Characterizing the Role of Adolescent Nicotine Exposure on the Mesocorticolimbic System and the Development of Mood and Anxiety Disorders

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Abstract

Adolescence is a period in which many are first introduced to cigarettes. With adolescence being a plastic neurodevelopmental period, exposure to addictive substances, like nicotine, may lead to abnormal neural development and consequently, behavioural and cognitive deficits. Importantly, nicotine exposure is linked to various psychiatric conditions including anxiety and depression. To assess the long-term neuropsychiatric-like effects of adolescent nicotine exposure, adolescent (PND 35-44) and early adult (PND 65-74) rats were exposed to nicotine. Once adults (PND 75), or later in adulthood (PND 105), rats were analyzed with a battery of behavioural, cognitive, molecular and cellular assays. Following adolescent, but not adult nicotine exposure, rats developed depressive and anxiety disorder phenotypes including temporal memory and social motivation deficits, relative to controls. Adolescent behavioural results were associated with a hyperdopaminergic, sub-cortical state at the level of the mesolimbic pathway and an upregulation in pERK1/2 and downregulation in D1DR expression levels in the PFC.

Keywords

Adolescent Neurodevelopment, Nicotine, Addiction, Affective disorders, Depression, Major Depressive Disorder, Anxiety Disorders, Ventral Tegmental Area, Prefrontal Cortex, Electrophysiology, D1DR, ERK1/2, Western Blots, Light-Dark Box, Open Field, Forced Swim Test, Sucrose Preference, Object Recognition, Object Recognition in Temporal Order, Social Interaction, Dopamine.
Co-Authorship Statement

Justine Renard, PhD. – Aided in behavioural paradigms by both teaching techniques as well as helping run various paradigms.

Laura G. Rosen, PhD.c – Contributed during extractions of tissue sample for molecular analyses.

Brian Pereira – Helped in slicing and performing histological analyses for placement of electrodes following In Vivo electrophysiology.

Walter J. Rushlow, PhD. – Aided in extractions of tissue samples, as well as full procedure of molecular analyses.

Steven R. Laviolette, PhD. – Supervisor, created the project and contributed in the extractions of tissue for molecular analyses.
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# Table of Contents

Abstract ................................................................................................................................. i  
Co-Authorship Statement ........................................................................................................ ii  
Acknowledgments ................................................................................................................... iii  
Table of Contents .................................................................................................................... iv  
List of Figures ........................................................................................................................ vi  

1 Introduction ......................................................................................................................... 1  
   1.1 Prevalence of Smoking in North America ................................................................. 4  
   1.2 Adolescent Neurodevelopment .................................................................................... 5  
   1.3 Affective Disorders ....................................................................................................... 7  
   1.4 Physiological and Neurological Effects of Smoking .................................................... 10  
   1.5 The Roles of DA D1 vs. D2 Receptors in the PFC ....................................................... 12  
   1.6 The Roles of ERK and CaMKII ................................................................................... 14  
   1.7 Epidemiology of Adolescent Smoking and Mood Disorders ...................................... 15  
   1.8 Preclinical Studies of Adolescent Nicotine Exposure ............................................... 16  
   1.9 Research Objectives and Hypothesis ......................................................................... 18  

2 Methods ............................................................................................................................... 20  
   2.1 Animal Models ............................................................................................................. 20  
   2.2 Behavioural and Cognitive Testing .............................................................................. 21  
      2.21 Behavioural Tests ................................................................................................... 22  
      2.22 Cognitive Tests ...................................................................................................... 26  
   2.3 Electrophysiology ......................................................................................................... 28  
   2.4 Molecular Analysis ....................................................................................................... 30  
      2.41 Extractions ............................................................................................................... 30
List of Figures

Figure 1: The Acute and Long Term Reward Pathways ............................................. 12
Figure 2: Timeline of Pretreatment ............................................................................. 21
Figure 3: Diagram of Behavioural Apparatuses ......................................................... 26
Figure 4: Schematic of Cognitive Testing in Open Field Arena ................................. 28
Figure 5: Adolescent Open Field and Locomotor Testing ........................................ 35
Figure 6: Adolescent Light-Dark Box Testing ............................................................ 38
Figure 7: Adolescent Social Interaction Testing ......................................................... 40
Figure 8: Adolescent Sucrose Preference Testing ....................................................... 42
Figure 9: Adolescent Forced Swim Testing ................................................................ 44
Figure 10: Adolescent Object Recognition Testing .................................................... 46
Figure 11: Adolescent Object Recognition in Temporal Order Testing .................... 48
Figure 12: Adolescent Electrophysiological Recordings from the VTA .................... 50
Figure 13: Adolescent Molecular Analysis of the PFC ............................................. 54
Figure 14: Adult Open Field and Locomotor Testing ................................................ 57
Figure 15: Adult Light-Dark Box Testing .................................................................. 59
Figure 16: Adult Social Interaction Testing ............................................................... 61
Figure 17: Adult Sucrose Preference Testing ............................................................. 63
Figure 18: Adult Forced Swim Testing ...................................................................... 64
Figure 19: Adult Object Recognition in Temporal Order Testing ............................. 67
1 Introduction

Smoking is one of the leading causes of preventable death in North America, (WHO, 2017) and is known to have a high co-morbidity with various neuropsychiatric disorders, including schizophrenia and mood disorders, such as major depression and anxiety (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Boden, Fergusson & Horwood, 2010). Although smoking rates in North American have decreased substantially with the emergence of research exposing the carcinogenic effects of smoking, smoking has again regained popularity in more recent years with the increased use of electronic cigarettes, which have been purported to be safer than traditional rolled cigarettes (Callahan-Lyon, 2014). The driving force behind the addictive nature of smoking both traditional and electronic cigarettes, is due to the effects of its main psychoactive component, nicotine. Like many other drugs of abuse, nicotine exerts its effects primarily on the mesocorticolimbic dopaminergic (DAergic) system. Nicotine’s effects on the DAergic system have been previously described through lesion studies by Corrigal and colleagues in 1992, which showed that lesions of the DA system were responsible for the reinforcing effects of nicotine, in rats that were chronically exposed. In this study, Corrigal and colleagues (1992), lesioned the dopaminergic efferents into the accumbens. Following these lesions, animals reduced the amount of self-administration of nicotine. In contrast, blockade of mesocorticolimbic DA signaling has been shown to dramatically potentiate the rewarding effects of nicotine in the drug-naïve state (Laviolette and van der Kooy, 2003). However, following chronic nicotine
exposure and dependence, DA signaling is required for the addictive properties of nicotine in the mesocorticolimbic system (Tan et al., 2009).

Adolescence is a period in which many people are first introduced to cigarettes. This is also a critical period of neurodevelopment, particularly in frontal cortical brain regions. During adolescence, the frontal cortex is still developing by way of ongoing myelination as well as synaptic pruning events (Knuesel et al., 2014). Based on the plastic nature of the brain during this period of development there is the potential for abnormal neurodevelopment and changes to mesocorticolimbic networks as a result of exposure to drugs of abuse, like nicotine. Previous research has described a host of cognitive, emotional and behavioural deficits that progress into adulthood following chronic exposure to nicotine during adolescence (Holliday & Gould, 2016; Counotte et al, 2009). Counotte and colleagues, for example, described cognitive deficits in the 5 choice serial reaction time task (5-CSRTT; 2009). Previous work has also described conflicting long-term behavioural effects of adolescent nicotine. With studies describing varying levels of anxiety and cognitive deficits, with some citing no deficits. Most evidence supports an increase in anxiety-like behaviours and increased reward sensitivity (Holliday & Gould, 2016). Nevertheless, little work has been done to understand the molecular and cellular changes that might underlie these deficits following adolescent nicotine exposure. Several previous studies have noted molecular alterations in various neural regions following neurodevelopmental nicotine exposure. For example, Trauth and colleagues (1999) noted an upregulation in nAChRs in the cortex and hippocampus of rats that were chronically exposed to nicotine 4 weeks after treatment. The same group also described a decrease in cholinergic activity following adolescent nicotine exposure.
(Trauth et al., 2000). Counotte and colleagues (2009), along with working memory deficits in a 5-choice serial recognition task, also described an increase in DA levels in the prefrontal cortex (PFC) when analyzing the tissue of adult rats that were chronically exposed to nicotine during adolescence. This suggests that neurodevelopmental nicotine exposure may be associated with persistent plastic alterations in mesocorticolimbic DA activity states.

In an interesting study by Boden and colleagues (2010), a causal link was reported between nicotine dependence and the development of depressive symptoms. In these experiments, a longitudinal approach was taken to assess the level of dependence on nicotine and the resulting depressive symptoms over time. The groups’ results best fit a causal model in which nicotine dependence directly resulted in increased risk of depression, as opposed to a model in which both stemmed from a common external factor (Boden et al., 2010). Furthermore, a review article by Jane-Llopis and Matysina (2006), describes the many epidemiological studies that have linked smoking to depression and anxiety disorders in various westernized regions around the world. In this review, nicotine was found to be related to psychiatric disease in a dose-response relationship (Jane-Llopis & Matysina, 2006).

Nevertheless, despite a large body of clinical literature identifying correlative links between smoking behavior and the development of mood disorders like anxiety and depression, there is virtually nothing known about the underlying, causal brain mechanisms that may be responsible for these co-morbidities. The primary aim of my research project was to use a multi-pronged pre-clinical neuroscience research approach aimed at identifying and characterizing how adolescent neurodevelopmental nicotine
exposure might lead to persistent alterations in neuronal and molecular states in the mesocorticolimbic DA system. Furthermore, my research project aimed to characterize how these neurodevelopmental alterations might lead to persistent symptoms of depressive and anxiety-like disorders in later adulthood, as they have been demonstrated in the above discussed epidemiological studies.

1.1 Prevalence of Smoking in North America

Smoking has been a practice in many religious ceremonies for thousands of years in ancient Egypt and Greece and in North America; tobacco has been growing for almost 8000 years in the wild (Cancer Council NSW, 2011). Smoking has, over the years, moved from a cultural/religious practice to a common practice with the development of cigarettes and increased promotional content. With increasing evidence of tobacco’s addictive and carcinogenic effects, the world health organization (2017), along with governing bodies, started to initiate mechanisms of tobacco control through public awareness campaigns. Over time, increasing public awareness of the dangers of tobacco exposure has led to a significant reduction in smoking behaviours, particularly in North America (WHO, 2017). Nevertheless, smoking remains the leading cause of preventable death worldwide.

In Canada, cigarettes as well as electronic cigarettes containing nicotine, are not approved for sale, importation or advertising to minors, under the Food and Drugs Act (Hamilton et. al, 2015). Canadian trends in both cigarette and e-cigarette use follow the same trends that are noted in the US. In both countries, there is a steady increase in high
school students that have tried some form of cigarettes in their life (Hamilton et. al, 2015; Schepis & Rao, 2005). In an Ontario study of drug use in 2015, almost 15% of students had used an e-cigarette and 24% of students had reported smoking tobacco cigarettes in their lifetime, with males from Caucasian backgrounds having greater odds of using e-cigarettes as opposed to females and non-white racial backgrounds (Hamilton et. al, 2015). All across Canada we are seeing trends of increased awareness and use of cigarettes among adolescents. In a Canada wide study from 2014, Czoli and colleagues found that close to half of 1188 youth and young adults had been exposed to e-cigarettes and just over 16% had reported trying them (Czoli et. al, 2014). E-cigarettes are a relatively new phenomenon, and still not as popular as traditional cigarettes, but both still contain nicotine, which is the compound of interest for this thesis, and one that has been previously associated with various neuropsychiatric disorders (Boden et al., 2010; Jane-Llopis & Matysina, 2006).

1.2 Adolescent Neurodevelopment

Adolescence is a period characterized by many behavioural and physical changes as a result of puberty. Puberty is usually characterized by the physical changes that take place as adolescents reach sexual maturity. Even though the trigger that represents the onset of puberty is not well understood, that trigger results in hormonal changes through the hypothalamic-pituitary axis that then cascades into physical changes and growth (HPA axis; Sisk & Foster, 2004). On average, in developed countries, puberty begins at the age of 13 for females and 15 for males, with differences found among varying countries and ethnicities within those countries (Parent et al., 2003). Adolescent brain development is
accompanied by a wide variety of behavioural and emotional changes and considerable research has examined changes in emotional processes as well as risk taking behaviours associated with these neurodevelopmental changes (Smith, Chein & Steinberg, 2014; Steinberg, 2008; Romer, 2009). The increase in risk-taking behaviours can ultimately lead to increases in inappropriate behaviours such as, smoking, drinking alcohol and risky sexual behaviour, to name a few.

Many of the critical neurodevelopmental changes during adolescence occur in higher cortical areas like the PFC, through the processes of myelination and synaptic pruning (Spear, 2013). During the adolescent period there is not only the expansion and regression of cortical areas, but reorganization of other areas, like reward processing mechanisms in the striatum and associated areas, such as the VTA and NAcc (Spear, 2013). In the mesocorticolimbic dopaminergic pathway there can be a 2 to 7-fold increase in dopaminergic activity (Spear, 2013). This increase in DA activity and decrease in associated DA receptors, could be the reason for changes in reward processing when a reward is anticipated, as well as explain the peak and following decrease in reward seeking behaviours in adolescence into adulthood (Spear, 2013). There is also evidence for a later development of neural responses to negative feedback as compared to positive feedback, leading to an insensitivity to negative stimuli during adolescence (Spear, 2013). This late opportunity for plasticity also gives way to sex-hormone dependent changes (Sisk & Foster, 2004). These changes alter social behaviour with respect to mate seeking and copulatory behaviours (Sisk & Foster, 2004). Thus, both hormone driven behaviours as well as neurodevelopmental changes could ultimately
result in a propensity to engage in smoking behaviours linked to a desire for social acceptance concomitant with the reinforcing effects of nicotine.

