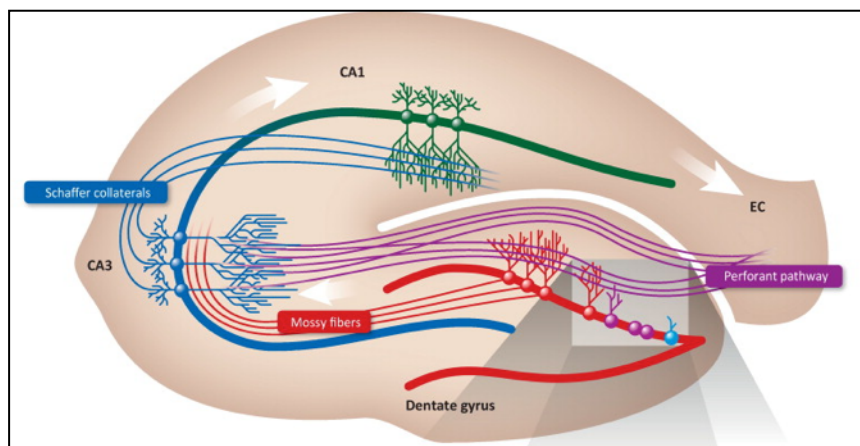


Figure 3. Perforant pathway of the hippocampal formation (Lucassen et al., 2013) showing the connection between cortex and hippocampus

Cellular markers

Cellular markers are molecular and cellular indicators of normal or altered biological state, detected in the blood and tissues. Examples include but are not limited to proteins, nucleic acids, lipids and ions. A number of widely used cellular markers in the neurotoxicology field enable us to evaluate the relationship between environmental exposure and development of disease. They are validated based on their ability to detect subtle changes in biological processes (sensitivity) as well as correctly identify the target in

question (specificity). They offer an inexpensive, rapid and objective measurement of cellular and molecular events, allowing us to better our understanding of prevention, intervention and therapeutic targets of central nervous system disorders caused by neurotoxicants. In the present study, histological evaluation was carried out in adult rats born to mothers exposed to nicotine. Cellular markers of interest included Nissl, GFAP and ER α .

i. Nissl

The Nissl stain is a histological stain of the cell bodies in the CNS (Figures 4, 5). It labels the ribosomal RNA in the endoplasmic reticulum of cells (Taupin, 2007, p.4). Although several different dyes have been developed for this purpose, cresyl violet is one that is widely used. And unlike other dyes, it is not selective for neuronal cell bodies but colors all nervous system cells (Zilmer, Spiers & Culberston, 2007, p.34) particularly neurons and glial cells (Taupin, 2007, p.4). The Nissl method's ability to map cell density has become particularly useful for two reasons. First, it can detect the distribution of cell bodies in specific areas of the brain (Bear, Connors & Paradiso, 2007, p.25). Second, it can be used to study cells both in normal and pathological conditions (Zilmer, Spiers & Culberston, 2008, p.35). Because cell bodies undergo physiological changes when injured, this procedure enables researchers to study the severity of the damage and resulting anatomical transformations.

Abou-Donia (2006) did a cresyl violet stain and found prenatal nicotine exposure led to a decrease in surviving neurons in the cerebellum of adult rat offspring (PND90). Similarly, Blutstein found that, despite having no acute effect, gestational nicotine significantly reduced NeuN-ir (neuronal marker) in the CA1 subfield of the hippocampus in guinea pigs when they reached adulthood.

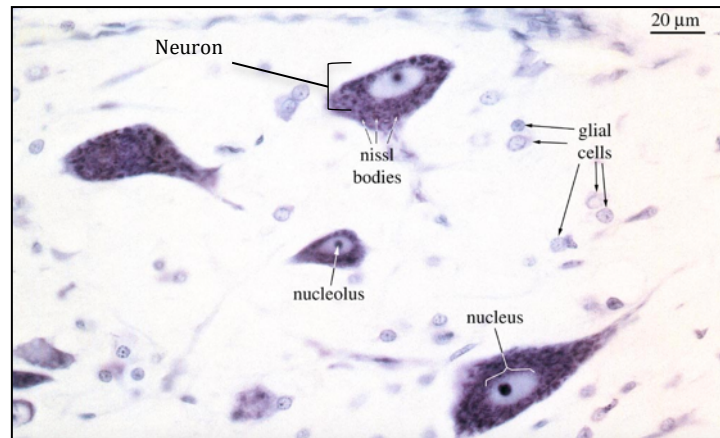


Figure 4. Nissl stained cell bodies showing the different cell types coloured by the Nissl procedure (Todt, W. 2009)

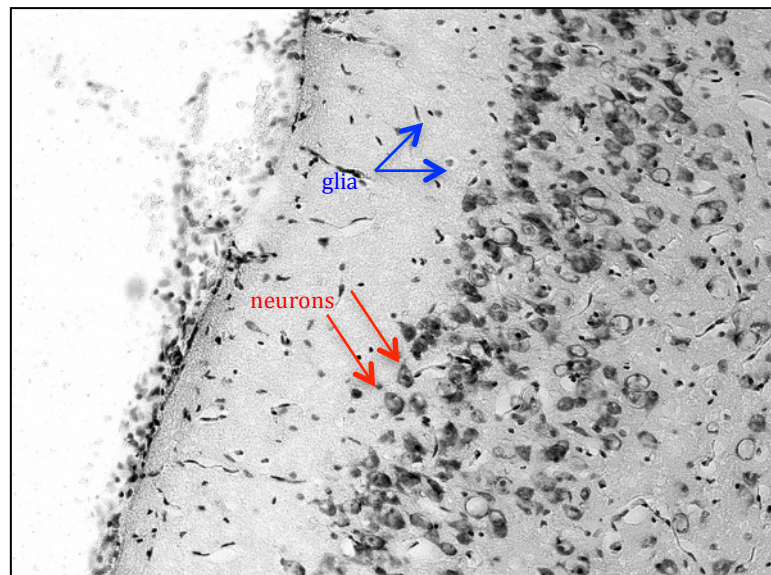


Figure 5. Nissl-stained section of the rat entorhinal cortex taken with a light microscope with a 10x objective

ii. GFAP

Astrocytes express two types of intermediate filaments. There is vimentin, a protein expressed predominantly in immature glia, and glial fibrillary acidic protein (GFAP), an astrocyte maturation marker (Bignami et al., 1972) (Figure 6). Although mature and immature astrocytes vary in terms of morphology, at present, no functional differences have been documented. In the course of astrocyte development, a switch occurs whereby vimentin is gradually replaced by GFAP (Pixley & Vellis, 1984; Wofchuk & rodnight, 1995). Mature astrocytes develop a stellate morphology with glial processes, and accumulate GFAP, the

major protein constituent of differentiated astrocytes in the CNS (Culican et al. 1990; Schmechel & Rakic 1979).

Astrocytes provide structural support in the brain and they are in close proximity to other cells of the CNS (neurons, microglia, oligodendrocytes and other astrocytes) and with blood vessels (Arevalo et al., 2011). This strategic location allows them to exert many essential complex functions in the CNS such as the formation of the blood brain barrier, metabolism of several neurotransmitters (Barres, 1991) and delivering neurotrophic signals involved in the survival, proliferation and differentiation of neurons (Gomes et al., 1999; Maxwell et al., 1996) and synaptic communication. Additionally, glial cells are key players of the cellular response to pathological insults (Panickar & Norenberg, 2005; Perea, Navarrete & Araque, 2009).

Astrocyte intermediate filaments can be seen as early as the end of gestation in rodents (Pixley & Vellis, 1984). According to transcriptional studies, there is a steady increase in GFAP expression starting at birth until day 15 of postnatal development, followed by a decrease through to day 55 (Riol, Fages & Tardy, 1992). A plateau occurs until the second year of adulthood proceeded by another GFAP mRNA increase in brain regions including the hippocampus, striatum and cortex (Laping et al., 1994), representative of a phenomenon referred to as “reactive gliosis” whereby there is a several-fold increase of GFAP expression which takes place during senescence, at least in rodents (Gomes, Paulin, Neto, 1999).

Reactive gliosis occurs in response to any CNS trauma and has become one of the most generalized markers of brain injury, aging and neurodegenerative disease. This involves the proliferation of astrocytes along with an increase in their size, larger and more tortuous processes, enhanced GFAP expression (Eng, 1985) and the release of pro-inflammatory cytokines (Cerciat, Unkila, Garcia-Segura & Arevalo, 2010; Kutsch, Oh, Nath & Benveniste, 2000). Over time, inflammatory molecules attract macrophages and T cells, cells of the immune system (Dong & Benveniste, 2001; Rebenko-Moll, Liu, Cardona & Ransohoff, 2006; Rostene, Kitabgi & Parsadaniantz, 2007) that further promote neural damage and exacerbate the inflammatory response, leading to neurodegeneration.

The identification of molecules that mediate reactive gliosis is key for the development of therapeutic strategies to prevent neuronal death as well as promote regeneration following CNS damage. Estradiol has been shown to suppress the release of pro-inflammatory

molecules by acting on its receptors (Lewis et al., 2008); estrogen receptors are commonly expressed on astrocytes (Dhandapani & Brann, 2007). Under pathological conditions, this expression is enhanced (Blurton-Jones & Tuszynski, 2001; Garcia-Ovejero et al., 2002; Lu et al., 2003; Sakuma et al., 2009) and estradiol suppresses the release of inflammatory molecules. Thus, binding of estradiol to its receptor suggests that a mechanism of estradiol-mediated neuroprotection is at play.

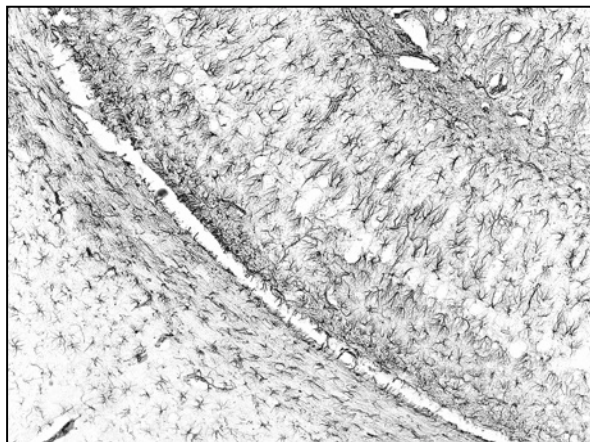


Figure 6. GFAP immunohistochemistry in the CA1 of the rat hippocampus taken with a light microscope with a 10x objective

iii. ER α

Two ER isoforms have been identified to date in the neonatal and adult brain (Su et al., 2001), ER- α (Koike, Sakai & Muramatsu, 1987) and ER- β (Kuiper et al., 1996). ER- α (nuclear receptor subfamily 3, group A, member 1) (Figure 7) and ER- β (nuclear receptor subfamily 3, group A, member 2) mediate the effects of estrogens in mammals (Ascenzi, Bocedi & Marino, 2006). A third ER is the more recently discovered GPR30, a g-protein-coupled receptor. While its role remains unknown, researchers have suggested that the membrane ER may play a role in mediating estradiol's effects in the hippocampus and cortex of rodents (Funakoshi et al., 2006; Khan, De Sevilla, Hadman & Brann, 2006; Perry & Cushing, 2006).

In their unliganded state, the classical ERs are generally located in the cell nucleus and cytoplasm (Ascenzi, Bocedi & Marino, 2006). They are associated with cytoplasmic chaperone proteins called heat shock proteins that maintain receptor conformation (Simoncini et al., 2004). Circulating estrogen passes through the phospholipid membranes of

the cell and binds to the ligand-binding domain, causing dissociation of the heat shock proteins and translocation of the ER-estradiol complex into the cell nucleus. The ER-estradiol complex then stimulates the transcription of target genes by binding to DNA sequences known as hormone response elements (i.e. genomic mechanism) located on the promoter regions of these target genes. ERs may also associate with cell surface membranes and can be rapidly activated by exposure to the hormone (i.e. nongenomic mechanism) (Spencer et al., 2008).

Exposure to estradiol begins prenatally via the mother's circulation and persists by means of circulating or locally produced androgens being synthesized to estradiol via neuronal aromatase. Estradiol is at its peak in the brain during the perinatal period of development before gradually declining to adult levels (MacLusky, Walter, Clark & Toran-Allerand, 1994; McCarthy, 2008; McEwen, Leiberburg, Chaptal & Lewis, 1977; Rhoda, Corbier & Roffi, 1984; Weisz & Ward, 1984). The latter is especially important in males, who secrete testicular androgens that are aromatized to estrogens locally within neurons (Weisz & Ward, 1984; Forest, 1979, Konkle & McCarthy, 2011).

Both forms of nuclear ER exist throughout the brain, particularly in areas involved in learning and memory such as the hippocampus (Shughrue, Lane & Merchenthaler, 1997). Estradiol synthesized *de novo* in the hippocampus (Fester et al., 2012) plays a central role in the modulation of synaptic plasticity and memory processes (Ooishi, 2012).

Rodent hippocampal cells express ERs in both neurons and astrocytes (Su et al., 2001). *In situ* hybridization and immunohistochemical studies have detected ERs in hippocampal neurons, specifically located in nuclei and cytoplasm (Wehrenberg, Prange-Kiel & Rune, 2001; Prange-Kiel et al., 2003). Similarly, astrocytes express ERs in various brain regions in sections derived from the guinea pig, rat, and human (Dhandapani & Brann, 2007) and this expression is enhanced under different pathological conditions (Blurton-Jones & Tuszynski, 2001; Sayaskan et al., 2001; Garcia-Ovejero et al., 2002; Lu et al., 2003; Takahashi et al., 2004; Sakuma et al., 2009). These are believed to aid in estradiol-mediated neuroprotection (Carbonaro, Caraci & Giuffrida, 2009). Estradiol and selective estrogen receptor modulators acting on glial cells via ER signaling is initiated at the nucleus, the membrane or the cytoplasm (Arevalo et al., 2010).

Nevertheless, the existence of at least two forms of the ER insinuates that estrogens mediate different events by binding to either one or a combination of both receptors. When slice cultures are supplemented with estradiol, ER α -ir but not ER β -ir was enhanced equally in male and female hippocampi (Rune et al., 2002) which would lead us to believe that ERs in both males and females respond similarly to estradiol upregulation. Nevertheless, there are discrepancies in the expression pattern across hippocampal development.

Western blot analysis revealed that ER α -ir is greater in the neonatal hippocampus than in the adult whereas ER β expression does not fluctuate (Su et al., 2001). This is consistent with findings from *in situ* hybridization in the rat (Shughrue et al., 1997) and by RT-PCR in the monkey (Register et al., 1998) that have demonstrated higher ER β mRNA expression in the adult hippocampus compared to ER α . Studies suggest that ER α expression patterns vary across structural and functional development of the hippocampus; it is not static, but rather responds to neuronal injury and neurodegeneration (Dubal et al., 2001; Vegeto et al., 2003). This is made clear not only by studies demonstrating that estrogen-mediated neuroprotection is lost in ovariectomized ER α knockout mice (Dubal, 2001; Merchenthaler, Dellovade & Shughrue, 2003) but also that ER α is upregulated in response to injury (in the presence or absence of estradiol) (Dubal et al., 1999). Numerous studies support the contention of estradiol's neuroprotective role in models of brain injury in the hippocampus and cerebral cortex (Culmsee et al., 1999; Dubal et al., 1998; Green & Simpkins, 2000; Green et al., 2001; Harms et al., 2001; Jover et al., 2002; Kuroki et al., 2001; Weaver, Park-Chung, Gibbs & Farb, 1997; Wilson, Dubal & Wise, 2000; Wilmer & Wilmer, 1989). While ER α expression is enhanced under pathological conditions and key developmental periods when neurogenesis is prioritized, studies examining ER α protein generally do not include many time points. Thus, little is known about its expression in healthy conditions or following a period of enhanced upregulation. According to Dietrich, Humphreys & Nardulli (2015) there is evidence to suggest that while mRNA levels decline in the female mice brain from PND5-18 months, protein levels remain stable.

In addition to its direct actions on neurons, estradiol and selective estrogen receptor modulators target ERs as a mechanism of neuroprotection in order to mitigate reactive gliosis and downregulate production and release of proinflammatory molecules by astrocytes (Cerciat, Unkila, Garcia-Segura & Arevalo, 2010). Reactive astrogliosis is characterized by

the secretion of neurotrophic factors and the production of pro-inflammatory cytokines such as interleukin-6, tumour necrosis factor α and interleukin-1 β (Farina, Aloisi & Meinl, 2007; Fisher et al., 2007; Kipp et al., 2008; Van Wagoner, Oh, Repovic & Benveniste, 1999) and chemokines such as monocyte chemotactic proteins 1 and 1 α and interferon- γ inducible protein-10 (also named CXCL10) (Bethel-Brown et al., 2011; Gorina et al., 2009). Activation of astrocytes is modulated by local and peripheral molecules transported from the blood (Garcia-Segura & Melcangi, 2006) such as estradiol, which not only reduces astrocyte proliferation but also mRNA expression of interleukin-6 and inducible protein-10 (Cerciat, Unkila, Garcia-Segura & Arevalo, 2010).

