

Role of Inflammatory Microglia in Mediating Periadolescent Nicotine-Induced Reward
Sensitization

by

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Nicotine exposure during the onset of adolescence (periadolescence) is demonstrated to sensitize the brain to the rewarding effects of various drugs of abuse. Nicotine enhances the expression of the transcription factor Δ FosB that may facilitate this process. It is suggested that microglia, the resident immunocompetent cells in the brain, may contribute to this sensitization. This study was conducted to determine whether suppression of inflammatory microglia impacts nicotine's ability at periadolescence to induce sensitization to cocaine in the adult rat. To achieve this, minocycline was administered at a dose of 30 mg/kg, 30 minutes prior to 10 once-daily doses of 0.4 mg/kg nicotine during periadolescence (postnatal days 35-44) in male Sprague Dawley rats. FosB-like immunoreactivity (FosB-ir) in key brain reward and memory areas, and conditioned place preference (CPP) were used to assess changes at the molecular and behavioral levels. Nicotine enhanced FosB-ir the dentate gyrus (DG) of hippocampus by 52% over vehicle control, while minocycline pretreatment attenuated the density of FosB-ir neurons in the DG by 34% compared to nicotine at PND 45. Similar, but not statistically significant reductions were observed in the nucleus accumbens (NAc), and medial prefrontal cortex (mPFC). This trend continued when the rats were allowed to mature into adulthood (PND 80) in both the NAc and mPFC, with minocycline pretreatment showing a significant decrease in FosB-ir by 36.7%

compared to nicotine in the mPFC. The significant reduction in FosB-ir observed at PND 45 in the DG with minocycline pretreatment was reversed at PND 80. CPP studies indicated that both nicotine and minocycline-prior-to nicotine enhanced the rat's anticipation for challenge cocaine following adult conditioning by over 100% with no significant difference between the two. These results suggest that nicotine-induced inflammatory (M1) activation of microglia may be important for the induction of FosB protein, but not related to the sensitization of the adult rat to cocaine. The results further suggest a mechanism separate from induction of FosB expression in producing the long-term changes induced by nicotine.

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Department of Pharmacology and Toxicology

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List of Abbreviations

NACHR	Nicotinic acetylcholine receptor
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate
ANOVA	Analysis of Variance
AP-1	Activator protein complex
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
cdk5	cyclin-dependent kinase 5
CD	Cluster domain protein
C5ar	Complement 5a Receptor
CK-2	Casein kinase-2
CNS	Central nervous system
CPP	Conditioned place preference
DAB	Diaminobenzadine
DAMP	Damage-associated molecular pattern
DAR	Dopamine receptors
DARPP	Dopamine- and cAMP regulated phosphoprotein
DAT	Dopamine Transporter
DG	Dentate gyrus
DOA	Drug of abuse
E-cigs	Electronic cigarettes
FosB	FBJ murine osteosarcoma viral oncogene homolog B
FosB-ir	FosB-like immunoreactivity, FosB-immunoreactive
Fra	Fos related antigens
GABA	γ -Aminobutyric acid
GDNF	Glia-derived neurotrophic factor
Iba1	Ionized calcium-binding adaptor molecule-1
IEG	Immediate early gene

IL	Interleukin
Ip	Intraperitoneal
kDA	Kilo dalton
LPS	Lipopolysaccharide
MDMA	Methylenedioxy-methamphetamine
MHCII	Major histocompatibility complex II
mPFC	Medial Prefrontal Cortex
MSN	Medium spiny neurons
mRNA	Messenger ribonucleic acid
NAc	Nucleus accumbens
NADPH	Nicotinamide adenine dinucleotide phosphate
NGS	Normal goat serum
NMDA	N-methyl-D-aspartic acid
NOS	Nitric oxide synthase
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PND	Postnatal day
ROS	Reactive oxygen species
SD	Sprague Dawley
TLR	Toll-like Receptor
TNF	Tumor necrosis factor
VTA	Ventral tegmental area
YRBS	Youth Risk Behavior Surveillance

Chapter 1: Problem Description

The epidemic of psychoactive drug abuse is a world-wide phenomenon that is burdensome not just to individuals and families, but to nations as a whole. Pathologies due to drug abuse result in elevated healthcare spending, untimely deaths, as well as losses in productivity. Drug use can occur at any age during an individual's lifespan, but exposure during adolescence is especially critical as the brain is undergoing structural and neurochemical plasticity in preparation for adulthood (Kandel 1975, Arain et al. 2013). Drug exposure during this time of rapid plasticity increases the probability of an individual becoming substance-dependent, or vulnerable to the rewarding effects of psychoactive substances in the future, a process referred to as 'sensitization' (Robinson and Berridge 2008). It is currently understood that the underlying bases for this effect are persistent structural and neurochemical changes that alter the subject's perception of psychoactive substances during the life of the individual. During this period, changes occur in the brain that increase the abuse liability of psychoactive substances, and thus sensitizes the brain in a way that makes the individual more vulnerable to the psychoactive effects of drugs (Kandel 1975, Kandel and Kandel 2014).

Nicotine, named after *Nicotiana tabacum* tobacco plant, is a psychoactive substance that is commonly available in tobacco-laced products and is conducive to enhancing sensitization in the brain (Koob et al. 2014). Although tobacco smoking has declined, nicotine continues to be widely accessed in the form of E-cigarettes (E-cig) or vaporizers by adults and teenagers. Nicotine use is widespread among the youth, not just in the United States, but world-wide. National data from the Youth Risk Behavior Surveillance (YRBS) system for 2015 indicate that youths in grades 9-12 on at least one day during the previous 30 days had smoked cigarettes (10.8%), used cigars or cigarillos (10.3%) or used an electronic vaporizer (24.1%): a total of

31.4% subjects had used one of these nicotine containing products. Further, 6.6% reported smoking a cigarette before age 13 (Arrazola et al. 2015). Clearly, tobacco and nicotine in vaporizers remain one of the most commonly abused drugs during the developmental period of adolescence. We and others, believe that this use is not without consequences. A 2012 survey conducted among US adults indicated that a large percentage of cocaine users (87.9%) used tobacco products prior to using cocaine (Kandel and Kandel 2014). This highlights the need to better understand how nicotine exposure during adolescence reorganizes the brain so that molecular targets can be further identified and therapeutic novelties to curb the brain's vulnerability to drugs of abuse in the future can be generated.

Chapter 2: Literature Review

2.1 Gateway Hypothesis

Adolescence is a vulnerable time for brain development as a child transitions into an adult.

During this critical period, the brain is undergoing maturation that is partly driven by changes in pubertal hormone levels. Perturbations during this vulnerable time window could result in long-lasting neuronal maladaptations that can alter an individual's behavior as an adult, or the way the individual responds to changing circumstances. The development of reward sensitivity is one such example. Exposure to drugs of abuse such as nicotine during adolescence can cause alterations in neuronal pathways that sensitize the brain so that it responds differently to psychoactive drug stimuli in the future. More importantly, an individual may develop a predisposition to experimenting with or abusing psychoactive substances in the future due to the development of persistent neuronal modifications resulting from previous experiences (Adriani et al. 2003, Casey et al. 2008). This 'development of predisposition' is an example of the 'Gateway' hypothesis of drug abuse (Kandel 1975). The concept is that adults who are addicted to powerful drugs such as cocaine or narcotics are likely to have started their journey as pre- or early teen-agers with drugs or substances that are inexpensive and available to the youth of that age. In addition to nicotine from tobacco and vaporizers, the youth can access alcohol, marijuana, and other cheaply accessible products that can be used for experimentation, leading some to progress on to more dangerous and potent drugs. Thus, it is important to understand why the early adolescent is vulnerable to these gateway drugs and the altered neuronal functions that increase the probability for developing a severe substance use disorder in the more mature subject

2.2 Vulnerability of brain during adolescence

During adolescence, the brain is vulnerable to perturbations by drugs and toxins, as the foundation is being laid for the refinement of neuro-behavioral circuits, especially those pertaining to sexual and social behaviors (i.e. maturation, Schulz and Sisk 2006). This window of development is characterized as a time for impulsivity, sensation-seeking, and experimentation (i.e. risk taking, Spear 2000). Many anatomical and physiological changes are occurring in the brain during this window that promote plasticity, or the ability of the brain to change or adapt, and this expectedly manifests into alterations of behavior (Arain et al. 2013). During this transitional epoch, the brain is not necessarily changing in volume, but in its organization. Different brain nuclei are maturing at different rates. Maturation typically commences with modifications of phylogenetically older circuits such as the limbic system, followed by maturation of circuits of the frontal and parietal cortex that are associated with impulse control and executive functioning (Gogtay et al. 2004, Yuan et al. 2015). The differential maturation of the limbic system, which includes the reward system, compared to that of frontal cortical systems is believed to be responsible for the impulsive behaviors observed in adolescents. Concurrent synaptic pruning and strengthening based on experience promotes the steady decline of gray matter and an enhancement in myelinated neurocircuitry or white matter as development progresses (Blakemore et al. 2010). Changes in neurochemistry over the course of adolescence allow for the transition of neural connectivity from a local to a wider span, in that the brain progresses toward more functional integration via recruitment of working memory-associated nuclei such as hippocampus and PFC (Fig. 2.1). The pubertal hormones play an intimate role in the remodeling of circuits to modify social, sexual, and reward behavioral circuits in the brain (Blakemore et al. 2010). Furthermore, such modifications persist long after

the hormonal surge (Schulz and Sisk 2006, Sato et al. 2008). Disruption of hormonal functioning during puberty can alter neuronal chemistry and functioning in a way that can alter the brain's responsivity to a variety of stimulants in the future (Varlinskaya et al. 2013). In the adolescent brain, gonadal steroids facilitate neurogenesis, gender dimorphism, neurite outgrowth, and brain organization that manifest into persistent behavioral changes (Arain et al. 2013).

2.2. A. Role of Pubertal hormones and maturation of CNS

Adolescence starts with the onset of puberty, and is marked by the reactivation of the hypothalamus-pituitary-gonadal (HPG) axis. Gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the release of sex hormones that facilitates neuronal plasticity and sexual dimorphism (Swerdloff and Odell 1975). Puberty in human males commences roughly by the age of 10 and is completed by the age of 15 with maturity of spermatogenesis (Vigil et al. 2016). This is commonly a time window when human teenagers expose themselves to nicotine, and according to Youth Risk Behavior Survey (YRBS), males are more likely to report use of nicotine-containing products than females (Arrazola et al. 2015). During this period, sex hormones such as estrogen, progesterone, and testosterone accelerate the maturation of a dormant neurocircuitry that was initiated during the perinatal stage (Gillies and McArthur 2010). Furthermore, sex hormones activate glia that contribute to myelination of neurons by acting as trophic factors (Schwarz and Bilbo 2012).

Pubertal onset is physically recognizable in males in both humans and rodents by a characteristic enlargement of testicles and rapid weight gain from increased food consumption (Soliman et al. 2014). The brains of adolescent males are enriched with androgen (AR) and estrogen (ER) receptors (Sato et al. 2008). Testosterone, produced primarily in the male testis, acts on ARs, and is necessary for the development of circuits that promote rewarding sexual and

reproductive behaviors in males (Sisk and Zehr 2005, Sato et al. 2008). Testosterone is aromatized to estradiol via brain aromatases that further promotes masculinization of the brain (Wu et al. 2009). Aromatases are prevalent in the male brain in regions such as amygdala, midbrain, cortex, and hippocampus (Barbieri et al. 1986, Gillies and Mc Arthur 2010). The importance of estrogen in neural circuit development is highlighted by aromatase knockout models that display a spontaneous apoptosis of dopaminergic (DA) neurons in the medial preoptic area (MPOA) and arcuate nucleus of hypothalamus of male mice (Hill et al. 2004, Gillies and McArthur 2010), lending support to the importance of hormonal homeostasis in neurogenesis and DA activity. Alterations in hormone synthesis and function due to xenobiotic exposure during adolescence can potentially impact plasticity, leading to aberrations in neurogenesis and ultimately behavior (Wu et al. 2009).

Exogenous influences such as drugs of abuse from different classes impact hormonal homeostasis and affect motivational and reward-seeking patterns. Opiates and cocaine, for example, are common substances that teenagers can access, and exposure to these substances can profoundly impact the HPG and hypothalamic-pituitary-adrenal (HPA) axes. Opiates such as methadone inhibit GnRH and DA activity in the hypothalamus and in effect reduce testosterone and upstream luteinizing hormone (Brown et al. 2006, Singh et al. 1982). Opioid use can also result in adrenal insufficiency and hypogonadism, which are associated with depression, impaired cognition, and drug abuse (Brown et al. 2006). Under *in vitro* conditions, nicotine, the drug of interest in this study, impacts the endocrine system and can alter circuit formation by blocking the conversion of the neuroactive androgen androstenedione to estrogen via nicotine's competitive inhibition of brain aromatases (Biegon et al. 2012, Villalba et al. 1999). Studies in male rats further demonstrate nicotine's ability to inhibit aromatase activity during fetal

development (Biegon et al. 2012). Whether or not aromatase activity contributes to sensitization in the context of gateway drugs is unclear.

2.2. B. Neurochemical changes make early adolescent brain vulnerable to exogenous insults

Neurochemical changes accompany structural changes that the brain undergoes during adolescence to better integrate the limbic, striatal and frontal regions during this time (Depue and Collins 1999). Drugs of abuse can have lasting consequences by interfering with these processes (Izenwasser 2005). Dopamine is a neurotransmitter commonly associated with locomotor sensitization, novelty seeking, and motivated behavior (Wingo et al. 2016). Activities that enhance such behaviors increase DA output in brain areas involved with the assessment of such activities including the ventral (NAc) and dorsal striatum (Wise 2004, Wingo et al. 2016). It is currently accepted that sensitization of these behaviors is due to an enhanced responsiveness of the DA system, as during the course of adolescence, dopaminergic neuronal connections in various nuclei such as NAc, hippocampus, and PFC become increasingly integrated to form the reward circuitry (Fig 2.2, Wahlstrom et al. 2010). Radioligand-binding assays of male rat brain homogenates reveal increments in the densities of DA receptors (DARs), notably D1 and D2, in the entire striatum and PFC during early adolescence (PND 40) that are subsequently pruned at late adolescence (PND 60-80) in both the PFC and dorsal striatum. In the NAc however both D1R and D2R densities remain relatively stable at late adolescence (Andersen et al. 2000). The temporal boost in DAR activity during adolescence is thought to facilitate synaptic connectivity, while their further pruning during late adolescence and adulthood especially in the striatum and PFC, allow for dopaminergic signaling that is progressively more efficient as the adolescent subject transitions into adulthood (Sisk and Zehr 2005, Andersen et al. 2000, O'Donnell et al. 2010).

During the transition from adolescence to adulthood, the DA system in the PFC is modified to allow for enhanced regulation of NAc functions (Yuan et al. 2015). Plasticity during this time allows for inhibitory interneurons to better regulate the excitability of pyramidal neurons (the major output neurons) in the PFC, which in turn regulate NAc activity. Both the pyramidal neurons and the regulatory GABAergic interneurons in the PFC contain D1 and D2 receptors. D1 receptors associate with N-methyl-D-aspartic acid receptors (NMDARs) and enhance their response, whereas D2 receptors associate with α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and attenuate the AMPA response. During early adolescence, the D1-NMDA as well as the D2-AMPA receptor interactions are less pronounced than during adulthood or even late adolescence, with the D1-NMDAR interaction taking prevalence over D2-AMPA interactions. Combined, in the presence of high levels of DA as expected in the presence of cues associated with drug-reward, this leads to an overall enhanced pyramidal cell activity without adequate GABAergic regulation. During adulthood, the D2 mediated GABAergic signaling becomes more refined and is thus better able to regulate pyramidal cell activity, in effect improving the signal-noise ratio (Fig. 2.3.A, O'Donnell 2010).

In the NAc, the same trend follows as in the PFC. Adolescents lack the mature D1-NMDAR and D2-AMPA interactions, as well as GABAergic signaling that is needed for interneuron mediated regulation of the accumbal medium spiny neurons (MSN). The weak interactions further lead to reduced interneuron excitability during adolescence, which progressively reverses as the individual matures into adulthood, and the D2-AMPA system is better able to interact with and recruit the GABA system (Yuan et al. 2015). These changes steer the brain toward the ability to discriminate and weigh reward with consequences as the subject matures (O'Donnell et al. 2010).