1.3 Affective Disorders

Affective disorders are among the most prominent types of psychiatric disorders in westernized society (Schulz & Arora, 2015; McCarron, Vanderlip and Rado, 2016). Affective disorders include, but are not limited to, bipolar disorders I and II, depressive disorders and anxiety disorders. For the purposes of this paper, depressive and anxiety disorders will be the focus based on their high rates of co-morbidity as well as their strong links to substance abuse problems (Schulz & Arora, 2015; McCarron et al., 2016; Stein & Sareen, 2015).

Depression is associated with huge economic costs and has been increasing in frequency with prevalence rates as high as 40% in certain populations (McCarron et. al, 2016). Many people will experience at least one depressive episode in their life, with certain populations, namely female, single individuals and those from low socioeconomic status (SES), being more susceptible to depression (Schulz & Arora, 2015). Depression is one of the most common psychiatric disorders both as a comorbid diagnosis or on its own (Schulz & Arora, 2015). It is a common presenting symptom in psychiatric disorders such as major depressive disorder (MDD), bipolar disorders, schizophrenia as well as in some neurodegenerative diseases (Schulz & Arora, 2015). Depression co-occurs frequently with anxiety disorders, substance abuse problems, impulse control disorders, anorexia nervosa and bulimia nervosa (Schulz & Arora, 2015). While specific causes of
depression are unknown, there have been associations with genetic factors as well as neuroanatomical variations such as decreased brain volume in the PFC, orbitofrontal cortex, cingulate cortex, hippocampus and striatum (Schulz & Arora, 2015). The diagnosis of depression requires patients to express 5 or more of the DSM-5 symptoms over the period of two weeks, with one symptom including a depressed mood or anhedonia (McCarron et. al, 2016). Other symptoms include disruptions in sleep, appetite, fatigue, large changes in weight, feelings of worthlessness, suicidal thoughts. Depression causes clinically significant distress or impairments in social, occupational or other areas of functioning (McCarron et. al, 2016). Depression can vary in severity with different treatment plans for the varying levels. Mild cases of depression can sometimes be treated with exercise and might not require medications, mild to moderate depression responds well to medication or psychotherapy and severe major depressive disorder patients benefit from medication or a combination of medication and psychotherapy (McCarron et. al, 2016). It is important to note that medications do not always work for every patient and there’s a large variety in the mechanism of actions for the different generations of currently prescribed antidepressants (McCarron et. al, 2016).

Anxiety disorders can be classified as a generalized anxiety disorder (GAD) or may be associated with a more specific cause of anxiety. More specific anxiety disorders include a variety of well-known psychiatric disorders such as obsessive compulsive disorder, social anxiety, panic disorders and post-traumatic stress disorder, to name the most common (Stein & Sareen, 2015).

The data describing the global prevalence of anxiety disorders is variable based on its co-morbidities and the overlap symptoms have with other psychiatric disorders such as
depression (Baxter et. al, 2013). A regresional analysis of data and trends worldwide estimates global prevalence at 7.3% of the population having ever suffered from GAD and 10.4% in European/Anglophone cultures (Baxter et. al, 2013). Risk factors and factors found to be responsible for the variability in the global data include age, gender, culture, conflict, SES and urbanity, with being female, coming from low SES, childhood adversity, abuse and substance abuse being risk factors (Baxter et. al, 2013; Stein & Sareen, 2015). To diagnosis GAD, a patient must have excessive anxiety and worry about various events that have persisted for 6 months with episodes or worry more days than not over that period of time (Stein & Sareen, 2015). The patient must find it difficult to control the worry and meet 3/6 of the symptoms listed in the DSM-5. Symptoms include disturbances in sleep, concentration, feelings of restlessness and muscle tension (Stein & Sareen, 2015). As mentioned, GAD is often a coexisting condition with depression, which has overlap in diagnoses symptomology (Stein & Sareen, 2015). Functional neuroimaging studies have demonstrated an increase in activation in the limbic system, decrease in activation in the PFC and diminished functional connectivity between these two regions (Stein & Sareen, 2015). Treatment for GAD is similar to that of MDD with a mix of pharmacotherapy (usually targeting the serotinergic system with drugs that are commonly used in treating depression) and psychotherapy (such as cognitive behaviour therapy (CBT), also used in depression) being the common choices for physicians (Stein & Sareen, 2015).

While the incidences of both of these disorders described are consistently increasing in the population, more research needs to be done with respect to the causes and
development of the two disorders to prevent further increases as well as better understand the etiology of them.

1.4 Physiological and Neurobiological Effects of Smoking

Beyond the well described effects of smoking on the body, such as the carcinogenic effects on lungs, throat and mouth, there are effects taking place on the brain through nicotine, the psychoactive compound found in cigarettes, that ultimately lead to the addictive quality of smoking. There are well-established acute and long-term effects of nicotine on the brain.

First time smokers will often describe feelings of nausea, coughing and dizziness, that then begin to subside with repeated nicotine exposure, as a tolerance to the negative effects develops (Laviolette & Van Der Kooy, 2004). First time smokers also have larger increases in heart rate, systolic and diastolic blood pressures as well as larger increases in plasma nicotine levels as compared to long-term smokers, with individual differences in sensitivity to nicotine (Pomerleau, 1995). This idea of individual differences with respect to sensitivity to nicotine is thought to play a role in the development of an addiction to nicotine, with those who are less sensitive developing an addiction (Pomerleau, 1995). The individual differences in sensitivity are thought to be related to genetics as well as differences in nicotinic receptor expression levels (Pomerleau, 1995).

Like many other drugs of abuse, nicotine acts on the mesocorticolimbic DA system, including the ventral tegmental area (VTA), a midbrain region containing the A10 DA neurons, which is involved in many functions including; learning, memory and reward
processing (Laviolette & Van Der Kooy, 2004). The VTA is predominately made up of DA neurons with a subpopulation of GABAergic interneurons (Swanson, 1982). The acute effects of nicotine are predominantly through nicotinic acetylcholine receptors (nAChRs) on the GABAergic neurons in the VTA (Laviolette & Van Der Kooy, 2004). Though these receptors desensitize rapidly, leading to a long lasting excitatory effect on the DA neurons by way of disinhibition from the now inactive GABA neurons (Laviolette & Van Der Kooy, 2004). This acute reward circuitry also involves the input of the pedunculopontine tegmental nucleus (PPT) on the VTA to produce the rewarding effects of nicotine (Laviolette et al., 2002). It is important to note that nAChRs are present throughout the body, in muscle, skin and organ cells, with different subunit conformations, each able to bind nicotine with varying affinities (Albuquerque et al., 2009). The initial effects on the GABAergic neurons, and then subsequent sensitization to nicotine, through the DAergic neurons, relates to the initial aversive effects of nicotine that then gives way to the rewarding effects.

Nicotine, unlike other drugs of abuse, isn’t immediately addictive, but produces a gradual state of dependence associated with a variety of neuroplastic adaptations, while other drugs such as opiates, are more acutely addictive (Laviolette & Van Der Kooy, 2004). Research suggests that nicotine addiction isn’t initially dependent on DA transmission through the mesocorticolimbic pathway, but through the efferents of the PPT on the VTA (Laviolette et al., 2002, Laviolette & Van Der Kooy, 2004, Tan et al., 2009). Long term, smoking functionally changes transmission of DA through the mesocorticolimbic pathway, increasing DA release through the pathway (Tan et al., 2009, Laviolette & Van Der Kooy, 2004). Initially described by Robinson & Berridge in 1993,
then applied to nicotine addiction by Laviolette & Van Der Kooy in 2004, the *Incentive Salience* of nicotine induces sensitization of DA pathways. This theory suggests that nicotine induces a sensitization of the DA pathway that coincides with an increase in salience of the drug (Laviolette & Van Der Kooy, 2004, Robinson & Berridge, 1993).

![Diagram of reward pathways](image)

**Figure 1.** The acute and long term reward pathways. Figure adapted from Laviolette and Van Der Kooy (2004).

### 1.5 The Roles of DA D1 vs. D2 Receptors in the PFC

The dopaminergic system, as described in the previous section, has been implicated in the rewarding properties of nicotine addiction. Not only is the PFC an important structure in the mesolimbic pathway, but it has also been implicated with morphological differences in patients suffering from depression and anxiety disorders (Schulz & Arora, 2015). This makes the receptors for that mesolimbic dopaminergic pathway, specifically within the PFC, of great interest for the purposes of this thesis.
Dopamine is a neurotransmitter that is implicated in a whole host of activities, from movement to learning and memory to reward processing, acting on a variety of receptors, throughout the brain. The two important, and very different classes of DA receptors are the D1 and D2 receptors. Interestingly, these two receptors have opposing actions, with the D1 receptor activating adenylyl cyclase and cascading pathways, while the D2 receptor inhibits the action of the adenylyl cyclase cascade (Trantham-Davidson, Neely, Lavin & Seamans, 2004). Activation of the D1 receptor results in increases in fast-spiking interneuron excitability and presynaptic modulation in the PFC (Trantham-Davidson et al., 2004). Conversely, activation of the D2 receptor results in reduced interneuron excitability (Trantham – Davidson et al., 2004).

Functionally, D1 receptors in the PFC, have been implicated in working memory and executive function while D2 stimulation has been demonstrated to not affect these two functions (Floresco, 2013). Results of patch-clamp experiments have described opposing effects of these two receptor subtypes, and different downstream molecular pathways in which they exert their neuronal effects (Trantham-Davidson et al., 2004). D1 receptors have been implicated upstream in the de-phosphorylation of ERK, moving ERK from the phosphorylated, active, state to the dephosphorylated, inactive, state (Girault et al., 2007; Beaulieu, Espinoza & Gainetdinov, 2014). Both D1 and D2 receptors have an effect on the function of CaMKII through IP₃ activity and therefore the movement of calcium within the cell, with the D2 receptor having a stronger relationship with CaMKII functioning (Beaulieu et al., 2014, Rosen et al., 2016). Previous work looking at opiate-induced adaptations to the D1/D2 signaling system, describe a D2-CaMKII-dependent signaling in the acquisition of opiate reward memories in drug naïve states, that
functionally reverses following chronic exposure (Rosen et al., 2016). Previously, the group had found similar results with D1-ERK-dependent signaling and a functional reversal in the BLA of opiate naïve and chronic exposure states respectively (Lyons et al., 2013). This research describes the importance of both the DA system and its downstream effector molecules in the processing of rewarding stimuli, the formation of memories associated with addictive stimuli and the changes that occur following chronic exposure to addictive substances.

1.6 The Functional Roles of ERK and CaMKII in Neuropsychiatric Disorders

As described in the previous section, ERK and CaMKII are activated upstream by D1 and D2 receptors respectively. CaMKII is directly activated by calmodulin with two bound calcium ions, as a result of calcium influx from calcium stores or extracellular sources (Shifman et al., 2006). CaMKII has been implicated in long term potentiation processes and, therefore, is critical to memory formation, as presented in the previous section with experiments by Rosen and colleagues (2016; Shifman et al., 2006). Furthermore, the beta isoform of CaMKII have been demonstrated to be up-regulated in the lateral habenula of animal models of depression, and furthermore, were found to be downregulated with the treatment of antidepressants (Li et al., 2013). ERK1/2 are activated when the Thr and Tyr residues are phosphorylated by MEK1/2 (Girault et al., 2007). Drugs of abuse (addictive substances) have previously been shown to activate ERK1/2 in stereotypic places such as the NAcc, striatum, central amygdala as well as deep layers of the PFC (Girault et al., 2007). Interestingly, ERK1/2 seems to be important in reconsolidation of information during various learning paradigms in animal models
(Girault et al., 2007). Disruptions to the ERK1/2 pathway during ‘reconsolidation’ phases of experiments, eliminates the conditioned response to drugs (Girault et al., 2007). Furthermore, ERK1/2 has been implicated in cell growth and differentiation as well as neuronal plasticity (Girault et al., 2007). Both molecules have been implicated in both affective disorders as well as substance abuse and are therefore important targets in understanding how long term nicotine addiction relates to depression and anxiety disorders.

1.7 Epidemiology of Adolescent Smoking and Mood Disorders

Previous studies of epidemiology and etiology of nicotine use among adolescence have explored the prevalence of smoking and how it may correlate with other psychosocial stressors, socioeconomic status, comorbid addictions, and the development of neuropsychiatric illnesses. Some risk factors that have been associated with greater risk of nicotine dependence, along with other drugs of abuse, is impulsivity, inattention, novelty seeking and disinhibition (Perkins et. al 2008). Childhood abuse, low socioeconomic status as well as nonspecific stress have all been described as significant risk factors for adolescent smoking (Schepis & Rao, 2005). Furthermore, parental and peer smoking within a social group has been consistently associated with increased risk in smoking (Schepis & Rao, 2005).

Smoking has previously been described to be strongly correlated with depression (Boden, Fergusson and Horwood, 2010; Schepis & Rao, 2005; Jané-Llopis & Matytsina, 2006). An interesting, and uncommon longitudinal study of adolescents in New Zealand,
describes a causal relationship between smoking and depressive symptoms (Boden et. al, 2010). In this study participants were questioned at ages 18, 21 and 25 and measured for frequency of smoking and a self-report questionnaire examining DSM-IV criteria for nicotine dependence as well as interviewed on a structured mental health interview used to assess aspects of mental health and psychosocial adjustment (Boden et. al, 2010). The study ultimately reported a significant comorbidity between major depression and nicotine dependence. The relationship couldn’t be described by a common source and was causal with nicotine leading to depression (Boden et. al, 2010). Along with the well-established depressive association, nicotine has been linked to a number of psychiatric disorders including anxiety disorders, somatoform disorders and schizophrenia (Jané-Llopis & Matytsina, 2006). Furthermore, smoking has also been associated with deficits in cognitive function with respect to working memory, attention and executive planning (Chamberlain et. al, 2012). Much of the epidemiological evidence relating smoking with psychiatric diagnoses is conflicting as to the nature of their relationship. Further research needs to be conducted to better describe these relationships.