Following brain injury, ER expression is upregulated and newly synthesized estradiol activates several neuroprotective-signaling mechanisms. It increases expression of anti-apoptotic genes and neuroprotective growth factors while reducing the expression of pro-apoptotic genes and pro-inflammatory molecules (Arevalo, Azcoitia & Garcia-Segura, 2014). The anti-inflammatory effects of ERs are exerted through the activation of extra cellular signal-regulated kinases 1 and 2 or the PI3K signaling pathways and inhibition of pro-apoptotic JUN amino-terminal kinase signaling (Arevalo, Azcoitia & Garcia-Segura, 2014). The activation of either the extra cellular signal-regulated kinases 1 or 2 or the PI3K signaling pathways by ERs results in the regulation of B cell lymphoma 2 (BCL-2) family members that are involved in the control of apoptosis (Wu, Wang & Chen, 2005; D'Astous et al., 2006; Wang, Ren, Guan & Zhang, 2011; Cardona-Rossinyol et al., 2013). Specifically, there is a decreased expression of B-associated death promoter, which is pro-apoptotic and an upregulation of B cell lymphoma 2, which is anti-apoptotic. While estradiol activates a series of neuroprotective signaling pathways, inhibition of a single one can abolish its neuroprotective actions, as seen in models of cerebral ischemia and stroke (Yang et al., 2010; Jover-Mengual, 2010).

Together, these data highlight the importance of ER α as a mechanistic component of estradiol-mediated neuroprotection, one of potentially many novel functions that reach far beyond estradiol's role in reproduction.

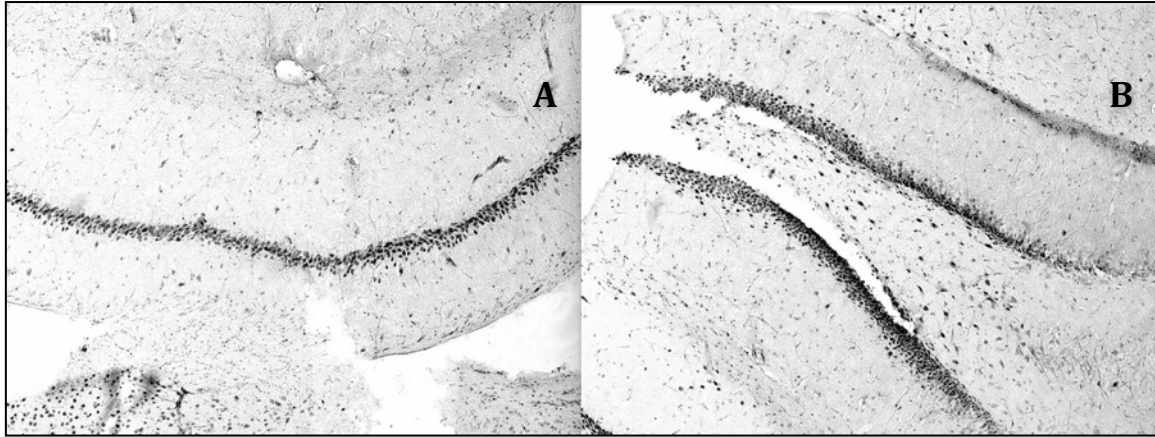


Figure 7. ER α immunohistochemistry in the CA1 (A) and dentate gyrus (B) of the rat hippocampus taken with a light microscope with a 10x objective

Objectives

Given the lack of knowledge regarding the effects of nicotine at doses mimicking NRT, we intend to:

1. Quantify neurons in the rodent hippocampal formation to elucidate the long-term effects of nicotine on neuronal survival following early life nicotine.
2. Quantify GFAP-ir in the rodent hippocampal formation to reveal the effects of nicotine on adult astrocyte populations following early life nicotine.
3. Quantify ER α -ir in the rodent hippocampal formation to elucidate the effects of nicotine-induced neurotoxicity on estrogen receptors following early life nicotine.

Hypotheses

Perinatal nicotine exposure at doses mimicking nicotine replacement therapy will interfere with proper brain development so that deleterious effects continue to emerge over the lifespan, made evident by neuron, astrocyte and ER α populations in adult offspring.

1. Nicotine treatment will prompt neuronal loss; it will produce an inflammatory response and abrogate endogenous estradiol-mediated neuroprotection, promoting

hippocampal damage. This neuron loss will endure in the postnatal period despite the discontinuation of nicotine.

2. Early life nicotine exposure will elicit an acute inflammatory response characterized by reactive gliosis and accumulation of GFAP, thus impairing normal astrocyte maturation in the developing rodent brain. This will in turn lead to increased GFAP immunoreactivity (ir) in adulthood.
3. Perinatal nicotine treatment will also produce long-lasting effects on ER α . These receptors, which are widely expressed in the rodent hippocampal formation, will be upregulated to attenuate the extent of injury and minimize neuronal death. Similar to astrocytes, this increase will be detectable in rodent offspring weeks after the termination of nicotine treatment.

CHAPTER 3. Method

Animals and treatments

All experiments were carried out with the approval of the Animal Research Ethics Board at McMaster University, in accordance with the guidelines of the Canadian Council for Animal Care. Note that these animals served as part of a larger study in collaboration with the Holloway laboratory at McMaster University.

Nulliparous female Wistar rats weighing 200-250g (Harlan, Indianapolis, IN) were housed under a 12L: 12D cycle with ambient temperature maintained at 22°C and allowed *ad libitum* access to food and water.

Dams were randomly assigned to a treatment group and injected subcutaneously with either a 0.09% saline solution or 1 mg/ kg body weight per day of nicotine bitartrate (SigmaAldrich, St Louis, MO), once daily from 2 weeks before mating until the time of offspring weaning, postnatal day 21 (PND21). This dose of nicotine results in maternal serum cotinine concentrations of 135.9 ± 7.86 ng/ml (Holloway, Kellenberger & Petrik, 2006), comparable to that of moderate female smokers (Lawson et al., 1998) and fetal cotinine serum concentrations of 26.2 ± 1.78 ng/ml (Holloway, Kellenberger & Petrik,

2006), similar to those observed in infants nursed by smoking mothers (Luck & Nau, 1985). On PND1, litters were culled to 6 to standardize number of pups in control and experimental litters. In order to eliminate possible confounds associated with the female reproductive cycle starting around puberty, only male offspring were utilized in this study. At the time of weaning (PND21), male offspring were placed 2 per cage (randomly by treatment). Body weights were assessed at time of sacrifice at 26 weeks.

Tissue collection

Offspring were euthanized at 26 weeks of age by CO₂ exposure (n=6 nicotine and n=6 vehicle/control). The brains were quickly removed from the skull and immersion fixed in 4% paraformaldehyde for 24 hours and stored in a 30% sucrose solution at 4°C until the time of sectioning. Part of the frontal cortex was cut off to expose the lateral ventricles, thus, allowing for better immersion fixation in cases where perfusion is not possible (Balthazart et al. 2008); the need to collect other tissues fresh (without fixative) prevented us from perfusing these animals.

Thirty µm coronal sections were collected at -22°C using a Leica model 1580 cryostat. Sections were deposited in 12-well dishes filled with 0.01M PBS. Tissue was collected serially into wells 1 through 11 while every 12th section was thaw-mounted onto a slide. Once the rostral half of the brain was complete, another 12-well dish labeled 13-23 was used for the remaining caudal part of the brain, with the 24th section being thaw-mounted on a slide. Once rinsed in PBS, the sections were placed into labeled microtubes filled with cryoprotectant (0.01M PBS, polyvinyl pyrrolidone, saccharose, ethylene glycol) and stored in a -20°C freezer until the time of immunohistochemistry. One set of brain sections consisting of consecutive sections 360 µm apart, served for each of the markers below.

Nissl stain

Sections that were mounted at the time of sectioning were colored using a Nissl stain. This prepared the tissue for quantitative analysis of Nissl bodies using light microscopy. The staining procedure consisted of sequentially dipping the slides in descending grades of

alcohol to remove lipids and fixation chemicals from the brain tissue, starting with 100% ethanol for 2 minutes, followed immediately by 70% ethanol for another 2 minutes. They were then placed in distilled water for 1 minute followed by 5-10 minutes in a 1:200 solution of Cresyl violet acetate : distilled water. The cresyl violet stains the Nissl bodies of the endoplasmic reticulum of cells to a purple color, which allows for the visualization of the brain's cytoarchitecture. Samples were removed from the staining bath and a differentiation step was then applied by placing the slides into a 95% ethanol/glacial acetic acid solution. This step was used to remove excess stain from non-Nissl containing structures, thus, lightening up the stain to the point where major fiber tracts turned white. The sections were then placed into distilled water for 1 minute, followed by ascending alcohol concentrations - 2 minutes in 50%, 70% and 95% ethanol, followed by two 100% ethanol washes to completely dehydrate the tissue. Once dehydrated, the sections were placed in two consecutive solutions of xylene for 1-5 minutes followed by cover slipping using mounting medium (Permount, BioPlus).

Immunohistochemistry

Immunohistochemistry for estrogen receptor α (ER α) and glial fibrillary acidic protein (GFAP) were performed on equivalent sets of sections for each of the 12 animals. For ER α : after 3x5min rinses in 0.01M phosphate buffer (PBS, pH:7.2-7.3) and 3x5min rinses in 1% PBS-T to permeabilize cell membranes, sections were incubated for 10 min in 5% dimethyl sulfoxide (DMSO) to maximize penetration of the reagents inside the cell nucleus. Another 3x5min rinses in 1% PBS-T took place prior to a 20 minute incubation in 0.6% H₂O₂ to block endogenous peroxidases. There were additional 3x5 min rinses in 1% PBS-T before sections were placed 30 minutes in 10% normal goat serum (Vector Laboratories) to limit background staining and block non-specific antibody binding. The tissue was then incubated in a rabbit anti-estrogen receptor α monoclonal antibody (Millipore: dilution of 1:5000 in 0.01M PBS-T) for one hour at room temperature and 48 hours at 5°C to bind to the target antigen.

Once the 48 hours elapsed, sections were rinsed 3x5min in 0.01M PBS-T and incubated in the biotinylated anti-rabbit made in goat secondary antibody (Vector Laboratories: dilution of 1:1000 in 0.01M PBS-T) for 2 hours to bind to the primary

antibody. Tissue received additional PBS-T rinses and was placed one hour in an avidin-biotin complex (Vector Laboratories Vectastain ABC kit) to amplify the target antigen signal and increase detection efficiency. Sections were once more rinsed 3x5min in 0.01M PBS and then let develop in 3,3-diaminobenzidine (Cedarlane DAB Chromagen tablets) in the fume hood. For GFAP, the procedure was similar with the omission of the DMSO step as the antibody does not require penetration inside the nucleus. The primary antibody used consisted of anti-GFAP made in rabbit (Millipore, 1:1000); all other steps were the same. The free-floating sections were mounted on slides in order, from the rostral to the caudal portion of the brain. The slides were dehydrated using 95 and 100% alcohol rinses and left to sit in xylene prior to coverslipping.

Microscopy and cell counting

i. Microscopy preparation

The Paxinos and Watson (2007) stereotaxic atlas was used to select a range within the hippocampus and entorhinal cortex that would ensure that the sections being quantified were consistent from one animal to the next. The Bregma is a rostral anatomical point on the skull where the coronal and sagittal sutures intersect perpendicularly, used as a reference point (value 0). The following values represent the distance from Bregma in millimeters (Table 2). The hippocampus is located posteriorly to the Bregma, thus explaining the negative values.

Brain area	Bregma rostral	Bregma caudal
CA1	-2.92 to -4.20	-4.80 to -6.24
CA3	-2.92 to -4.20	-4.80 to -5.88
DG	-2.92 to -4.20	-4.80 to -6.24
Entorhinal cortex		-5.04 to -6.48

Table 2. Bregma values from the Paxinos and Watson (2007) rat brain atlas used to photograph brain areas of interest for quantification

Cell counts were done blindly: the labels on each of the slides were re-coded by a second experimenter to eliminate potential experimenter bias that could arise during the cell counting procedure.

ii. Digital image capture

A Zeiss Axio Imager microscope was used to visualize the sections. Black and white photographs were captured with the attached AxioCam (Zeiss) digital camera using the 10x, 20x or 40x objective based on optimal image resolution. For smaller, more condensed cellular markers, a higher magnification was ideal to accurately delineate each individual cell whereas for larger, more spread out cellular markers, a smaller magnification was favored.

Anatomical structures of interest (CA1, CA3 and DG of the hippocampus and entorhinal cortex) were identified with the help of the Paxinos and Watson (2007) atlas. These areas were photographed in the same region across animals and divided into rostral and caudal for each animal to ensure a consistent and comparable sampling of immunopositive cells (see Table 1).

iii. Cell counting

The quantitative analysis consisted of counting immunopositive cells in a 3.58 by 5.37 inch (90.9mmx136.4mm) surface area of the entorhinal cortex and each hippocampal subregion (CA1, CA3, DG). Since these brain areas are bilateral, counting was done for each of the right and left portions of the coronal sections for each animal.

Nissl-stained neurons, ER α -ir and GFAP-ir cells in the hippocampus proper and the entorhinal cortex were counted with the help of the computer software, Image J. The average cell count for two or three rostral sections and two caudal sections per animal (n=6 nicotine, n=6 saline) were utilized for quantitative analysis in the hippocampus and three sections per animal were utilized for the enthorhinal sections (see Table 3). As cell numbers were counted for each picture, the immunopositive count was entered into a Microsoft Excel spreadsheet for future statistical analyses.

Brain region	Cellular marker	Subregion	Location	# sections/ animal
hippocampus	ER	CA1	Rostral	3
		CA3		
		DG		
		CA1	Caudal	3
		CA3		
		DG		
	Nissl	CA1	Rostral	3
		CA3		
		DG		
		CA1	Caudal	2
		CA3		
		DG		
	GFAP	CA1	Rostral	3
		CA3		
		DG		
		CA1	Caudal	2
		CA3		
		DG		
entorhinal	ER			3
	Nissl			3
	GFAP			3

Table 3. Distribution of brain sections for counting of cellular markers and subsequent statistical analysis

Statistical analysis

The average immunoreactive positive counts were analyzed separately by brain region and for each cellular marker. The values were calculated for each individual animal before the means and standard errors of the means (SEMs) were determined for the total number of animals in the nicotine and saline treated groups separately - once the animals were decoded. A Levene's test for homogeneity of variance was first conducted. When results of the Levene's test revealed no heterogeneity of variances, the data were then analyzed by unpaired Student's *t* test allowing us to compare the average cell count for the two groups. A one-tailed *P-value* of <0.05 was considered statistically significant. When variances were heterogeneous, the data were analyzed non-parametrically, using the Mann-Whitney U test.

Body weights were similarly analyzed for the nicotine and saline treated groups.

CHAPTER 4. Results

Body weight

Nicotine-exposed offspring had similar body weights to saline treated animals at the time of sacrifice (583.6 ± 6.0 saline, 585.3 ± 11.6 nicotine) (Figure 8).

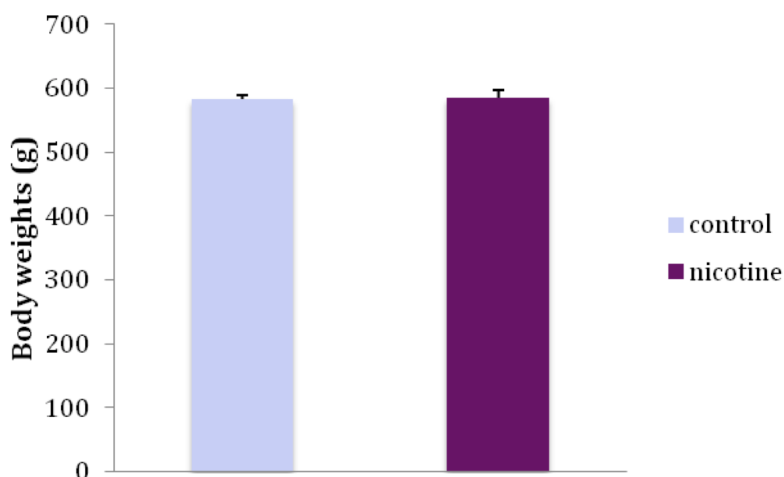


Figure 8. Body weights represented by mean $\pm$ SEM ($n=6$ /group). No significant difference in treatment was found ($p < 0.05$).

Nissl stain in the hippocampal formation

There was no observable difference in neuron counts in the hippocampus of animals that were exposed to nicotine *in utero* versus their control counterparts. This is consistent throughout all three subfields, including the CA1 (24.2 ± 1.3 saline, 24.4 ± 0.8 nicotine) (Figure 9), CA3 (18.6 ± 1.1 saline, 16.6 ± 0.6 nicotine) (Figure 10) and DG (10.5 ± 1.1 saline, 8.5 ± 1.3 nicotine) (Figure 11).