2.3. Neuronal effects of nicotine exposure

Nicotine, a sympathomimetic alkaloid that selectively binds subunits present in nicotinic acetylcholine receptors (nAChR), can have a significant impact on limbic circuitry that is undergoing plasticity during adolescence. Nicotine's actions on nicotinic receptors can alter the excitation and release of catecholamines, including DA, that integrate circuits underlying motivated behavior (Dwyer et al. 2009). Nicotinic receptors mediate the actions of acetylcholine (ACh) that plays an important role in brain maturation and synaptic transmission by activating various combinations of nAChR subunits, with the most abundant being the $\alpha_4\beta_2$ combination (Dani 2001, Dwyer et al. 2009). Nicotinic receptors comprised of the α_4 and β_2 subunits are the primary high-affinity nicotine binding sites in the brain, and are involved in mediating nicotine reward, as mice null in either of the subunits do not respond to nicotine with the same efficacy as their wild type counterparts (Dwyer et al. 2009, Pons et al. 2008). The $\alpha_4\beta_2$ subtype is upregulated both during adolescence and adulthood with chronic exposures to nicotine (Doura et al. 2008). Furthermore, the enhanced activity of this receptor subtypes is due to post-translational mechanisms in rodents, not so much by increased subunit assembly or trafficking, but via a gradual enhancement in the stability of the subunits that allow for reductions in receptor turnover and desensitization with subsequent exposures to nicotine (Vallejo et al. 2005). It is also reported that chronic exposure to nicotine increases the affinity of the receptor for nicotine, and this could further contribute to increased sensitivity to nicotine (Marks et al. 1992, Vallejo et al. 2005). Intriguingly, the $\alpha_4\beta_2$ subtype is also present during late adolescence, and chronic nicotine exposure at late adolescence does not promote heterologous sensitization in rats to cocaine or diazepam when challenged with these drugs during adult testing (Doura et.al. 2008, McMillen et al. 2005, James-Walke et al. 2007). Whether or not there are modification in the

activity of $\alpha_4\beta_2$ subtype at late adolescence the lead to reduced conditioned responsiveness at adulthood to challenge drugs is unclear (McMillen et al. 2005, James-Walke et al. 2007).

In mediating the rewarding and motivational properties of nicotine, the β_2 subtype plays a critical role in the depolarization of the DA cell bodies in the VTA that feed into the NAc to mediate the rewarding effect of nicotine (Picciotto et al. 1998, Arias-Carrión et al. 2010). In mouse β_2 -subunit knockout models, the mice fail to self-administer nicotine in a fixed ratio operant paradigm. Additionally, these mice become less sensitive to nicotine, and there is a reduced efflux of DA into the extracellular fluid in the NAc when measured by DA microdialysis from nicotine exposure (Picciotto et al. 1998). Furthermore, the mesencephalic DA neurons become unresponsive to nicotine, thus lending support to β_2 subunit's involvement in nicotine reinforcement.

2.4. Rat model for studying sensitization from periadolescent exposure to nicotine

The rat system is a widely used animal model to mimic human behavior as the window for adolescence is comparatively shorter in rats (days as opposed to years in humans), which facilitates experimentation (Spear and Brake 1983). The testosterone surge that marks the onset of puberty in the male rat occurs at about postnatal day (PND) 37, and by PND 45 the testes have descended, the male rat is rapidly gaining weight, and is able to mate with females (Dohler and Wuttke 1975, Spear and Brake 1983). The specific age for adulthood is unclear, however, several laboratories accept PND 70 or later as a suitable time for adult testing (Adriani et al. 2003, Spear and Swartzwelder 2014). Adolescent rats, much like their human counterparts, show an increased propensity to act impulsively and seek novelty as demonstrated by elevated maze tests (Laviola et al. 1999, 2003, Spear 2000).

2.4. A. Behavioral effects of nicotine-induced sensitization

Sensitization can be assessed by a variety of methods or paradigms in animal models. Behavioral paradigms for testing sensitization allow for the assessment of changes in cue-reward associations that characterize the subject's motivation for obtaining reward (Robinson et al. 2014). In the context of nicotine priming during early adolescence, rats show enhanced sensitivity towards test-drugs including stimulants such as cocaine, and anxiolytics such as benzodiazepines during later life when primed with nicotine at early adolescence. Behavioral paradigms that assess sensitization in animal models (demonstrated by heightened responsiveness to a sub-maximal dose of a test substance due to prior exposure to a priming substance) are operant conditioning, and conditioned place preference (CPP) (Adriani et al. 2003, McMillen et al. 2005, James-Walke et al. 2007).

Most behavioral studies that utilize operant conditioning for testing psychoactive drug sensitivity make use of different schedules of reinforcement, and the tests are typically carried out in operant chambers where the subject performs actions necessary to obtain reinforcement. Actions may involve pressing a lever one time to attain reinforcement, or the requirement could be increased to 3 or more presses, to make attaining reinforcement less obtainable. Enhanced responses with increased work requirement signify a stronger rewarding effect of the drug, implying sensitization (Staddon and Cerrutti 2003).

Conditioned place preference (CPP), on the other hand, is typically a 3-chamber paradigm with two conditioning chambers and a third neutral chamber where the animal is not subjected to treatment. This paradigm relies on classical conditioning, where the animal or subject is conditioned with a test drug in a chamber that is initially less preferred. If a test drug can induce a rewarding effect, it can be expected that the initially lesser preferred chamber will

increase in preference following conditioning. The subject's preference for the drug-paired chamber as opposed to a placebo (vehicle)-paired chamber can be assessed by the time spent in the drug-paired chamber (Prus et al. 2009). Studies indicate that priming of rats with nicotine during early adolescence increases preference (or time spent) for a chamber paired with sub-maximal (or subthreshold) doses of DOAs such as diazepam and cocaine following conditioning. Furthermore, CPP allows for the measurement of total frequency of chamber entries that can be used to estimate locomotion (James-Walke et al. 2007, McMillen et al. 2005).

2.4. B. Nicotine exposure: Periadolescence vs. late/post adolescence

The difference between periadolescence and post (or late) adolescence in the context of drug-induced sensitization to other drugs of abuse becomes apparent with behavioral tests for sensitization. Adriani and colleagues (2003) demonstrated that when rats are primed with nicotine using a schedule of once-daily for 10 days at a dose of 0.4 mg/kg during periadolescence, the rats increasingly self-administered nicotine *i.v.* by lever-pressing to operate an infusion pump at adulthood over different work requirements in an operant conditioning paradigm. This heightened incentive-salience, or drug seeking behavior, however was substantially curbed when the rats were first exposed to nicotine during late adolescence, indicating a weakened sensitization, or responsiveness, when priming occurred at late adolescence (Robinson and Berridge 2008, Adriani et al. 2003).

CPP studies indicate similar patterns for the development of sensitization with early-life nicotine exposure. McMillen and colleagues (2005) demonstrated enhanced responsiveness in adult rats to subthreshold doses of cocaine when the rats were previously exposed to 10 once-daily injections of 0.4 mg/kg nicotine during periadolescence. A subthreshold dose, or a dose that normally would not elicit a behavioral response, was chosen to demonstrate that nicotine

priming during periadolescence produced long-lasting changes in the brain that led the rats to become more susceptible to the rewarding effects of cocaine. When the rats were primed at late adolescence (PND 60-69), however, this sensitization effect was diminished, and the time spent in the cocaine-paired chamber was like that of the vehicle-treated cohort. The same pattern was observed when the challenge drug was a subthreshold dose of diazepam (James-Walke et al. 2007). The enhanced sensitization from priming at periadolescence as opposed to late adolescence imply that changes occur in the brain with periadolescent exposure that is more conducive to lasting sensitization. The relative lack of sensitization at adulthood due to late-adolescent priming could be due to the enhanced feedback regulatory system between PFC and NAc as discussed previously.

2.4. C. Molecular effects of nicotine-induced sensitization

Behavioral tests can estimate the strength or degree of development of sensitization. They however do not shed light into the changes occurring at the molecular and neuronal levels that facilitate this phenomenon. The study by Adriani and colleagues (2003) demonstrated that priming with nicotine during periadolescence increased the mRNA levels of $\alpha 5$, $\alpha 6$, and $\beta 2$ nicotinic receptor subunits in the VTAs of rat brains when analyzed at adulthood (PND 75). However, when a comparative group of rats were primed with nicotine during late adolescence (PND 60-69), there was a reversal in the mRNA levels of the aforementioned subunits compared to control, thus indicating an age-dependent molecular effect of nicotine exposure that manifested as disparate behavioral outcomes, in this case nicotine self-administration. Changes in the expression patterns of nAChR subunits in the VTA, as a result of periadolescent exposure to nicotine, may be more conducive to plasticity in the DA signaling system in the NAc due to altered DA neurotransmission and changes in the activity of molecular factors such as immediate

early genes (IEGs) and their downstream targets discussed later (Adriani et al. 2003, Picciotto et al. 1998). The underlying molecular bases for time or age-dependent plasticity needs further exploration to better enable therapeutic interventions to preempt the adverse consequences of an individual's future encounters with psychoactive drugs such as dependence and relapse.

2.4. C. 1. Significance of IEGs and FosB in facilitating long-term sensitization and memory

Various studies of the effects of nicotine and other psychoactive substances in animal models have implicated the DA system's involvement in the rewarding properties of these drugs (Picciotto et al. 1998), yet how sensitization of the reward system develops from these drugs remains unclear. Exaggerated responsiveness during later life to cue or stimuli that was associated with past experiences suggests the development of intrinsic modifications occurring at the cellular and molecular levels from the previous experiences (Robinson et al. 2014). In a neurochemical context, studies demonstrate that immediate early genes (IEGs) from different families mediate plasticity via induction of plasticity-related proteins (PRPs) to enhance associative memory formation that can facilitate learning (Minatohara et al. 2015). Changes in the levels of expression of IEGs from DOA stimulation can thus be expected to have an impact on long-term neuronal modifications lasting into the future that can outlast the temporary psychopharmacological effects observed during the time of drug exposure via synaptic modifications (Abraham et al. 1991). This can further facilitate formation of memory trace and recall (Minatohara et al. 2015).

IEGs belonging to the *fos* family of transcription factors have gained recent notice for their putative roles in mediating drug reward and dependence. In a study involving nicotine pretreatment followed by cocaine challenge, FosB and its spliced isoform Δ FosB, referred to by some as a 'molecular switch for addiction' (Nestler et al. 2001), was demonstrated to be

increasingly active during cocaine conditioning in C57BL/6J mice that were previously primed with nicotine. It was further demonstrated that nicotine priming enhanced subsequent cocaine's ability to inhibit histone deacetylase (HDAC) activity in the entire striatum, which led to the hyperacetylation of FosB promoter regions resulting in the accumulation of FosB protein. This effect was accompanied by an enhanced locomotor response to cocaine (Levine et al. 2011).

Another study performed in rats by Soderstrom and colleagues (2007) demonstrated that 24 hours following 10 once-daily injections of 0.4 mg/kg nicotine during periadolescence, there was an increase in the density of neurons expressing the protein FosB in key memory and reward areas of the rat brain, which included the NAc, mPFC, and dentate gyrus (DG) of the hippocampus. Furthermore, this change was sustained into adulthood (PND 80), especially in the NAc and the DG. Similar trends in the density of FosB-immunoreactive (FosB-ir) neurons were observed in the brains of the rats when analyzed 24 hours following the same injection schedule at late adolescence (PND 60-69, Soderstrom et al. 2007), thus indicating that FosB response to nicotine is not temporally dependent, but rather an accumulation of the protein from repeated nicotine exposure. It is further important to mention here that the antibody used in that study recognized all variants of FosB protein including the stable splice variant Δ FosB.

Δ FosB is a transcription factor that is a product of the *fosb* gene and is formed by the C-terminal truncation of full-length FosB (Fig. 2.4). It dimerizes with c-Jun to form the AP-1 transcription complex (Dobrazanski et al. 1991). The AP-1 complex then binds AP-1 sites on promoter sequences of several target genes to induce transcription of key genes implicated in synaptic connectivity in brain areas associated with drug seeking and dependence (Nestler 2001). The C-terminal truncation of FosB confers to Δ FosB a unique stability that allows the protein to accumulate with subsequent psychoactive drug exposures. FosB, on the other hand, is a transient

protein that degrades within hours of a single drug exposure (Nestler 2001). The dimerization of Δ FosB with c-Jun appears to play a necessary role in the sensitization process, as selective deletion of c-Jun in the ventral (NAc) and dorsal striatum reduces the rewarding effects of cocaine (Peakman et al. 2003). The role of FosB in mediating the rewarding and sensitizing properties of DOAs appears to be primarily focused in the NAc. Furthermore, studies indicate that FosB may recruit the opioid system to mediate behavioral sensitization. This was demonstrated in a study where Δ FosB was selectively overexpressed in the dynorphin-containing MSNs in the striatum of adult mice, and this resulted in increased behavioral sensitization of the animal to drugs of abuse such as morphine (Zachariou et al. 2006). It is currently held that Δ FosB enhances the activation of several down-stream target genes that may contribute toward a ‘relative permanence’ in the brain’s sensitization to drugs of abuse (Nestler et al. 2001).

Δ FosB induction from repeated exposure to stimuli promotes neuronal plasticity through a variety of mechanisms and targets. One important downstream target is the AMPA glutamate receptor subunit (*GluR2*). GluR2 appears to be the only AMPA receptor subtype whose expression is increased in the NAc due to Δ FosB overexpression, and overexpression of GluR2 in the NAc engenders reward phenotypes for cocaine consistent with that of Δ FosB (Kelz et al. 1999). Another downstream effector of Δ FosB is cdk5 (cyclin dependent kinase 5), which is involved with neuronal growth and survival (Bibb et al. 2001). The inhibition of cdk5 in the NAc by roscovitine reduces cocaine’s ability to induce dendritic spine development in NAc core and shell (Norrholm et al. 2003). Furthermore, cdk5 activity regulates dopamine and cyclic AMP-regulated phosphoprotein 32 (DARPP-32) phosphorylation, which plays a key role in DA signaling. These studies suggest a link between Δ FosB and development of neuronal plasticity

that may pertain to drug-reward sensitization. It is further suggested that these morphological and functional changes may outlast the Δ FosB protein itself (Norrholm et al. 2003).

2.5. Involvement of microglia

A variety of mechanisms that may lead to the development of sensitization from DOA exposure at an early age have been investigated. Which pathways are exactly responsible for establishing sensitization that persist into later life from early adolescent exposure to DOAs is still unclear, although FosB activation appears to play a key role (Nestler et al. 2001). Furthermore, whether FosB acts solo or in concert with other factors is currently of interest, as FosB modulates several downstream genes that regulate dopamine in memory and reward-related circuits (Grueter et al. 2013). Since FosB appears to be disconnected from the reward sensitization resulting at adulthood from early adolescent nicotine exposure (Soderstrom et al. 2007, Adriani et al. 2003, McMillen et al. 2005), other factors that could contribute to this sensitization effect need to be investigated. Microglia, the resident immunocompetent cells in the brain, have received attention in the attempts to understand the pathways for sensitization due to their importance in wiring of the neuronal circuits, and could be a putative contributor to long-term sensitization (Paolicelli et al. 2011). Microglia display a variety of morphologies depending on the microenvironment (Fig. 2.5). Additionally, microglia activation and involvement have been ascribed to the sensitization effect of various psychoactive substances such as morphine and cocaine (Schwarz and Bilbo 2013, Chen et al. 2009).

2.5. A. Importance of microglia during adolescence

Microglia play key roles throughout the brain development process and are sensitive to changes in brain homeostasis and xenobiotic insults (Nayak et al. 2014). For example, microglia

are responsive to fluctuations in sex hormone levels in the brain, as they express cell surface receptors recognizing androgens and estrogens (Nissen 2017). As adolescence is the transition from childhood to adulthood, led by gonadal hormonal surge, microglia can be expected to influence synaptic plasticity related to behavioral changes induced by nicotine and other drugs that can impact hormonal levels (Spear 2000, Peper and Dahl 2013, Biegon et al. 2012).

Microglia activity is crucial for neuronal connectivity and is intimately involved in the pruning of synapses by acting as highly motile phagocytes (Kabba et al. 2018). In recent years, errant microglia activity has been implicated in pathologies such as Alzheimer's and Parkinson's diseases, where they are involved in cytokine secretion, and subsequent neuroinflammation in response to pathogen accumulation (Tejera and Heneka 2016). Such actions often lead to apoptosis and phagocytosis of neurons that result in removal of dopaminergic and cholinergic neurons. In the context of drug sensitization that is a form of associative learning, there is the possibility that activation of microglia can lead to the pruning of synapses that can consequently strengthen long-term cue-reward associations, which may further impact reward motivation or salience (Schwarz and Bilbo 2013, Robinson and Berridge 2008). Chronic stimulation of DARs on microglial surface, as expected with chronic exposures to nicotine in rodents, is reported to enhance microglial migratory activity (Färber et al. 2005). Microglia's role in memory consolidation and learning is further highlighted by a study on the adult transgenic mouse brain in which depletion of microglia led to a weakening of synapses in the motor cortex that are normally associated with learning tasks (Parkhurst et al. 2013).