1.8 Preclinical Studies of Adolescent Nicotine Exposure

Preclinical studies refer to the use of animal models to better understand the mechanism of action of a drug or disorder before moving to clinical studies and trials using humans. The use of animal models in long term studies is also preferable for the speed in which researchers are able to see long term effects of a drug. A rat, for example, has a much shorter life cycle as compared to humans, have similar neural structures responsible for motivation, reward and learning as well as social structures, making them
ideal experimental subjects to investigate neural substance abuse mechanisms. Preclinical studies are important for understanding both acute and lasting effects of nicotine, especially during the critical period of neurodevelopment taking place in adolescence. The acute effects of nicotine were described in section 1.4 of this introduction “Physiological and Neurological Effects of Smoking”, therefore the preclinical analyses of long lasting effects of adolescent nicotine use will be further described in this section.

Previous preclinical work has suggested that exposure to nicotine during periadolescence increases the sensitivity to addictive properties of nicotine during adulthood, as described in a bar press self-administration experiment (Goriounova & Mansvelder, 2012). Other experiments have described changes in grooming, locomotor and rearing activity during and following adolescent nicotine treatment (Trauth, Seidler & Slotkin, 2000). Further behavioural changes such as anxiogenic and changes in fear behaviour have also been reported (Dwyer, McQuown & Leslie, 2009). The most heavily reported long term effect is the anxiogenic effects of chronic adolescent nicotine exposure (Dwyer, McQuown & Leslie, 2009; Goriounova & Mansvelder, 2012; Slawecki, Thorsell, Khoury, Mathe & Ehlers, 2005; Slawecki, Gilder, Roth & Ehlers, 2003). Short term, nicotine can have a pro-cognitive effect, while long term nicotine has been shown to have negative cognitive effects as demonstrated through visuospatial attentional tasks and serial recognition tasks (Dwyer, McQuown & Leslie, 2009; Counette et al., 2009). Furthermore, in these tasks there was an increase in premature responses in adolescent nicotine treated rats, suggesting an increase in impulsivity among those exposed to nicotine during adolescence (Dwyer, McQuown & Leslie, 2009; Counette et al., 2009).
There have been fewer studies examining the cellular and molecular changes following chronic adolescent nicotine exposure. One such study describes an increase in DA release at the boutons in the PFC followed electrical stimulation of the axons of the DA neurons innervating the PFC (Counette et al., 2009). Other articles have described changes in DA transmission through an upregulation of transporter densities and increased DA content in the striatum (Dwyer, McQuown & Leslie, 2009). Further changes in nAChRs and serotonergic signaling have been described (Dwyer, McQuown & Leslie, 2009). The changes described in the cholinergic activity may impact a variety of neurotransmitter systems that are modulated by ACh (Dwyer, McQuown & Leslie, 2009).

The preclinical studies described each look at an aspect of adolescent nicotine use and have varying methodologies used within each set of experiments. This could lead to some of the varying results that are present in the literature. Further research should be done using a specific dose and exposure procedure to better understand the effects of adolescent nicotine exposure in terms of cellular and molecular changes and the resulting behavioural deficits.

1.9 Research Objectives and Hypothesis

From this brief review of the literature concerning the links between adolescent neurodevelopment, smoking behaviours and mood disorders, it is clear that there are many unanswered questions concerning how and why adolescent smoking may contribute to complex mood disorders like MDD or anxiety. The primary research
objective of my thesis was to characterize and identify the effects of adolescent, neurodevelopmental exposure to nicotine on the developing mesocorticolimbic DA system, focusing on the VTA and PFC regions. Pursuant to this, my objective was to examine how these alterations may subsequently lead to neuronal, molecular and behavioural symptoms of mood disorders, using pre-clinical behavioural pharmacological modelling in rats.

Given the previously described research implicating the role of the PFC and mesocorticolimbic DA system in the effects of both nicotine addiction and mood disorders, my overarching hypothesis is that adolescent neurodevelopmental exposure to chronic nicotine will cause long-term and persistent neuropathological alterations in the mesocorticolimbic DA system associated with symptoms of depression and anxiety and associated neuronal and molecular adaptations in the PFC-VTA circuitry. I have addressed this research hypothesis with the following specific AIMS:

**AIM 1:** Characterize the effects of adolescent neurodevelopmental nicotine exposure on later adulthood measures of affective processing, anxiety, cognitive and depressive-like symptoms.

**AIM 2:** Characterize the molecular effects of adolescent neurodevelopmental nicotine exposure on later adulthood measures of DA D1, D2 and their downstream signaling pathways, ERK 1/2 and CaMKII directly in the PFC.

**AIM 3:** Characterize the effects of adolescent neurodevelopmental nicotine exposure on later adulthood measures of baseline changes in the neuronal activity states of VTA DA neurons.
2 Methods

2.1 Animal Models

Groups of adolescent and adult male Sprague-Dawley rats were obtained from Charles River (Quebec, Canada) at postnatal days (PND) 30 and PND 60, respectively. Rats were housed in pairs in a temperature controlled 12-hour light-dark cycle facility. Housing consisted of a plexi-glass box with a corn bedding base and environmentally enriched with paper towel, paper nesting material as well as a wooden block to chew. Rats were left to habituate to the facility and housing for four days prior to being handled. Food and water were available ad libitum for the duration of the experiments except when otherwise specified. The two days leading up to their pre-treatment, rats were handled and habituated to the procedure of injections (without needle/syringe). Rats were then pretreated with subcutaneous injections of 0.4 mg/kg of nicotine (nicotine bitartrate, Sigma-Aldrich Chemicals; s.c.) or a phosphate buffered saline vehicle solution. Injections were done three times a day (10:00/13:00/15:00) for 10 consecutive days (PND 35-44 or PND 65-74; Figure 2) as previously described by Counette and colleagues (2004). Following the pre-treatment, rats were left to develop with no other interventions until PND 75 or 105 for the adolescent and adult groups respectively (Figure 2). All experiments followed approved government and institutional standards of care.
Figure 2. Timeline of Pretreatment. Animals arrived at PND 30/60 for the adolescent/adult cohorts to allow animals to habituate to their new housing/facility. From PND 35-44/65-74 the adolescent/adult cohorts received 0.4 mg/kg of nicotine three times per day. At PND 75/105 animals began the testing as described in section 2.

2.2 Behavioural and Cognitive Testing

Once PND 75, for the adolescent cohort, or PND 105, for the adult cohort, were reached, a battery of behavioural and cognitive assays were conducted to examine the long term effects of nicotine exposure. It is important to note that a minimum of 3 days were left between each experiment and experiments were ordered from least to most stressful to attempt to minimize any confounding variables. Furthermore, after each rat testing, chambers, cages, and all other testing objects and tools were cleaned with 50% ethanol to avoid olfactory cue biases. In all behavioural and cognitive tests there is an n
value of 10 animals per group (control and nicotine) with the exception of social interaction testing for the adolescent cohort, in which there is an n value of 20.

2.21 Behavioural Tests

The cognitive tests are as follows:

**Light Dark Box Test** – The light dark box is an apparatus used as an unconditioned test of anxiety. The apparatus consists of two (50 x 25 x 37 cm) plexi-glass compartments divided in two by a wall that has a door (10 x 10 cm) on the bottom center of the wall. One side of the box has opaque black walls and a lid to create a fully dark space, the other side consists of opaque white walls and an open top, which a light is placed over to create a light open side with a 1500 lux at floor level (Figure 3a). The premise of this test is that one side is an aversive environment – the light side, and one is a non-aversive environment – the dark side. The level of anxiety in the animal is measured based on the spontaneous novelty-induced exploratory behaviour of the brightly illuminated and open areas as rodents have an innate aversion to those spaces (Kulesskaya & Voikar, 2014). In these experiments rats were habituated to the room in which the testing took place for 60 minutes prior to testing. Testing consisted of 8 minutes of exploration. Rats were placed in the middle of the light side of the box with their head facing away from the door separating the two sides. Measures taken include the latency taken for the rat to enter the dark side from the beginning of the test in the light side (Latency 1), the latency for them
to return back from the dark side into the light side after entering the dark side for the first time (Latency 2), the total transitions between the sides and the time spent in each side.

**Social-Interaction Testing** – Social interaction, as previously described by Renard *et al.*, (2016), analyzes two aspects of social behaviour. This task evaluates social affiliation/motivation and social recognition memory in phases 1 and 2 respectively. The apparatus consists of 3 chambers, the center being an open space used for habituation to the apparatus, with two identical interaction chambers on either side separated by plexi-glass walls with removable doors. In the center of each interaction chamber were individual metal cages for naïve rats to be explored by the rats in the experiments. Walls of all chambers are clear plexi-glass with removable doors between the center habituation area and two interaction chambers (Figure 3d). Rats were habituated to the test arena for 13 minutes, 24 hours before testing and then day of, rats were habituated to the room, in which the testing took place, for 30 minutes prior to testing. The test consisted of a habituation period (5 minutes) followed by 2 testing phases (each 8 minutes). The habituation period was 5 minutes in which the rat was able to explore the center chamber. At the end of the 5-minute habituation, the doors were opened and the rat was able to explore an empty cage in one interaction chamber or a novel conspecific in the other interaction chamber. This first phase of testing analyzed social motivation, that is, the propensity to spend time with an unfamiliar rat. The second phase then analyzed social recognition. For the second phase of testing, a rat was placed in the previously empty cage, leaving the experimental animal with the choice to explore a new conspecific or the
conspecific from the first phase of testing. In this situation, control rats will spend significantly more time with the novel stranger, demonstrating a natural preference for social novelty. The locations of stranger versus novel rats in the left versus right side chambers were counterbalanced between trials.

**Locomotor/Open Field testing** – Rats were placed in an automated open field activity chamber (Figure 3c; Med Associates, San Diego, CA, USA). Activity patterns were recorded for 60 minutes for the adolescent cohort and then shortened to 30 minutes for the adult cohort as adolescent animals spent most of the final 35 minutes sleeping and no longer moving. Analyses were performed over the total recording as well as over the first 5 minutes of the recording as that is what is typical in the literature. Results presented here describes the first 5 minutes of the recording. Analyses looked at stereotypic movement, and time spent in the center, zone 1 area, of the open field chamber as well as the time spent along the walls, and residual area. Time spent in the zone 1 area is similar to the time spent in the light side of the light dark box, a test of anxiety-like behaviour, as rats have a natural aversion to the open and more vulnerable space (Ohl, 2003). This testing therefore also demonstrates the anxiogenic levels of the rats tested.

**Porsołt Forced Swim Test** – In this well-established pre-clinical test for depressive-like phenotypes, rats were placed in a 70 x 19 cm cylinder filled ¾ of the way up with water for 15 minutes and behaviours were analyzed (Figure 3b). The main behaviours analyzed is immobility. Immobility is characterized by floating in the water with only movements
necessary to keep the nose above the surface (Bogdanova et al, 2013). This behaviour is a
resigned behaviour that is considered a depressive-like behaviour in animal models of
psychiatric disorders. The longer time spent immobile equates to a more depressive
phenotype. A consideration that is important for the purposes of this thesis is the body
weight of the rats. Previous work has described the significant effect of age and weight
on the behaviours observed in testing (Bogdanova et al, 2013).

_Sucrose Preference Testing_ – The sucrose preference test is a measure of a rat’s intrinsic
preference for sweet tasting substances, which serve as natural reward cues. The
measurement of sucrose consumption therefore serves as a proxy for hedonic sensitivity,
with a decrease in sucrose preference used as a proxy for anhedonia. For 24 hours before
the test, rats were habituated to the procedure of being moved to a cage identical in size
and shape to their own with fresh corn cob bedding and two bottles of water. They were
habituated to these testing parameters for 1 hour. For 12 hours prior to testing, rats were
water restricted to ensure rats were thirsty for testing. For testing, rats were moved to a
cage identical in size and shape to their home cage with fresh corn cob bedding and two
bottles, - one containing water and one containing a 2% sucrose solution. The amount of
each consumed over a 1-hour period was measured and preference for sucrose solution
was calculated. Sucrose preference testing is used in measures of anhedonia, a key
characteristic in depression. Anhedonia describes the reduced ability to experience
pleasure or rewarding stimuli, like a sucrose solution.
Figure 3. Diagram of behavioural apparatuses. A. Light dark box. B. Forced Swim test. C. Locomotor/Open field. D. Social Interaction – consisting of phase 1 and phase 2 each 8 minutes long as described in methods.

2.22 Cognitive Tests

The cognitive tests were both conducted in an 80 x 80 cm open roofed arena. Rats were habituated to the arena 24 hours prior to testing for two sessions of 10 minutes, and then day of testing left to habituate to the room in which testing was performed for 30 minutes prior to testing. Objects used in these tests were previously validated, and systematic biases to either object through the testing phase were counterbalanced for place preference. Tests were as follows:
**Object Recognition** – For these experiments, rats were placed in the open field arena with two identical objects and rats were allowed to explore them for 5 minutes for an acquisition phase. One hour later rats were tested with an identical object from the acquisition phase and a novel object. In the testing phase rats were allowed to explore the two objects for 3 minutes (Figure 4a). In this situation, control rats will spend significantly more time with the novel object, demonstrating a natural preference for novelty. Object recognition (OR) is a working memory task and requires the rat to recognize the previously explored object and is widely used to assess object recognition memory (Hannesson, Howland & Phillips, 2004).

**Object Recognition in Temporal Order** – Similar to OR, object recognition in temporal order (ORITO) adds a second acquisition phase and tests two previously explored objects. In these experiments, acquisition phase one allows rats to explore two identical objects for 5 minutes. An hour later, two different identical objects are explored in a second 5-minute acquisition phase. One hour after that, the animal is tested with one object from each of the acquisition phases for 3 minutes (Figure 4b). In this situation, control rats will spend significantly more time with the object from the first acquisition phase, demonstrating a natural preference for less recently explored objects. ORITO is a higher order memory task also known as recency discrimination. On this test trial, normal rats spend more time exploring the old familiar object rather than the more recent familiar
object, requiring the animal to both recognize the previously explored objects as well as to discriminate their relative recency (Hannesson et al, 2004).