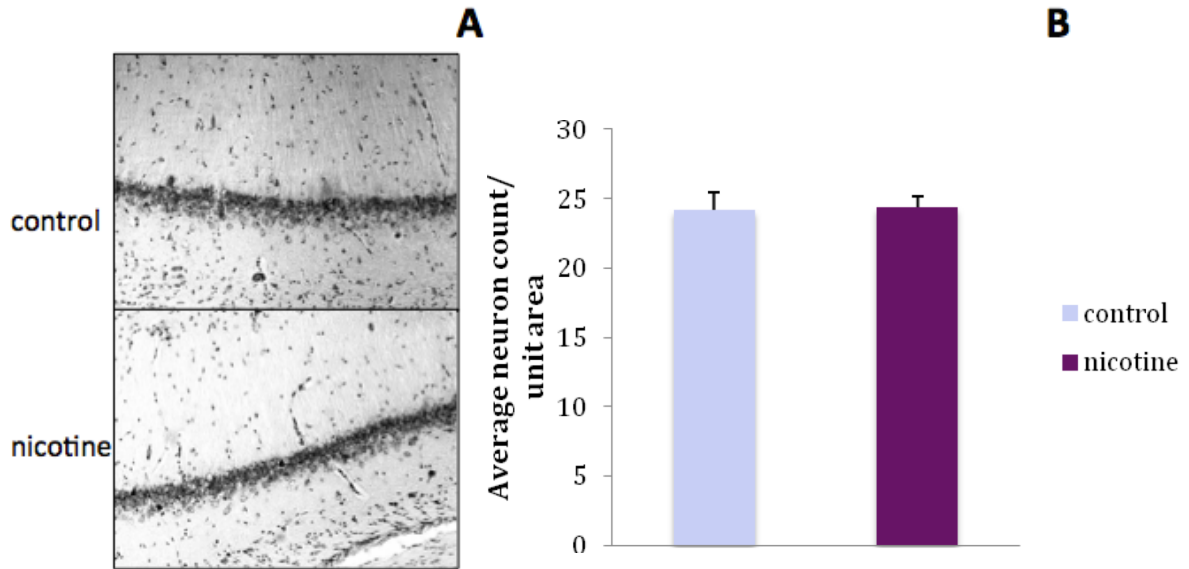


Figure 9. Cresyl violet staining in the CA1 subregion of the hippocampus. (A) Representative photomicrographs of Cresyl violet staining in the CA1 region in controls and nicotine exposed animals taken with a 10x objective. Images used for counting were taken with a 40x objective. (B) Nissl body count in neurons in the CA1 represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p < 0.05$).

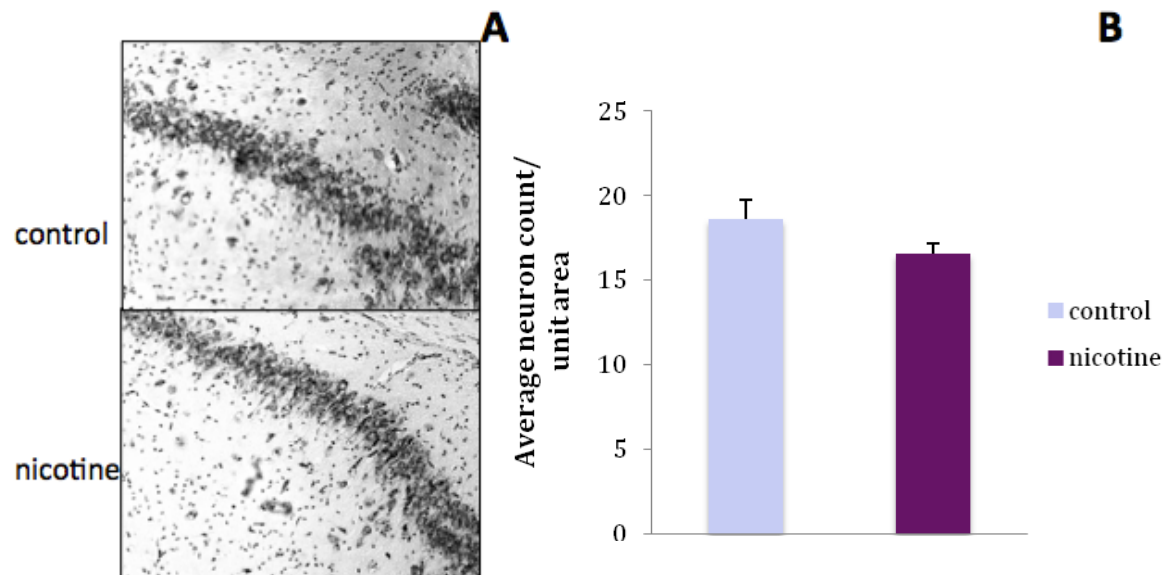


Figure 10. Cresyl violet staining in the CA3 subregion of the hippocampus. (A) Representative photomicrographs of Cresyl violet staining in the CA3 region in controls and nicotine exposed animals taken with a 20x objective. Images used for counting were taken with a 40x objective. (B) Nissl body count in neurons in the CA3 represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p < 0.05$).

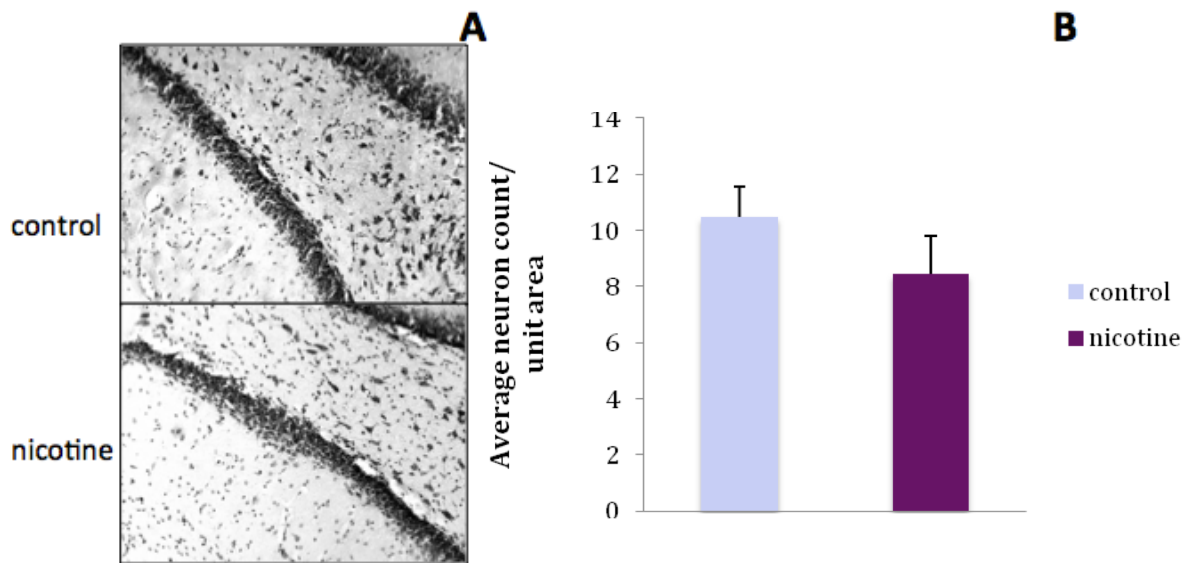


Figure 11. Cresyl violet staining in the DG region of the hippocampus. (A) Representative photomicrographs of Cresyl violet staining in the DG region in controls and nicotine exposed animals taken with a 10x objective. Images used for counting were taken with a 40x objective. (B) Nissl body count in neurons in the DG represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p < 0.05$).

The number of neurons in the entorhinal cortex did not vary either between both groups of Nissl-stained brain sections (8.2 ± 0.4 saline, 8.3 ± 0.4 nicotine) (Figure 12).

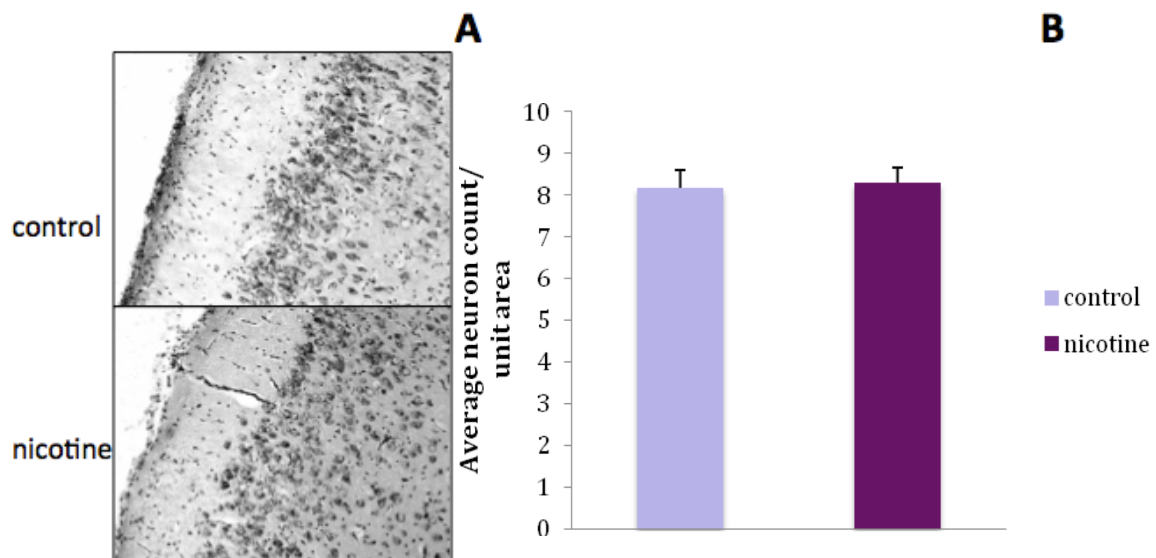


Figure 12. Cresyl violet staining in the entorhinal cortex. (A) Representative photomicrographs of Cresyl violet staining in the entorhinal in controls and nicotine exposed animals taken with a 10x objective. Images used for counting were taken with a 40x objective. (B) Nissl body count in neurons in the entorhinal represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p < 0.05$).

GFAP immunoreactivity in the hippocampal formation

While there appeared to be fewer astrocytes in the hippocampi of nicotine treated animals, nicotine did not significantly impact GFAP immunoreactivity in the CA1 (27.3 ± 1.3 saline, 25 ± 1.3 nicotine) (Figure 13), CA3 (22.8 ± 1 saline, 19.5 ± 2.1 nicotine) (Figure 14) and DG (30.7 ± 1.8 saline, 30.5 ± 1.5 nicotine) (Figure 15).

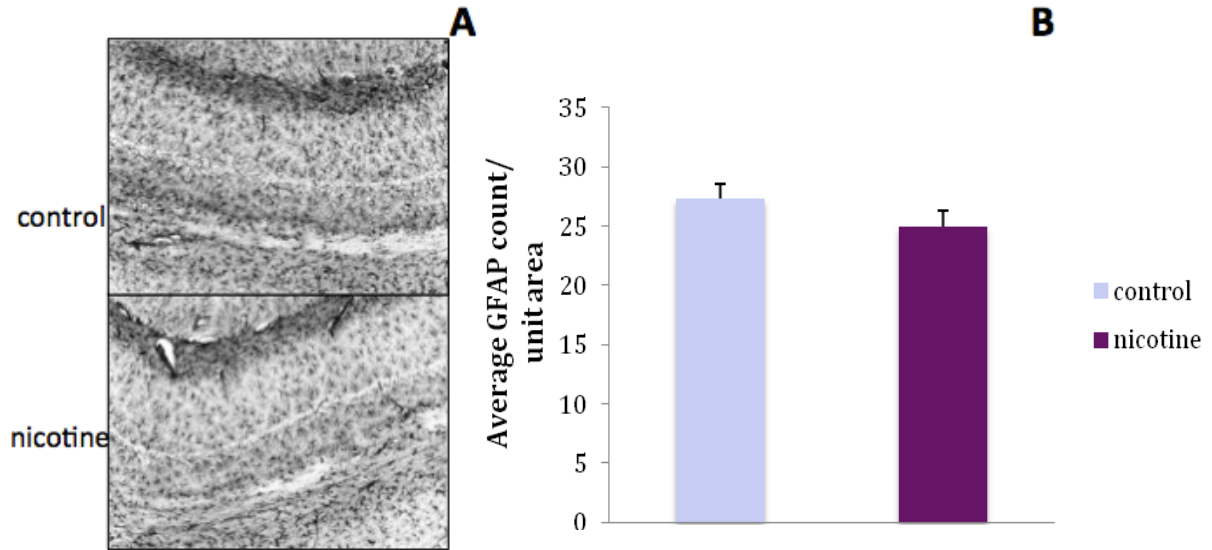


Figure 13. GFAP-ir in the CA1 subregion of the hippocampus. (A) Representative photomicrographs of GFAP-ir in the CA1 in controls and nicotine exposed animals taken with a 10x objective. Images used for counting were taken with a 10x objective. (B) GFAP count in the CA1 represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p < 0.05$).

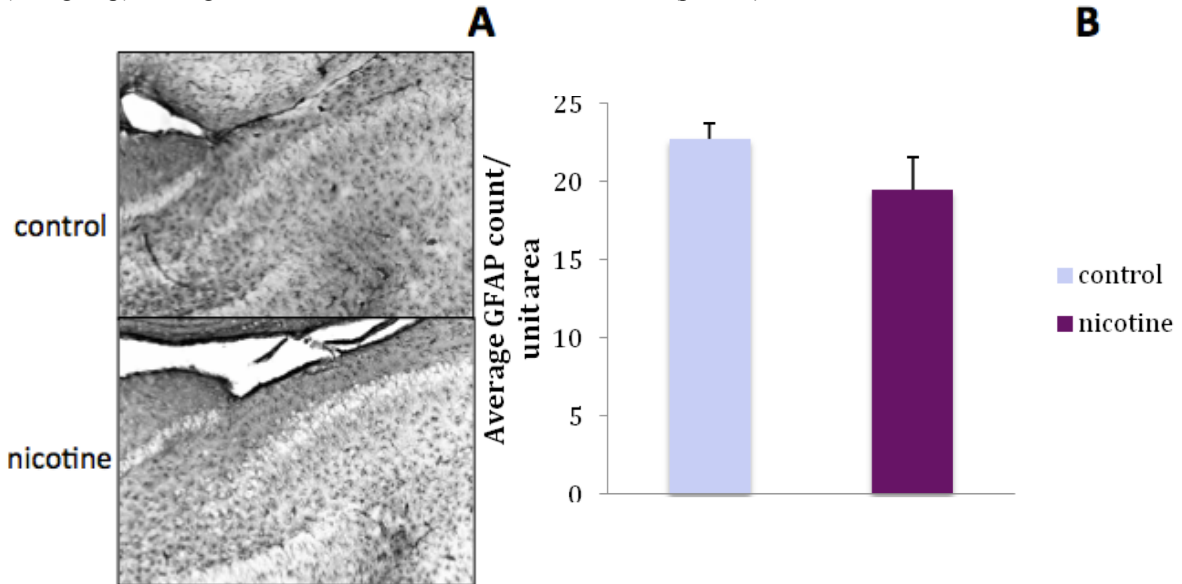


Figure 14. GFAP-ir in the CA3 subregion of the hippocampus. (A) Representative photomicrographs of GFAP-ir in the CA3 in controls and nicotine exposed animals taken with a 10x objective. Images used for counting were taken with a 10x objective. (B) GFAP count in the CA3 represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p < 0.05$).

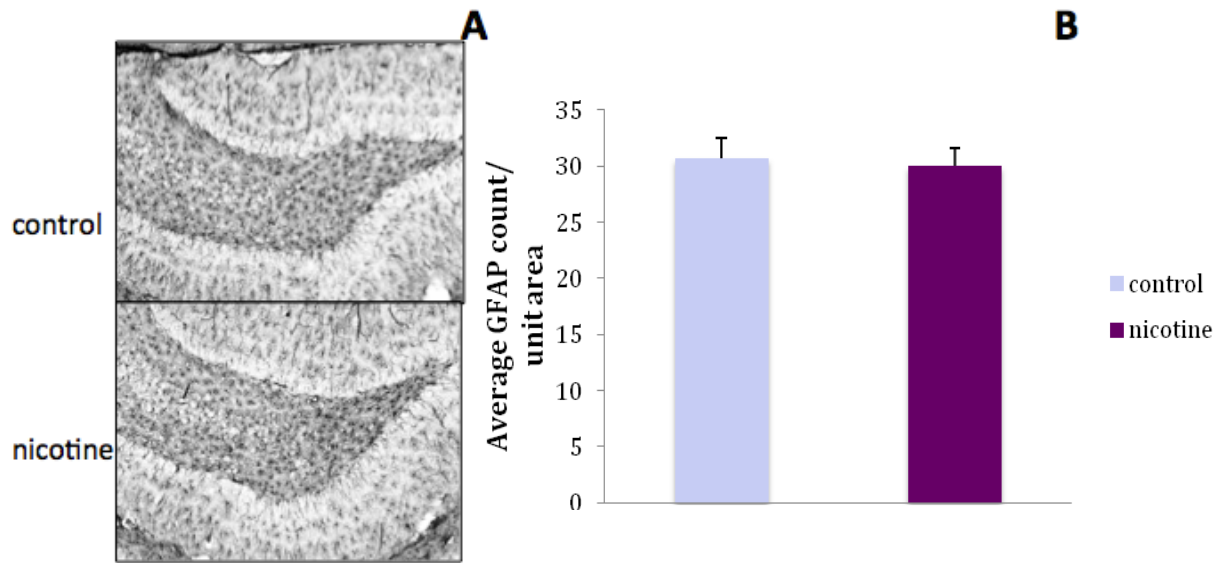


Figure 15. GFAP-ir in the DG subregion of the hippocampus. (A) Representative photomicrographs of GFAP-ir in the DG in controls and nicotine exposed animals taken with a 10x objective. Images used for counting were taken with a 10x objective. (B) GFAP count in the DG represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p < 0.05$).