2.5. B. Microglia Functions

Perturbations in microglia activity during development are linked to defects in the wiring of the brain, as microglial processes make direct contact with synapses (Paolicelli and Ferretti

2017). Microglia intimately regulate synapses and are sensitive to neuronal signals. Exposure of microglia to pathogens during critical periods of development result in priming of microglia that is further exacerbated by later exposure to same or different pathogens, an indication of formation of ‘microglial memory’. This can further instigate excessive pruning of circuitry relevant to learning and memory, and neuroinflammation characteristic of autism-like symptoms (Williamson et al. 2011). Several molecules facilitate microglia’s ability to prune leading to synaptic plasticity. One example is the neurotrophic factor BDNF, the deletion of which leads to deficits in learning and synaptic remodeling associated with learning (Parkhurst et al. 2013).

Microglia express various receptors for complement proteins and immunogens on their outer surfaces that regulate microglia-neuron interactions. Microglia express pattern recognition receptors that sense pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) that allow microglia to prime and react efficiently to noxious stimuli, one example being the Toll-like receptors (TLRs). Under physiological conditions, neuron-microglia interactions via signaling molecules such as fractalkine and its receptor (CX3CR1), as well as other anti-inflammatory cytokines, neurotrophins, and prostaglandins secreted by neurons keep microglia in a relatively benign surveilling state rather than an activated (cytotoxic) state (Kabba et al. 2018). Pathogens and other factors, including cytokines and neurotrophins, promote activation of microglia that can be either pro- or anti-inflammatory depending on context and severity of insult (Kabba et al. 2018).

2.5. B. 1. Microglia polarization

The polarization or shifting in function of microglia appears to play a key role in the induction of drug sensitivity as agents selective for certain polarizations can impact the development of sensitization both in the short and long term (Chen et al. 2009, Schwarz and

Bilbo 2013). Since microglia are sensitive to their surroundings, the cells can polarize into the basic M1 (proinflammatory) or M2 (anti-inflammatory) classification or in between to react to stimuli or changes in the microenvironment. M1 polarization typically results from actions of pathogens such as lipopolysaccharides (LPS) on Toll-like receptors (TLRs) that further promote the secretion of proinflammatory cytokines such as TNF- α , IL-1 β , interferon- γ (IFN- γ), nitric oxide (NO), as well as cell surface antigens CD68 and CD86 to name a few (Crews et al. 2017). M2 polarization is induced by molecules that promote interleukin-4 (IL-4) or IL-13 cytokines that direct microglia to play a role in repairing damaged tissue (Kabba et al. 2018). As certain agents can induce a particular polarization of microglia, there are also agents that can inhibit polarization patterns. Kobayashi and colleagues (2013) demonstrated *in vitro* that the tetracycline antibiotic minocycline selectively prevents the M1 polarization of mouse cortical microglia via inhibition of the transcription factor NF κ B. Furthermore, the same study demonstrated that minocycline did not affect the expression of molecular markers characteristic of M2 polarization such as CD206, arginase 1, IL-4, IL-10, and Ym-1 following LPS stimulation. Thus, minocycline provides a useful tool for exploring how inflammatory polarization of microglia can impact sensitization of the brain by drug priming.

2.5. B. 2. DOAs and microglia

The question of whether or not DOAs can activate microglia to mediate sensitization is under continued investigation. Several studies suggest that microglia may play a role in the process as they are impacted by drugs of abuse from different categories. One report demonstrated that rats exposed to alcohol at binge levels 3 times/day for 4 days resulted in microglia displaying an activated morphology in the hippocampus that lasted for an entire month (McClain et al. 2011). Another study demonstrated that in rats initially primed with morphine at

early adolescence, microglia play a role in reinstatement of sensitization to morphine following abstinence from initial challenge doses of morphine during later life. In that study, ibudilast was used to suppress the activation of microglia during the abstinence phase, and this led to an inhibition of morphine reinstatement. The authors further concluded that the mechanism for activation of microglia due to morphine was microglial TLR4 activation as PCR experiments demonstrated an increase in TLR4 mRNA levels in microglia isolated from adult brains (Schwarz and Bilbo 2013).

Microglia polarization may also play a role in the brain's sensitization to DOAs such as cocaine and methamphetamine. A study performed in adult mice suggests that the activation of microglia may mediate locomotor-sensitization to cocaine, although microglia were not directly implicated in the establishment of sensitization. In that study, minocycline at a prophylactic dose of 40 mg/kg administered 3 hours prior to 10 mg/kg cocaine over 4 days impaired cocaine's ability to stimulate locomotor hyperactivity with later cocaine challenge. This effect of minocycline was however negated once sensitization to cocaine had already been established, a result indicating microglia action in conjunction with cocaine in inducing locomotor sensitization (Chen et al. 2009). Since minocycline selectively inhibits M1 polarization of microglia without impacting M2 polarization, (Kobayashi et al. 2013), it appears that M1 microglia plays a role in synaptic remodeling in drug-associated learning and memory formation. Whether or not minocycline has an impact on curbing periadolescent nicotine-induced sensitization leading to cocaine-seeking at adulthood is unclear and therefore this dissertation project was designed to address that question.

2.6. Significance, innovation, and hypothesis

From several studies, it appears that the M1 activation of microglia contributes to the psychoactive effect of various DOAs, as sensitization whether locomotor or reward, was inhibited with agents known to suppress M1 polarization (Chen et al. 2009, Schwarz and Bilbo 2013). Studies further indicate that activation of microglia occurs with exposure of laboratory animals to drugs of abuse such as alcohol, cocaine, and morphine (McClain et al. 2011, Guo et al. 2015, Schwarz and Bilbo 2013). No study to our knowledge have demonstrated whether nicotine activates microglia in the context of nicotine-induced cross sensitization to heterologous classes of psychoactive substances. Furthermore, there is no comprehensive understanding of the modifications persisting into adulthood that may enhance a subject's vulnerability to seeking psychoactive drugs in the future due to periadolescent exposure to nicotine. As discussed previously, the induction of FosB, the putative 'sustained molecular switch for addiction', is not confined to a specific time window for nicotine exposure (Nestler et al. 2001, Soderstrom et al. 2007), thus necessitating the exploration of other factors that can facilitate FosB activity. Microglia is a likely candidate as they are reactive to the androgen surge that is expected at adolescence onset in males (Nissen et al. 2017). Also, the suppression of inflammatory microglia activity during early adolescence curbs the reinstatement effect of DOAs such as morphine during adulthood when the rats were initially morphine-primed at adolescence, thus raising the question if microglia can also mediate sensitization from nicotine exposure during the onset of adolescence.

This study aims to determine whether nicotine exposure during periadolescence promotes M1 activation of microglia in the brains of rats leading to lasting sensitization. To demonstrate this, minocycline at a dose of 30 mg/kg is injected daily 30 minutes prior to a periadolescent

nicotine schedule of 10 once daily injections of nicotine for the 10 days bracketing the onset of adolescence. For injection and analysis schedule please see (Fig 2.6). With minocycline, the role of M1 microglia in promoting nicotine-induced plasticity that translates into behavioral vulnerability to drugs of abuse (cocaine) in the future can be better understood. To test the effectiveness of minocycline, microglia activation will be assessed using morphologies characteristic of activated or ramified microglia, following Iba1 immunodetection. Iba1, or ionized calcium-binding adapter molecule 1, is an antigen that is uniquely expressed by microglia throughout their cell-surface, including processes (Ito et al. 1998). Reductions in density of Iba1 stained microglia displaying an 'activated' morphology would indicate efficacy of minocycline pretreatment (Fig. 2.5). The impact of minocycline on nicotine-induced sensitization at the molecular level will be evaluated by relative FosB-ir, both during early adolescence (PND 45) and adulthood (PND 80) in the NAc, DG of hippocampus and the PFC, three brain nuclei involved in memory and reward perception (Soderstrom et al. 2007).

This dissertation study will attempt to answer the following questions: (1) Does neurotoxic (M1) activation of microglia play a role in sensitizing the brain's reward and motivation system that is stimulated by nicotine exposure during the onset of adolescence? This will be assessed at the molecular level by suppressing the M1 activation of microglia with minocycline pretreatment 30 minutes prior to the periadolescent nicotine schedule discussed previously, (PND 35-44), and then immunostaining rat brain sections for FosB 24-hours (PND 45) and 35 days (PND 80) after the final set of injections. If FosB-ir is attenuated in minocycline pretreated rats, then one can infer that M1 activation of microglia does mediate FosB activity, as induction of FosB is both a short and long-term consequence of periadolescent nicotine exposure (Soderstrom et al. 2007). In the long term, this study tests if M1 activation of microglia from

periadolescent nicotine exposure mediates heterologous-sensitization to other psychoactive compounds at adulthood, leading to the second question: (2) Does minocycline pretreatment prior to each nicotine exposure during the periadolescence window attenuate heterologous-sensitization to cocaine at adulthood? CPP experiments with cocaine conditioning will determine whether nicotine-only rats are more anticipatory of cocaine reward than the rats that have received the minocycline pretreatment. Furthermore, if the suppression of M1 activation microglia has negligible impact on nicotine's ability to induce FosB-ir, and there is responsivity to cocaine similar to that of the nicotine group, then it can be inferred that periadolescent nicotine enhanced sensitivity to cocaine at adulthood is independent of the M1 activation.

2.7. Possible outcomes from this study

This study which combines CPP and molecular methods can result in three possible outcomes that will move our understanding of the periadolescent nicotine induced sensitization paradigm forward. **Outcome (1):** Periadolescent 10-day nicotine injection induces inflammatory activation of microglia along with FosB-ir. If this is accompanied by an increased preference for cocaine during adulthood, then this suggests that both M1 microglia and FosB are involved in promoting sensitization. **Outcome (2):** Minocycline blocks M1 activation of microglia, but no changes in FosB-ir. If this result is accompanied with increased cocaine preference during adulthood, then neither are conducive to sensitization. **Outcome (3):** Minocycline blocks M1 microglia, but there is the increase in FosB-ir accompanied by increase in cocaine preference, then we know FosB, but not microglia are important to the sensitization process. The ensuing chapters will examine the three possibilities discussed above in more detail.

2.8. Hypothesis:

Once daily administration of nicotine at a dose of 0.4 mg/kg i.p. during the 10 days that bracket the onset of adolescence in rats (PND 35-44) will induce inflammatory activation of microglia and increase expression of the transcription factor FosB in reward-specific brain regions 24-hours post final injection (PND 45), with this effect sustaining till adulthood (PND 80). Moreover, periadolescent nicotine will sensitize the rats at adulthood (PND 80) to subthreshold doses of cocaine. Pretreatment with minocycline will weaken this sensitization via suppression of M1 activation of microglia and reduction in FosB-ir both during periadolescence and adulthood, and this will manifest as reduced sensitivity to subthreshold doses of cocaine. For testing this hypothesis, this dissertation study was divided into three specific aims (SA).

SA 1: With the use of IHC determine whether inflammatory activation of microglia contributes to the increase in FosB protein expression at PND 45 in select brain regions (NAc, mPFC, and DG) from 10 once-daily injections of nicotine during periadolescence (PND 35-44).

- a. Determine whether the densities of activated microglia (measured by counting microglia displaying ‘activated’ morphology following surface Iba1 immunoreaction) and neurons that express FosB-ir protein, increase in brain areas associated with drug reward and memory 24 hours following the periadolescent nicotine injections.
- b. Determine whether minocycline pretreatment reduces the density of activated microglia from nicotine exposure during periadolescence, and if this treatment attenuates the increase in FosB-ir in the select brain regions.

SA 2: Determine via classical conditioning whether the inflammatory activation of microglia contributes to periadolescent nicotine-induced cocaine seeking behavior at adulthood (PND 80).

- a. Use CPP to determine whether the minocycline pretreatments 30 minutes prior to nicotine during preadolescence reduces the time spent in cocaine-paired chamber during adulthood following cocaine conditioning.

SA 3: With the use of IHC determine whether changes in the density of microglia displaying the activated morphology, as well as neurons expressing FosB due to periadolescent treatments are sustained into adulthood (PND 80), the time when behavioral testing is performed.

- a. Determine if in rats that received nicotine at periadolescence, there is a sustained increase in the density of microglia displaying the activated morphology, as well as increased expression of FosB protein in the select brain regions when brains are analyzed at adulthood.
- b. Determine if minocycline pretreatment during periadolescence lead to sustained reduction in densities of microglia displaying activated morphology, as well as neurons expressing FosB protein.

Fig. 2.1: A sagittal rat brain cross section depicting nuclei of the limbic system in the brain pertaining to this study. The nuclei marked are medial prefrontal cortex (or mPFC), Nucleus accumbens (NAc), and Dentate gyrus of hippocampus (DG). Dashed lines indicated the regions counted in the areas of interest. Scale bar 500 μ m.

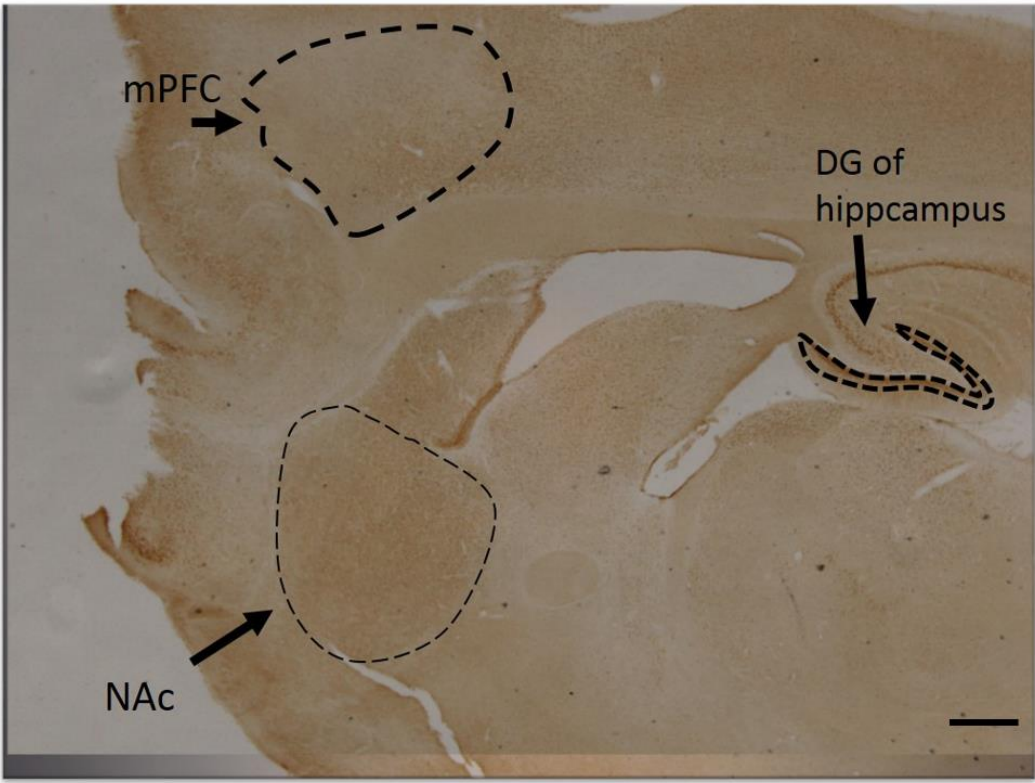


Fig. 2.2: Figure delineating the mesolimbic reward pathway. The two main components of the mesolimbic reward system are ventral tegmental area (VTA) and nucleus accumbens (NAc), with inputs from different nuclei. Dopaminergic (DA) neurons (blue) from the VTA synapse with the medium spiny neurons (MSNs) in the NAc, as well as neurons of PFC, hippocampus, and amygdala (AMG). The PFC, hippocampus, and AMG send excitatory glutamatergic inputs (green) to the MSNs of the NAc. The NAc in turn send GABAergic projections to the VTA. Nicotine primarily acts on the $\beta 2$ subunits in the VTA in combination with $\alpha 4$, $\alpha 6$ subunits of the nicotinic receptor and increases DA levels via increasing the firing rate by producing phasic bursts into the NAc and PFC. Figure adapted from Russo and Nestler (2013), Figure 1.