All tests with the exception of sucrose preference and locomotor testing were video recorded with webcams (Logitech) and analyzed using a video-tracking system (ANY-maze; Stoelting) on PC computers using windows operating systems.

![Figure 4. Schematic of cognitive testing in open field arenas. A. Object Recognition. B. Object Recognition in Temporal Order Testing.](image)

2.3 **In Vivo** Neuronal Electrophysiology

*In Vivo* neuronal electrophysiological recordings were performed unilaterally in the VTA, as previously described (Renard et. al, 2016). Rats were anesthetized with urethane (1.3 g/kg, i.p.) and placed in a stereotaxic frame with a body temperature maintained at 37 °C. A scalp incision was made and a hole was drilled in the skull
overlying the VTA, as well as a small hole for a screw in the skull overlaying the cerebellum. A grounding wire was then attached to the screw. For recordings, a glass microelectrode (with an average impedance of 6-8 MΩ), filled with a 2% pontamine sky blue solution in KCl, were lowered using a hydraulic micro-positioner (Kopf640) into the VTA. Coordinates were determined using the Paxinos and Watson rat atlas (1986) to the following coordinates: anterior/posterior: -5.2 mm from bregma, lateral: ±0.8-1 mm and dorsal/ventral: -6.5 to -9 mm from the dural surface. Extracellular signals were amplified using a Multiclamp700B amplifier (Molecular Devices) and recorded through a Digidata1440A acquisition system (Molecular Devices) using a pClamp10 software. Recordings were filtered at 1 kHz and sampled at 5 kHz. Analyses were performed offline using the Clampfit10 (Molecular Devices) software package.

Predetermined track patterns were used to sample spontaneously active DA neurons in the VTA by moving 200 µm between each track. In each track presumed DA neurons were isolated and its spontaneous activity was recorded for 5 minutes. DA neurons within the VTA were identified according to previously established electrophysiological features (Ungless & Grace, 2012): 1) relatively long action potential, with a duration of >1.1 ms from the start to the negative trough; 2) a slow spontaneous firing rate (~2-5 Hz), and 3) a single irregular or bursting firing pattern. Non-DAergic cells were, conversely, higher in frequency with shorter AP durations. The two parameters of activity that were assessed were the basal firing rate and the proportion of spikes fired by the DA neurons that occurred in bursts. The onset of a burst was defined as the occurrence of 2 spikes with an inter-spike interval of 80 ms (Grace & Bunney, 1983). The percentage of spikes in bursts was calculated by dividing the number of
spikes occurring in the bursts by the total number of spikes over the recorded period. Following recordings, recording electrode locations were marked with iontophoretic deposit of pontamine sky blue (-20 µA for 15 minutes). Animals were euthanized using Euthanyl (made in a 1:1 dilution of euthanyl-pentobarbital and ddH2O), decapitated and brains were removed, and stored in 10% formalin. Brains were sliced using a microtome at sections of 60 µm that were stained with neutral red to confirm the neuronal recording site. Cells recorded outside the anatomical boundaries of the VTA were excluded from data analysis.

2.4 Molecular Analysis

2.4.1 Extractions

Rats were injected with 0.8 mL of Euthanyl (made in a 1:1 dilution of euthanyl – pentobarbital and ddH2O). Once the animal was on a surgical plane, (ex. no longer responded with reflexes to hind leg pinch or cornea touch) the animal was decapitated, the brain was quickly removed and blocked into coronal sections. Areas of interest were collected – targets based on the rat brain atlas (Paxinos and Watson, 1986). Tissue samples were placed in a homogenizer with lysis buffer (350 uL for mPFC, 600 uL for STR and 200 uL for SN). The tissue and liquid was then transferred to labelled eppendorf tubes. All tubes were rotated in the fridge for 30 minutes and then centrifuged at 10,000g for 10 minutes at 4°C. The pellet created from placing samples in the centrifuge was discarded and supernatant was transferred to a clean eppendorf tube. Of this liquid, aliquots were taken and placed in new eppendorf tubes, a loading buffer was added in a
1:1 ratio, boiled for 5 minutes and placed in the -80°C freezer to be used in blots. Left over liquid was labeled as count to further analyze for total protein count and stored in the -80°C freezer.

### 2.42 Total Protein Count

Before running western blots, total protein count was analyzed in each of the samples in order to determine the volume of sample to be used to standardize total protein count across all samples. Two rows of standard protein concentrations were pipetted into the first two rows of wells on a 96-well loading assay plate followed by varying dilutions of the sample protein. Standard BSA protein concentrations ranged from 0-2000 ug. The samples were then diluted, incubated and quantified. The samples were quantified based on the colour of the sample as compared to the standard curve.

### 2.43 Western Blots

Gels were cast, usually a 10% gel was made with some variation depending on the size of the protein of interest. Using the chart created following the total protein count, specific volumes of the total sample were aliquoted into a new eppendorf tube, 2xLB buffer was then added to a final volume of 15 uL. Gels were assembled into the Western blot chamber with the fist running buffer, samples were loaded, the first well was the standard ladder on both gels and then samples alternated between control and nicotine. Gels were run at 125 V until the loading buffer reached the bottom of the gel.
After samples were run through the gel, they were transferred to nitrocellulose. The whole transfer apparatus was placed in a bucket surrounded by ice to control temperature. The gels were run at 75 V for 60-70 minutes. Once removed from the transferring process the nitrocellulose was cut down to the size of the gel. It was then placed in a container with TBS-T on a rocker to wash the transfer buffer away. The blot was then moved to a blocking solution, which was left on the rocking nitrocellulose for 45 minutes - 1 hour. From here the primary antibody was made up in either a dehydrated milk/TBS-T solution or a BSA/TBS-T solution depending on the antibody. After washing the blocking solution from the blot, the primary antibody was put on the nitrocellulose and it was left to rock in the fridge overnight. Primary antibody dilutions were as follows: α-tubulin (1: 120 000; Sigma-Aldrich), phosphorylated ERK1/2 [T202/Y204] (pERK1/2; 1: 2000; Cell Signaling Technology), total ERK1/2 (tERK1/2; 1:5000, Cell Signaling Technology), phosphorylated CaMKIIα [T286] (pCaMKIIα; 1:10 000; Cell Signaling Technology), total CaMKIIα (tCaMKIIα; 1:5000; Cell Signaling Technology), D1R (1:100; Santa Cruz Biotechnology), and D2R 1:100; Santa Cruz Biotechnology). The next morning the rocker with all of the nitrocellulose/primary antibody containers was brought out to return to room temperature. Once room temperature was again reached, the primary antibody was washed off the blot and the secondary antibody was applied and left on the rocking blots for one hour. Secondary antibodies (Thermo Scientific) were all used at a dilution of 1:20 000. The secondary antibody was prepared in a BSA/TBS-T solution. The blots were washed a final time and at this point ready to be read by the Licor processing machine to visualize the protein bands and eventually be quantified. Once the bands were visualized
and turned into a .tiff image, the band intensity was quantified and normalized to the alpha tubulin loading control. Normalized samples were then compared as a function of change from control samples.

2.5 Statistical Analyses

Behavioural and electrophysiological data were analyzed with ANOVAs and t-tests, where appropriate. All statistical analyses were run using SPSS Statistics software and only values with $p \leq 0.05$ were considered as significant and outliers of more than 2 standard deviations away from the mean were excluded from analyses.

3 Results

3.1 The Effects of Adolescent Nicotine Exposure

3.1.1 Behavioural Results

The first set of experiments performed looked at behavioural differences that resulted from adolescent nicotine exposure. Behavioural paradigms were all run 31 days after the chronic exposure to nicotine or a saline control, at PND 75. There were no pharmacological interventions after the adolescent protocol.
a. Locomotor & Open Field Testing

Rats were placed in an open field arena and left to explore, and freely move through arena over 60 minutes. Movement patterns and distances were recorded and analyzed using an automated activity monitor. Over the full 60 minutes of the recording there were no statistical differences in the total movement of the two groups of rats. The total distance travelled by each over the 60-minute test was 3651 cm for the vehicle group (SD = 375.85) and 2726.89 cm for the nicotine group (SD = 430.31). As the test progressed, rats spent a larger proportion of the time immobile and ultimately ended up sleeping in a corner of the apparatus, as described in figure 5A. Due to the amount of time spent immobile in the second half of the test, as well as the standard of analyzing the first 5 minutes of an open field test, only the first block of time was examined further for behavioural differences. Over the first 5 minutes, the two groups did not differ from one another with total movement (M = 846.3 cm, SD = 120.81 for nicotine, M = 1031.23, SD = 90.16 for vehicle). In the first 5-minutes, the adolescent nicotine group spent significantly less time, and travelled less distance in the zone one area as compared to the vehicle group (t (18) = 2.787, p = 0.012; t (18) = 2.522 p = 0.021 respectively; Figure 5). Conversely, the adolescent nicotine group also spent significantly more time in the residual area (t (18) = 2.704, p = 0.015; Figure 5). The nicotine group spent about 4x more time in the residual area as compared to the vehicle group. They also spent about half the amount of time in the zone one area as compared to the vehicle group (Figure 5).
Figure 5. Adolescent Open Field and Locomotor Testing. In all charts Blue = adolescent vehicle, Grey= adolescent nicotine. N = 10 per group. A. Ambulatory distance in 5 minute blocks for the full 60 minutes of testing (each block represents 5 minutes of testing; Block 1 = 0-5 minutes, block 2 = 6-10 minutes etc.). There were no statistically significant differences between the adolescent nicotine and adolescent vehicle groups, with respect to distance traveled, at any of the time points through testing. B. Total distance travelled in zone 1 (the center portion of the open field apparatus) over the first 5 minutes of locomotor testing. Adolescent nicotine rats travelled significantly less distance in the zone 1 area ($p = 0.021$). C. Total distance travelled in the residual area (the surrounding wall portion of the open field apparatus) over the first 5 minutes of locomotor testing. Rats did not significantly differ in distance travelled in the residual area ($p = 0.236$). D. Total time spent in zone 1 (the center portion of the open field apparatus) over the first 5 minutes of locomotor testing. Adolescent nicotine rats spent significantly less time in the zone 1 area ($p = 0.012$). E. Total time spent in the residual area (the surrounding wall portion of the open field apparatus) over the first 5 minutes of locomotor testing. Adolescent nicotine rats spent significantly more time in the residual area compared to the vehicle group ($p = 0.012$). F. Pictogram of adolescent nicotine animal’s movement patterns over 60 minutes of locomotor testing. G. Schematic of open field apparatus with zone 1 and residual areas labelled. H. Pictogram of adolescent vehicle animal’s movement patterns over 60 minutes of locomotor testing. Error bars represent mean ±SE for all figures.
b. Light-Dark Box Testing

Rats were initially placed in the light side of the light-dark box. The first latency, the time it took from the beginning of the testing, to the time the animal entered into the dark side of the box, was taken. There was a trend that the adolescent nicotine pretreated rats took longer to run into the dark side, as compared to the vehicle group ($t_{(10.176)} = 0.052, p = 0.052$; Figure 5D). The vehicle group took about 10 seconds to run into the dark side (SD = 6.62), while the nicotine group took just under 20 seconds to run into the dark side (SD = 12.11). The second latency calculated was the time taken from arriving in the dark side of the box to then re-enter the light side. In this measure, there was no statistical difference between the two groups, as the nicotine group demonstrated quite a bit of variability in their responses ($t_{(9.218)} = 1.476, p = 0.173$; Figure 6E). The vehicle group took just over 20 seconds, on average, to return back to the light side (SD = 20.17), while the nicotine group took more than twice the amount of time at almost 1 minute to return to the light side (SD = 76.96; Figure 6E). It is important to note here, that one animal from the nicotine group was excluded from the analysis as he did not return to the light side, spending the rest of the test in the dark side. Over the 8 minutes of testing, the rats in the nicotine group, on average, spent significantly more time in the dark side, and conversely significantly less time in the light side of the box, as compared to the vehicle group ($t_{(18)} = 2.240, p = 0.038$; $t_{(18)} = 2.294 p = 0.034$ respectively; Figure 6B-C). The adolescent nicotine group spent, on average, 100 seconds in the light side of the box (SD = 59.65) and 350 seconds in the dark side (SD = 59.97), compared to the vehicle group, that spent 160 seconds in the light side (SD = 41.69) and about 300 seconds in the dark side (SD = 39.73; Figure6B-C). Over the course of the 8-minute test, the groups did not
differ in their transitions between the two compartments (Figure 6F). Both groups transitioned between the two compartments between 15 and 20 times over the course of the testing (M = 19.4, SD = 4.33 for vehicle, M = 15.3, SD = 9.04 for nicotine).
Figure 6. Adolescent Light–Dark Box Testing. In all charts Blue = adolescent vehicle, Grey = adolescent nicotine. N = 10 per group. 

A. Schematic of light dark box arena. 

B. Average time spent in the dark side of the light-dark box over the 8 minutes of testing. The adolescent nicotine group spent significantly more time in the dark side of the light-dark box ($p = 0.034$). 

C. Average time spent in the light side of the light-dark box over the 8 minutes of testing. The adolescent nicotine group spent significantly less time in the light side of the light-dark box ($p = 0.034$). 

D. Latency 1 describes the time it took the animal to run from the light side into the dark side of the box from the beginning of the test. The two groups did not statistically significantly differ from one another on this measure. 

E. Latency 2 describes the time it took the animal to run from the dark side into the light side of the box from the beginning of the test. The two groups did not statistically significantly differ from one another. 