Similarly, the number of astrocytes remained relatively unaffected by nicotine in the entorhinal cortex (9.9 ± 0.5 saline, 9.7 ± 0.6 nicotine) (Figure 16).

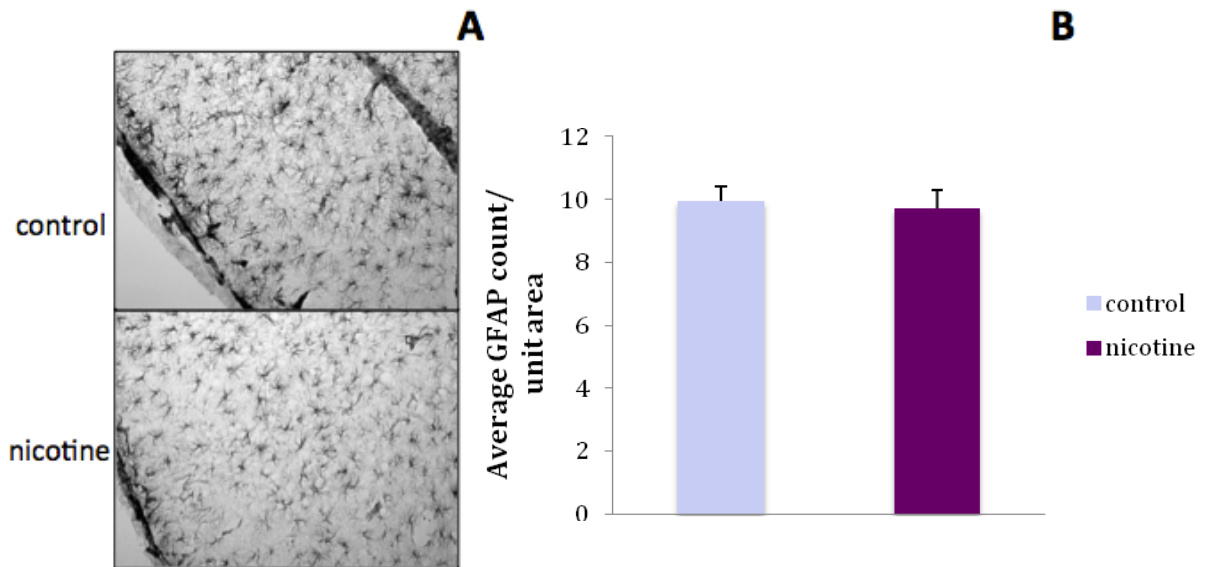


Figure 16. GFAP-ir in the entorhinal cortex. (A) Representative photomicrographs of GFAP-ir in the entorhinal cortex in controls and nicotine exposed animals taken with a 20x objective. Images used for counting were taken with a 20x objective. (B) GFAP count in the entorhinal cortex represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p < 0.05$).

ER α immunoreactivity in the hippocampal formation

ER α positive cells were observed in all three subfields of the hippocampus and there appeared to be a trend whereby the nicotine treated animals had increased ER α immunoreactivity compared to the control animals.

In the CA1 the average number of receptors in the nicotine group was much more elevated than the control group (0.1 ± 0.1 saline, 7.1 ± 4.3) (Figure 17). Though both groups had median values of 0, the saline group had between 0 and 8 receptors per animal (also 0-8 per bilateral section), while the nicotine cohort had 0 to 159 receptors per animal (0-64 per bilateral section). For the saline group, one animal out of six had immunopositive cells in the CA1, whereas in the nicotine group, three animals had large counts that ranged from 51 to 159. Due to the large difference in variance between the groups (Levene's test $p < 0.05$), we conducted a non-parametric test on these data; results of the Mann-Whitney U did not reveal any effect of the nicotine treatment.

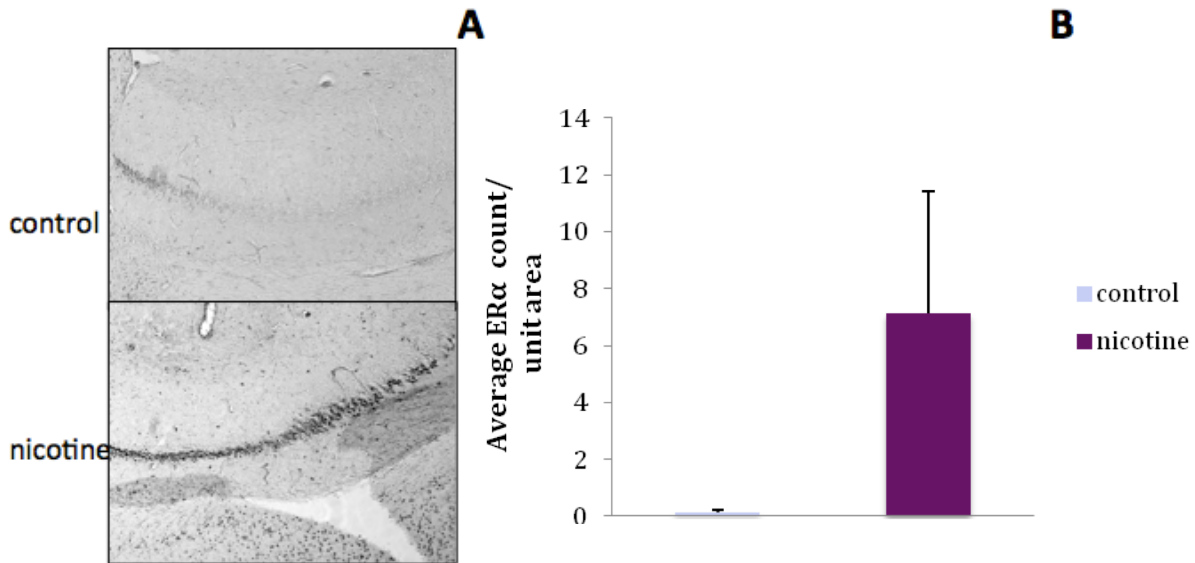


Figure 17. ER α -ir in the CA1 subregion of the hippocampus. (A) Representative photomicrographs of ER α -ir in the CA1 in controls and nicotine exposed animals taken with a 10x objective. Images used for counting were taken with a 20x objective. (B) ER α count in the CA1 represented by mean $\pm$ SEM ($n=6$ /group). No significant difference in treatment was found ($p < 0.05$).

As for the CA3, the number of receptors was increased in nicotine exposed animals, but did not reach statistical significance (0.95 ± 0.95 saline, 4.7 ± 1.9 nicotine) (Figure 18).

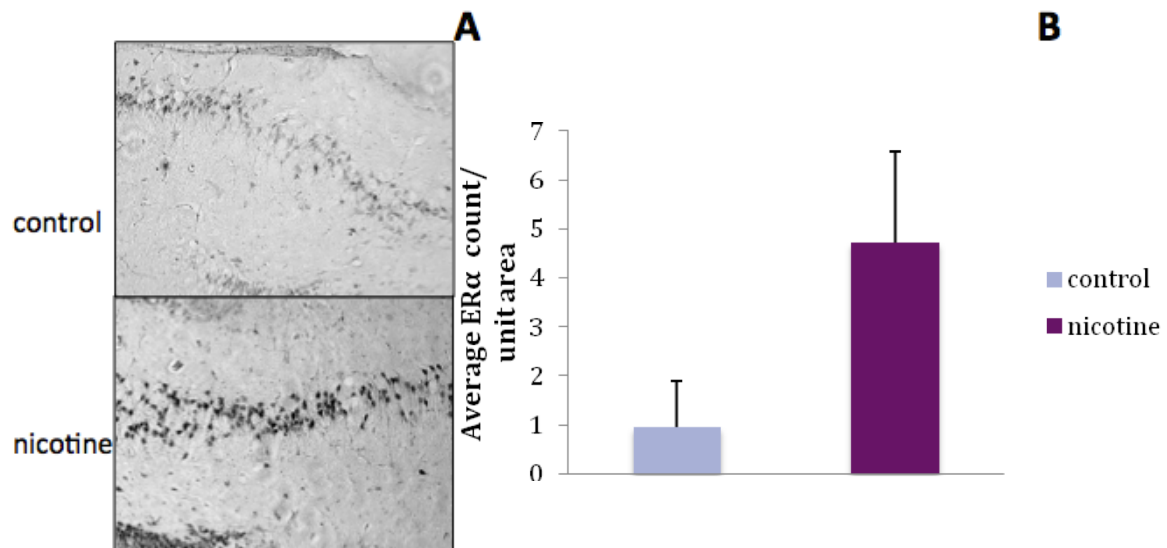


Figure 18. ERα-ir in the CA3 subregion of the hippocampus. (A) Representative photomicrographs of ERα-ir in the CA3 in controls and nicotine exposed animals taken with a 20x objective. Images used for counting were taken with a 20x objective. (B) ERα count in the CA3 represented by mean ± SEM (n=6/group). No significant difference in treatment was found ($p < 0.05$).

In the DG, 26-week old male rats showed significant upregulation of ERα following early life nicotine exposure (8.7 ± 3.8 saline, 20.1 ± 4.5 nicotine; $p < 0.05$) (Figure 19).

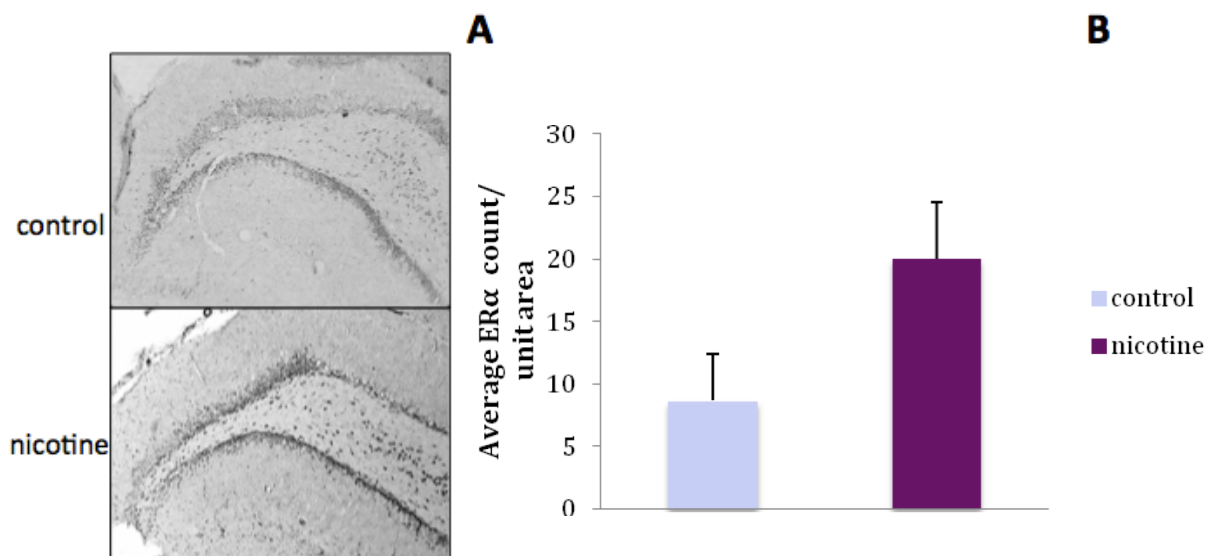


Figure 19. ERα-ir in the DG subregion of the hippocampus. (A) Representative photomicrographs of ERα-ir in the DG in controls and nicotine exposed animals taken with a 10x objective. Images used for counting were taken with a 20x objective. (B) ERα count in the DG represented by mean ± SEM (n=6/group). The average count of ERα+ cells was greater in the nicotine vs. control group ($p < 0.05$).

As for the of the entorhinal cortex, ER α immunoreactivity did not differ significantly between the saline (7.2 ± 2.1) and nicotine groups (6.1 ± 2.6) (Figure 20).

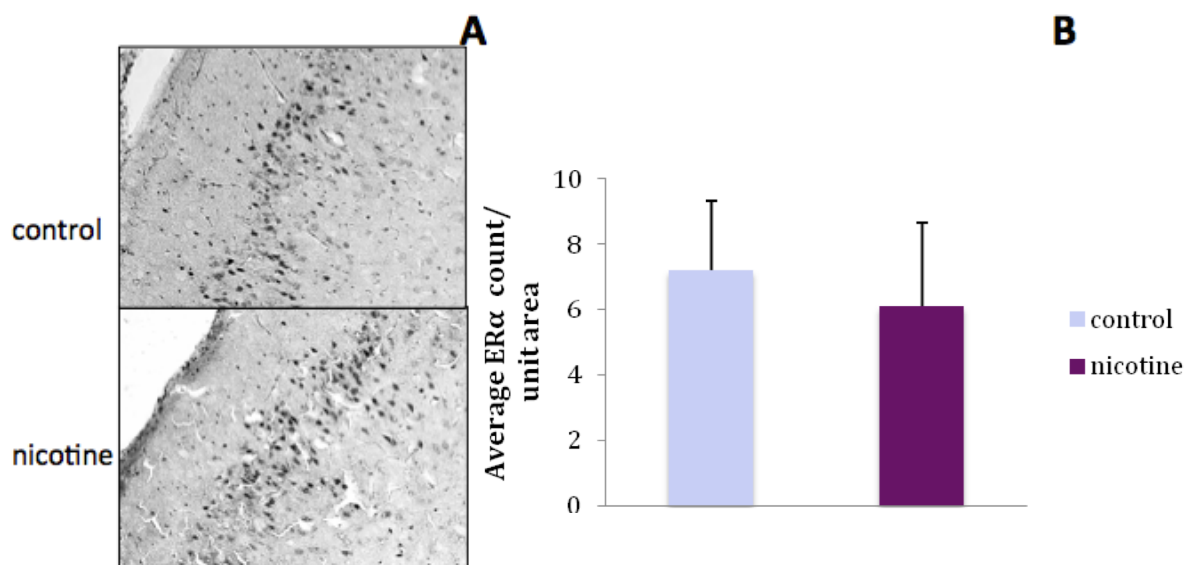


Figure 20. ER α -ir in the entorhinal cortex. (A) Representative photomicrographs of ER α -ir in the entorhinal cortex in controls and nicotine exposed animals taken with a 20x objective. Images used for counting were taken with a 20x objective. (B) ER α count in the entorhinal cortex represented by mean $\pm$ SEM (n=6/group). No significant difference in treatment was found ($p < 0.05$).

CHAPTER 5. Discussion

Smoking in pregnancy is an ongoing public health challenge associated with adverse cognitive and behavioral outcomes in offspring. The long-term ramifications of maternal smoking on offspring neurologic development impose a considerable economic burden on society. The fact that nicotine is the chief component in tobacco responsible for many of these negative health effects poses a problem in the use of NRT for smoking cessation (Dempsey & Benowitz, 2001). If nicotine itself is injurious to the developing brain, then nicotine substitution will not eliminate the deleterious health outcomes of maternal smoking.

There is evidence to suggest that early life nicotine exposure perturbs the programming of neurodevelopmental events at the cellular level. This may, in turn, form the basis for functional deficits that may appear over time as subtle neurodevelopmental and psychobehavioral deviations.

Currently, there are no prospective epidemiological studies addressing the correlation between NRT use during pregnancy and onset of disease in adult offspring. However,

considerable insight into the long-term effects of perinatal nicotine exposure can be gained from animal models. The use of animal models can better our understanding of the manifestations of perinatal nicotine exposure throughout the lifespan of the animal, and perhaps most importantly, the underlying causes of these abnormalities.

Using a rat model, we assessed the effects of nicotine at doses mimicking NRT on the adult male hippocampal formation. In the present study, 26-week old offspring were evaluated for alterations in neuron, astrocyte and ER α expression following perinatal exposure to nicotine.

Nicotine treatment does not alter long-term body weight gain

We cannot speak to the weight gain in our particular study, given the lack of information on body weight and litter specificity at birth. Nevertheless, body weights in adulthood were similar between groups, suggesting that birth weight was not likely a confounding factor when assessing the effects of early life exposure in adulthood.

In keeping with earlier reports (Abdel-Rahman et al., 2004, 2005; Abou-Donia et al., 2006; Roy, Seidler & Slotkin, 2002), postnatal weights in offspring measured at 26 weeks was unaffected by nicotine treatment. In a similar animal model, dams were subcutaneously injected with 1mg/ kg body weight per day of nicotine, during gestational days 4-20 (Abdel-Rahman, 2004). The neonatal weights of offspring were recorded and there were no significant differences between the control and nicotine-treated groups. There was also no significant difference in body weight gain throughout postnatal development (until PND60). This particular group also found that the body weights remained unchanged when the dams were exposed to a dose of 3.3mg/ kg body weight per day via osmotic minipump (Abdel-Rahman, 2005).