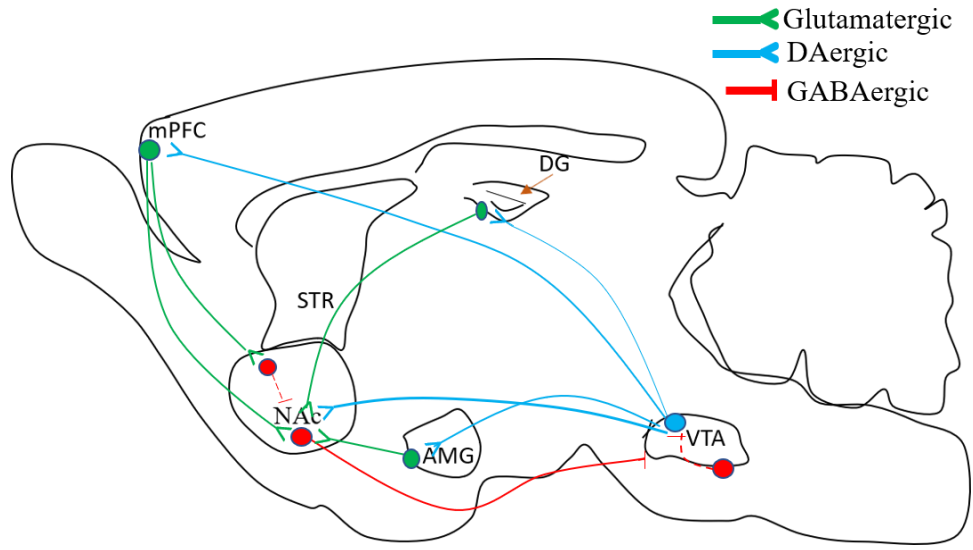
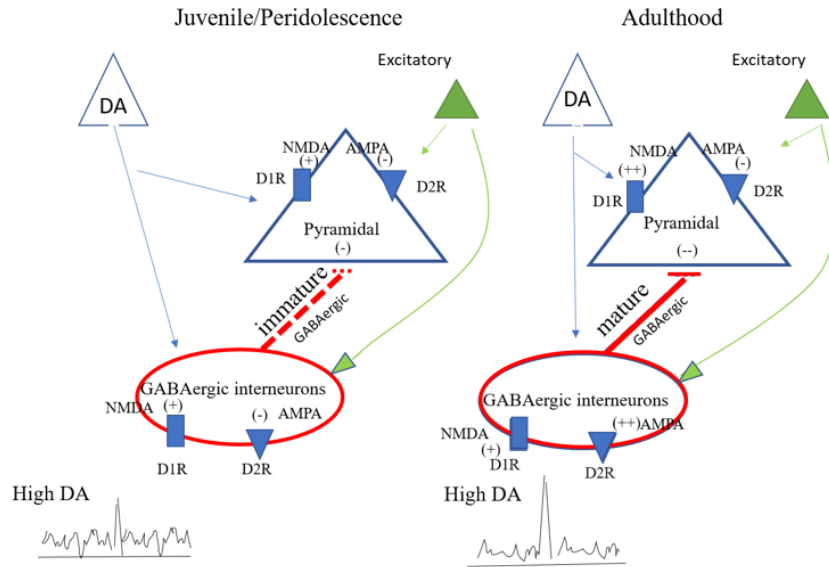


Fig. 2.3: Figure illustrating the adolescent to adult transition in the PFC and NAc. The interaction between the PFC and NAc become more integrated as the subject transitions from early to late adolescence or adulthood. The communication between the NAc and PFC becomes more refined as the subject ages. (A) During periadolescence, the synergistic association between D1R and NMDA receptors produce an enhanced pyramidal cell firing, while the D2 AMPA response is under construction. Furthermore, the GABAergic interneurons (red) that suppress spurious excitation of the pyramidal neurons are still under development. Result of this effect is higher background of excitatory signals to the NAc. At late adolescence, the D2 AMPA activity is heightened in the pyramidal cells with enhanced GABAergic interneuron activity resulting in enhanced regulation of the excitatory outputs of pyramidal neurons and a more refined signal emanating from the PFC. (B) In the NAc, similar trends are observed. Primary DA source to the NAc is the VTA. Periadolescents also lack the mature D1-NMDA and D2-AMPA receptor interactions, initially resulting in immature synaptic communication with the PFC. Additionally, cell excitability of medium spiny neurons (MSN) is low due to weaker DA-excitatory neurotransmitter interactions. As the subject ages, GABAergic interneurons are better able to refine the excitability of the MSNs thus allowing the accumbal MSNs to respond more efficiently to PFC inputs. Figure adapted from Yuan and colleagues (2015), and O'Donnell (2010).

A



B

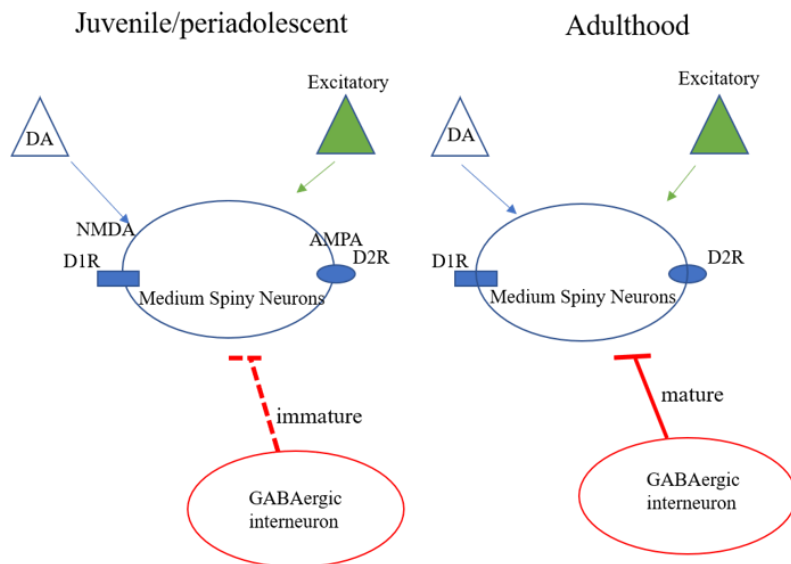


Fig. 2.4: Figure delineating the difference between full-length FosB and its spliced variant Δ FosB. Both moieties contain a DNA binding domain and dimerization domain that allow both isoforms to associate with Jun factors to form the AP-1 transcription complex. The full-length FosB contain two domains that allow for proteosomal and non-proteosomal degradation. The truncation of these two domains allows Δ FosB enhanced stability. The Δ FosB isoform is smaller in size from the full length variant by 101 amino acids (aa). Furthermore, phosphorylation at aa site S27 by casein kinase 2 (CK2) and at site S17 by other kinases confer structural stability to the Δ FosB variant. Figure adapted from Nestler (2008), Figure 1.

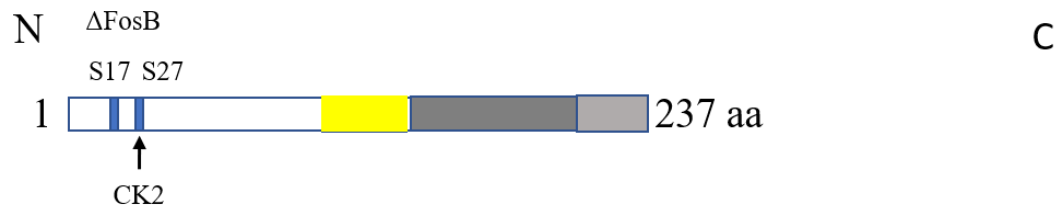
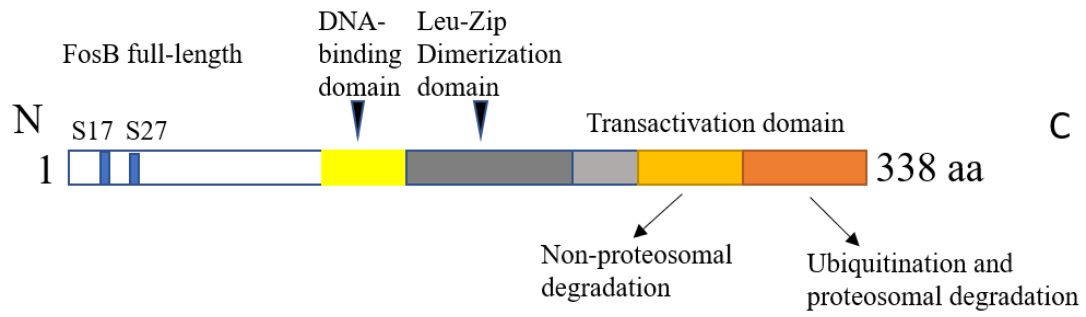
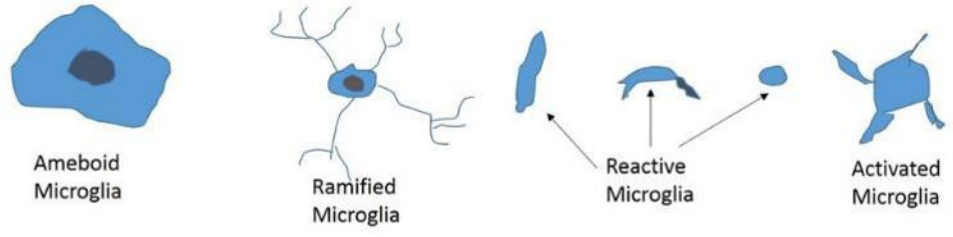


Fig. 2.5: Illustrations depicting characterization of microglia based on morphological features.

A. Amoeboid microglia tend to have a larger cell body without processes. Ramified microglia have small to midsize cell bodies, and thin processes. Reactive microglia have distorted cellular shapes and may contain a few thickened processes. Activated microglia have thicker and fewer processes, with less branching as compared to ramified. **B.** A sample immunohistological Iba1 stain of rat brain section showing microglia morphologies in the NAc at 200X magnification. Larger arrows indicate reactive microglia, while arrowhead shows ramified microglia. Scale bar **B**, 100 μm . Panel **A** inspired by Walker et al. (2014), Figure 6.

A



B

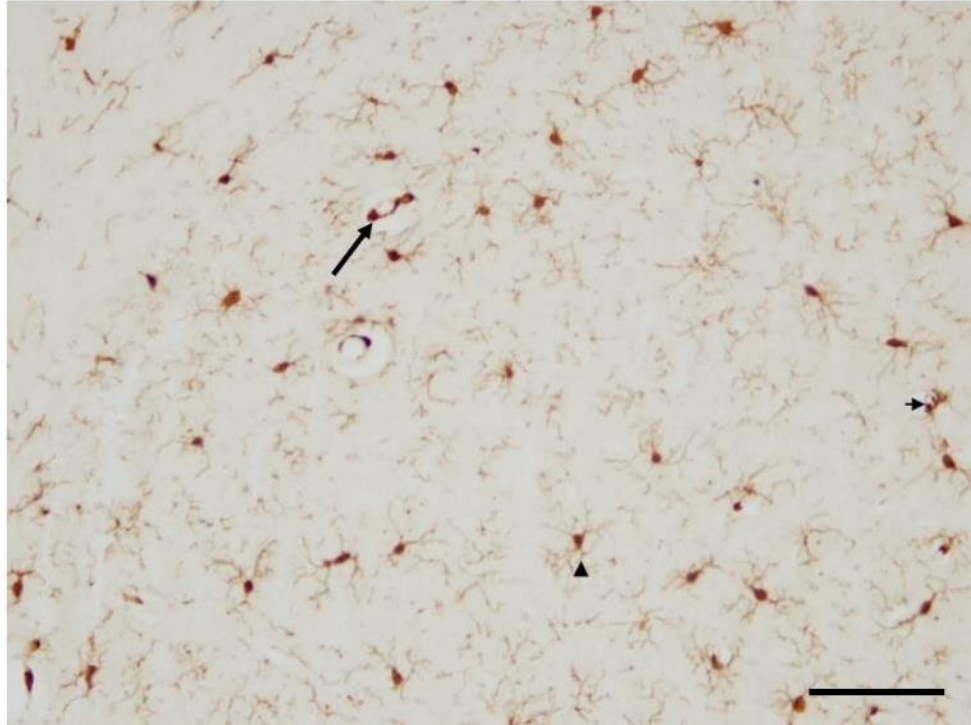
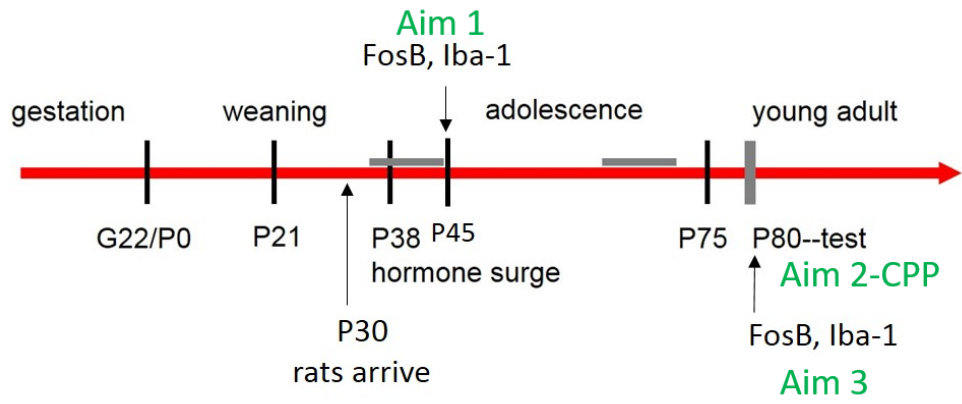


Fig. 2.6: Timeline for experimentation. Male rats are obtained from Charles River, initially weaned (i.e. separated from dam) between post-natal days (PND) 20-22. At PND 30, the Sprague Dawley rats arrive, and are pair-housed. Rats are handled three times prior to commencing with injections. On PND 35, once daily injections begin, and end at PND 44. For Specific Aim 1, rats are euthanized, and brains are fixed on PND 45 (24 hours after final injection) to examine FosB expression and microglia activation status (Iba1). For Specific Aim 2, same protocol is followed as in Specific Aim 1, but rats are allowed to mature to adulthood (PND 80) when cocaine-conditioning begins. For Specific Aim 3, same injection protocol is followed as in Specific Aim 1. Rats are allowed to mature to PND 80. Rats are then euthanized, and brains are fixed and harvested to examine FosB and microglia.



Chapter 3: Materials and Methods

3.1.A. Groups and Treatment

Male Sprague Dawley (SD) rats were obtained from Charles River Laboratories at the age of PND 30. The rats were housed in pairs under a 12-hour light/dark cycle. Food and water were available *ad libitum*. The rats were randomly divided into four treatment groups, namely vehicle (Veh), nicotine (Nic), minocycline control (Min), and minocycline 30 minutes prior to nicotine (NM), with n = 5-8 per group for PND 45 testing (SA 1), n = 4-5 for PND 80 testing (SA 3), and n = 8-12 for conditioned place preference (CPP) studies (SA 2) (please refer to Fig. 2.5 for experimental timeline). Nicotine hydrogen tartrate (Sigma; 0.4 mg/kg as the free base), saline, or minocycline-HCl (PCCA, USA; 30 mg/kg as the free base) were injected intraperitoneally (i.p.) once every 24 hours for a 10-day period during the light cycle from PND 35-44. The weights of the rats were used to determine the volume to be injected. Rats were sacrificed at either PND 45, or allowed to mature till PND 80 without any treatment during the interim period for adult brain analysis and CPP testing. All procedures for testing rat behavior and brain isolation were approved in advance by the East Carolina University Institutional Animal Care and Use Committee and complied with the *Guide to the Care and Use of Laboratory Animals* from the National Research Council.

3.1.B. Transcardial perfusion and immunohistochemistry (IHC)

To prepare tissues for the immunodetection of FosB or microglia at PND 45 and PND 80, rats were first euthanized using a lethal dose of sodium pentobarbital (100 mg/kg). Volume of the pentobarbital solution was adjusted so that 1 mL was appropriate for 1 kg body weight. On ensuring adequate sedation by toe-pinching, the chest cavity was opened, and 50 mL of saline

were pumped into the left ventricle of the heart using a peristaltic pump. Subsequently, 200 mL of cold buffered 4% paraformaldehyde solution was pumped into the left ventricle for systemic fixation of the entire animal. Rats were then decapitated, and brains were harvested and post-fixed overnight in 4% paraformaldehyde solution at 4°C. The brains were then stored in 30% buffered sucrose solution at 4°C until sectioning. The prepared brains were sectioned in the sagittal plane from 2 mm lateral-to-midline, and 40 µm sections were collected using a cryostat slicing in the lateral-medial direction, so that the nucleus accumbens (NAc), medial prefrontal cortex (mPFC), and the dentate gyrus (DG) of the hippocampus could all be visualized in the same section. Sections were separated into two groups, with each group containing sections adjacent to that in the other group, for single antibody detection. Free-floating sections were incubated in buffered 0.3 % hydrogen peroxide to quench endogenous peroxidases, followed by blocking in 5% normal goat serum (NGS) dissolved in phosphate buffered saline (PBS) containing 0.3% Triton X-100 surfactant. Antibodies for immunodetection were directed at FosB (SC48: 1:1000) and Iba1 (Wako, USA: 1:10,000), an antigen commonly expressed throughout the surface of microglia including processes. During the early stages of this project, an antibody from Santa Cruz Biotechnology (FL-147) was used to detect Iba1 expression on microglial cell surface. This antibody however was not selective for microglia, as other neuronal types were also detected, and the antibody, and data generated from that antibody were consequently excluded. Antibody selective for Iba1 was obtained from Wako, USA based on recommendations from various publications. The new antibodies revealed microglia in brain sections that were distinctly identifiable with negligible background staining.