F. Total transitions over the 8-minute testing period.
c. Social Interaction Testing

Social Interaction Testing is made up of two 8-minute testing phases. In the first phase, the animal has the option to explore either an empty cage or a rat that they had never interacted with before. Comparing exploration of the novel conspecific versus an empty cage in phase one, a two way repeated measures ANOVA revealed a significant effect of treatment ($F_{(1,33)} = 5.319, p = 0.028$). Post hoc comparisons revealed that the difference between the two groups being the adolescent nicotine group spent less time exploring the stranger rat as compared to the adolescent vehicle pretreated group ($p = 0.05$; Figure 7). The vehicle group spent, on average, 30 seconds more exploring the stranger in phase one than the nicotine group ($M = 127.61, SD = 31.68$ for vehicle, $M = 105.46, SD = 29.45$ for nicotine; Figure 7). Furthermore, in each phase, both the nicotine and vehicle pretreated groups demonstrated a main effect preference, for the stranger rat as opposed to an empty cage ($p < 0.001$). The second phase of testing takes place immediately after the first. For the second phase of the testing, the animal has the option to explore either the rat from the first phase of experiments or a new stranger rat placed in the previously empty cage. In this 8-minute test, results from a two way repeated measures ANOVA revealed a significant effect of treatment ($F_{(1,33)} = 5.303, p = 0.028$). Again, there was a difference between the two groups with respect to the amount of time they explored the novel rat. Post hoc comparisons, describe the adolescent nicotine group spent less time exploring the novel rat as compared to the vehicle group ($p < 0.05$; Figure 7). The vehicle group spent, on average, more than 40 seconds more, than the adolescent nicotine group, exploring the newest conspecific ($M = 122.25, SD = 50.37$ for vehicle, $M$
= 85.68, SD = 30.47 for nicotine; Figure 7). Again there was a main effect of preference for the novel conspecific (p < 0.001).

![Figure 7. Adolescent Social Interaction Testing](image)

Figure 7. Adolescent Social Interaction Testing. Blue = adolescent vehicle, Grey = adolescent nicotine. N = 20 per group. Adolescent nicotine rats spent significantly less time exploring the stranger rats in both the first and second phases of the experiment (p = 0.05, p = 0.046 respectively). In both phases of the test, both groups demonstrated a preference for the novel conspecific (p < 0.001).

**d. Sucrose Preference Testing**

After 12 hours of water deprivation, rats were placed in a cage with two water bottles, one bottle containing a 2% sucrose solution and one bottle containing tap water. After 1 hour of exploration and liquid consumption, total liquid changes were calculated. In terms of average 2% sucrose solution consumed, the groups did not statistically differ in terms of amount consumed ($t_{(18)} = 0.827, p = 0.419$; Figure 8A). It appears, though, that the adolescent nicotine group, on average, consumed less of the sucrose solution than the vehicle group (M = 11.55, SD = 6.05 and M = 14.6, SD = 4.2, respectively). Conversely,
the adolescent nicotine group consumed significantly more water than the adolescent vehicle group over the course of testing (M = 6.81, SD = 5.25 and M = 2.8, SD = 2.57 respectively; t(18) = 2.645, p = 0.016; Figure 8B). Through the testing, the groups consumed similar amounts of liquid, about 18 mL, with the group differences being in which liquid they preferred to consume (M = 17.4, SD = 2.99 for vehicle, M = 18.36, SD = 3.93 for nicotine). The vehicle group consumed 15 mL (SD = 4) of the 2% sucrose solution and 3 mL (SD = 2.5) of the water, while the nicotine group consumed 11 mL (SD = 6) of the 2% sucrose solution and 7 mL (SD = 5) of the water (Figure 8). There was a significantly lower preference for the 2% sucrose solution in the adolescent nicotine group as compared to the adolescent vehicle group (t(18) = 2.393, p = 0.028; Figure 8D). The adolescent nicotine group had about a 62% (SD = 28) preference for the sucrose solution as compared to the vehicle group that had an 83% (SD = 18) preference for the sucrose solution (Figure 8D).
Figure 8. Adolescent Sucrose Preference Testing. In all charts Blue = adolescent vehicle, Grey = adolescent nicotine. N = 10 per group. A. The amount of the 2% sucrose solution consumed over the 1-hour test. The two groups did not statistically differ from one another, while there was a trend in the data suggesting the adolescent nicotine rats consumed less of the solution ($p = 0.173$). B. The amount of the tap water consumed over the 1-hour test. The adolescent nicotine group consumed significantly more water than the vehicle group ($p = 0.016$). C. The total amount of liquid consumed over the 1-hour test. The two groups consumed similar amounts of liquid in total through the test. D. The preference for the 2% sucrose solution. The adolescent vehicle group had a significantly greater sucrose preference as compared to the nicotine group ($p = 0.028$).
e. **Forced Swim Testing**

In forced swim testing, the animal is placed in the tube of water for 15 minutes and the resulting behaviours were analyzed. The main behaviours analyzed are time spent swimming, time spent climbing the walls and time spent immobile (Bogdanova et al., 2013). Over the test, the adolescent nicotine group spent significantly less time swimming, as compared to the vehicle group ($t_{(18)} = 3.140, p = 0.006$; Figure 9). The vehicle group spent, on average, 559.82 seconds swimming (SD = 75.64), while the nicotine group spent 432.23 seconds swimming (SD = 103.87; Figure 9). Both groups spent less time immobile than swimming but the two groups significantly differed from one another with the adolescent nicotine pretreated rats spending significantly more time immobile, compared to the adolescent vehicle pretreated rats ($t_{(18)} = 2.549, p = 0.02$; Figure 9). The adolescent nicotine group spent, on average, 341.06 seconds immobile over the test (SD = 124.46), while the vehicle group spent, on average, 221.09 seconds immobile (SD = 77.34). Both groups performed similarly when the climbing behaviour was analyzed. Both groups spent around 115 seconds climbing (M = 110.92, SD = 26.71 for vehicle, M = 123.41, SD = 79.85 for nicotine). The behaviour of climbing was typical to the beginning of the test as the initial behaviour observed. This usually lasted for the first few minutes of the test until the rats began the swimming behaviour and exploratory dives.
Figure 9. Adolescent Forced Swim Testing. Blue = adolescent vehicle, Grey = adolescent nicotine. N = 10 per group. The amount of time over a 15-minute testing period spent swimming, climbing or immobile. The adolescent nicotine pretreated group spent significantly less time swimming ($p = 0.006$) and significantly more time immobile ($p = 0.02$) over the testing. Both groups spent similar time climbing.

3.12 Cognitive Results

The next set of experiments performed, looked at cognitive differences that resulted from adolescent nicotine exposure. Cognitive paradigms were all run 31 days after the chronic exposure to nicotine or a saline control, at PND 75. There were no pharmacological interventions after the adolescent protocol.

a. Object Recognition

To assess memory cognition, object recognition testing was performed. Object recognition assesses memory and is linked to hippocampal activity (Mumby, 2001). Object recognition plays on the rats’ natural preference for novel things. Control rats
typically display similar amounts of time exploring the pair of identical objects during the training phase, and then a longer time exploring the novel object during the testing phase of this paradigm. Both the adolescent nicotine as well as the adolescent vehicle groups performed at the same level (Figure 10). In the training phase, a two way repeated measures ANOVA describes no effect of treatment on time spent exploring the two identical objects ($F_{(1, 17)} = 0.005, p = 0.946$; Figure 10). During the testing phase, a two way repeated measures ANOVA again does not describe a treatment effect on the time spent exploring the two objects ($F_{(1, 17)} = 0.016, p = 0.901$). The ANOVA did however describe a significant main effect with both the vehicle and nicotine groups spending significantly more time exploring the novel object ($F_{(1, 17)} = 20.817, p < 0.001$; $M = 27.20$, SD = 12.62 and $M = 28.36$, SD = 6.72, respectively) as compared to the object identical to those in the training phase ($M = 15.07$, SD = 5.49 for vehicle, and $M = 16.88$, SD = 6.60 for nicotine; Figure 10). The groups performed at the same level in all aspects of this task.
Figure 10. Adolescent Object Recognition Testing. Blue = adolescent vehicle, Grey = adolescent nicotine. N = 10 per group. In the training phase of the testing, both groups spent similar time exploring an identical set of objects. In the testing phases of the test, both groups demonstrated a preference for the novel object (p = 0.02 for vehicle, p = 0.006 for nicotine).

b. Object Recognition in Temporal Order

To further explore cognitive memory, object recognition in temporal order was performed on all rats. This test takes the requirements from object recognition, explained previously, and adds a second training phase with a different pair of objects. Finally, the testing phase consists of one of each of the objects previously explored. Adding in the second training phase forces the animal to not only remember the first object but the second as well as integrating that information in the testing phase of this task. In training phase one, both the adolescent nicotine and adolescent vehicle groups explored both sets of the object equally, there were no differences between the groups (for vehicle: M = 20.67, SD = 7.61 and M = 21.43, SD = 9.82, for nicotine: M = 24.24, SD = 9.43 and M = 19.28, SD = 11.33; Figure 11A). A two way repeated measures ANOVA describes no
changes no effect of treatment on exploration during the first phase ($F_{(1, 17)} = 0.368, p = 0.552$). In the second phase of training, there was no treatment effect between the two adolescent groups, nor was there a difference between the time spent exploring the two identical objects ($F_{(1, 17)} = 0.205, p = 0.656$; for vehicle: $M = 22.35$, $SD = 7.33$ and $M = 15.89$, $SD = 8.68$; for nicotine: $M = 15.24$, $SD = 11.47$ and $M = 10.46$, $SD = 7.70$; Figure 11B). In the testing phase of the task, a two way repeated measures ANOVA describes an effect of treatment on the exploration ($F_{(1,17)} = 4.654, p = 0.046$). The adolescent nicotine and adolescent vehicle groups spent similar amounts of time exploring the object from the second phase of testing ($M = 9.54$, $SD = 7.90$ for vehicle, $M=11.33$, $SD = 7.47$ for nicotine), but the adolescent nicotine group spent less time exploring the first object ($M = 18.12$, $SD = 12.90$ for vehicle, $M = 9.91$, $SD = 5.99$ for nicotine; Figure 11C). The adolescent nicotine group spent around the same amount of time exploring both the object from the first and the object from the second phases of testing, while the adolescent vehicle group spent significantly more time exploring the object from the first phase of testing when compared to the amount of time spent exploring the object from the second phase of testing ($p = 0.041$; Figure 11D). In the object recognition in temporal order testing the two groups only differed on the testing phase of the task, the adolescent vehicle group spent significantly more time exploring the object from the first training phase as compared to both the second training phase and the adolescent nicotine group (Figure 11).
Figure 11. Adolescent Object Recognition in Temporal Order Testing. Blue = adolescent vehicle, Grey = adolescent nicotine. N = 10 per group. A. Training phase 1 of the task, two identical objects were explored. Bars represent the amount of time spent exploring the two identical objects. The vehicle and nicotine group did not differ in their time spent exploring the two objects. B. Training phase 2 of the task, two different identical objects were explored. Bars represent the amount of time exploring the two identical objects. The vehicle and nicotine group did not differ in their time spent exploring the two objects. C-D. The testing phase of the task represented as he two objects (one from the first and one from the second phase of training) and then as the two groups (adolescent nicotine and adolescent vehicle) to best visualize the differences. The adolescent nicotine group spent similar amounts of time exploring the two objects while the vehicle group spent significantly more time exploring the object from training phase 1 as compared to training phase 2 ($p = 0.041$). D. Black = object from the first phase of training, Grey = object from second phase of testing.

3.13 Electrophysiology Results

*In Vivo* electrophysiology was performed in the VTA to measure baseline firing patterns of dopaminergic neurons within the VTA. Electrophysiological paradigms were all run
31 days after the chronic exposure to nicotine or a saline control, at PND 75. There were no pharmacological interventions after the adolescent protocol. Over the 5 minute recordings, the cell frequency and bursting behaviour was analyzed. Results from these recordings indicated an increase in dopaminergic cell frequency as well as bursting in the adolescent nicotine group as compared to the adolescent vehicle group. In cell frequency, the adolescent group pretreated with nicotine, had more than a 3x greater frequency at 4.2 Hz (SD = 2.98) as compared to the adolescent group pretreated with a vehicle solution 1.5 Hz (SD = 0.79; Figure 12C). This describes a significantly more active cell population with respect to action potentials in the adolescent nicotine group ($t_{(9.116)} = 2.655, p = 0.026$). Furthermore, the adolescent nicotine group also had a significantly higher burst rate ($M = 43.73, SD = 32.04$ for nicotine, $M = 13.62, SD = 9.86$ for vehicle) and a significantly higher average bursts in the recordings ($t_{(9.502)} = 2.695, p = 0.023, t_{(13.288)} = 2.047, p = 0.023$, respectively; Figure 11D-E). In burst rate, the adolescent nicotine group again had a value more than three times the size of that in the adolescent vehicle group. The adolescent nicotine group had a burst rate of 43.7 bursts while the adolescent vehicle group only had a burst rate of 13.6 bursts (Figure 12D). Over the course of the recording, the average number of bursts was calculated for each treatment group. The adolescent nicotine group averaged 218.7 bursts during the entire recording (SD = 160.20), while the adolescent vehicle group averaged 68.1 bursts over the recording period (SD = 49.30). This describes a marked difference in both bursting and cell firing behaviours between the adolescent nicotine group and the adolescent vehicle group.
Figure 12. Adolescent Electrophysiological Recordings from the VTA. Blue = adolescent vehicle, Grey = adolescent nicotine. N = 9 cells per group obtained from 3 nicotine and 2 vehicle animals. A. Representative baseline single cell baseline recordings of adolescent nicotine and adolescent vehicle rats demonstrating the increase in firing among the adolescent nicotine group. B. Average cell frequency over the duration of the recording. Adolescent nicotine pretreated rats had a significantly higher baseline firing frequency when compared to the adolescent vehicle group ($p = 0.026$). C. Average burst rate. The adolescent nicotine group had a significantly higher burst rate as compared to the adolescent vehicle group ($p = 0.023$). D. The average bursts over the duration of the recording. The adolescent nicotine pretreated group again had a significantly greater number of bursts over the recording as compared to the adolescent vehicle group ($p = 0.023$). E. Diagram of the location of the In Vivo electrophysiological recordings as well as a representative stained section describing the location of the final recording.
3.14 Molecular Results

Due to the results found in the In Vivo electrophysiology experiments, further molecular analyses were performed to better characterize some of the long term changes in the PFC, a structure innervated by the mesolimbic pathway, that originates in the VTA. Therefore, tissue from the PFC was analyzed using a western blot technique. Molecular analyses were all run 31 days after the chronic exposure to nicotine or a saline control, at PND 75. There were no pharmacological interventions after the adolescent protocol. Data was normalized to the loading control and is presented as a percentage change from the vehicle group. Based on the changes in DA firing, dopaminergic receptor densities were analyzed. The adolescent nicotine and adolescent vehicle groups did not significantly differ from one another in terms of D2 receptor levels (Figure 13C). With the adolescent vehicle group representing 100% (SD = 9.9), the nicotine group is at a level of 96% (SD = 12.7) meaning the D2 receptor levels in the two groups were relatively similar. The two groups did, however, differ in D1 receptor concentrations. There was a significant downregulation in D1 receptor levels in the adolescent nicotine levels. The adolescent nicotine group was at 77% (SD = 11.5) of the adolescent vehicle group, demonstrating that, when compared to the vehicle group, the adolescent nicotine group had a significant reduction in the total levels of D1 receptors in a one tailed test ($t_{(6)} = 2.822, p = 0.015$, Figure 13B).