In humans, findings have been inconsistent. One study examining the use of NRT during pregnancy concluded that nicotine gum, while unable to increase smoking cessation rates, increased birth weight in the infants of mothers who smoked (Oncken et al., 2008). On the contrary, a study revealed that the risks of low birth weight and preterm birth were highest in women using NRT (Gaither, Huber, Thompson & Huet-Hudson, 2008). A recent review of NRT interventions in pregnancy determined that there were no statistically significant differences in birth weights between NRT and control groups (Coleman et al.,

2012). To my knowledge, there have been no studies conducted to determine whether weight gain remained consistent through to adulthood, to corroborate our findings.

Perinatal nicotine exposure did not significantly impact neuronal populations

According to our data, neither the hippocampus proper nor the entorhinal cortex showed significant changes in the number of neurons in adulthood following perinatal nicotine exposure. At the same dose (1 mg/kg), a similar histopathological evaluation using cresyl violet staining also found no significant alterations in hippocampal neuron counts in adolescent rats (PND60) prenatally exposed to nicotine (Abdel-Rahman et al., 2004).

This group achieved considerably different results when they increased the nicotine dose threefold (3.3mg/kg) (Abdel-Rahman et al., 2005). PND30 and PND60 offspring exhibited a significant reduction in the number of surviving neurons in the CA1 subfield of the hippocampus. Additionally, neurobehavioral performance on the beam-walk and forepaw grip were significantly impaired, indicating that maternal nicotine exposure may have put the offspring at risk for long-term adverse health effects. Taken together, these findings suggest that the dosage of nicotine is negatively associated with neuron counts.

Neuron-type targeting of perinatal nicotine exposure

While quantitative measurements in neuronal counts did not differ between groups, a morphological examination of cells in the hippocampus suggests that nicotine targets specific cell types as opposed to selective brain regions. This coincides with previous findings whereby nicotine induced damage was worse in cells with postnatal birth dates (Roy, Seidler & Slotkin, 2002); pyramidal cells within the CA1 were less affected than the same cell type in the CA3 and cell properties in the CA1 were entirely restored by adolescence.

Additionally, despite the unvarying total neuronal counts, the proportion of pyramidal to non-pyramidal neurons might be altered; within these two types of neurons, there exist different subtypes, depending on the neurotransmitters and neuropeptides they synthesize. In the rodent, differentiation of telencephalic regions essential for learning and memory such as the hippocampus, occur during the late prenatal period and early postnatal

period (Levin et al., 1996; Lichtensteiger et al., 1988). Within the hippocampus, granule cells and pyramidal cells have widely disparate birth dates. Hippocampal CA1 and CA3 pyramidal neurons are generated prenatally, with CA3 pyramidal cell migration continuing later in development, and most of the granule cells of the hippocampus appearing even later during postnatal development (Altman and Bayer, 1990a,b). As such, animal studies have shown that nicotine exposure towards the end of prenatal development reduces performance on cognitive tasks as well as increases anxiety and hyperactivity (Ankarberg, 2001; Eppolito, 2010; Levin et al., 1996).

This is further corroborated in humans where mid- to late- gestation is the period when the developing fetus is most vulnerable to the harmful effects of nicotine (Prabhu et al., 2010). For this reason, it is generally recommended that women be nicotine free by the third trimester to limit the risks of adverse effects in the fetus (Bergsjö, Bakketeig & Lindmark, 2007; Bernstein et al., 2000; Lampl, Kuzawa & Jeanty, 2003; SOGC, n.d.; Zaren, Lindmark & Bakketeig, 2000).

GFAP-ir in the adult rat brain is unchanged following perinatal nicotine exposure

Earlier studies have mainly focused on the persistent changes that perinatal nicotine exposure has on neurons because they more directly underlie cognitive and behavioral problems. Only recently has work begun to address the contribution of nicotine-induced changes in glial cells, specifically astrocytes (Abdel-Rahman et al. 2004, 2005; Abou-Donia et al. 2006; Blutstein et al., 2013; Roy et al. 2002).

Our study demonstrated that the glial population in 26-week-old offspring exposed *in utero* and postnatally to nicotine did not greatly differ from control animals. Though animals exposed to nicotine *in utero* had slightly fewer GFAP-ir cells across all regions of the hippocampal formation, none of these findings reached statistical significance.

Blutstein et al. (2013) arrived at the same conclusion. First, guinea pigs experienced a robust accumulation of GFAP following gestational nicotine exposure (GD 52-62) (200µg/ml), which is synonymous with acute reactive gliosis. But once offspring reached adulthood (PND90), GFAP-ir was significantly reduced in the CA1 subfield of the hippocampus. Similarly, a post-mortem analysis examined the hippocampus and entorhinal cortex of elderly individuals with a smoking history. It revealed a significant reduction in

GFAP-ir cells in smokers and ex-smokers compared to individuals with no known smoking history (Teaktong et al. 2004). This goes to show that the consequences of nicotine exposure are in fact long lasting.

Conversely, Nissl staining of brain sections by Roy, Seidler & Slotkin (2002) revealed that rats exposed to nicotine prenatally (GD 4-21) had elevated numbers of glia in all subregions of the hippocampus (CA1, CA3, DG) during the juvenile and adolescent stages of development (PND21 and PND30). Also in adolescent rats (PND30 and PND60), gestational nicotine exposure (GD4-20) (3.3mg/kg) led to an increase in GFAP-ir in the CA1 hippocampal subfield (Abdel-Rahman et al., 2005). But, this same group also discovered that GFAP-ir was unaffected in the hippocampus following smaller nicotine doses (1mg/kg) (Abdel-Rahman et al., 2004).

Thus, the lower dose of nicotine used in our study likely failed to generate persistent changes in astrocytes; it may have been more telling to look at differences across the lifespan. It should be noted that most animal studies to date have focused on replicating maternal smoking and not utilizing nicotine at doses mimicking NRT. Thus, the nicotine dosage could have played an important role in interpreting these results. Rats that displayed increased GFAP-ir were exposed to much higher levels of nicotine (2-3.3mg/kg) (Abdel-Rahman et al., 2005; Roy, Seidler & Slotkin, 2002) compared to what is typically found in NRT (1mg/kg) (Abdel-Rahman et al., 2004). It may be that nicotine at doses mimicking NRT was not sufficient to activate reactive gliosis and the associated accumulation of GFAP nor to prompt the secretion of pro-inflammatory molecules that often result in neuronal damage - though these were not evaluated herein.

An alternative explanation could be that a mechanism of estradiol-mediated neuroprotection was at play, restoring homeostasis in the hippocampus. An inflammatory response is known to occur in the CNS under pathological conditions. In both immature and adult brains, acute reactive gliosis is triggered in response to insults such as neurotoxicant exposure and is characterized by an accumulation of GFAP. GFAP aids in the long-term maintenance of brain cytoarchitecture (Liedtke et al., 1996), proper functioning of the blood-brain barrier (Pekny et al., 1998) and modulation of neuronal function (Shibuki et al., 1996). In order to mitigate this glial cell response and prevent progressive neurodegeneration, protective molecules such as estradiol can intervene to modulate the inflammation.

Based on these observations, we can infer that, while it is possible that GFAP upregulation from acute reactive gliosis is apparent in juvenile and adolescent brains (Abdel-Rahman et al. 2004, 2005; Roy et al. 2002), it does not persist chronically into adulthood.

ER protein expression inconsistent in the CA1 of the hippocampus

Estradiol-mediated neuroprotection occurs via activation of ER α . Despite the clear role of this receptor in neuroprotection, its response to perinatal nicotine exposure remains unknown – both acutely and chronically. For this reason, we tested whether nicotine treatment altered the expression of ER α in rats whose mothers were exposed to nicotine at doses mimicking NRT.

Histopathological findings showed that ER α upregulation was uniform across all subfields of the hippocampal formation in offspring of mothers treated with nicotine. Despite not reaching significance in the CA1 of the hippocampus, ER α -ir was considerably heterogeneous in the nicotine-treated animals. As for the control group, there was a single outlier that contained a minimal amount of receptors. This was observed exclusively in the CA1 subfield of the hippocampus, signifying that it was unlikely the result of non-specific staining during immunohistochemistry.

Researchers have stated that ER α is relatively low in the hippocampal formation compared with the ER β which appears to be the major ER expressed in the hippocampus (Kuiper et al., 1997; Shughrue et al., 1997; Laflamme et al., 1998; Mitra et al., 2003). Furthermore, ER α mRNA expression is predominant in the CA3 and DG of the hippocampus and observed to a much lesser degree in the CA1 subfield in adult rats (Rai & Jeswar, 2012, Rune et al., 2002). In humans, nuclear ER increase in the DG and CA3 (Ishunina, Fischer & Swaab, 2007) while they tend to decline in the CA1 during aging (Tohgi et al., 1995; Ishunina, Fischer & Swaab, 2007).

This potentially highlights the variability of ER α expression patterns across structural and functional development of the hippocampus; it is not static, but rather can be induced by neuronal injury and neurodegeneration (Dubal et al., 2001; Vegeto et al., 2003). Thus, the generous upregulation of ER α levels in some nicotine-exposed offspring was, in fact, a response to a threatening event occurring in the CNS.

Overall, while the consensus seems to be that both ER subtypes are highly expressed in a variety of cell types throughout the rat hippocampus, researchers agree that the literature pinpointing their exact location is both confusing and contradictory (as stated in Rune et al., 2002) (see Shughrue, Lane & Merchenthaler, 1997; Shughrue & Merchenthaler, 2001). Significant variability in commercially available ER antibodies may account for some of these discrepancies, further highlighting the need for additional research to better our understanding of these steroid receptors, as well as the standardization of scientific protocols to ensure reproducibility and consistency of results.

What we can also learn from this unusual variation in staining pattern is that internal positive control samples are extremely important for evaluation of steroid receptor expression analysis. Including a positive control such as the use of endometrium sections, would have allowed us to ensure the specificity of the immunohistochemical labeling.

Perinatal nicotine significantly upregulated ER α -ir in the DG of the hippocampus

Exposure to nicotine only elicited significant ER α -ir in the DG. This would indicate that perinatal nicotine treatment produces long-lasting effects on ER α , with significant increases still apparent in male rats weeks after the termination of drug exposure.

This provides further support to the claims that ER α is the critical mechanistic component that mediates the neuroprotective effects of circulating estradiol during brain injury (Dubal et al., 2001). This also provides additional evidence that trauma causes ER α upregulation both in the presence or absence of estradiol (Dubal et al., 1999), a steroid that has been suggested to be reduced by nicotine (Barbieri, Gochberg & Ryan, 1986; Biegon et al., 2010; Kitawaki et al., 1993). Even under conditions of low estradiol levels, ER α remains the main receptor mediating transcription (Foster, 2012).

The increased number of ER α that we have witnessed is comparable to early postnatal development in the brain, a period typified by widespread neurogenesis and differentiation when ER numbers are elevated, especially up to PND2 (Shughrue et al., 1990; Toran-Allerand, Miranda, Hochberg & MacLusky, 1992). This would suggest that under pathological conditions such as nicotine exposure seen here, a mechanism of action was triggered whereby ER α expression was enhanced to replicate the developmental actions of estradiol and minimize potential neurological insult.

ER α is important for maintaining hippocampal function when estradiol levels are low (Foster et al., 2008). Nicotine has been said to reduce estradiol levels by targeting key steroidogenic enzymes such as aromatase (Barbieri et al., 1986; von Ziegler, Schlumpf & Lichtensteiger, 1991). Male rats that have been subjected to aromatase inhibition for the first 3 weeks of postnatal development show higher ER-ir than controls in the preoptic area and hypothalamus at 6 months of age (Bakker et al., 1997). This suggests that low neonatal estrogens, derived from the aromatization of testosterone, permanently alter the expression of ERs in the adult male brain. This coincides with studies that have shown that the half-lives of unliganded ER α proteins are longer than their ligand-occupied counterparts (Lonard et al., 2000, Nardulli & Katzenellenbogen, 1988). Therefore, declining estradiol levels as a result of nicotine exposure may lead to longer receptor half-lives and this decreased receptor turnover may contribute to maintaining protein levels throughout adulthood.

Because estradiol levels at certain points in development are said to permanently alter ER α expression, further evidence that this particular animal model inhibits estradiol synthesis would have added to our results. Although nicotine's anti-estrogenic properties have been discussed at length in the literature, quantifying DNA of key enzymes involved in estradiol synthesis (such as aromatase and the steroidogenic acute regulatory protein) via polymerase chain reaction may have shown that low-level nicotine does in fact antagonize estradiol synthesis, resulting in longer receptor half-lives (see appendix for a detailed account of the PCR challenges encountered over the past year).

Studies revealing the importance of hippocampal ER α receptors in memory function are abundant in both human and animal literature: ER α polymorphisms have been associated with age-related memory deficits and an increased incidence of Alzheimer's disease in women (Corbo, Gambina, Ruggeri & Scacchi, 2006; Ji et al., 2000; Olsen et al., 2006). Furthermore, the knockout of ER- α in rodents resulted in learning and memory impairments, as seen in performance of hippocampus-dependent tasks (Fugger, Cunningham, Rissman & Foster, 1998; Fugger, Foster, Guftafsson & Rissman, 2000).

Mounting evidence suggests that increased ER α expression is involved in memory processes in the hippocampus and can delay the onset of age-related memory impairments. In fact, better memory is linked to this increased expression in the hippocampus (Iivonen et al., 2006; Foster et al., 2008; Pawluski et al., 2010; Rodgers et al., 2010; Su et al., 2010). As

such, age-related changes could arise through the disruption of the hormone/receptor system through a loss of hormone, or uncoupling of receptor-transcriptional activity or decreased receptor expression (Foster, 2005). The latter not being the case in this particular study, suggesting that enhanced ER α could maintain memory function thus halting cognitive decline typically experienced in animals who have been subjected to perinatal nicotine exposure.

ER α upregulation as a potential mechanism of neuroprotection following nicotine exposure

Though estrogen's involvement in neuroprotection is widely accepted, its specific underlying molecular and cellular mechanisms are still unknown. Accumulating evidence suggests that estrogen-mediated neuroprotection is activated via glial cell interaction, mitigating inflammation and protecting neurons.

Overall, this study alludes to the fundamental molecular mechanism by which ER α expression is enhanced, potentially protecting the brain against injury, neurodegeneration and cognitive decline. Even at small doses, developmental nicotine exposure elicits this effect in the DG of the hippocampus, a brain area involved in learning in memory.

While we can speculate as to the neuroprotective role of estradiol-mediated neuroprotection in an animal model of NRT, there is a lack of evidence allowing us to extrapolate a precise mechanism of action at play. Above all, it is evident that nicotine treatment upregulates ER α in the DG of adult male rats following maternal nicotine exposure. In addition, astrocyte and neuron populations in 26-week old offspring were not significantly altered. Thus, is it possible that this is the result of the neuroprotective effects of estradiol-mediated neuroprotection mitigating gliosis and halting neuronal death. Unfortunately, in order to report on these parameters, additional information regarding the acute effects of perinatal nicotine exposure on cellular markers of interest is necessary.

Most important would be to report on the acute effects of nicotine exposure to allow us to determine whether or not there is indeed variability of cellular marker expression over time. Also, studies are needed to better our understanding of the implications of enhanced ER α expression in the brain. The addition of a behavioral component to this study might

have enabled us to observe the potential functional manifestations of any anomalies, specifically testing of memory and learning engaging the hippocampal formation.

In conclusion, though no significant alterations in neuronal and glial cell numbers were observed, it is likely that the severity of deleterious outcomes are responsive to dosage and timing of exposure. Furthermore, this study has provided evidence that even relatively small amounts of nicotine during CNS development can produce changes that persist into adulthood. While the implications of this remain unknown, it is clear that there is in fact, no such thing as a safe dose of nicotine. Consequently, if nicotine itself is responsible for adverse effects of smoking, then nicotine substitution may not eliminate the deleterious health outcomes of maternal smoking on the offspring.

Finally, if a mechanism of estradiol-mediated neuroprotection is in fact at play, additional research could carry far-reaching implications for the selective targeting of ERs in the treatment of disease states associated with cognitive decline and neurodegeneration.

CHAPTER 6. Interdisciplinary Approach to NRT

Determinants of Health

Although the proportion of women who smoke during pregnancy in high-income countries has declined, it remains an international public health priority. The economic burden of tobacco-related morbidity and mortality is substantial (Green et al., 2005; Miller Villar, Hogue & Sivapathasundaram, 2001), contributing significantly to socioeconomic inequalities in stillbirths and infant deaths (38% and 31% respectively) as shown in a retrospective cohort study of mothers with varying degrees of socioeconomic deprivation (Gray et al., 2009). This is in accordance with an observation made by Bauld, Judge & Platt (2007) whereby despite low overall efficacy, smoking cessation services have had a disproportionate effect in the most disadvantaged groups, possibly reducing the social gradient. Unfortunately, there is little information available regarding smoking cessation treatment efficacy in pregnancy and the few relevant studies provide conflicting results (for review, see Coleman, Chamberlain, Cooper & Leonardi-Bee, 2010).