Antibodies for detecting FosB were obtained from Santa Cruz Biotechnology based on the article by Soderstrom and colleagues (2007). This antibody detects FosB full-length protein

and reacts with the shorter splice variant Δ FosB. Thus, what is detected is FosB-like immunoreactivity or FosB-ir. Primary antibodies were further probed using the Vectastain ABC signal amplification kit (PK 4001, Vector Labs). The stain was further developed using 0.05% diaminobenzidine (Sigma D12384) in 50 mM Tris buffer pH 7.5. Immunolabeled sections were photographed using Olympus BX50 light microscope and images were analyzed with Image Pro software 6.3 (Media Cybernetics). Neurons expressing FosB and microglia displaying an activated morphology were counted and presented as density (cells or reactive nuclei / mm²). Efforts were undertaken to include all treatment-groups in IHC experiments to minimize variance due to antibody assay conditions.

3.1.C. Analysis of immunostained sections

Following immunostaining of PND 45 and PND 80 rat brain sections, micrographs were acquired of specific brain regions associated with reward, learning, and drug seeking behavior (hippocampus (DG), NAc, and the mPFC) from the different treatments. For sections assayed for FosB, punctate reactive nuclei in specified drawn-in areas were counted by an individual blinded to the treatments. Data are presented as the number of punctate or reactive nuclei per unit area (mm²). Similar procedure was followed for microglia (number of microglia displaying an ‘activated’ morphology’ per unit area [mm²]). A microglial cell was deemed activated when the morphology of the cell presented itself as round/amoeboid, or one with thick stout processes, according to the classifications by Walker and colleagues (2014) (Fig. 2.4). Images were acquired, and the treatment-groups were handled as uniformly as possible.

3.1.D. Statistical analysis for IHC analysis

On the data generated from both sets of experiments described previously (FosB and Iba1), several statistical analyses were performed to compare treatments. One-way ANOVA was the primary mode of analysis with the main effects of treatment (independent variable). Dunnett's t-test for multiple comparison was used for the post-hoc analysis of the ANOVA results. P value < 0.05 ($\alpha=0.05$, 2-tailed, parametric) was deemed statistically significant. Furthermore, effect sizes, compared to nicotine, were calculated to analyze biological relevance. All statistical analyses were performed using the GraphPad Prism software or Microsoft Excel.

3.2.A. Cocaine induced conditioned place preference (CPP)

Periadolescent rats were treated according to the injection schedule discussed under **3.1.A**, and allowed to mature until adulthood or PND 80. The CPP protocol followed was similar to that described by McMillen and colleagues (2005). The CPP apparatus consisted of a three-chamber gray wooden box with distinct visual and tactile cues. The two larger chambers, one black with a white circle and the other white with a black stripe, were separated by a smaller gray neutral chamber with guillotine doors on each side. On days 1, 2, and 3, each rat was placed in the neutral chamber and the guillotine doors were lifted. Each rat was allowed to explore the entire apparatus for 15 minutes. On the 3rd day, chamber entries were recorded using the BEHAVIOR program (written by Prof. L.W. Means) and each rat's chamber preference determined. An entry into a chamber was scored when the rat inserted 3 paws into the chamber. To minimize inherent chamber bias, any rat that did not explore each chamber of the apparatus for at least 50 seconds per chamber on day 3 were not considered for conditioning. On days 4, 6, 8, and 10, each rat received an injection of 3.0 mg/kg of cocaine (cocaine-HCl as the base, Sigma USA, dissolved in vehicle) and then immediately confined to its less-preferred chamber for 15 minutes. On days 5, 7, 9, and 11, the rats received an i.p. injection of 0.01 M citrate buffer (i.p.,

pH 6.5) and then immediately confined to its preferred chamber for 15 minutes. On day 12, no injections were administered, and each rat was placed in the neutral chamber and the doors were lifted. Chamber-entries were again recorded for 15 minutes for each rat using the software BEHAVIOR. The time spent in the least preferred chamber was recorded, as that was the chamber for cocaine reinforcement, as well as total chamber entries (or frequency of entry) as a measure of locomotion.

3.2.B. Statistical analysis for CPP

On the data generated from the CPP experiments described previously, statistical analyses were performed to compare the differences among the treatments. Repeated measures, two-way ANOVA was the primary mode of analysis with the main effects of treatment i.e. drug and time point (pre- vs. post- conditioning). Bonferroni multiple comparison test was used for post-hoc analysis of the ANOVA results, as we are comparing before *vs* after or pre- *vs* post-conditioning. A p-value ≤ 0.05 was deemed as significant. All statistical analyses were performed using the GraphPad Prism software or Microsoft Excel.

Chapter 4: Results

4.1. Activated Microglia and FosB-ir 24 hours following periadolescent exposure

The purpose of specific aim (SA 1) of this study was to investigate links between the activation of microglia and the neuronal expression of the IEG FosB measured as FosB-like immunoreactivity (FosB-ir) 24 hours following periadolescent nicotine exposure. The tetracycline antibiotic minocycline was used to suppress the M1 (proinflammatory) polarization of microglia. Adjacent sections among the four treatment groups (vehicle, nicotine, minocycline, minocycline 30 minutes prior to nicotine) were probed for FosB and Iba1 using selective antibodies. One day following the last of 10 days of injections of nicotine at a dose of 0.4 mg/kg i.p. (PND 35-44), the density of activated microglia (as determined by morphology) in the NAc was increased by 88% when compared to the vehicle treatment group ($F_{3,16} = 11.06$, 95% CI, $p < 0.001$, nicotine vs vehicle) (Fig. 4.1.5 A). Minocycline pretreatment 30 minutes prior to each daily dose of nicotine reduced the density of activated microglia compared to vehicle by 6.7% (minocycline and nicotine vs vehicle, $p > 0.05$), and was significantly less than the density observed with the nicotine group. Minocycline control reduced the density of activated microglia by 13.5% ($p > 0.05$) when compared to vehicle (Fig. 4.1.5 A). In the mPFC, the 10-day exposure to nicotine increased the density of activated microglia by 82% over vehicle ($F_{3,17} = 5.19$, 95% CI, $p < 0.01$, nicotine vs vehicle, $p < 0.05$), whereas in the minocycline pretreatment group the density of activated microglia was increased by 24% ($p > 0.05$) when compared to vehicle but less than nicotine-alone by 32 % ($p > 0.05$, Fig. 4.1.5 B).

In harmony with a previous report (Soderstrom et al. 2007), the density of neurons expressing FosB-ir proteins in rats that had received ten once daily injections of 0.4 mg/kg i.p. nicotine increased in limbic structures and reached significance in the DG at percentages that

were similar to the previous report (Fig. 4.1.6 **E**). In the NAc, there was an 18% increase ($p > 0.05$, nicotine vs vehicle) one day following the last dose of nicotine when compared to vehicle, but the effect did not reach statistical significance ($F_{3,24} = 1.10$, $p > 0.05$) (Fig. 4.1.5 **C**). Furthermore, minocycline pretreatment reduced FosB-ir neurons by a modest 10% ($p > 0.05$) from the nicotine group in the NAc (Fig. 4.1.5 **C**).

In the mPFC, nicotine treatment increased the density of FosB-ir neurons by 32% when compared to vehicle ($F_{3,19} = 1.20$, $p > 0.05$) (Fig. 4.1.5. **D**). Minocycline pretreatment reduced density of FosB-ir neurons by 28% ($p > 0.05$) from the nicotine group. In the DG, nicotine treatment increased the density of FosB-ir neurons by 52 % over vehicle control and was significantly different from the other groups ($F_{3,24} = 6.3$, $p < 0.05$) (Fig. 4.1.6 **E**). Minocycline pretreatment reduced the density of FosB-ir neurons by 34% ($p < 0.05$) in the DG. In all three areas, the density of FosB-ir neurons in rats that had received both minocycline and minocycline-prior-to-nicotine were less than that obtained for rats that received nicotine only.

Effect sizes were calculated to assess biological relevance of treatments in the NAc and PFC (Table 4.1). Again, only the changes in the DG were statistically significant, but the effect sizes of the other groups, especially in the PFC, indicate that responses were biologically significant.

4.2. Conditioned place preference

To test for changes in locomotion as a result of the treatments, the total number of chamber entries (entry into the three chambers) were tallied both pre- and post-conditioning (days 3 and 12 respectively) (Table 4.2). There was an increase in the total number of entries after conditioning among the 4 groups with the minocycline pretreatment group showing the

most percent increase (73%), while the nicotine group showed a 46% increase between pre- and post-conditioning. The vehicle and minocycline groups showed a modest increase of 30% pre- vs. post-conditioning. There was no significant difference in changes in total number of entries during either pre-conditioning or post-conditioning due to the large variances. These data demonstrated that the different exposures had not altered baseline activity or ‘liking’ for the different chambers.

To test for changes in responsivity of adult rats to cocaine due to the various treatment schedules at periadolescence, the CPP paradigm was utilized. Minocycline pretreatment was used to selectively suppress the cytotoxic (M1) polarization of microglia in order to assess the impact of inflammatory microglia activation in facilitating the sensitization process. A dose of 3.0 mg/kg i.p. cocaine was chosen based on previous work that examined the dose-response relationship for cocaine in the CPP paradigm (Jones and McMillen 1995). The expectation was that this dose would be at the threshold for a significant effect. Indeed, the vehicle group did demonstrate an increase of 71% ($p>0.05$) in the cocaine-paired chamber between pre- and post-conditioning (Fig. 4.2.1 **A**). When the time spent in the least-preferred chamber prior to conditioning for each rat was subtracted from the rat’s post-conditioning time, the differences between the exposures became more apparent (Fig. 4.2.1 **B**). Both the nicotine-only and minocycline pretreatment groups exhibited a greater than 100% further increase in time spent in the cocaine-paired chamber. An increase of 118% ($p<0.05$) in the difference-of-time spent in the cocaine-paired chamber (pre- vs post-conditioning) was observed in seconds for the nicotine treatment group when compared to the vehicle-only group (Fig. 4.2.1 **B**). Interestingly, minocycline pretreatment group also showed a similar increase of 125% ($p<0.05$) when compared to vehicle. No significant differences (pre- vs post-conditioning) were observed

between the minocycline control group and vehicle control group, which indicates that minocycline alone had no impact on sensitization to cocaine (Fig. 4.2.1 **A and B**). Furthermore, all groups on average spent an equal time in the cocaine-paired chamber prior to conditioning (Fig. 4.2.1 **A**).

4.3. Activated Microglia and FosB-ir at PND 80 following periadolescent injection schedule

Previously, Soderstrom and colleagues (2007) demonstrated that nicotine exposure during the ten days that bracket the onset of puberty in rats (periadolescence, PND 35-44) significantly increased the density of FosB-ir neurons in the DG, and the NAc. Furthermore, this increase persisted into adulthood (PND 80). The purpose of this study was to extend the aforementioned study to investigate how suppression of inflammatory microglia during periadolescence affects the induction of FosB in the brain of adult rats that were previously exposed to nicotine during periadolescence. For this purpose, 30 mg/kg minocycline was administered 30 minutes prior to each 0.4 mg/kg nicotine injections once daily for the 10 days of periadolescence (PND 35-44). After allowing the rats to mature to PND 80, brain sagittal sections from all the treatments were analyzed using IHC, with antibodies directed at FosB or Iba1.

In the NAc, Iba1 staining of PND 80 brain sections indicated that nicotine exposure during periadolescence increased the density of activated microglia (as determined by morphology) by 28% when compared to vehicle ($F_{3,12} = 2.9$, $p > 0.05$, nicotine *vs* vehicle) (Figs. 4.3.1, 4.3.5 **A**). Minocycline treatment 30 minutes prior to each daily dose of nicotine during periadolescence led to a decrease of 15.4% from the vehicle (minocycline-prior-to-nicotine *vs* vehicle, $p > 0.05$) at adulthood. The minocycline control reduced the density of activated microglia by 25% from the vehicle (minocycline control *vs* vehicle, $p > 0.05$) (Figs 4.3.1, 4.3.5

A). An unexpected observation was that the total microglia density was reduced for the minocycline control by 54% when compared to vehicle ($F_{3,12} = 3.5$, $p > 0.05$, Fig. 4.3.7 **A**), and a significant reduction when compared to nicotine ($p < 0.05$). Nicotine and vehicle showed an almost identical total microglia density. Minocycline pretreatment prior to nicotine also resulted in a modest decrease of 19.6% compared to nicotine-alone ($p > 0.05$, Fig. 4.3.7 **A**) for total microglia density.

Similar trends in activated and total microglia density emerged in the mPFC during adulthood 35 days following periadolescent injection schedule. Nicotine exposure from periadolescence increased the density of activated microglia during adulthood by 19.3% compared to vehicle ($F_{3,12} = 5.8$, $p > 0.05$, Figs. 4.3.2, 4.3.5 **B**). Minocycline pretreatment had little impact on the density of activated microglia compared to the vehicle group, a reduction of 9.6% ($p > 0.05$), but compared to the nicotine group, the reduction was 24.2% ($p > 0.05$). The nicotine group however showed a significant increase of 31% in the density of activated microglia when compared to the minocycline control group ($p < 0.05$). The total density of microglia in the mPFC was consistent among the various treatments, with the exception of the minocycline control which showed 21% fewer microglia on average compared to the vehicle control ($p > 0.05$, Fig. 4.3.7 **B**). Total microglia density was significantly lower than the nicotine treatment group by 24.4% ($F_{3,12} = 3.6$, $p < 0.05$, Fig. 4.3.7 **B**).

In the case of FosB-ir, results obtained were in harmony with a previous report (Soderstrom et al. 2007) in that the once-daily for 10 days nicotine exposure increased the density of FosB-ir neurons in all the regions of interest, namely NAc, mPFC, and DG of the hippocampus (Fig 4.3.6 **E**). In the NAc, periadolescent nicotine treatment increased the density of FosB-ir neurons by 25.4% when compared to vehicle ($F_{3,16} = 0.43$, $p > 0.05$, Fig. 4.3.3, 4.3.5

C). Minocycline pretreatment reduced density of FosB-ir neurons by 21.6% from the nicotine group ($p>0.05$). In the mPFC, there was a significant increase of 96.8% in the density of FosB-ir neurons when compared to vehicle control ($F_{3,16} = 4.5$, $p<0.05$, Figs. 4.3.4, 4.3.5 **D**).

Minocycline pretreatment reduced the density of FosB-ir neurons from nicotine group by 36.7% ($p<0.05$), whereas in minocycline control, FosB-ir neuron density differed from vehicle by 51.3% ($p>0.05$). Finally, in the dentate gyrus, periadolescent nicotine treatment resulted in a very modest increase of 15.6% ($F_{3,16} = 0.25$, $p>0.05$, Fig. 4.3.6 **E**) when compared to vehicle at adulthood, whereas minocycline pretreatment increased the number of FosB positive neurons in the DG by 12.7% ($p>0.05$) compared to vehicle. Thus, the patterns were similar to the data obtained at PND 45, but not as robust.

Table 4.1 Effect sizes to compare the effects on FosB-ir at PND 45 by the different periadolescent treatment groups to nicotine control (Veh- vehicle, Nic- nicotine, Min- minocycline, NM- minocycline 30 minutes prior to nicotine).

NAc	Effect Size
Veh	0.44
Min	0.89
NM	0.28
PFC	Effect Size
Veh	0.66
Min	0.74
NM	0.77

Fig 4.1.1: Images of microglia in the NAc following immunoreaction with Iba1 antibody in PND 45 rat brain sagittal sections 24-hours following 10 once-daily periadolescent injection schedule. Panels **A-C** show vehicle (Veh) treatment, **D-F** show nicotine (Nic) treatment, **G-I** show minocycline (Min) treatment, and **J-L** show minocycline 30 minutes prior to nicotine (Nic + Min). 200 X magnification images for each treatment are represented by insets in panels **A, D, G, and J**. Arrows indicate microglia displaying activated morphology, arrowheads indicate ramified microglia. Scale bar for 12.5 X is 500 μm ; for 100 X and 200 X are 100 μm respectively.