Based on previous work in our lab, regarding changes in the PFC following adolescent THC exposure, similar electrophysiological results were obtained (Renard et al., 2016). Based on molecular pathways examined and found to have changed in these results as well as knowledge of the functions of these molecules and their respective
pathways, ERK1/2 and CaMKII were both quantified in the samples. Looking at both the total CaMKII and just the phosphorylated form of CaMKII, there were no significant changes in the levels between the adolescent nicotine and adolescent vehicle groups. CaMKII is activated when phosphorylated, therefore, it is important to look at both the phosphorylated as well as total levels to see any potential changes in the ratios of active/inactive levels. In these experiments, there were no changes in either phosphorylated nor total levels of CaMKII, therefore no changes to this ratio. Total levels of pCaMKII for the nicotine group were at 99.5% (SD = 20.0) when compared to the levels found in the vehicle group (M = 100, SD = 6.5; Figure 13D). In total CaMKII levels, the nicotine group was at 97% (SD = 8.4) to the 100% (SD = 8.1) vehicle levels (Figure 13D). When looking at the results from ERK, the two groups significantly differed from one another. Again, ERK is a molecule that is activated through phosphorylation, therefore, we must look at both the phosphorylated as well as total levels of ERK. ERK also consists of two substrates noted ERK1 and ERK2. Both serve similar functions but appear as two distinct bands on western blots due to their difference in size (Figure 13A). In terms of total levels of ERK1/2 the adolescent nicotine group did not significantly differ from the vehicle controls (Figure 13A). For ERK1 the two groups only differed by 0.5% (M = 99.5, SD = 8.7 for nicotine, M = 100, SD = 6.2 for vehicle), and for ERK2, the nicotine group was at 98% (SD = 5.0) of the vehicle levels (M = 100, SD = 6.1; Figure 13A). Neither of these differences were significant as they were too similar, especially given the spread of the data. The adolescent nicotine pretreated group did however differ from the adolescent vehicle pretreated group in levels of activated (phosphorylated) ERK1/2. In pERK1, the nicotine group expressed a 4-fold increase (M
= 447 for nicotine and M = 100 for vehicle), and in pERK2, they showed a 5-fold increase in a one tailed test (M = 531, SD = 310 for nicotine and M = 100, SD = 39.9 for vehicle; \( t_{(6)} = 2.300, p = 0.03 \) and \( t_{(6)} = 2.747, p = 0.016 \) respectively; Figure 13A). With total levels of ERK1/2 remaining the same, this demonstrates a large difference in the ratio of activated/inactivated ERK levels. This demonstrates that adolescent nicotine rats had persistent changes in pERK1/2 levels with a substantial increase in the activated form of ERK1/2.
**Figure 13. Adolescent Molecular Analysis of PFC.** Blue = adolescent vehicle, Grey = adolescent nicotine. N = 4 per group. A. Relative concentrations of phosphorylated ERK1/2, total ERK1/2 in nicotine rats compared to the vehicle pretreated group. The nicotine group had a significantly larger concentration of phosphorylated ERK1 and ERK2 (\( p \text{ (one tail)} = 0.03 \) and \( p \text{ (one tail)} = 0.016 \) respectively). There were no significant differences between the groups with respect to total ERK1 or ERK2 concentrations. **B.** Relative concentrations of D1 receptors in nicotine rats compared to the vehicle group. The adolescent nicotine group had a significantly smaller concentration of D1 receptors as compared to the vehicle group (\( p \text{ (one tail)} = 0.015 \)). **C.** Relative concentrations of D2 receptors in nicotine rats compared to the vehicle group. There were no significant differences between the groups with respect to total D2DR concentrations. **D.** Relative concentrations of total and phosphorylated CaMKII. There were no significant differences between the nicotine and vehicle groups with respect to total as well as phosphorylated concentrations.
3.2 The Effects of Nicotine Exposure in Adult Rats

3.21 Behavioural Results

Following the robust changes found following adolescent nicotine exposure, it was important to then further describe the changes as being a function of the developmental period as opposed to a general long term result of nicotine. Behavioural paradigms were run 31 days after the chronic exposure to nicotine or a saline control, at PND 105. There were no pharmacological interventions after the adult exposure protocol.

a. Locomotor & Open Field Testing

Rats were placed in an open field arena and left to explore and freely move through arena over 30 minutes. Movement patterns and distances were recorded and analyzed using an automated activity monitor. Over the full 30 minutes of the recording there were no statistically significant differences in the total movement of the two groups of rats (M = 2118.01, SD = 212.27 for vehicle, M = 2364.79, SD = 177.49 for nicotine). In all measures, there was no statistical differences between the nicotine or vehicle groups. Over the first 5 minutes, the two did not differ in total distance travelled (M = 675.187, SD = 98.43 for vehicle and M = 774.82, SD = 87.42 for nicotine; t (18) = 0.757, p = 0.459). Furthermore, the nicotine and vehicle groups did not differ from one another in the distance travelled in zone 1 (M = 182.83, SD = 109.74 for vehicle, M = 208.96, SD = 110.41 for nicotine; t (18) = 0.686, p = 0.502) nor in the residual area (M = 492.36, SD = 229.89 for vehicle, M = 565.86, SD = 249.02 for nicotine; t (18) = 686, p = 0.502; Figure 14B-C). Furthermore, the two groups spent similar amounts of time exploring and resting.
in the zone 1 area (M = 55.5, SD = 34.97 for vehicle, M = 49.9, SD = 25.12 for nicotine; t_{(18)} = 0.411, p = 0.686) as well as in the residual area (M = 286.5, SD = 39.16 for vehicle, M = 283.9, SD = 28.02 for nicotine; t_{(18)} = 0.411, p = 0.686; Figure 14D-E). The pictograms in figure 14 demonstrate movement patterns of a vehicle and nicotine animal over the course of testing. In this case it appears that both rats move in similar non-specific patterns through the residual and zone one areas. In general, rats will spend more time in the residual area but both groups spent a similar amount of time exploring the zone 1 area.
Figure 14. Adult Open Field and Locomotor Testing. In all charts Orange = adult vehicle, Yellow = adult nicotine. N=10 per group. A. Ambulatory distance in 5 minute blocks for the full 60 minutes of testing (each block represents 5 minutes of testing; block 1 = 0-5 minutes, block 2 = 6-10 minutes etc.). There were no statistically significant differences between the adult nicotine and adult vehicle groups, with respect to distance traveled, at any of the time points through testing. B. Total distance travelled in zone 1 (the center portion of the open field apparatus) over the first 5 minutes of locomotor testing. There were no statistical differences between the two groups in distance travelled through zone 1. C. Total distance travelled in the residual area (the surrounding wall portion of the open field apparatus) over the first 5 minutes of locomotor testing. There were no statistical differences between the two groups in distance travelled through the residual area. D. Total time spent in zone 1 (the center portion of the open field apparatus) over the first 5 minutes of locomotor testing. There were no statistical differences between the two groups in time spent in zone 1. E. Total time spent in the residual area (the surrounding wall portion of the open field apparatus) over the first 5 minutes of locomotor testing. There were no statistical differences between the two groups in time spent in the residual area. F. Pictogram of adult nicotine animal’s movement patterns over 60 minutes of locomotor testing. G. Schematic of open field apparatus with zone 1 and residual areas labelled. H. Pictogram of adult vehicle animal’s movement patterns over 60 minutes of locomotor testing.
b. Light Dark Box Testing

Rats were placed in the light side of the light-dark box. The first latency, the time it took from the beginning of the testing to going into the dark side of the box, was taken. There weren’t any significant differences between the two groups while the nicotine group tended to take longer to go into the dark side (Figure 15D; $t_{(18)} = 1.887, p = 0.075$). In general, the two groups took longer times than the adolescents appeared to.

The adult vehicle group took, on average, 32 seconds to run into the dark side (SD = 23.06), while the nicotine group took, on average, 56.3 seconds to run into the dark side (SD = 33.57; Figure 15D). The second latency calculated is the time taken from arriving in the dark side of the box to then re-enter the light side. There again were no significant differences between the two groups ($t_{(18)} = 0.216, p = 0.832$). Both groups took about 70 seconds to return to the light side of the box from the dark side (M = 67.2, SD = 88.45 for vehicle, M = 74.5, SD = 60.29 for nicotine; Figure 15E). Over the 8 minutes of testing, both groups spent over 300 seconds in the dark side (M = 303.93, SD = 81.26 for vehicle, M = 318.04, SD = 32.55 for nicotine) and less than 200 seconds in the light side of the box (M = 173.73, SD = 79.89 for vehicle, 158.7, SD = 29.93 for nicotine; Figure 15B-C). Neither group significantly differed from the other ($t_{(18)} = 0.557, p = 0.584$ for time spent in light side, $t_{(11.85)} = 0.510, p = 0.620$). In general, both the adult nicotine and adult vehicle groups performed at similar levels in all measures of the testing. Both groups were similar in total transitions between the two compartments, with the vehicle group transitioning 14.1 times throughout testing (SD = 4.07), and the nicotine group transitioning 12.2 times throughout testing (SD = 4.78; $t_{(18)} = 0.957, p = 0.351$).
Figure 15. Adult Light-Dark Box Testing. In all charts Orange = adult vehicle, Yellow = adult nicotine. A. Schematic of light dark box arena. B. Average time spent in the dark side of the light-dark box over the 8 minutes of testing. Adult nicotine and vehicle groups did not significantly differ from one another. C. Average time spent in the light side of the light-dark box over the 8 minutes of testing. Adult nicotine and vehicle groups did not significantly differ from one another. D. Latency 1 describes the time it took the animal to run from the light side into the dark side of the box from the beginning of the test. The two groups did not statistically significantly differ from one another. E. Latency 2 describes the time it took the animal to run from the dark side into the light side of the box from the beginning of the test. The two groups did not statistically significantly differ from one another. F. Total transitions through testing.
c. **Social Interaction Testing**

Social Interaction Testing is made up of two 8-minute testing phases. In the first phase, the animal has the option to explore either an empty cage or a rat that they had never interacted with before. In this, both the nicotine and vehicle pretreated groups demonstrated a preference for the stranger rat as opposed to an empty cage. In the first phase of testing, a two way repeated measures ANOVA reveals no effect of treatment on exploration ($F_{(1, 13)} = 0.763, p = 0.398$). Both adult pretreated groups spent similar amounts of time exploring both the stranger ($M = 118.38, SD = 44.69$ for vehicle, $M = 138.36, SD = 56.03$ for nicotine), and the vehicle group spent more time exploring the empty cage ($M = 56.03, SD = 14.62$ for vehicle, $M = 24.17, SD = 20.41$ for nicotine; Figure 16). Both groups spent more than 120 seconds exploring the stranger rat and less than a minute exploring the empty cage, with both groups showed a significant preference for the novel conspecific ($p < 0.001$; Figure 16). The second phase of testing takes place immediately after the first. For the second phase of the testing, the rats have the option to explore either the rat they previously explored in the first phase of experiments or a new stranger rat placed in the previously empty cage. In this 8-minute test, both the nicotine and vehicle pretreated groups again demonstrated a preference for the newer conspecific. Again, there was no treatment effect with respect to the amount of time the groups explored the novel rat ($F_{(1, 13)} = 0.031, p = 0.862$). Both adult groups spent similar amounts of time exploring the newer rat ($M = 118.5, SD = 50.39$ for vehicle, $M = 109.8, SD = 40.50$ for nicotine) as well as the familiar rat ($M = 53.18, SD = 20.12$ for vehicle, $M = 41.17, SD = 20.41$ for nicotine). Both spent more than 120 seconds exploring the novel rat and again less than 60 seconds exploring the familiar rat,
demonstrating a significant preference for the novel rat for both the vehicle and nicotine groups \((p < 0.001; \text{Figure 16})\).