The results of the largest NRT randomized control trial conducted thus far has suggested that smoking cessation treatment failure in pregnancy may stem from the >90% dropout rate (Coleman et al., 2012). This outcome has been corroborated in numerous

smaller studies, where mean duration of NRT use rarely exceeded one month (Wisborg et al., 2000; Pollack et al., 2007; Coleman, Chamberlain, Cooper & Leonardi-Bee, 2011). Although experts agree that the increased nicotine clearance from the body experienced during pregnancy is partly to blame for lack of compliance to an NRT regime, and higher doses of nicotine may be required to optimize efficacy (Dempsey et al., 2002), this unilinear and oversimplified approach overlooks important predictors of non-adherence. There are a number of complex reasons for women's smoking patterns that studies blatantly disregard. Barriers are multiple and interacting psychosocial, cultural, economic, and biological influences, which are further accentuated in pregnancy and postpartum also factor in to the lifestyle choice that goes beyond physiological addiction. Spontaneous quitters tend to be more highly educated, less addicted and less likely to have partners who smoke (Klesges et al., 2001). Unfortunately, these are rarely accounted for in interventions, which focus on individual behaviour change (Greaves et al., 2011).

Maternal smoking rates are much higher for women from lower socioeconomic groups as depicted by low-income levels, lower education attainment and low occupation status. Other determinants of treatment attrition include: degree of addiction, social support, culture, mental illness and health services. These women who are generally part of socially disadvantaged and economically marginalised groups, are especially susceptible to smoking during pregnancy and yet, remain under-acknowledged in research and intervention development. To fully capture the effectiveness of interventions initiated during pregnancy and avoid producing biased estimates of risk, future studies must control for a full range of psychosocial factors.

Socioeconomic Status

High-income countries have seen a significant decline in maternal smoking rates since the 1980s (Cnattingius 2004; US DHHS 2004). Unfortunately, this is not uniform across all sectors of society. Low socioeconomic groups have experienced a much slower rate of decline relative to those of higher socioeconomic standing (US DHHS 2004).

In Canada, smoking prevalence is higher among pregnant women with low household income, who are less than university educated and who have not recently held a job (Cui et al., 2014). While all three of these variables are not always represented, there is a clear pattern arising from Canadian studies (Al-Sahab, Saqib, Hauser & Tamim, 2010;

Erickson & Arbour, 2012; Johnson et al., 2004). Regardless of how social status is operationalized (low income, low educational attainment, low occupational status), these findings are also consistent with those documented in other developed countries including Australia (Hoekzema et al., 2014; Mohsin, 2005), Iceland (Erlingsdottir, 2014), USA (Gyllstrom, Hellerstedt & Hennrikus, 2012; Holtrop et al., 2006; Ockene et al., 2002), Scotland (Tappin, 2010) and Finland (Räsänen, 2014).

Cigarette smoking is a marker of social disadvantage in high-income countries and has been cited as one of the most important contributing factors of health inequality between the rich and poor (Wanless 2004). The World Health Organization's report into the *Social Determinants of Health* acknowledges that disadvantaged people are more likely to use substances in response to their circumstances (CSDH, 2008) and yet, up until recently, smoking-cessation strategies and interventions failed to address the social barriers to participation among these high-risk groups. For example, a Canadian study conducted by Stewart and colleagues (1996) found that only 23 percent of treatment programs geared towards women were appropriate for, or accessible to, disadvantaged smokers. Higher cigarette consumption, lower educational level, higher confidence in ability to quit on one's own and multiparity are some of the predictors of treatment non-compliance that have caused interventions to be less effective among pregnant women with low socioeconomic status (SES) (Wen, Miller, Lazev, Fang & Hernandez, 2013).

This blatant overrepresentation of women with lower SES among pregnant smokers warrants the need for approaches that address risk factors of non-adherence. Though the development and implementation of such smoking cessation strategies could be challenging, it is imperative that health professionals be aware of and acknowledge the difficulties that underserved minority pregnant smokers encounter. In order to so, anti-smoking campaigns will need to adopt a positive rather than punitive approach and respect individual values, capabilities and circumstances to achieve compliance in women (Bond, Brough, Spurling & Hayman, 2012).

Degree of addiction

A retrospective study conducted in British-Columbia reported that smoking during pregnancy is a significant dose-dependent risk factor for adverse birth outcomes including

small-for-gestational age, low birth weight at term and intra-uterine growth restriction (Erickson & Arbour, 2012).

Researchers agree that smoking during pregnancy is more prevalent among smokers with a higher level of addiction prior to pregnancy (Cui et al., 2014; Hoekzema et al., 2014; Schneider, Huy, Schütz, & Diehl, 2010). Johnson et al. (2004) found that Canadian women smoked on average 9.6 cigarettes daily during the first trimester, exceeding the 7.5 cigarettes smoked by women who quit when they found out they were pregnant. This would suggest that spontaneous quitters have a lower degree of nicotine dependence and therefore have less difficulty quitting than heavy smokers (Mohsin & Bauman, 2005; Solomon & Quinn, 2004). The reported number of cigarettes smoked across pregnancy was however lower than the average daily consumption reported by non-pregnant female smokers in Ontario (15.2 cigarettes) (Monitoring the Ontario Tobacco Strategy, 2002). These data could be skewed due to bias – whether recall or underreporting due to social stigmatization – or it could be an accurate representation of smoking status among women who, although unable to quit, reduce tobacco consumption as a precaution while pregnant (Johnson et al., 2004).

Studies also suggest that heavy smoking is a potential marker for lifestyle risk factors that, in combination with smoking, influence birth outcomes. Heavier smoking mothers are more likely to also fall into the following categories: Aboriginal status, low socioeconomic status (Mohsin & Bauman, 2005), low level of education, single parent, drug or alcohol use (Erickson & Arbour, 2012), poor antenatal care attendance and multiparity (Erickson & Arbour, 2012; Mohsin & Bauman, 2005)

Social Support

Numerous studies have highlighted the importance of social support networks in helping pregnant women reduce or cease smoking. In particular, marital status (an indicator of social support) is highly predictive of successful smoking cessation during pregnancy. There is a significant trend whereby an increasing risk of maternal smoking is associated with decreasing parental bonding (Kiernan & Pickett, 2006). Thus, the extent of maternal smoking is lowest in married mothers, followed by cohabiting mothers, then solo mothers who were closely involved with the father at the time of the birth and finally, solo mothers not in a relationship with the father who have the highest risk of continuing to smoke.

Al-Sahab and colleagues (Al-Sahab, Saqib, Hauser & Tamim, 2010) have indeed shown that single mothers are at an increased risk of smoking during pregnancy. The Millennium Cohort Study conducted among 18 225 women in the UK also came to this conclusion, revealing that married women have much lower rates of smoking throughout pregnancy compared to cohabitating and single mothers (Pickett, Wilkinson & Wakschlag, 2009). The presence of a partner reduces the amount smoked by 36 percent compared to women who did not report any partner involvement (Martin, McNamara, Milot, Halle, & Hair, 2007).

In addition to the presence of a partner, the amount of support a woman receives from her significant other is predictive of her likeliness to quit smoking. A supportive husband or stable partner will increase the chances of successful smoking cessation for pregnant women (Fingerhut, Kleinman, & Kendrick, 1990; Lu, et al., 2001; Nafstad, et al., 1996, Smedberg, Lupattelli, Mardby & Nordeng, 2014). Accordingly, there is evidence that pregnant smokers are more likely to have problematic relationships and that this pattern of problematic behavior may interfere with the effectiveness of current public health cessation interventions (Wakschlag et al., 2003).

The importance of partner support has been further substantiated by the many studies documenting higher use of tobacco, alcohol, and illicit drugs in pregnant women subjected to physical abuse (Heaman & Chalmers, 2005; Perreira & Cortes, 2006), suggesting that heightened conflict in a relationship may intensify the urge to smoke and hinder cessation attempts. Problematic interpersonal relationships within the family of origin, peers and neighbours are also more prevalent among smokers compared to quitters and non-smokers (Pickett, Wilkinson & Wakschlag, 2009); a single problem within interpersonal relationships increases the likelihood of a pregnant woman becoming a persistent heavy smoker by 67%.

Provided that women experience positive social relationships, living with others who smoke is one of the primary barriers to smoking cessation in pregnancy (Fang, et al., 2004; Ward, et al., 2006) because the other smokers provide easy access to cigarettes (Thompson, Parahoo, McCurry, O'Doherty, & Doherty, 2004). Approximately 80% of pregnant smokers are partnered with an expectant father who continues to smoke (Everett et al., 2005), 78% have a smoker in the household and 95% have a smoker among friends (Hoekzema et al., 2014).

In view of the high smoking rates among other household members and friends, smoking cessation and relapse prevention interventions must also include those closely associated with the expectant mothers, particularly partners. On the contrary, existing interventions disregard the utility of partner inclusion during research and intervention development. A systematic review of interventions to enhance partner support as part of stop-smoking therapies revealed a lack of effective programs that include or target partners (Hemsing, Greaves, O'Leary, Chan & Okoli, 2012). Quit smoking programs do not address the complex psychosocial context of tobacco use and rely on women having the social resources to independently implement and sustain the necessary behavioural strategies that lead to quitting (Pickett, Wilkinson & Wakschlag, 2009). They fail to consider that persistent smokers generally have fewer social resources on which to draw and those available to them might be destructive.

Culture

High maternal smoking rates among Indigenous minority groups are largely patterned by their social and material deprivation. Numerous qualitative studies recognize this health disparity, even more so in Canadian (Heaman & Chelmers, 2005; Wenman, Joffres, Tataryn & the Edmonton Perinatal Infections Group, 2004) and Australian (Li, Zeki Hilder & Sullivan, 2010; Mohsin & Bauman, 2005) indigenous populations.

In Alberta - a province with one of the highest concentrations of Aboriginal people in Canada - 61.2 percent of Aboriginal women (First Nations, Métis and Inuit) reported smoking during pregnancy, compared to 26.2 percent of non-Aboriginal women (Heaman & Chalmers, 2005). This coincides with maternal smoking rates in Australia where one in two (49.3%) Aboriginal and Torres Strait Islander women smoke while pregnant (Li, Zeki Hilder & Sullivan, 2010) compared to non-Indigenous Australian mothers.

Although systemic reviews promoting tobacco interventions are readily available, Ivers (2004) suggests that further research is required to assess transferability of evidence from other populations to Aboriginal people prior to implementing such programs. In order to assuage the burden maternal smoking poses on the healthcare system, the development of effective smoking cessation programs geared towards high-risk populations must be prioritized.

Borland et al. (2013) investigated the adequacy of smoking cessation support available to pregnant and postpartum women in Ontario and found that Northern and rural communities encountered many barriers impeding service availability and utilization. Firstly, inadequate funding for tobacco control and cessation activities relative to larger urban communities was one of the main shortcomings as told by key informants. Secondly, the geographical dispersion of residents inhabiting these remote locations posed a serious threat to the accessibility to resources. Finally, participants articulated the importance of implementing culturally sensitive practices and tailored interventions. This would mean adopting a perspective that addresses traditional tobacco use distinguishing between typical cigarette smoking and ceremonial and medicinal tobacco use (Health Canada, 2010) and adopting a holistic approach to wellbeing, inclusive of the family and community (Borland et al., 2013).

In addition to ensuring accessibility and engagement, it is important to have a clear understanding of the determinants of health that lead to the initiation and continuation of tobacco use in women of reproductive age. This requires tailoring programs to meet the social and economic pressures experienced by minority groups. Poverty, social stigma and misconceptions regarding the safety of stop-smoking aids are only some of the barriers hindering the success of stop-smoking interventions (Borland, 2013). Difficult life circumstances and high levels of stress are equally common among Aboriginal pregnant smokers (Gould, Munn, Watters & McEwen, 2013) and take precedence over smoking cessation. Furthermore, widespread use of tobacco products among Indigenous people make it particularly difficult for pregnant women to avoid other smokers and obtain support from family and partners when attempting to quit (Gould, Munn, Avuri, Hoff, Cadet-James et al., 2013).

Mental Health

A number of studies have compared mental health parameters between pregnant smokers and non-smokers, and found higher rates of depression (Blalock, Fouladi, Wetter & Cinciripini, 2005; Linares Scott et al. 2009; Orr et al. 2012; Smedberg & Lupatteli, 2014) and a history of mental health problems (Holtrop et al., 2010; Kodl & Wakschlag, 2004) to be important predictors of maternal smoking.

Pregnant smokers report significantly more symptoms of depression and anxiety than spontaneous quitters and are more likely to exhibit social withdrawal (Linares Scott et al., 2009). Higher scores of depression in smokers remain despite controlling for confounding variables, suggesting that depressive symptoms might be an independent contributor of persistent smoking among expectant mothers. Another more recent study of 4 295 pregnant women from 15 European countries reported approximately twice the prevalence of continued smoking among depressed subjects across all European regions (Smedberg et al., 2014).

While some studies suggest that a history of depression is associated with a greater difficulty quitting smoking (Howard et al. 2013), others show no such relationship (Holtrop et al. 2010; Ludman et al. 2000). For example, Gyllstrom & Hellerstedt (2012) found that maternal mood did not affect ability to quit, but experiencing stressful life events was a strong determinant. Pregnant women who reported three or more stressful life events were only half as likely to quit smoking as women who reported no stressful events in the previous year. This gives weight to the belief that smoking before pregnancy is a habituated response to the circumstances of women's lives, such as: unsupportive partners, caring for young children, unstable jobs, domestic situation, and economic vulnerability (Flemming et al. 2013). Women may be more likely to smoke as a means to self-medicate using nicotine's powerful mood-altering properties to temporarily alleviate affective symptoms.

Overall, there appears to be a lack of robust evidence linking depression and difficulty quitting smoking, warranting the need for further inquiry. Shortcomings in study design have greatly impacted the quality of the available evidence. The majority of literature investigating mental health in pregnant smokers has relied on a cross-sectional design that does not allow for conclusions related to temporality or causality. Methods of data collection are also vulnerable to biases inherent in self-reporting, including recall and social desirability bias. For this reason, cotinine measurements in women should have been an integral component in all studies to verify smoking status but were only discussed in a fraction of them. In addition, relatively small sample sizes make it difficult to extrapolate larger conclusions regarding the applicability of findings.

Nonetheless, prenatal depression is an important precursor for postnatal depression (Bennett et al. 2004) and may put the fetus at high risk for prematurity and low birth weight

(Marcus et al. 2003; Marcus 2009) in addition to the many adverse health outcomes of smoking during pregnancy. Knowledge about these high-risk groups will have meaningful implications for antenatal care improvement. Even though smoking initiation is multifactorial in origin, several sources point towards negative affect as a vital contributing factor (Baker, Brandon, Chassin, 2004). These individuals might be more susceptible to nicotine addiction because of its mood altering properties caused by excessive stimulation and reorganization of the brain's reward circuitry (Benowitz, 2010).

It is becoming increasingly clear that smoking and depression have a complex comorbid relationship whereby nicotine intake affects mood, which in turn, increases dependence. If this is indeed the case, then interventions geared towards women of childbearing age should endeavor to reduce depression rates thereby diminishing smoking prevalence among pregnant women.

Health Services

Pregnancy is regarded as a window of opportunity for quitting smoking, and so, prioritizing successful and enduring tobacco cessation strategies during pregnancy is an ongoing public health concern. Establishing the proper conditions to facilitate tobacco cessation has proven to be an obstacle for health care providers. Health interventions designed over the last three decades have had a poor success rate partly due to the fetus-centric perspective they have adopted (Greaves et al., 2011). Because approaches to smoking cessation have focused primarily on benefiting fetal health, interventions have been confined to the period of pregnancy and dismissive of women's wellbeing. In order to ensure treatment adherence, a thorough understanding of the barriers in all areas of antenatal care provision must be addressed: patients, health professionals and health system.

Barriers to pregnant smokers:

The negative views of women towards healthcare services are an important barrier preventing women from quitting. The judgmental manner in which healthcare professionals behave make them reluctant to discuss their current smoking behaviour (Ingall & Cropley, 2010). One in two smokers report that their healthcare providers are either unaware of their smoking or do not discourage smoking during pregnancy (Hoekzema et al., 2014). While their motivation to quit is high, their confidence to quit is low (Hoekzema et al., 2014;

Ussher, Etter, & West 2006), suggesting the need for support from health professionals. Seeing as a lack of antenatal care in the first trimester is strongly associated with increased risk of smoking in pregnancy (Mohsin & Bauman, 2005), investment into improving health service delivery is warranted.

Barriers to health professionals and health system:

National guidelines recommend that clinicians provide effective smoking cessation counselling to pregnant women who smoke, utilizing the 5A's model (Fiore, Keller, & Curry, 2000). This model based on the Agency for Healthcare Research and Quality (AHRQ) guidelines, follows a manualized protocol that includes scripted material and the following steps: Ask about smoking status, Advise to quit smoking, Assess readiness to quit, Assist to quit, and Arrange follow-up. According to a 2013 Cochrane review, behavioural interventions such as the 5A's increase the smoking abstinence rate by 5% during pregnancy (Chamberlain et al., 2013). The 5A's are currently a best practice approach in the USA for all patients, including pregnant women during prenatal care (Dorfman, 2008). Similar approaches have been adopted in Britain (Raw, McNeill & West, 1998), Canada (OMA, 2003) and Australia (National Preventive and Community Medicine Committee, 2002).