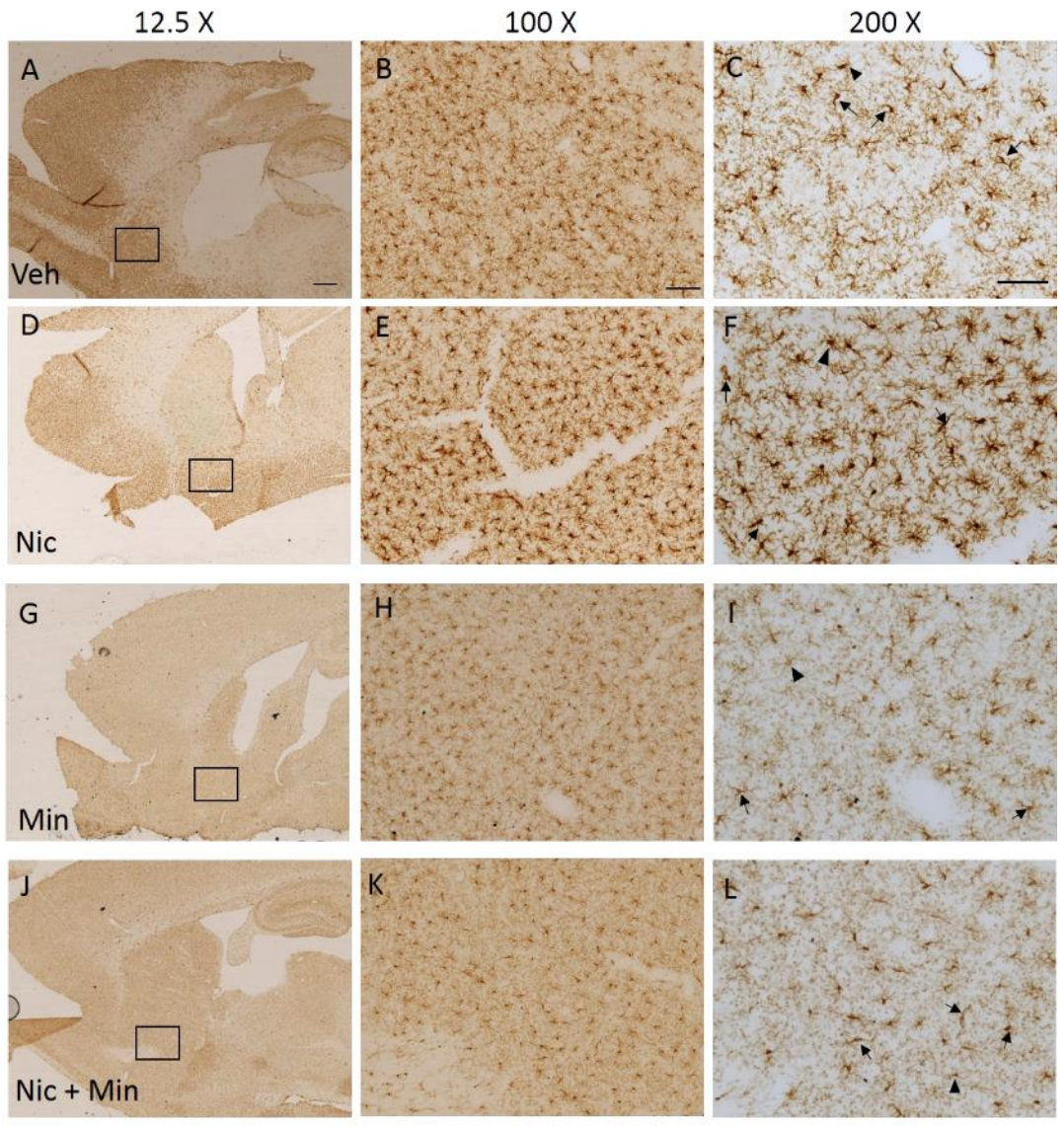


Fig 4.1.2: Images of microglia in the mPFC following immunoreaction with Iba1 antibody in PND 45 rat brain sagittal sections 24 hours following periadolescent injection schedule. Panels **A-C** show vehicle (Veh) treatment, **D-F** show nicotine (Nic) treatment, **G-I** show minocycline (Min) treatment, and **J-L** show minocycline 30 minutes prior to nicotine (Nic + Min). 200 X magnification images for each treatment are represented by insets in panels **A, D, G, and J**. Arrows indicate microglia displaying activated morphology, arrowheads indicate ramified microglia. Scale bar for 12.5 X is 500 μm ; for 100 X and 200 X are 100 μm respectively.

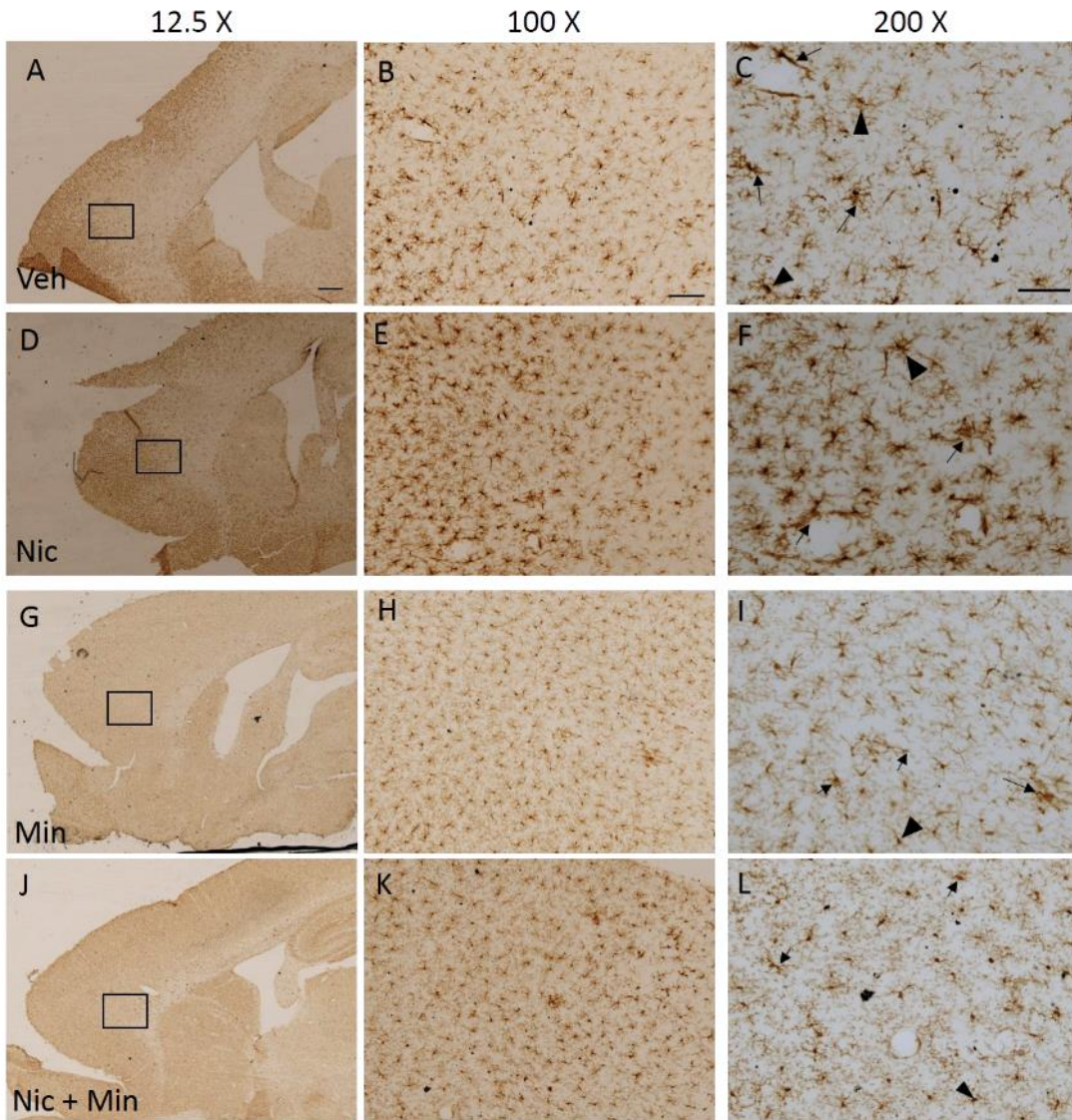


Fig 4.1.3: Images of FosB-ir neurons in the NAc following immunoreaction with FosB antibody in PND 45 rat brain sagittal sections 24 hours following periadolescent injection schedule. Panels **A-C** show vehicle (Veh) treatment, **D-F** show nicotine (Nic) treatment, **G-I** show minocycline (Min) treatment, and **J-L** indicate minocycline 30 minutes prior to nicotine (Nic + Min). 100 X magnification images for each treatment are represented by insets in panels **A, D, G, and J**. Scale bar for 12.5 X is 500 μm ; 5X for 50 X and 100 X are 100 μm respectively.

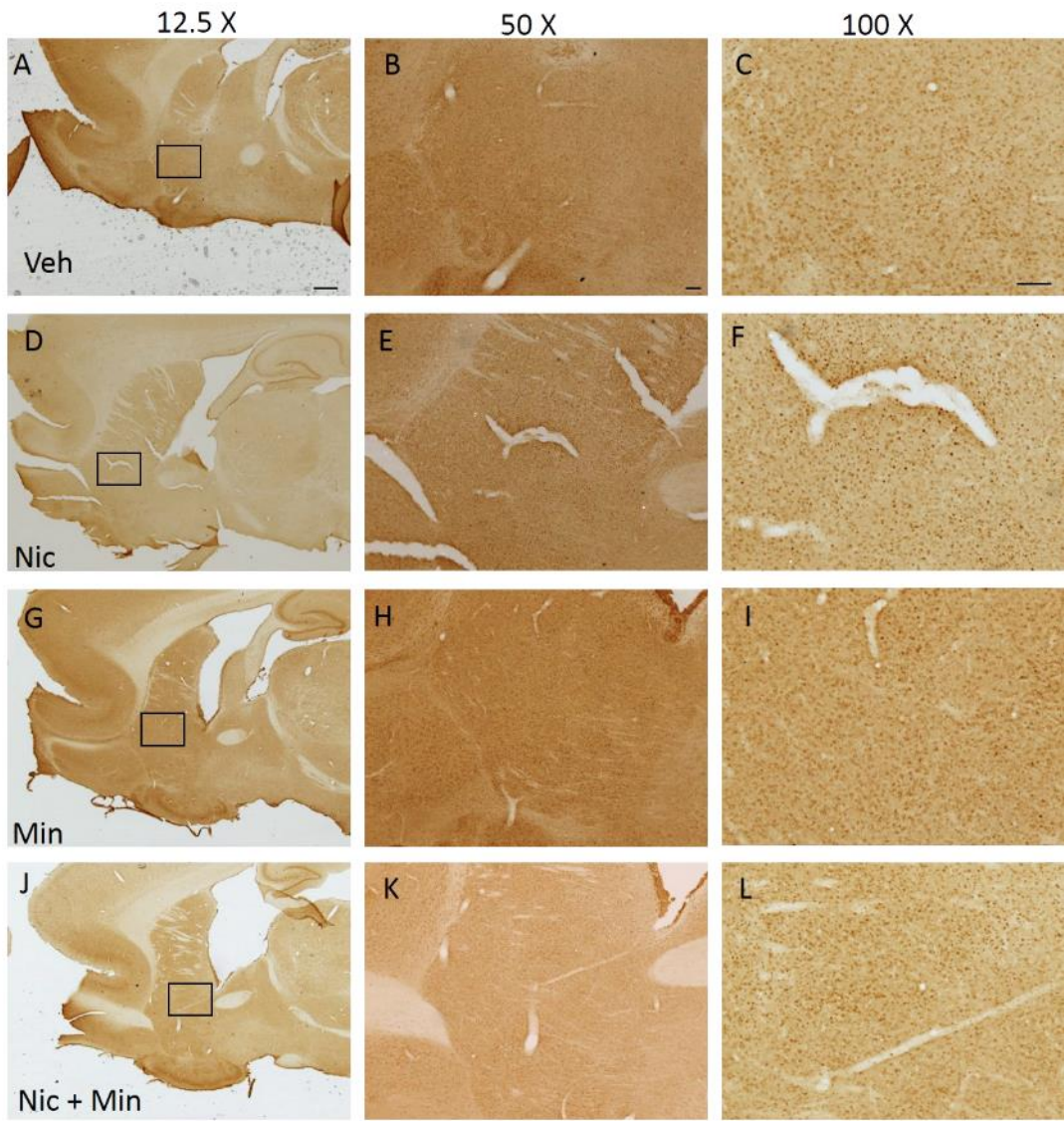


Fig 4.1.4: Images of FosB-ir neurons in the mPFC following immunoreaction with FosB antibody in PND 45 rat brain sagittal sections 24-hours following periadolescent injection schedule. Panels **A-C** show vehicle (Veh) treatment, **D-F** show nicotine (Nic) treatment, **G-I** show minocycline (Min) treatment, and **J-L** show minocycline 30 minutes prior to nicotine (Nic + Min). 100 X magnification images for each treatment are represented by insets in panels **A, D, G, and J**. Scale bar for 12.5 X is 500 μm ; for 50 X and 100 X are 100 μm respectively.

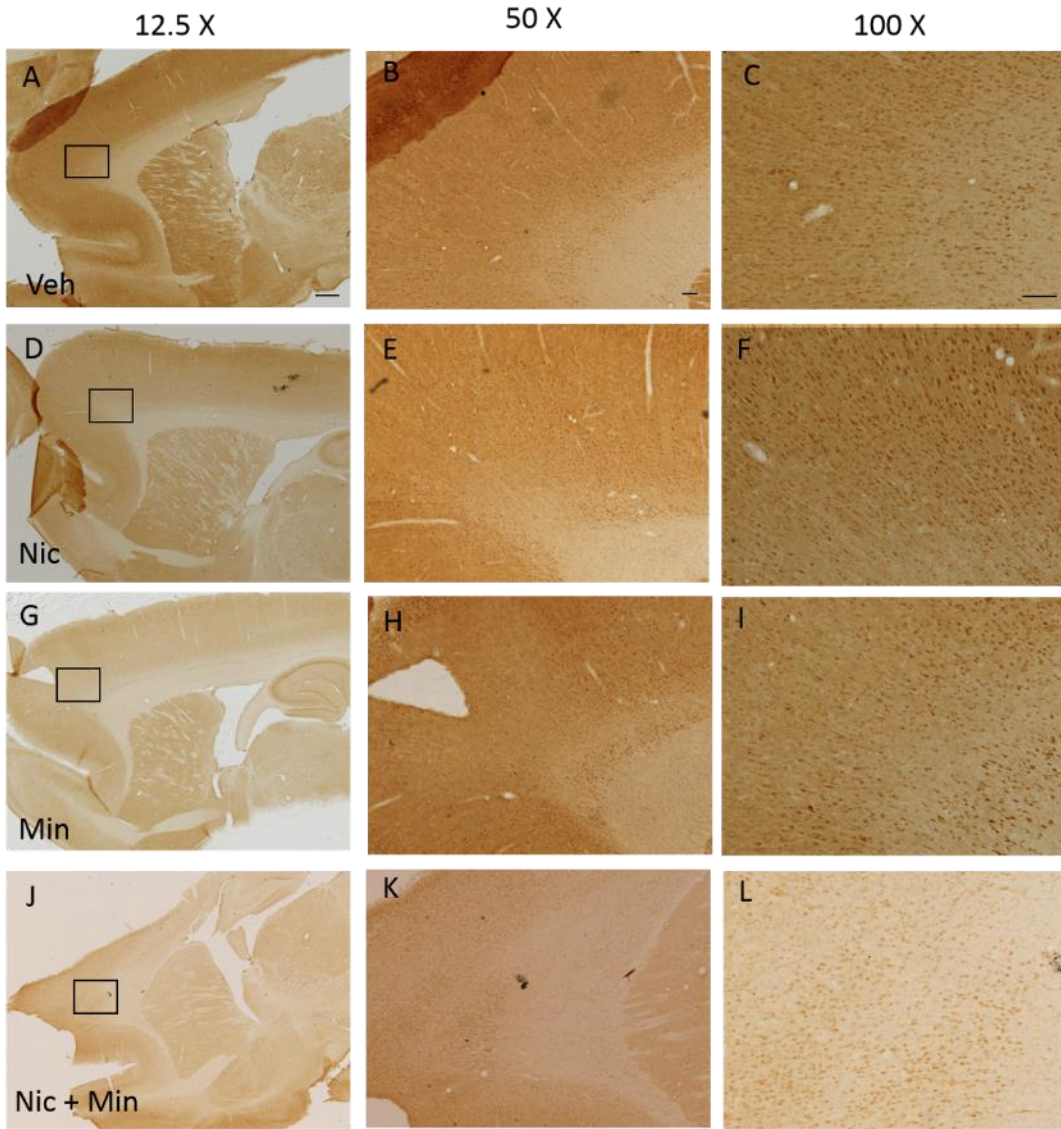


Fig 4.1.5: Bar graphs representing densities of microglia displaying activated morphology and FosB-ir in the NAc and mPFC among the 4 treatment groups at PND 45 following periadolescent injection schedule. Density of microglia displaying activated morphology in the NAc (**A**) and mPFC (**B**). Bar graphs in panels (**C**) and (**D**) represent densities of FosB-ir neurons (Reactive nuclei / mm²) in NAc and mPFC respectively. (* in (**A**) different from all treatment groups (p<0.05, Dunnett's t-test)), (* in (**B**) different from Veh and Min (p<0.05, Dunnett's t-test)).