**Figure 16. Adult Social Interaction Testing.** Orange = adolescent vehicle, Yellow = adolescent nicotine. N = 10 per group. Both the adult nicotine and adult nicotine groups performed at similar levels. In the first phase of the test, both groups demonstrated a preference for the novel conspecific \((p < 0.001)\). In the second phase of the test, again both groups demonstrated a preference for the newest conspecific \((p < 0.001)\).

d. **Sucrose Preference Testing**

After 12 hours of water deprivation, rats were placed in a cage with one bottle containing a 2% sucrose solution and one bottle containing tap water. After 1 hour of exploration and liquid consumption, total liquid changes were calculated. In terms of average 2% sucrose solution consumed, the groups did not statistically differ in terms of amount consumed \((t_{(18)} = 0.608, p = 0.551; \text{Figure 17})\). Though the adult nicotine groups on average consumed less sucrose \((M = 13.6, SD = 8.39)\) and more water \((M = 7.7, SD = \text{Figure 17})\).
than the vehicle controls (M = 16.2, SD = 6.75 for sucrose solution, M = 3.9, SD = 2.38 for water), there were no significant differences between the two groups ($t_{(18)} = 0.763, p = 0.455, t_{(18)} = 1.705, p = 0.105$ respectively; Figure 17). Through the testing, the groups consumed similar amounts of liquid, just under 20 mL. The vehicle group consumed 16 mL of the 2% sucrose solution and 4 mL of the water, while the nicotine group consumed 14 mL of the 2% sucrose solution and 7 mL of the water (Figure 17). There was no significant difference in preference for the 2% sucrose solution in the adult vehicle and nicotine groups (Figure 17D). The adolescent nicotine group had a 62% preference (SD = 33.7) for the sucrose solution as compared to the vehicle group that had an 77% preference (SD = 21.94) for the sucrose solution (Figure 17D). Though there does appear to be a trend that may suggests the nicotine group has a decrease in preference for the sucrose solution, but again it was far from statistically significant ($t_{(18)} = 1.157, p = 0.263$).
Figure 17. Adult Sucrose Preference Testing. In all charts Orange = adult vehicle, Yellow = adult nicotine. N = 10 per group. A. The amount of the 2% sucrose solution consumed over the 1-hour test. The two groups did not statistically differ from one another. B. The amount of the tap water consumed over the 1-hour test. The two groups did not statistically differ from one another. C. The total amount of liquid consumed over the 1-hour test. The two groups consumed similar amounts of liquid in total through the test. D. The preference for the 2% sucrose solution. The two groups did not statistically differ from one another.

e. Forced Swim Testing

In forced swim testing, the animal is placed in the tube of water for 15 minutes and the behaviours are analyzed. The main behaviours analyzed are time spent swimming, time spent climbing the walls and time spent immobile. Over the test, the two groups did not statistically differ from one another (Figure 18). The vehicle group spent, on average,
447.05 seconds swimming (SD = 69.37), while the nicotine group spent 416.91 seconds swimming (SD = 68.19; Figure 18). Both groups spent slightly less time immobile than swimming and the two groups did not significantly differ from one another in either measure ($t_{(18)} = 0.586, p = 0.565$, $t_{(18)} = 0.980, p = 0.340$ respectively; Figure 18). The adult nicotine group spent 352.89 seconds immobile over the test (SD = 89.92), while the vehicle group spent 333.16 seconds immobile (SD = 57.01). Both groups performed similarly when the climbing behaviour was analyzed. Both groups spent around 125 seconds climbing ($M = 119.78$, SD = 36.49 for vehicle, $M = 130.20$, SD = 46.43 for nicotine). The behaviour of climbing was typical to the beginning of the test as the initial behaviour observed. This usually lasted for the first few minutes of the test until the rats began the swimming behaviour and exploratory dives.

![Figure 18. Adult Forced Swim Testing](image-url)

**Figure 18. Adult Forced Swim Testing.** Orange = adult vehicle, Yellow = adult nicotine. The amount of time over a 15-minute testing period spent swimming, climbing or immobile. The adult vehicle and adult nicotine group did not significantly differ on any measure analyzed, including swimming and climbing behaviours and time spent immobile.
3.22 Cognitive Results

Cognitive paradigms were all run 31 days after the chronic exposure to nicotine or a saline control, at PND 105. There were no pharmacological interventions after the adolescent protocol.

a. Object Recognition in Temporal Order

Object recognition in temporal order takes the requirements from object recognition, explained in the adolescent section as well as methods, and adds a second training phase with a different pair of objects. Finally, the testing phase consists of one of each of the objects previously explored. Adding in the second training phase forces the animal to not only remember the first object, but the second as well as integrating that information in the testing phase of this task. A two way repeated measures ANOVA doesn’t describe an effect of treatment on exploration during the first phase of training (F (1, 16) = 1.114, p = 0.307). Both the adult nicotine and adult vehicle groups explored both sets of the object equally (for the vehicle group: M = 22.18, SD = 8.72, M = 26.21, SD = 10.69; t (9) = 1.014, p = 0.337, and for the nicotine group: M = 21.72, SD = 10.80, M = 19.2, SD = 14.56; t (7) = 0.488, p = 0.641), there were no differences between the groups (Figure 19A). In the second phase of the task, a two way repeated measures ANOVA reveals no effect of treatment on exploration (F (1, 16) = 1.788, p = 0.200). The two groups spent similar time spent exploring the two identical objects (for the vehicle group: M = 23.72, SD = 12.83, M = 24.05, SD = 12.27, and for the nicotine group: M = 23.52, SD = 9.41, M = 25.46, SD = 6.10; Figure 19B). A two way repeated measures ANOVA assessing the
testing phase again describes no treatment effect on exploration ($F_{(1, 16)} = 0.066, p = 0.800$). During the testing phase both adult groups spent more time exploring the object from the first phase of training than with the object from the second phase of testing. *Post hoc* tests described a trend towards a preference for the object from the first training phase as opposed to the second, though data did not reach significance ($p = 0.067$ for the vehicle group and $p = 0.165$ for the nicotine group). The adult nicotine and adult vehicle groups spent similar amounts of time exploring the object from the second phase of testing ($M = 10.72, SD = 5.43$ for vehicle, $M = 8.26, SD = 5.54$ for nicotine) as well as from the first phase ($M = 18.15, SD = 10.94$ for vehicle, $M = 14.34, SD = 7.89$ for nicotine; Figure 19C). In this case it is important to note that the two groups did not reliably differ from one another in any of the phases of testing with both groups behaving in stereotypic manors.
Figure 19. Adult Object Recognition in Temporal Order Testing. Orange = adult vehicle, Yellow = adult nicotine, Black = object from first training phase, Grey = object from second phase. A. Training phase 1 of the task, two identical objects were explored. Bars represent the amount of time spent exploring the two identical objects. The vehicle and nicotine group did not differ in their time spent exploring the two objects. B. Training phase 2 of the task, two different identical objects were explored. Bars represent the amount of time exploring the two identical objects. The vehicle and nicotine group did not differ in their time spent exploring the two objects. C-D. The testing phase of the task represented as the two objects (one from the first and one from the second phase of training) and then as the two groups (adult nicotine and adult vehicle) to best visualize the differences. The vehicle and nicotine group did not differ in their time spent exploring the two objects.
4 Discussion

The present series of experiments demonstrates that chronic nicotine during a specific critical period of adolescent neurodevelopment can induce an affective disorder-like phenotype as demonstrated through behavioural, molecular and neuronal analyses. These significant changes in behaviour, cellular signaling as well as molecular abnormalities are specific to the adolescent period and are not present when rats were exposed to nicotine during adulthood.

Behaviourally, the adolescent nicotine group differed from the vehicle group in some measure with each assay run. In the locomotor testing there were no significant differences in total distance travelled. Drugs of abuse have previously been described to have progressive and long lasting enhancement on locomotor sensitivity with repeated exposure (Vaijent et. al, 2010). Acutely, 0.4 mg/kg of nicotine increases basal locomotor activity and, like other drugs of abuse, can lead to locomotor sensitization (Zaniewska, McCreary & Filip, 2009). In our results, there was no indication of locomotor sensitivity nor increased basal activity for neither adolescent nor adult pretreated rats. It is important to keep in mind, at the point of testing, there has been a 30-day drug washout period. So, at this point there is no effect of the drug on locomotor activity, which could suggest that the rats are no longer in a state of withdrawal or dependence on nicotine. While it is possible that an increased sensitivity to nicotine would be present during a re-administration test, at our testing point, it appeared that nicotine did not have a long-term effect on basal locomotor activity states. This furthermore suggests that any differences
in other behavioural paradigms involving exploration are not merely a function of locomotor artifacts, but instead are likely due to selective differences in underlying affective, cognitive and/or neuronal and molecular phenotypes induced by nicotine exposure.

**Adolescent Nicotine Exposure Induces a Persistent Anxiety-Like Behavioural Phenotype**

As demonstrated in both open field testing and light dark box testing, there was an anxiogenic phenotype found in the adolescent nicotine pretreated rats as compared to their vehicle controls. In both tests, adolescent nicotine rats tested at adulthood spent significantly less time in the open, more vulnerable areas of each testing apparatus (Figures 5 & 6). In these types of tests, rodents tend to avoid the unprotected areas of a novel environment, the light/open side and the center portion of the open field (Ohl, 2003). Rodents, in these novel environments, are conflicted between the drive to explore the unknown area and the motivation to avoid potential danger (Ohl, 2003). Nicotine pretreated rats spent even less time in these unprotected areas displaying a higher level of unconditioned anxiety as compared to the adolescent vehicle pretreated rats. When looking at the adult cohort, there were no differences between the groups as both groups spent similar amounts of time exploring these unprotected areas, and therefore, displayed similar levels of unconditioned anxiety. Altogether, results from the light-dark box and open field testing describe a persistent anxiety-like phenotype in the adolescent nicotine pretreated rats but not in the adult cohort pretreated with nicotine.
Furthermore, in social interaction testing, adolescent rats pretreated with nicotine displayed a decrease in exploration of strangers in two different phases of testing. All groups (vehicle and nicotine) of adolescent and adult rats, displayed a preference for the newest conspecific as compared to an empty cage and more familiar animal (Figure 7 & 16). Adolescent nicotine pretreated rats, but no other group (adolescent vehicle, adult nicotine and adult vehicle) displayed social withdrawal, when it came to exploration of the newest conspecific (in both phases). It is important, at this point, to note that there were no significant differences in locomotor testing for total movement, nor in object recognition for exploration or recognition (Figures 11 & 19). This suggests that the differences in exploration of the stranger and novel rats, in the adolescent nicotine group, are due to anxiety and social withdrawal as opposed to differences in exploration of novel objects or differences in total movement. Again results from both the adolescent and adult cohorts describe a persistent anxious and social deficit in the adolescent nicotine pretreated rats but not in the adult cohort pretreated with nicotine.

As described in the introduction, the most heavily reported long-term effect of nicotine exposure is the anxiogenic effects (Dwyer, McQuown & Leslie, 2009; Goriounova & Mansvelder, 2012; Slaweki et al., 2003). For example, in 2003, Slaweki and colleagues described the anxiogenic effects of adolescent nicotine exposure using variations of an open field protocol. Two weeks after 31-36 day old rats were exposed to nicotine, rats were tested in two variations of open field testing, in which different aspects of exploratory behaviour were analyzed. Results described an anxiogenic effect of nicotine on the exploratory behaviour of the rats treated (Slaweki et al., 2003). Anecdotally, in humans, smokers use cigarettes to calm down when life stressors make
them anxious, suggesting an anti-anxiety property to smoking, while many smokers are highly anxious people. Anxiety disorders, and mood disorders like depression have strong links to substance abuse problems, including nicotine dependence (Schulz & Arora, McCarron et al., 2016; Stein & Sareen, 2015). Supporting the preclinical studies as well as the high occurrence of smoking and anxiety disorders, results from this set of experiments concurs with the current body of literature linking nicotine-dependence to anxiety disorders. Furthermore, we have described a persistent anxiogenic phenotype, that lasts longer than the 2 weeks described by Slaweki and colleagues in 2003. Our results describe an anxious phenotype after a drug washout period of over a month. Furthermore, our results describe this persistent anxiogenic phenotype as being a function of developmental period, not just a result of nicotine dependence.

**Adolescent Nicotine Exposure Induces Persistent Depressive-Like Symptoms**

Both sucrose preference and forced swim testing can be used to assess two symptoms of depressive-like behaviour in an animal model. In both tests adolescent nicotine rats displayed depressive-like symptoms, while the adult nicotine group did not significantly differ from the control group (Figure 9 & 17). In sucrose preference testing, control rats will typically demonstrate a preference for the sucrose solution, especially after a period of water deprivation (D’Aquila, Brain & Willner, 1994). This is because the sucrose solution is innately more appetitive and rewarding (D’Aquila et al., 1994). In these experiments, the adolescent nicotine rats had a significantly decreased preference for the 2% sucrose solution as compared to the water (Figure 8). This is proposed to be an
indication of anhedonia, an inability to feel pleasure, which is a key symptom in depression (Schulz & Arora, 2015). In the adult cohort, there was no statistically significant differences between the nicotine and vehicle pretreated groups (Figure 17). There seemed to be a trend in the data for the adult cohort that may indicate a level of anhedonia as compared to the vehicle group, but, it is first and foremost, not as pronounced as the deficit seen in the adolescent cohort, and secondly, not statistically significant so cannot be reliably described as a deficit (Figure 17).

In the forced swim test, one of the most important behaviours to analyze is the amount of time spent immobile. This time spent immobile describes a level of resigned behaviour in which the animal has given up on swimming and theoretically survival (Bogdanova et al., 2013). With the adolescent nicotine pretreated group, there was a significant increase in time spent immobile compared to the vehicle control (Figure 9). This suggests that the adolescent nicotine group, but not the vehicle group, shows persistent depressive-like behaviours. The adult cohort (vehicle and nicotine) spent a larger amount of time immobile (more similar to the adolescent nicotine group; Figure 17). Age and weight play a major factor in behaviour in forced swim testing. The rats used in the adult cohort were quite large (close to 1 kg at the time of testing), and therefore were much larger than rats used in other studies using forced swim test paradigms (Bogdanova et al., 2013). The higher fat content and stabilized ability to float could account for the significant differences and increases in time spent immobile (Bogdanova et al., 2013). Based on the many articles addressed in this review from 2013, comparing results from young adults to much older and larger adults, is inappropriate and should not be done. Based on this we can compare the results from each group to their
control. With that we see that the adolescent nicotine but not the adult nicotine demonstrates persistent depressive symptoms in the forced swim test.