Unfortunately, a review of health care providers' engagement in smoking cessation with pregnant smokers found that although more than 50% ask women about their smoking status and advise them to quit, fewer than 50% use all components of the 5A's to address smoking (Okoli, Greaves, Bottorff & Marcellus 2010). Factors influencing the provision of smoking cessation counselling based on the 5As model include time constraints (Coleman-Cowger, Anderson, Mahoney & Schulkin, 2014; Colomar et al., 2014) and inadequate knowledge of cessation interventions and training to implement a brief counselling intervention during pregnancy (Colomar et al., 2014; Price, Jordan, & Dake, 2006). In line with this, a recent survey of midwives and gynaecologists found that their knowledge of NRT use in pregnancy, especially in midwives, was insufficient and thus not recommended (De Wilde et al., 2015). "Ask", "Advise" and to a lesser extent "Assist" of the 5A's model were implemented in smoking cessation communication between these health care providers and their clients, but there were important barriers to providing counselling which included lack of time, lack of communication skills in sensitive topics such as smoking cessation, and

dealing with resistance. When used properly this systematic approach can increase the likelihood that tobacco use is addressed in the healthcare system (Fiore et al., 2007). Active screening of smoking status among pregnant women during each healthcare visit with accurate methods is crucial to continue and expand efforts. The probability of quitting and readiness to quit increases significantly when asked about smoking by two or more types of health professionals (An, Foldes, Alesci et al., 2008), lending support to the routine enquiry about smoking status. Fiore (2007) also suggests that such strategies be evaluated and lessons shared to allow such data to provide optimal patient care and inform policy decisions.

In addition, health professionals involved in the care of pregnant women must play a more proactive role when consulted on matters of smoking cessation. By providing them with the knowledge and tools to aid in smoking cessation, they can better educate smokers and motivate them to quit. Healthcare workers would therefore benefit from additional University-accredited certificate programs such as the Training Enhancement in Applied Cessation Counselling and Health (TEACH) Project funded by Smoke Free Ontario (CAMH, n.d.). A significant number of obstetrician-gynecologists feel the need for additional training on smoking cessation (Coleman-Cowger, Anderson, Mahoney & Schulkin, 2014), thus, this should be a mandatory part of their training so that they are better equipped when conducting smoking cessation interventions.

Finally, encouraging collaboration between antenatal care providers and non-medical professionals is a valuable step in providing salient cessation support in the outpatient settings of maternity hospitals and other antenatal settings (Hoekzema et al., 2014).

Recap - Determinants

In Coleman's randomized trial of NRT in pregnancy, the rate of smoking cessation was higher at 1 month in the NRT group compared to the placebo group. This was short-lived as only 7.2% of women assigned to nicotine-replacement therapy and 2.8% assigned to placebo reported using trial medication for more than 1 month (Coleman et al., 2012), making it difficult to accurately measure NRT efficacy in pregnancy. This leads to the inevitable conclusion that before one designs yet another study whereby the researchers

simply require a higher dose schedule of NRT, determinants related to the lack of adherence to treatment must be identified.

Existing studies have adopted a narrow view of what is in fact a complex behaviour. The emphasis has concentrated primarily on individualistic behaviour, excluding social determinants entirely. If the majority of pregnant women who quit do so without intervention, it is evident that the advice and programming directed at pregnant women should take a different focus. Characteristics such as socioeconomic status, degree of addiction, social support, culture, mental illness and health services should be considered when developing and implementing effective promotional strategies to prevent smoking long before pregnancy all the way through to the postnatal period.

Smokeless Tobacco

The downward trend in the national smoking rate has been attributed to smoke-free spaces legislation and bylaws, education campaigns, taxation and regulations restricting the sale and display of cigarettes (Heart and Stroke Foundation, 2011). Quitting smoking is one of the best things expectant mothers can do for themselves and their unborn children. While NRT and counselling are generally recommended to aid in smoking cessation, could the newest tobacco substitute be setting us back?

E-cigarettes are stainless steel or plastic devices that come in a disposable and a rechargeable model, the latter more closely mimicking cigarette smoking (Berlin, 2014). They contain a battery-operated heating element, a cartridge and an atomizer that converts the contents into an inhalable vapour. The cartridge contains water, flavouring, propylene glycol and sometimes nicotine.

Health Canada issued an advisory warning to Canadians not to purchase or use electronic smoking products in March of 2009 due to safety, quality and efficacy concerns (Government of Canada, 2009). Though products containing nicotine fall within the scope of the Food and Drugs Act and require authorization by Health Canada prior to being imported, advertised or sold (Health Canada, 2009), convenience stores, gas stations and other retail stores have been openly selling them (Czoli, Hammond & White, 2009). However, online retailers remain the largest source of e-cigarette sales in the country.

E-cigarette popularity appears to be overtaking NRT, with smokers choosing e-cigarettes as their first smoking cessation option rather than government approved pharmacological aids (Britton & Bogdanovica, 2014). While the majority of e-cigarette users are said to be adults trying to reduce or stop smoking (Farsalinos et al., 2014), usage among youth has increased dramatically in recent years (Bunnell et al., 2014). Because of the lack of regulation, the dose of nicotine delivered differs considerably. An FDA analysis found that not only did nicotine doses vary in between puffs (26.8-43.2 micrograms) but it was also detected in products labelled as nicotine-free (Westenberger, 2009).

Smoke-free bylaws don't apply to e-cigarettes and laws regulating their use in public places are not under Health Canada's jurisdiction but rather are the responsibility of provincial, territorial or municipal governments. Ontario introduced legislature to regulate the sale of e-cigarettes in November 2014. Bill 45, which would take effect in 2016, would ban the sale of e-cigarettes to youth under the age of 19 and prohibit their use in restaurants and public buildings (*Bill 45*, 2014).

Stress associated with smoking cessation

When all is said and done, is nicotine the culprit or is the stress associated with quitting smoking causing the most harm to the fetus?

In non-pregnant women, nicotine has long been associated with marked neuroendocrine alterations. Not only is cigarette smoking responsible for acute increases in cortisol levels (Kirschbaum, Scherer, & Strasburger, 1994; Kirschbaum, Wust, & Strasburger, 1992), it also leads to higher basal cortisol concentrations throughout the day (Badrick, Kirschbaum, & Kumari, 2007; Direk, Newson, Hofman, Kirschbaum, & Tiemeier, 2011; Field, Colditz, Willett, Longcope, & McKinlay, 1994; Steptoe & Ussher, 2006).

During pregnancy, the placenta and fetus contribute to circulating cortisol, particularly in late gestation (Challis & Patrick, 1983; Challis, Sprague & Patrick, 1983). Unfortunately, few studies have assessed glucocorticoid levels in pregnant women who smoke, with some excluding this group entirely from sampling or analysis (e.g., Kaasen et al., 2012; La Marca-Ghaemmaghami et al., 2013; Voegtline et al., 2013), or controlling for smoking without further inquiry (e.g., Bolten et al., 2011).

While fetal cortisol is required for the maturation of organ systems required for postnatal extra-uterine survival (Challis et al., 2001), there is evidence implying that excessive maternal cortisol levels (associated with a response to stress) in pregnancy could predict deleterious outcomes in offspring. For example, elevated levels of cortisol and corticotrophic-releasing hormone during gestation increase the risk for preterm delivery (Sandman et al., 2006). Also, Bolten et al., (2011), recently found that newborns of mothers with higher cortisol levels had lower birth weights for gestational age and were shorter at birth. Evidence accumulated over the last 20 years is generally supportive of the hypothesis that elevated levels of glucocorticoids *in utero* predisposes offspring to low birth weight (Reynolds, 2013); birth weight is an important indicator of a newborn's chance for survival, growth and long-term health (Bale, Stoll & Lucas, 2003). This brings up a whole other set of issues such as increased risk for diabetes, coronary heart disease and impaired cognitive development in adulthood (Bhargava et al., 2004; Barker, 2003).

There is also preliminary evidence suggesting that maternal smoking alters fetal programming of the stress response, although much of the research is conflicting. Newborns exposed *in utero* to tobacco smoke have increased cord blood concentrations of cortisol (Varvarigou et al., 2006, 2009). McDonald (2006) on the other hand found that umbilical arterial cortisol levels did not differ while adrenocorticotrophic hormone levels (which stimulates the production of cortisol) were significantly elevated in smoke exposed infants. Recently, researchers from the Miriam Hospital in Rhode Island discovered that prenatal nicotine exposure attenuated basal and reactive cortisol levels over the first postnatal month and altered DNA for a gene that regulates passage of stress hormones from mother to fetus (Stroud et al., 2014). However, in rodents, prenatal nicotine increased fetal blood corticosterone and glucocorticoid receptor expression (Xu et al., 2012). Alternatively, a recent study found that acute tobacco abstinence significantly lowers salivary cortisol levels (Wong et al., 2014), supporting previous findings (Al'Absi, Hatsukami, Davis & Wittmers, 2004; Frederick et al., 1997). Similarly, Steptoe and Ussher (2006) monitored 112 smokers treated with behavioral support and 15 mg nicotine patches and observed a sharp decline in cortisol levels which was sustained over the 6-week abstinence period.

What's more is that an animal study found that brain region weights and fetal body weights were affected significantly more in the animals in whom nicotine administration

continued into the postnatal period compared to those who underwent withdrawal at 21 days gestation. Even better, animals undergoing withdrawal on gestational day 13 showed no weight abnormalities whatsoever (Slotkin, Lappi & Seidler, 1993). These findings suggest that nicotine withdrawal has very little impact on fetal growth and brain development but rather timing of exposure appears to be the most important factor to consider.

To our knowledge, researchers have not studied the benefits of NRT in comparison with abrupt withdrawal and its ensuing withdrawal/ stress effects on offspring. Although there is a lack of evidence that would allow researchers to make definite conclusions, the general consensus is that abrupt withdrawal is safer than exposure to nicotine and its teratogenic effects.

CHAPTER 7. Conclusion

The adverse effects of maternal smoking on neurological outcomes in offspring are a major scientific and public health concern. In industrialized countries, 13 to 27% of women will smoke while pregnant (Colman & Joyce, 2003; Connor & McIntyre, 1999; Penn & Owen, 2002; Schneider, Huy, Schutz & Diehl, 2010; Schneider & Schutz, 2008). Approximately 75% of them will report a desire to quit (Ruggiero, Tsoh, Everett, Fava & Guise, 2000) but only 20-30% will successfully do so and half will relapse within 6 months of giving birth (Ebert & Fahy, 2007).

There exists a myriad of options available for women who wish to quit, from behavioral therapy to prescription medicines, but NRT is by far the most common approach. While NRT is highly effective for smoking cessation in non-pregnant smokers (Stead et al., 2012), the safety and efficacy of these products in pregnant women have not been rigorously validated (Coleman et al., 2011).

Health agencies such as the OMA in Canada and the Committee on Safety of Medicines (CSM) and Medicines and Healthcare Regulatory Authority (MHRA) in the United Kingdom agree that the risks to the fetus of continued smoking outweigh any potential adverse effects of NRT (Action on Smoking and Health, 2005; OMA, 2008). Thus, they continue to advocate the use of NRT in pregnant and breastfeeding mothers despite acknowledging that there is no safe dose of nicotine during pregnancy (OMA, 2008).

Unfortunately, perinatal nicotine exposure, even in small doses, may interfere with brain maturation at the cellular level. This may, in turn, result in functional deficits that appear over time as subtle neurodevelopmental and psychobehavioral deviations in offspring.

Using a rat model, we assessed the long-term effects of perinatal nicotine exposure at doses mimicking NRT, on the hippocampal formation. Early life nicotine interfered with proper brain development, in the form of enhanced ER α in the CA1 of the hippocampus in 26-week old rats.

Taken together, the results of this study along with previous animal studies, strongly point to nicotine as a teratogenic chemical involved in mediating long-term neurological effects in offspring, regardless of dosage. Overall, NRT is of questionable safety and potentially comes at a cost to the health of the offspring.

Nicotine-induced morbidity from impaired cognitive and neurobehavioral function is an important public health concern. The scientific community, along with health agencies worldwide can agree that smoking during pregnancy imposes a substantial economic burden on the healthcare system. Unfortunately, this issue will not be resolved simply by advocating NRT use.

Public health measures to reduce smoking rates have oversimplified the complex psychosocial context of tobacco use in pregnancy and overlooked important predictors of non-adherence to quit-smoking programs. There are a number of reasons for women's smoking patterns that include psychosocial, cultural, economic, and biological influences that go beyond nicotine addiction, which are further accentuated in pregnancy and postpartum. Rather, it is wrongly assumed that women have the social resources to independently implement and sustain the necessary behavioural strategies that lead to quitting (Pickett, Wilkinson & Wakschlag, 2009).

More importantly, characteristics such as socioeconomic status, degree of addiction, social support, culture, mental illness and health services should be considered when developing and implementing effective strategies to prevent smoking long before pregnancy. Smoking cessation is most effective if implemented prior to pregnancy or the initiation of prenatal care (Tong, England, Dietz & Asare, 2008). Thus, rather than treating tobacco use

in pregnant women, eliminating smoking among women of childbearing age should be the number one public health priority.

Appendix A

Over the course of my MSc., I conducted a lot of additional work and learned a wide range of important laboratory techniques. The objective of my research project was originally to quantify steroidogenic enzyme levels in the rodent limbic system following perinatal nicotine exposure. Nicotine has been reported to inhibit many enzymes involved in estradiol synthesis, from human placental aromatase (Barbieri et al., 1986), to forebrain aromatase (von Ziegler, Schlumpf & Lichtensteiger, 1991), 17 α -hydroxylase and 17,20-lyase of adult rat Leydig cells (Yeh et al., 1989) (Figure 1). We also have preliminary data showing that early life nicotine exposure reduces aromatase activity in select areas of the neonatal brain (Figure 2). However, difficulties in working out some unexpected difficulties altered the course of my work and led to expanding the portion on the immunohistochemical analyses instead; note that immunohistochemical analyses of these enzymes in the rat has not yet been shown reliably.

RT-qPCR was to be conducted on adult rat brain tissue, from cortex to hypothalamus and hippocampus. Markers of interest for assessing estrogenic effects included the steroidogenic acute regulatory protein and p450 aromatase genes while housekeeping genes included 18s rRNA and Glyceraldehyde 3-phosphate dehydrogenase.

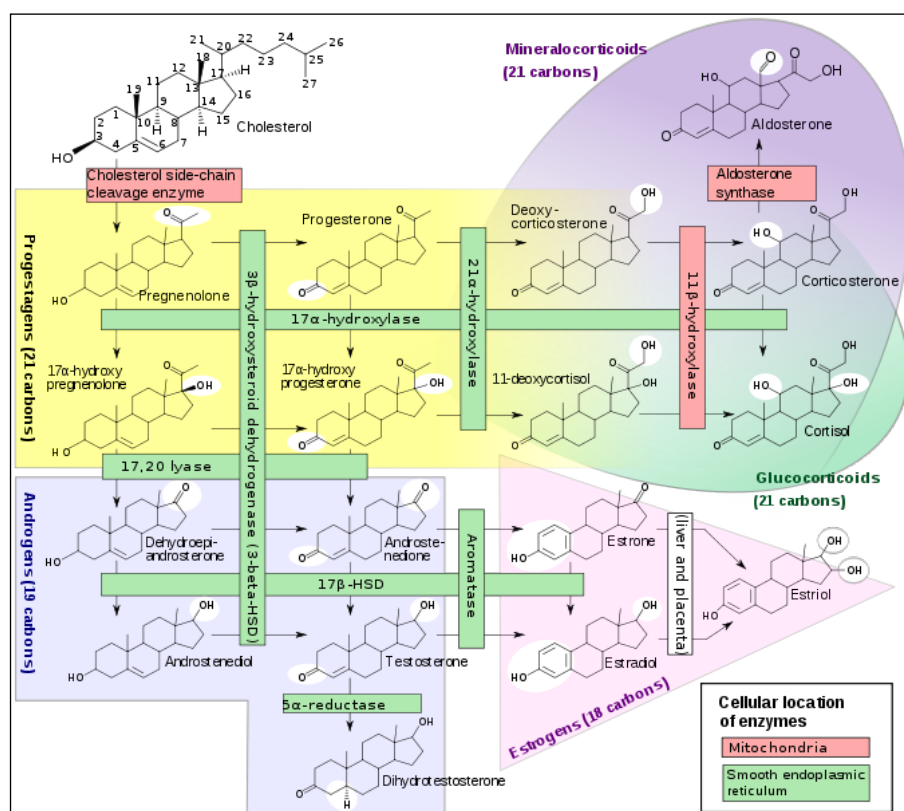


Figure 1. Steroidogenesis pathways from the cholesterol precursor

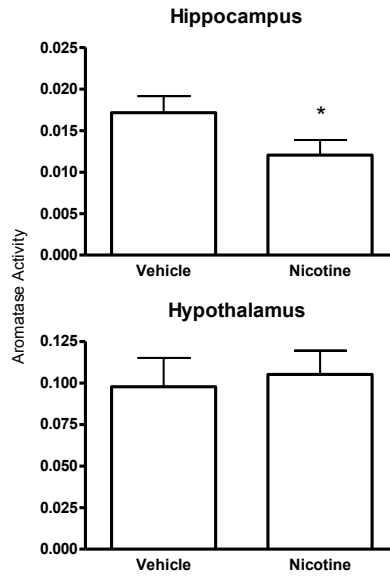


Figure 2. Aromatase activity in the hippocampus and hypothalamus of males at PND3, following two days of subcutaneous treatment with nicotine bitartrate (2mg/kg).