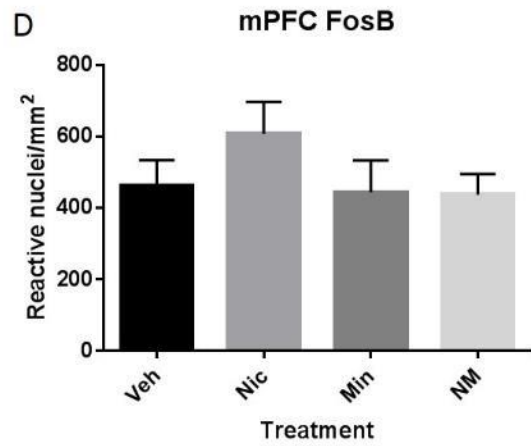
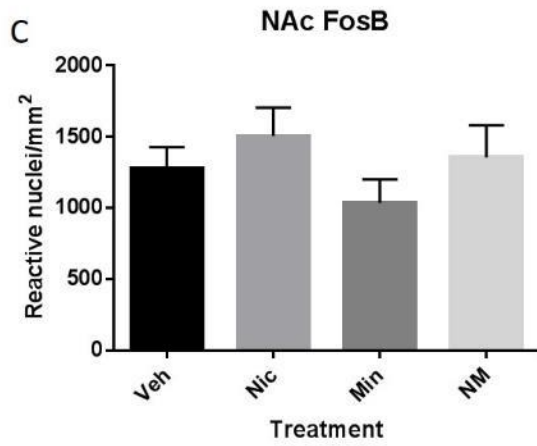
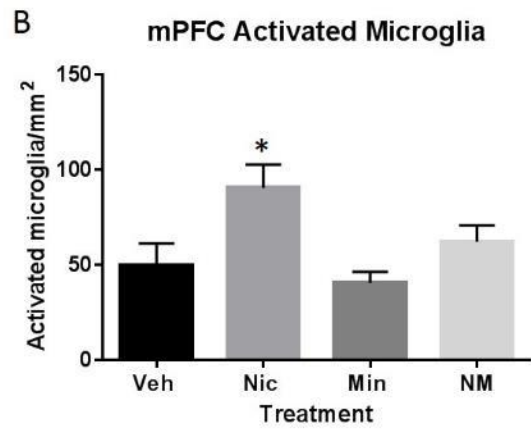
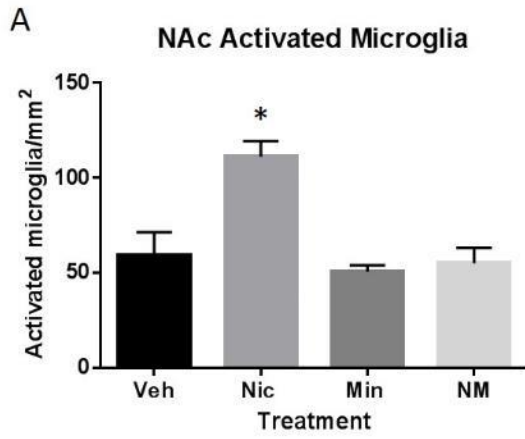


Fig 4.1.6: Images displaying FosB-ir in the DG of the hippocampus in PND 45 rat brain sagittal sections 24-hours following periadolescent treatment schedule. **A-D** The different treatment groups: vehicle, nicotine, minocycline, minocycline 30 minutes prior to nicotine respectively. 100X magnification. **E** Bar graph depicting density of FosB-ir neurons (Reactive nuclei / mm²). (*different from all treatments, p<0.05, Dunnett's t-test). Scale bar in **D** 50 μm.

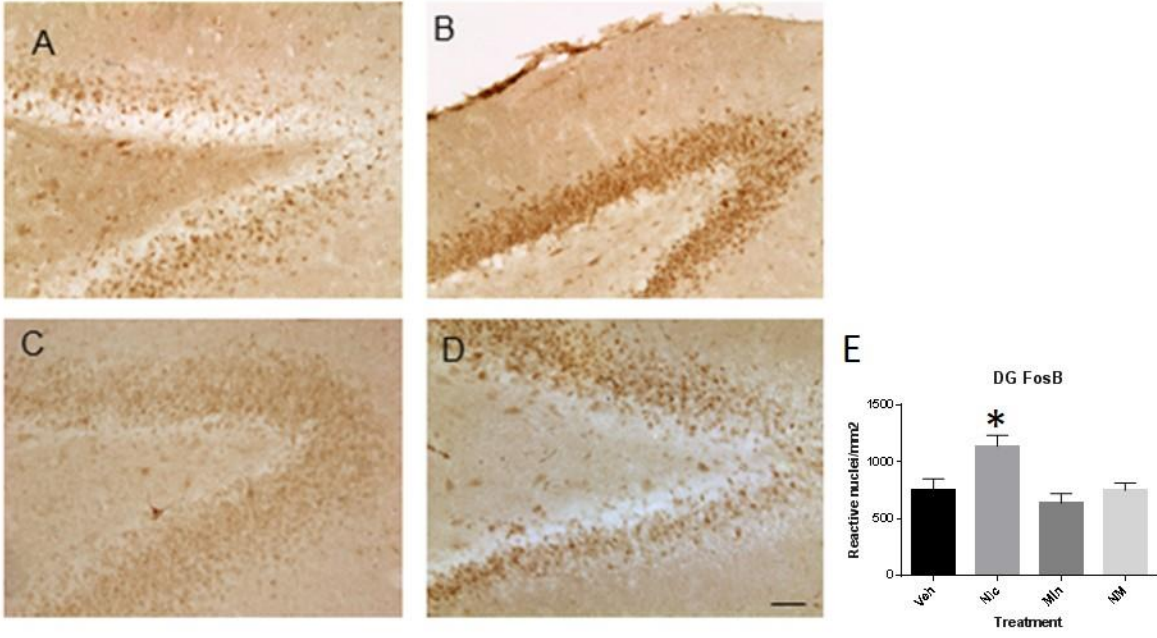


Table 4.2

Total CPP apparatus chamber entries for pre-conditioning and post-conditioning with cocaine.

Group	Pre-conditioning	Post-Conditioning
Vehicle (11)	36 ± 5.7	47 ± 8.2
Nicotine (11)	30 ± 4.2	44 ± 8.2
Minocycline (8)	27 ± 6.7	35 ± 6.1
Minocycline+Nicotine (10)	23 ± 5.0	40 ± 6.2

Fig 4.2.1: Graphs of conditioned place preference showing the time spent (seconds) in the least preferred or cocaine-paired chamber at PND 80. **A** Time spent in the least preferred chamber during pre-conditioning and post-conditioning [* represents significant difference ($p < 0.05$) between pre and post]. **B** Time difference (pre-conditioning subtracted from post conditioning, (* show significant difference from vehicle group). $n=8-11$. Mean \pm SEM. Post hoc analysis Dunnett's t-test.

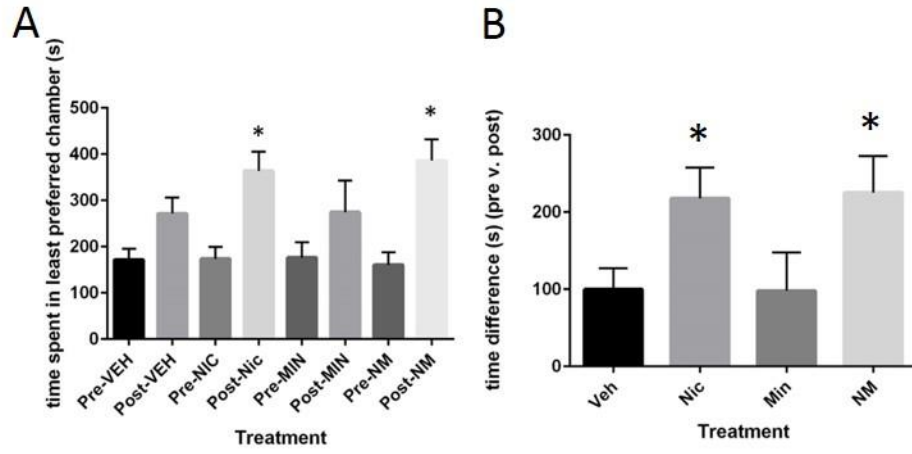


Fig 4.3.1: Images of microglia in the NAc following immunoreaction with Iba1 antibody in PND 80 rat brain sagittal sections following periadolescent injection schedule. Panels **A-C** show vehicle (Veh) treatment, **D-F** show nicotine (Nic) treatment, **G-I** indicate minocycline (Min) treatment, and **J-L** indicate minocycline 30 minutes prior to nicotine (Nic + Min). 200 X magnification images for each treatment are represented by insets in panels **A, D, G, and J**. Arrows indicate microglia displaying activated morphology, arrowheads indicate ramified microglia. Scale bar for 12.5 X is 500 μm ; for 100 X and 200 X are 100 μm respectively.

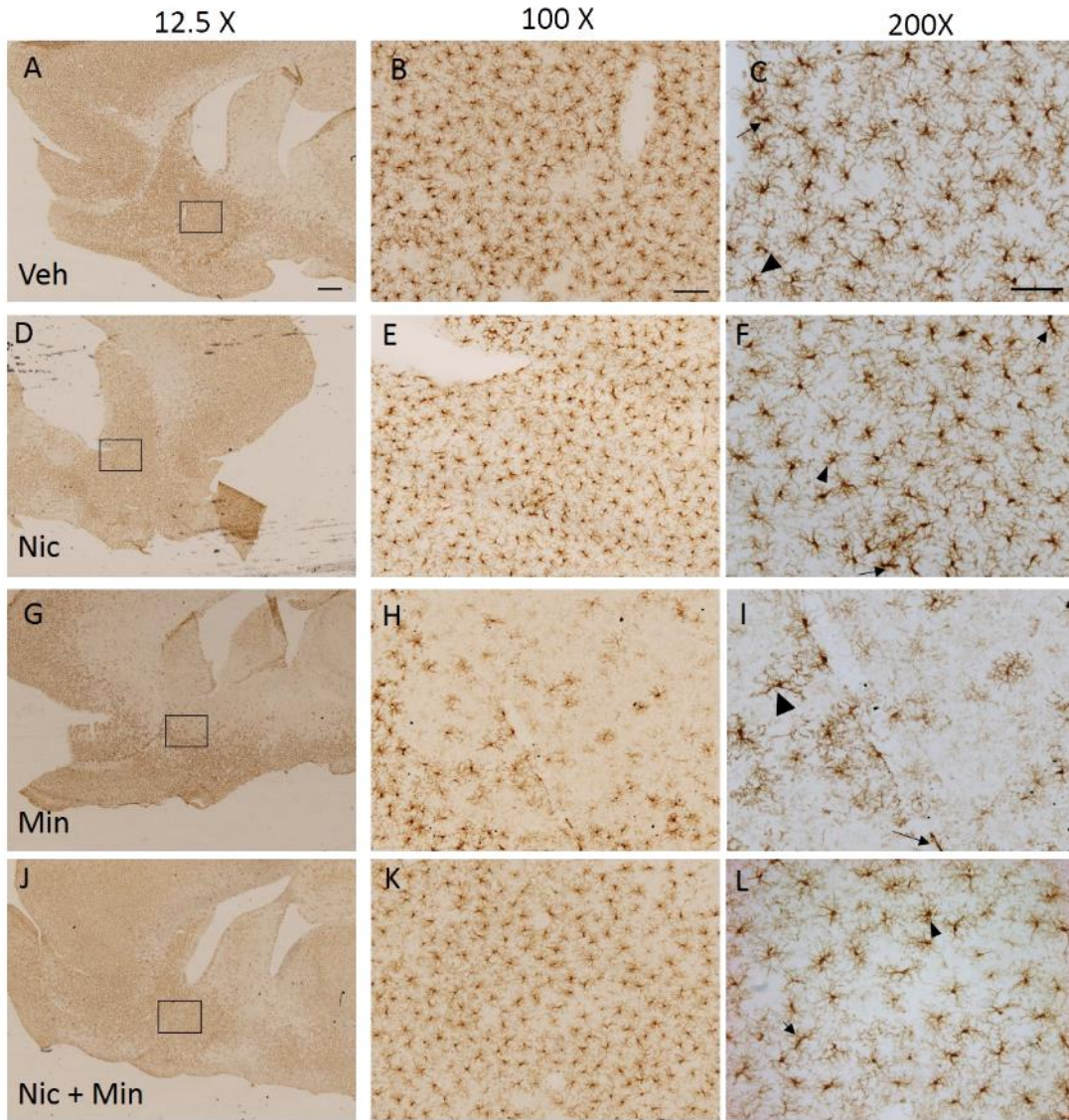


Fig 4.3.2: Images of microglia in the mPFC following immunoreaction with Iba1 antibody in PND 80 rat brain sagittal sections following periadolescent injection schedule. Panels **A-C** show vehicle (Veh) treatment, **D-F** show nicotine (Nic) treatment, **G-I** indicate minocycline (Min) treatment, and **J-L** indicate minocycline 30 minutes prior to nicotine (Nic + Min). 200 X magnification images for each treatment are represented by insets in panels A, D, G, and J. Arrows indicate microglia displaying activated morphology, arrowheads indicate ramified microglia. Scale bar for 12.5 X is 500 μm ; for 100 X and 200 X are 100 μm respectively.

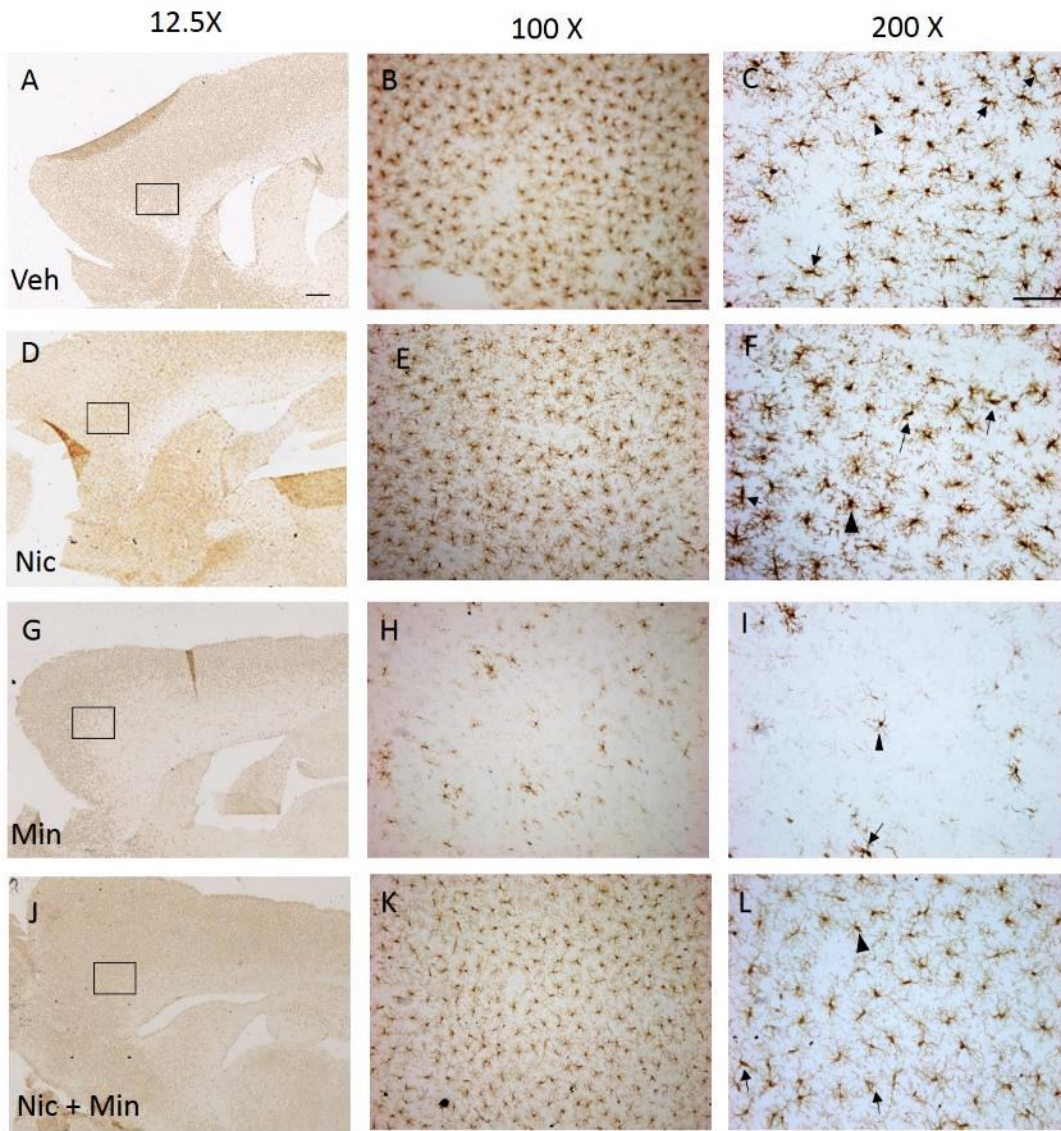


Fig 4.3.3: Images of FosB-ir neurons in the NAc following immunoreaction with FosB antibody in PND 80 rat brain sagittal sections following periadolescent injection schedule. Panels **A-B** show vehicle (Veh) treatment, **C-D** show nicotine (Nic) treatment, **E-F** indicate minocycline (Min) treatment, and **G-H** indicate minocycline 30 minutes prior to nicotine (Nic + Min). 100 X magnification images for each treatment are represented by insets in panels **A, C, E, and G**. Scale bar for 12.5 X is 500 μm ; for 100 X is 100 μm .