Our results from the depressive experiments are also in accordance with epidemiological studies of adolescent nicotine use. In 2010, Boden and colleagues described a causal link between adolescent smoking and the later development of depression. The group followed a longitudinal experimental design in which they followed adolescents from birth, 1 year and then annually until the age of 16 and then three more times at ages 18, 21 and 25 with a battery of questionnaires and assessments to analyze their smoking habits as well as their depressive symptoms (Boden, 2010). They found that the higher smoking rates were related to greater symptoms of depression. Using statistical modelling methods, the group describes a causal link between smoking frequency, or nicotine dependence, and major depression (Boden, 2010). Results from the analyses presented in this thesis, not only support the causal link found in the human model of nicotine dependence and smoking, but further describe this link as being a function of developmental period. Similarly, to the findings presented by Boden and colleagues (2010), we found that chronic exposure to nicotine during adolescence led to persistent depressive symptoms in adulthood, as described through assays performed during adulthood, or later on in development. Furthermore, we describe here, that the development of depressive symptoms is a function of the developmental period in which the animal was exposed to nicotine. When rats were exposed to nicotine during adulthood, they did not differ from their vehicle age-matched cohort, while those that were exposed to nicotine during adolescence, significantly differed from their age-matched vehicle counterparts.
Adolescent Nicotine Exposure Induces Selective Deficits in Higher Cortical Cognitive Tasks

Depression and anxiety-related disorders have been associated with cognitive deficits, particularly with episodic memory and learning, processes that are highly dependent of PFC function (Austin, Mitchell & Goodwin, 2001). In this set of experiments, two variations of an object recognition test were used to analyze long-term cognitive functions of rats. In the object recognition task, we analyze the ability of the animal to distinguish between familiarities of previously encountered objects. In this task, it is expected that the rats will spend more time with a novel object, as opposed to a familiar object previously encountered (Hannesson et al., 2004). This is a very basic memory task that has been linked to hippocampal function (Hannesson et al., 2004). Only the adolescent cohort was tested in this task, as following the testing and analysis, no deficit was found suggesting there is no cognitive impairment with respect to this task following adolescent nicotine exposure. Due to not finding any deficits following the adolescent nicotine exposure, the neurodevelopmental period of interest for these studies, no adult experiments were performed.

Next, we used, the object recognition in temporal order task, which is a more complex memory task to measure the episodic memory. In this task, rats have to discriminate between the order of presented objects. Typically, rats will spend more time exploring the object they were first exposed to, which involves the perirhinal cortex as well as the medial PFC (Hannesson et al., 2004). In this task, both the adolescent and
adult cohorts were tested. In both the first and second training phases of the task, all
groups (vehicle and nicotine) of adolescent and adult rats show similar times exploring
the two identical objects, suggesting that there was no preference for one area of the
testing apparatus or side bias (Figures 11 & 19). In the testing phase of the task, the
adolescent nicotine pretreated group spent an equal time exploring both the more recent
and less recent objects. This could be thought of as remembering both objects, but does
vary from stereotypic behaviour as demonstrated by both the adolescent vehicle group,
both the nicotine and vehicle group in the adult cohort as well as previous literature
(Figures 10 & 18, Hannesson et al., 2004). All results considered, again it appears as
though adolescent, but not adult, chronic nicotine exposure leads to long term changes to
episodic memory.

Results from cognitive tasks, are in accordance with the current body literature,
regarding both preclinical and epidemiological studies. For example, long-term cognitive
deficits have been observed in rats pretreated during adolescence with nicotine and then
tested in the 5-choice serial recognition task during adulthood (5-CSRT; Counette et al.,
2009). The 5-CSRT is an operant based task used to study attention and impulse control
in rodents. In this task, rats must watch for a light and press the lever associated with
said light, there are 5 choices for levers to press. First the animal must be trained on the
paradigm and learn the rules of the task, and then respond correctly to the stimuli. In the
5-CSRT, authors showed that the rats pretreated with nicotine during adolescence, had
significantly higher premature responses, as well as a significantly higher proportion of
incorrect responses to the task when tested in adulthood suggesting impulsivity and
attentional deficits (Counette et al.,2009). Furthermore, Dwyer, McQuown & Leslie
(2009), describe deficits in both spatial and serial recognition tasks in animal models. Indeed, these cognitive deficits found following nicotine exposure are not limited to preclinical studies of nicotine use. For example, a clinical study supported the association between smoking and cognitive problems in young people (Chamberlain et al., 2011). Through a battery of neuropsychological tasks, adults between the ages of 18 and 29 were assessed with groups being split into smokers and non-smokers (Chamerlain et al., 2011). Authors described the significant cognitive impairments on sustained attention, spatial working memory, executive planning as well as risk evaluation. In line with results from this clinical study, results from our set of experiments, show persistent deficits in executive function following adolescent nicotine exposure. As demonstrated in the ORITO task, during integration of information from the two training phases of testing, as well as in memory, but in temporal memory as opposed to spatial.

**Adolescent Nicotine Exposure Induces a Persistent Hyper-Dopaminergic State in the Midbrain Ventral Tegmental Area and Associated Molecular Adaptations in the Prefrontal Cortex**

The adolescent cohort was further analyzed at the cellular and molecular levels. Using *In Vivo* electrophysiological recordings, we analyzed baseline firing patterns of DA neurons in the VTA (Figure 12). Adolescents chronically exposed to nicotine had, in adulthood, a significantly higher VTA DA neuronal activity as well as a significant increase in bursting compared to the chronic vehicle group. This suggests that the adolescent nicotine group were in a hyperdopaminergic state within the mesolimbic DA
pathway that projects from the VTA to multiple areas, including the PFC and NAcc (Swanson, 1983). Due to the innervation of the PFC from VTA efferents, the implications of both structures (and DA) in depression, in the motivational effects of nicotine, and in higher order cognitive tasks, molecular analyses of the PFC were run (Swanson, 1982; Laviolette et al., 2008; Schulz & Arora, 2015; Hannesson et al., 2004). To begin the molecular analyses, D1 and D2 receptor levels were analyzed in the PFC (Figure 13). Our results demonstrate a significant downregulation in PFC D1 but not D2 receptor levels in adult rats treated with nicotine during adolescence. PFC D1 receptors, have been implicated in working memory and executive functioning, while PFC D2 stimulation does not greatly affect these two functions (Floresco, 2013). Furthermore, D1 receptors have been implicated upstream in the de-phosphorylation of ERK, moving ERK from the phosphorylated, active, state to the dephosphorylated, inactive, state (Girault et al., 2007). This function of D1 receptors makes sense as there is not only a downregulation in D1 receptors, but also a large increase in phosphorylated ERK1/2 levels (Figure 13). Interestingly, we found changes in PFC phosphorylated ERK1/2 levels, but not in total ERK1/2 levels in adult rats treated with nicotine during adolescence. This suggests that there is not a deficit in the production or elimination of ERK1/2, only in the de-phosphorylation state. The ERK signaling pathway has previously been implicated in addiction, and is involved in many different cell functions, including cell growth and differentiation as well as neuronal plasticity (Girault et al., 2007). Therefore, a large upregulation in ERK1/2 function could potentially have vast effects on PFC functioning and efficiency. Bruchas and Chavkin (2010), previously described the role of ERK, through the kappa opioid receptor system, in the stress-induced responses including both
anxiety and depression. In contrast, CaMKII is an effector protein that is implicated in long-term potentiation of a synapse and ultimately activated through NMDA-type glutamate receptors, which allow an influx of Ca\(^{2+}\) (Shifman et al., 2006). In our results there were no significant changes in CaMKII levels in the adult PFC of adolescent nicotine pretreated rats, lending to the idea that there is a disturbance in the DA system following chronic adolescent nicotine that leads to changes in the DA-D1-ERK receptor linked pathways, but not in the DA-D2-CaMKII related signaling pathway. Based on its role in cell proliferation, it’s not surprising that CaMKII has also been implicated in mood and anxiety disorders. In 2004, Du and colleagues described the role of CaMKII in depression and anxiety disorders through modification by serotonin reuptake inhibitors (SSRIs). The group describes the increased auto-phosphorylation and therefore activity of CaMKII in the hippocampus following treatment with selective serotonin reuptake inhibitors (SSRIs; Du et al., 2004). It is important to note that SSRIs are commonly prescribed on their own or in conjunction with therapy in the treatment of both depression as well as anxiety disorders. Furthermore, both pathways have been previously implicated in reward processing/reward memory following exposure to drugs of abuse, such as opioids and nicotine (Lyons et al., 2013; Rosen et al., 2016). Both molecular pathways analyzed, have been previously associated with the affective disorders described in behavioural experiments, though only the D1-ERK1/2 pathway was implicated in our experiments, within the PFC.

All results considered together describes a depressive, anxious phenotype with corresponding social and cognitive deficits. Considering depression in a general sense, it is commonly diagnosed as co-morbid with anxiety disorders, substance abuse problems
as well as with compulsive control disorders (Schulz & Arora, 2015). Furthermore, symptoms include anhedonia, suicidal thoughts, feelings of worthlessness (in animal models can be thought of as resigned behaviours in FST), impairments in social functioning and integration cognitive deficits (McCarron et al., 2016; Hannesson et al., 2004). Neuroanatomically, associations have been made between the PFC, cingulate cortex, hippocampus and striatum (Schulz & Arora, 2015). In our results, behaviours describe a good portion of symptoms described in the DSM-5 for diagnosis of depression in humans. Ultimately, the present results demonstrated that in adolescent rats chronically exposed to nicotine, a persistent depressive and anxiety-like phenotype takes place that is present at least until adulthood. These same long term changes were not found when an adult cohort was chronically exposed to nicotine and tested following the same latency between exposure and testing. Results from our adult cohort lends to the conclusion that the deficits and changes found in our adolescent cohort are a function of being exposed to nicotine during a critical and plastic period of development and not a function of chronic nicotine exposure itself. Furthermore, these results are supported by significant molecular and cellular changes in the adolescent nicotine cohort. A month following the chronic nicotine protocol, adolescent rats were found to have persistent changes in DA cell activity and resulting D1 receptor and ERK1/2 concentrations. 

Chronic nicotine-dependence is maintained through the dopaminergic pathway (Laviolette & Van Der Kooy, 2004). Nicotine, in chronic dependence, results in an increase in DA transmission through the mesolimbic pathway (Laviolette & Van Der Kooy, 2004). Results from In Vivo electrophysiology during adulthood demonstrate that this increase in DA release in the mesolimbic pathway persists into adulthood. This
persistent increase in DA release, resulted in a downregulation in D1 receptors on the postsynaptic membrane, which then downstream lead to an upregulation in the phosphorylated form of ERK1/2 in the PFC. The change in dopamine transmission, described through both In vivo electrophysiology as well as western blots, underlies the depression and anxiety phenotype. Major depressive disorder is associated with diminished dopaminergic neurotransmission by way of diminished DA release or impaired signal transduction (Dunlop & Nemeroff, 2007). Similarly, DA polymorphisms have been implicated in anxiety disorders, describing a deficit in transmission of DA (Gallagher & Schrag, 2011). In our experiments the anxious and depressive phenotype was due to the impaired signal transduction from the downregulation of D1 receptors. Furthermore, we know from previous studies described, that phosphorylated ERK1/2 is also implicated in depression and anxiety disorders, a result that is supported by this study. Finally, the mesolimbic pathway is important in learning and memory, along with ERK and CaMKII. Dysregulation and disruption to this pathway, as we found with our experiments, resulted in cognitive deficits in higher order cognitive tasks.

**Future Directions**

Based on results from this set of experiments, further cellular and molecular analyses should be performed on other structures of interest, such as the BLA, vHipp, NAcc and PFC. Based on results from the molecular analyses in this study, with the PFC. Further cellular analyses using In Vivo electrophysiological recordings could further describe the role and relationship of the PFC in the vast changes that take place following
adolescent nicotine exposure. Furthermore, other areas of interest could be the vHipp, because of its’ role in memory and our deficits found in object recognition in temporal order, the NAcc, because of its’ role in the mesolimbic pathway and reward processing, as well as the BLA due to its’ role in emotional regulation, processing and memory. Assessing molecular changes, with respect to the up and downregulation of key molecules in cellular process pathways, as well as changes in baseline firing in these structures, could further lend to what long term changes underlie the significant behavioural changes described in this thesis. Furthermore, pharmacological reversals should be attempted to further understand and begin treatment options for those suffering from nicotine addiction, and the associated deficits that have resulted from adolescent nicotine use. A start for pharmacological reversals would be micro-infusions of ERK1/2 inhibitor into the PFC. Based on results described in this thesis, we would expect that blocking ERK1/2 in the PFC of a nicotine pretreated animal would lead to a reversal of behavioural deficits. Based on the role of ERK1/2 in anxiety and mood disorders, we would expect a reversal of the anxiety and depressive symptoms. Furthermore, we would expect a reversal in the cognitive deficits found in ORITO, as ERK1/2 is involved in neuronal plasticity and the PFC is important in integration of information.

Limitations of the Current Studies

A clear limitation to this work is the difficulty of describing depression, a highly complex mood disorder, in an animal model because of the difficulty describing a feeling or mood in an animal model. By using a combination of behavioural, cognitive, In Vivo electrophysiology and western blot techniques, that characterize different aspects of the depression, we have attempted to best describe the model as a depressed-phenotype to
meet the diagnosis criteria set by the DSM-5. Though there is still much criticism on animal models of depression, as some of the core features of depression may be ‘un-modellable’ in an animal model (Matthews et al., 2005).

Another limitation to our study is the method in which the rats received the nicotine. The nicotine was injected subcutaneously into the animal, as opposed to a self-administration paradigm. This means that the rats were stressed when infused with the nicotine solution as opposed to administering it themselves. Furthermore, this limits our knowledge as to whether or not the animal was addicted to nicotine or if nicotine acted as a stressor to the animal.
References


Curriculum Vitae

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Adolescent nicotine exposure induces long-term anxiety and depression-like symptoms through molecular and neuronal dysregulation of mesocorticolimbic dopamine D1 and ERK1/2 signaling pathways.

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Long-term effects of chronic adolescent nicotine exposure on anxiety and social interaction: Implications for psychiatric disorders

Long-term effects of chronic adolescent nicotine exposure: Implications for psychiatric disorders.

Publications