Primer Design

Rat genomic sequences for StAR, aromatase, 18s rRNA and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the rat were found using NCBI Nucleotide. Appropriate primers were then designed using Primer-BLAST (Table 1). Additional primer sequences were also found in the existing literature and verified using Primer-BLAST.

Oligonucleotide Data Sheet

Primer	Sequence	Length	Tm	GC
18s rRNA	F-CTTAGTTGGTGGAGCGATTGTCTG	25	64.6	48.0%
	R-GTTATTGCTCAATCTCGGGTGGC	23	64.6	52.2%
GAPDH	F-CTGAGGAGTCCCCATCCCAA	20	64.5	60.0%
	R-GGTATTTCGAGAGAAGGGAGGGC	20	66.4	59.1%
StAR1	F-CATCCAGCAAGGAGAGGAAG	20	62.4	55.0%
	R-CGTGAGTTTGGTCTTTGAGG	20	60.4	50.0%
StAR2	F-CGAGGCCTGTACATGCTGAC	20	64.5	60.0%
	R-CTTTGTGCACACACTGCCTT	20	60.4	50.0%
StAR3	F-CCAGATAGAGTTCGCCAGCC	20	64.5	60.0%
	R-ACAGCTAGTGGGAGACACTG	20	62.4	55.0%
StAR4	F-CCCAGATAGAGTTCGCCAGC	20	64.5	60.0%
	R-GCACGCCATTGGAAGTTCTC	20	64.5	60.0%
aromatase1	F-CTGTCGGGGTAACACCTT	20	62.4	55.0%
	R-CTGTCGGGGTAACACCTT	20	59.9	55.6%
aromatase2	F-ATGGGGATTGGAAGTGCCTG	20	62.4	55.0%
	R-TCATGAAGAAAGGGCGGACC	20	62.4	55.0%
aromatase3	F-GGAGCCACAGCCAACTACT	20	62.4	55.0%
	R-CACAGTTTCCGGGACCATGA	20	62.4	55.0%
aromatase4	F-TGACGTCCTGACAACTCGG	20	62.4	55.0%
	R-TGTCAGGTCTCCACGTCTCT	20	62.4	55.0%

Table 1. Oligonucleotide data sheet

Extraction

Following dissection, RNA extraction was done as per the RNeasy Mini Kit's manufacturer's specifications (Qiagen). Extractions were completed for all 15 week and 26 week male offspring (n= 7 animals at 15 weeks and n=8 at 26 weeks per treatment group) hypothalamus and hippocampus as well as some cortex practice tissue.

RNA purity was determined with the help of the Thermo Scientific NanoDrop 2000 spectrophotometer. Afterwards, reverse transcriptase occurred in a two-step process: genomic DNA wipeout and reverse transcription, using the QuantiTect Reverse Transcription kit (Qiagen). Newly synthesized cDNA aliquots were kept in a -80 freezer until the samples are used for qPCR.

Polymerase Chain Reaction

Gene expression of the key steroidogenic enzymes were assessed following instructions of the BioRad SsoFast Eva Green Supermix PCR kit (Biorad).

PCR performance was consistent for 18s rRNA and GAPDH, as evidenced by the values of efficiency, precision (between replicates), R^2 and sensitivity. Unfortunately, despite experimenting with varying cDNA concentrations and primer concentrations,

amplification was low for StaR and aromatase. We also experimented with different brain tissue including hippocampus, hypothalamus and cortex that were either part of the control group or nicotine-treated, but amplification was still not optimizable. Below you will find a table showing some of the challenges that were encountered and the troubleshooting techniques that I learned and undertook over the course of the year (Table 2).

Observation	Possible Cause	Troubleshooting
Multiple or non-specific products	Contamination with impurities	Rearrange serial dilution on plate with <i>No Template Control</i> further away
		Setup dedicated work area and pipettes for experiment
		Aliquot solutions in small portions and store in designated area
		Use fresh solutions
		Use filtered pipette tips
		Exercise caution when placing sealing tape over plate
		Wear fresh gloves
		Include <i>No Reverse Transcription</i> to control for genomic DNA contamination
		Include <i>No Template Control</i> to control for non-specific DNA contamination
Variability in amplification of serial dilution triplicates	Poor pipetting	Test different pipetting techniques to mix solutions
Low product amplification	Poor primer design	Design additional primers
		Consult literature for recommended primer design
	Insufficient primer concentration	Test primer concentration range
	Incorrect annealing temperature	Test annealing temperature gradient
	Insufficient number of cycles	Increase number of cycles
	Poor expression in tissue	Use various brain tissues including cortex, hippocampus, hypothalamus
	Ineffective Taq Polymerase	Purchase new SsoFast supermix

Table 2. PCR Troubleshooting

The following serve as examples of some of the multiple qPCR runs that I conducted over the years (Figures 3-6); however, the amount of work conducted is not accurately depicted by these simple examples. These also serve as a starting point for future analyses of the effects of nicotine on this steroid system.

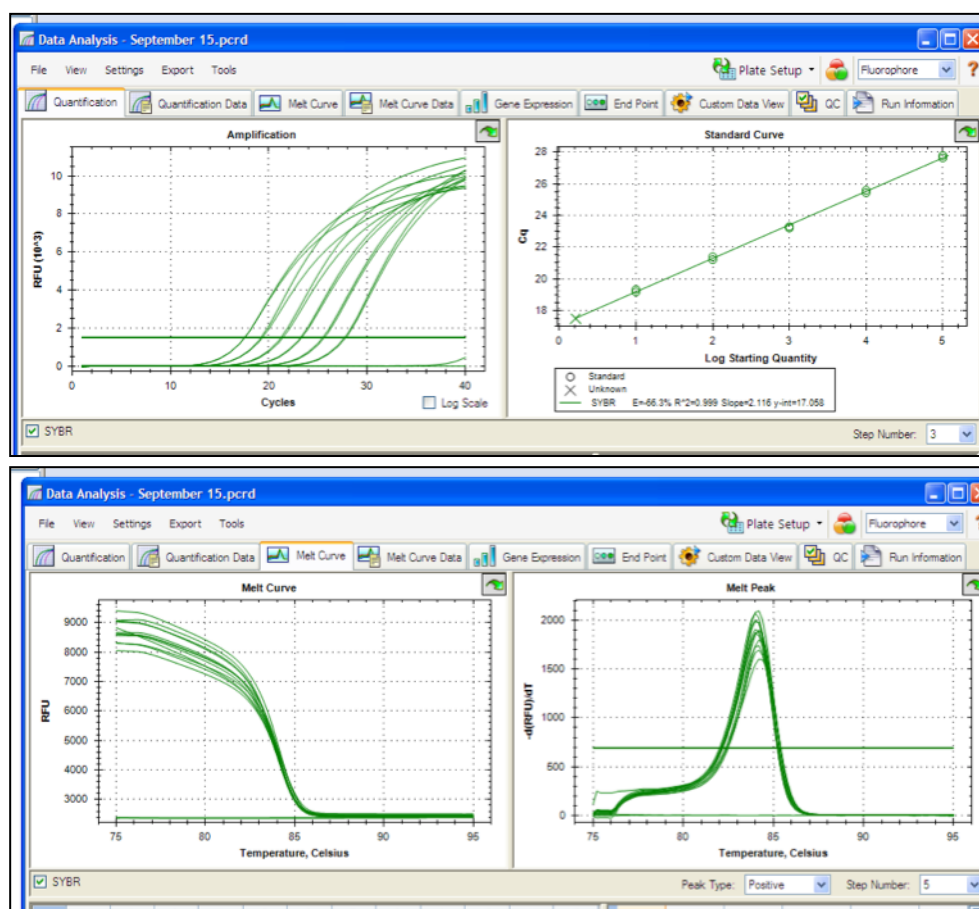


Figure 3. PCR with 18s rRNA. A stable housekeeping gene that remained unchanged regardless of nicotine treatment.

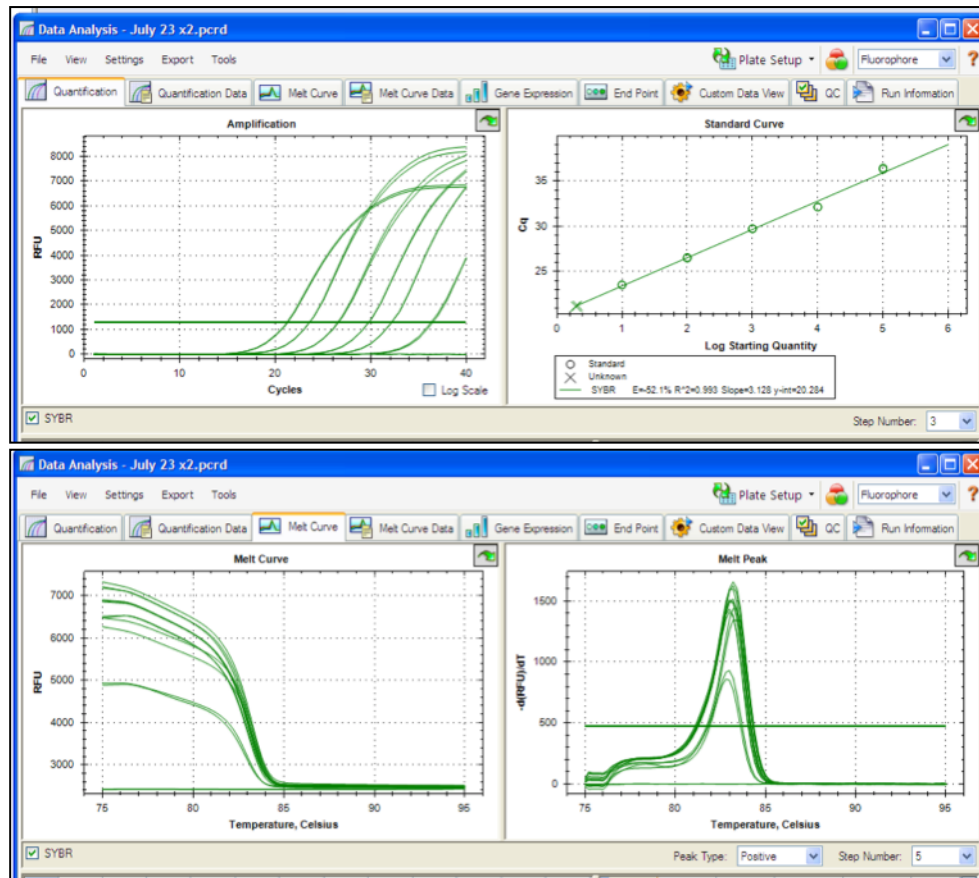


Figure 4. PCR with GAPDH. A second housekeeping gene used to normalize q-PCR data.

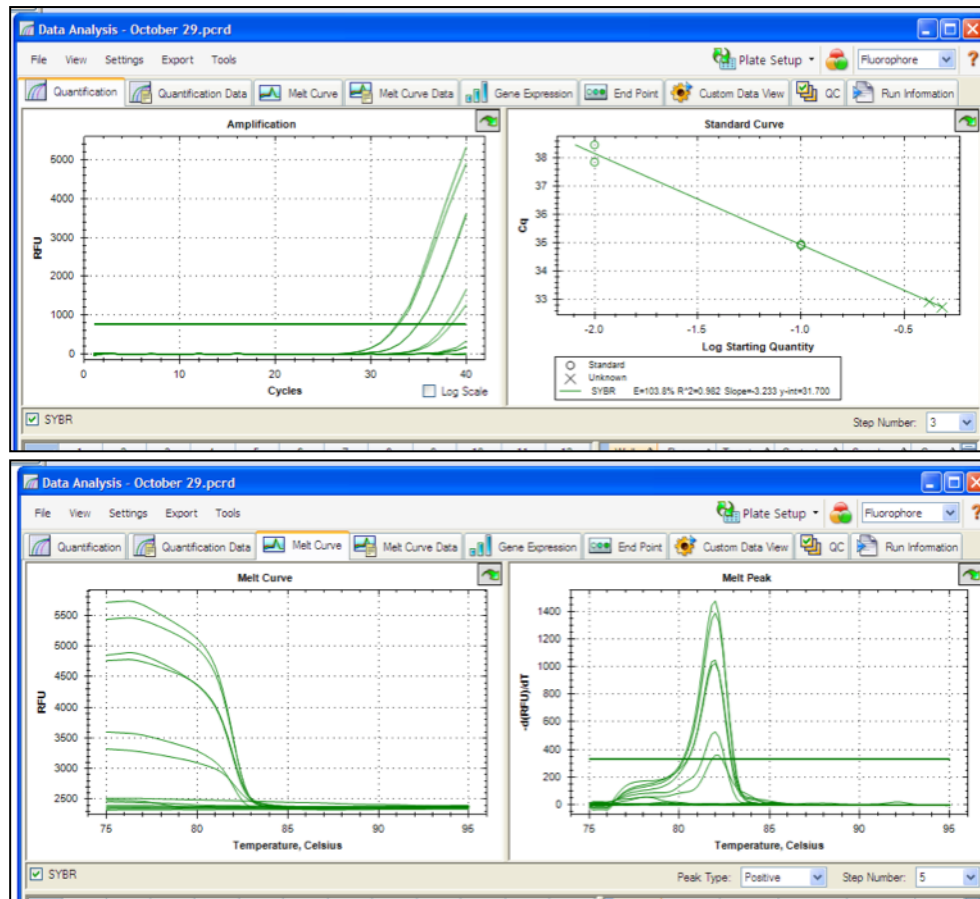


Figure 5. PCR with Steroidogenic acute regulatory protein. As part of the first step of the estradiol synthesis pathway, steroidogenic acute regulatory protein transfers cholesterol from the outer to the inner mitochondrial membrane.

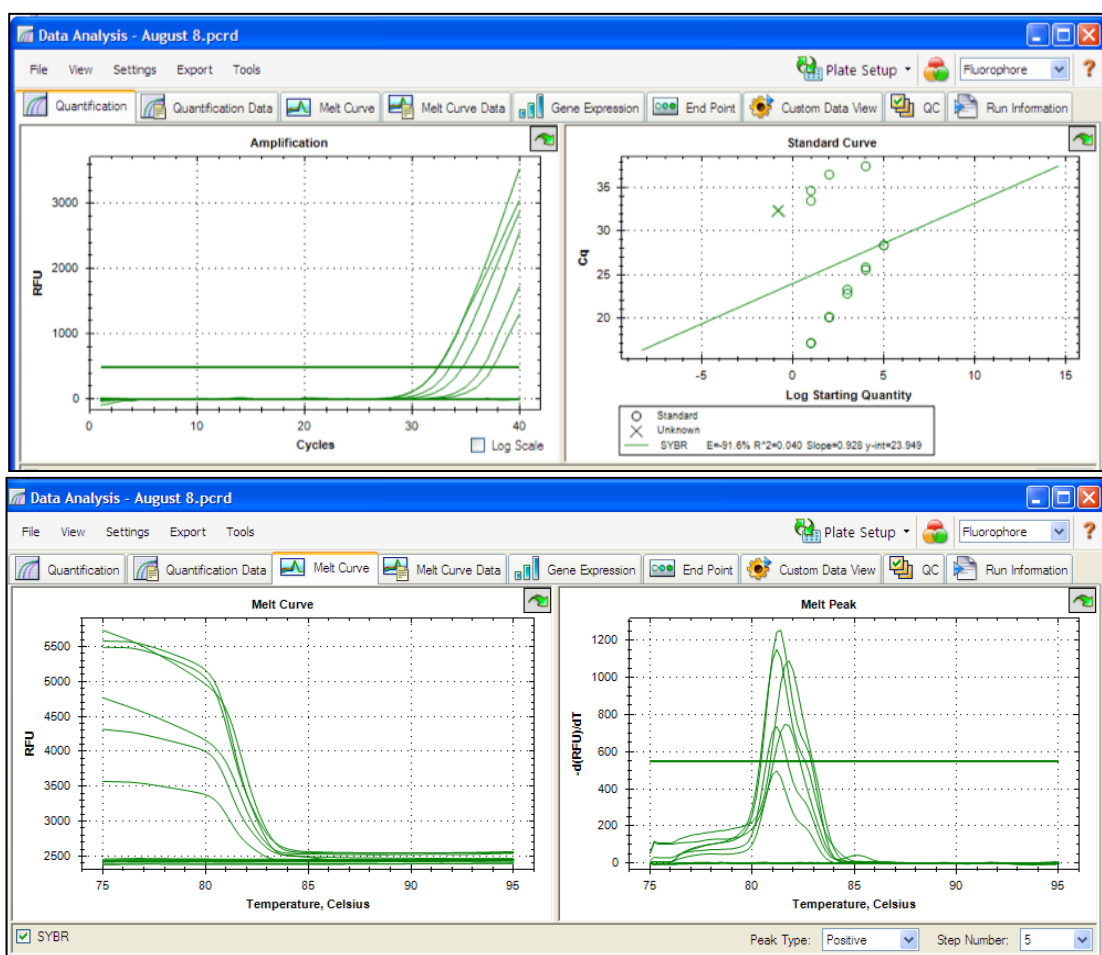


Figure 6. PCR with aromatase p450. The final step involved in estradiol synthesis uses cytochrome P450 aromatase to convert androgens to estrogens.

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