12.5 X

100 X

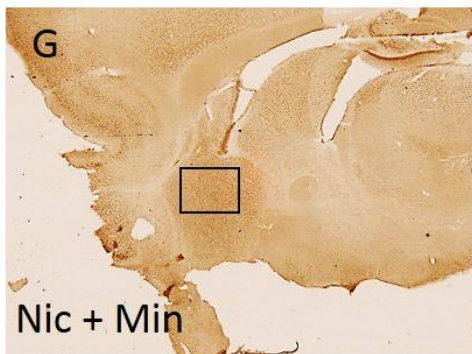
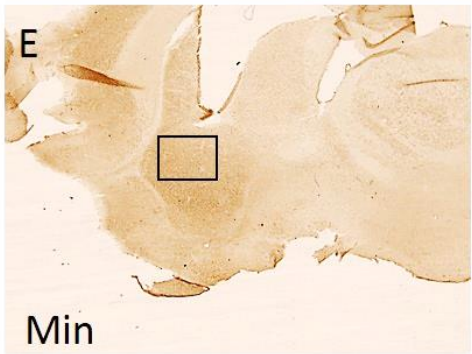
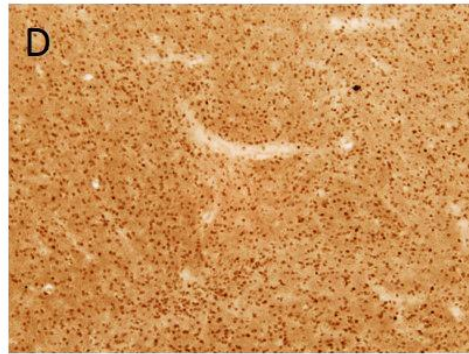
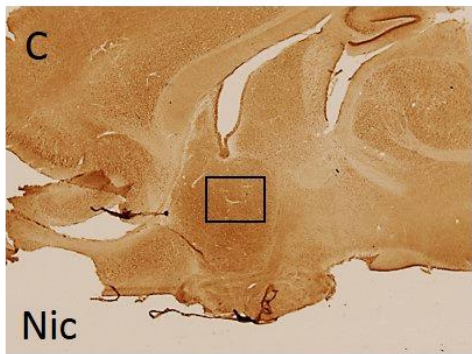
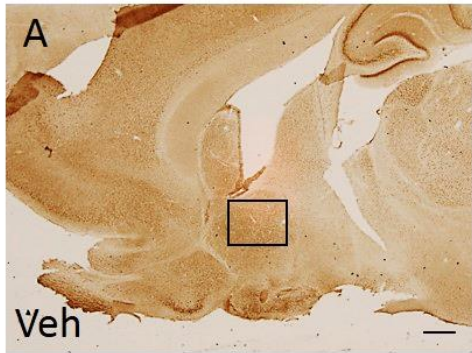


Fig 4.3.4: Images of FosB-ir neurons in the mPFC following immunoreaction with FosB antibody in PND 80 rat brain sagittal sections following periadolescent injection schedule. Panels **A-B** show vehicle (Veh) treatment, **C-D** show nicotine (Nic) treatment, **E-F** show minocycline (Min) treatment, and **G-H** show minocycline 30 minutes prior to nicotine (Nic + Min). 100 X magnification images for each treatment are represented by insets in panels **A, C, E and G**. Scale bar for 12.5 X is 500 μm ; for 100 X is 100 μm .

12.5 X

100X

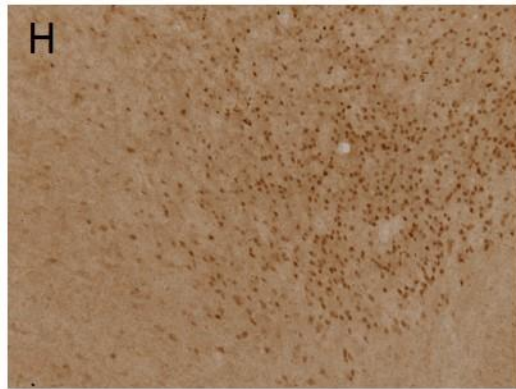
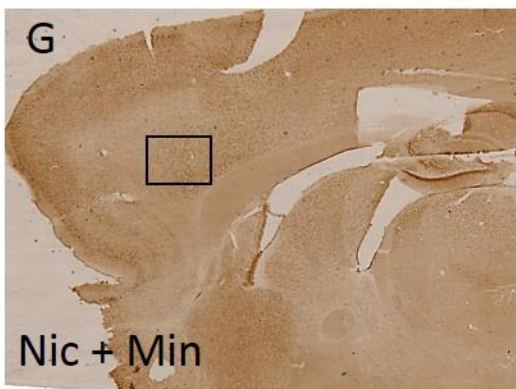
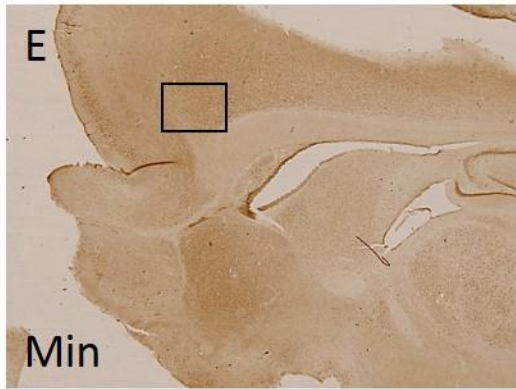
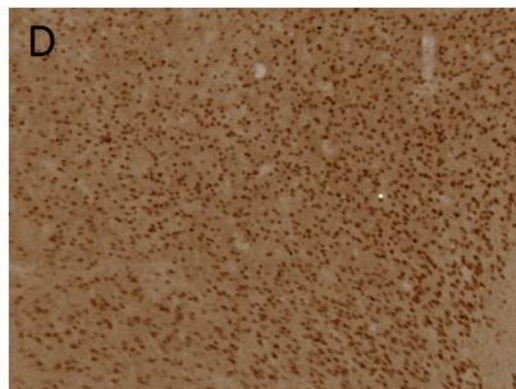
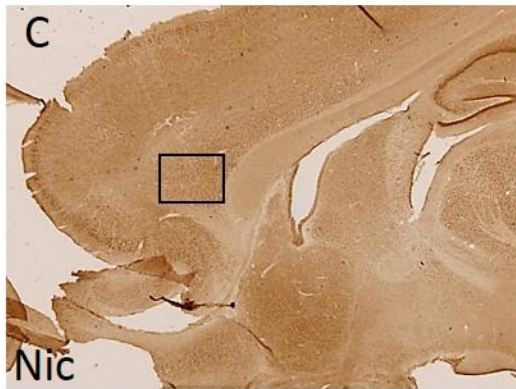
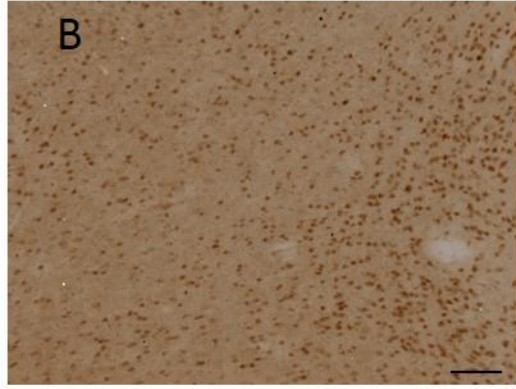
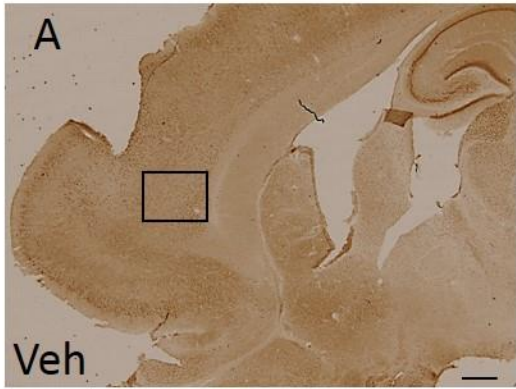


Fig 4.3.5. Bar graphs representing densities of microglia displaying activated morphology and FosB-ir in the NAc and mPFC among the 4 treatment groups at PND 80 following periadolescent injection schedule. Bar graphs in panels (C) and (D) represent densities of FosB-ir neurons in NAc and mPFC respectively between the 4 treatment groups. (* in (A and B) different from minocycline (MIN) group ($p < 0.05$, Dunnett's t-test), * in (D) different from vehicle (VEH) and (NM) groups ($p < 0.05$, Dunnett's t-test)).

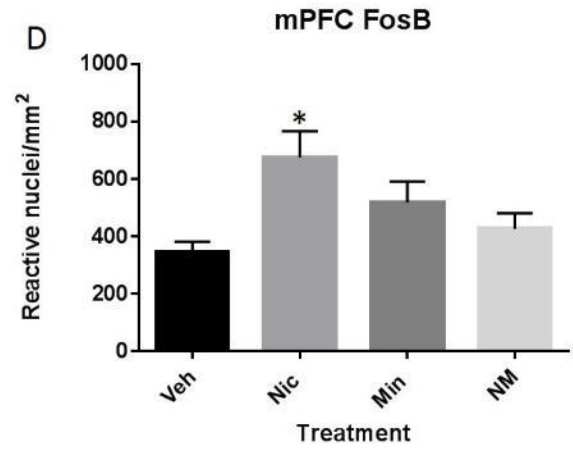
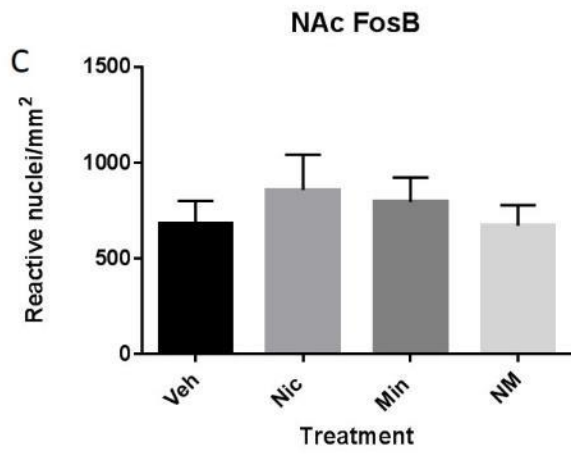
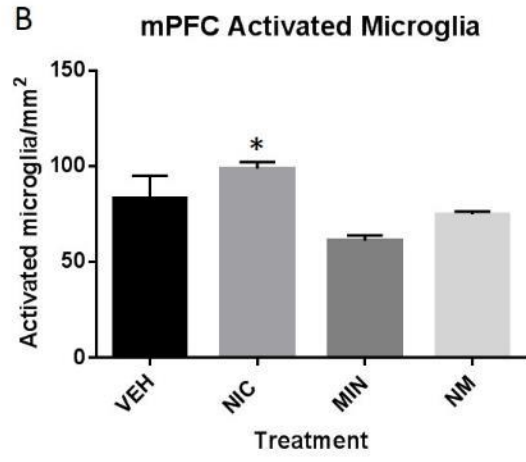
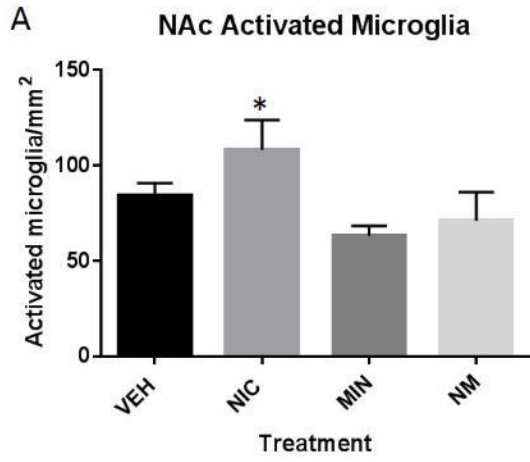


Fig 4.3.6. Images of FosB-ir neurons in the DG of the hippocampus in PND 80 rat brain sagittal sections following 10 once-daily periadolescent treatment schedule. **A-D** The different treatment groups: vehicle, nicotine, minocycline, minocycline 30 minutes prior to nicotine respectively. **E** Bar graph depicting relative densities of FosB-ir neurons among the 4 treatment groups. (No significant change observed between groups, $p > 0.05$, Dunnett's t-test). Scale bar in **D** 50 μm .

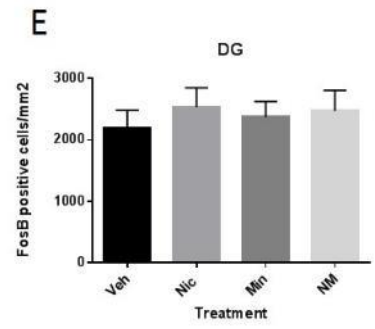
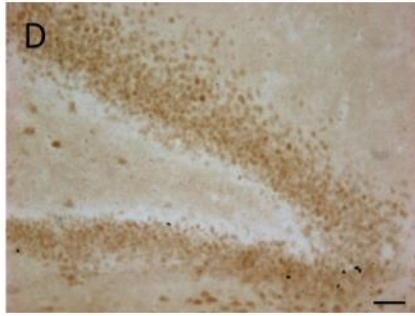
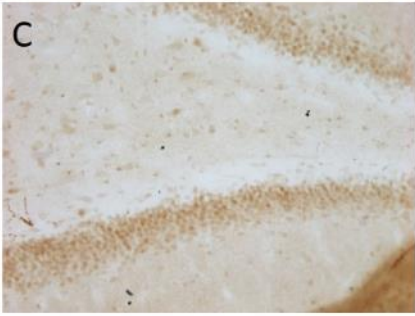
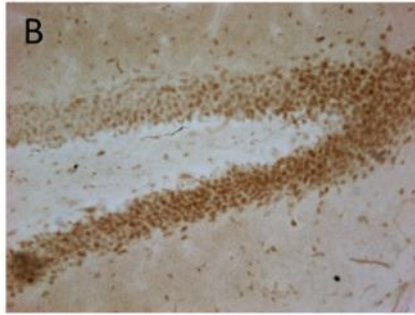
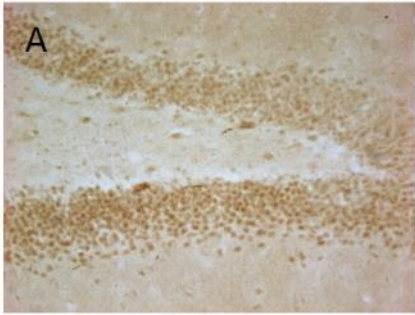
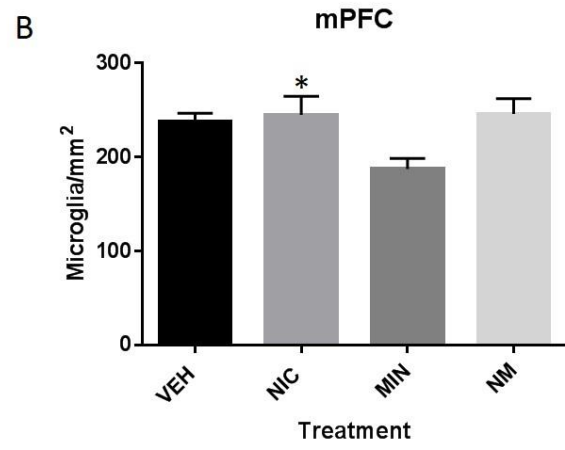
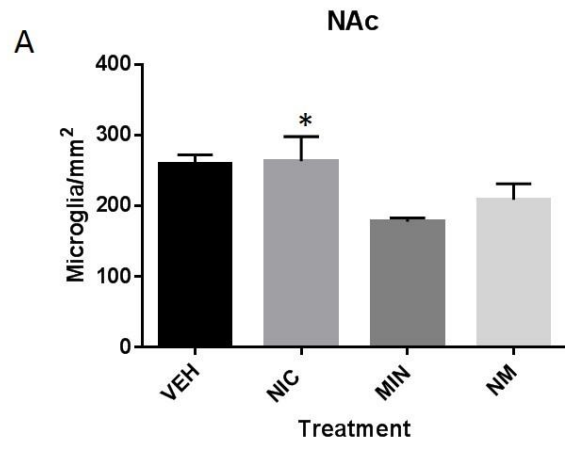


Fig 4.3.7: Bar graphs depicting total density of microglia in NAc and mPFC among the 4 treatment groups. **(A)** NAc **(B)** mPFC at PND 80. (***A** and **B**, different from minocycline control).



Ch 5. Discussion

5.1. Purpose of Dissertation

The purpose of this dissertation study was to investigate the role of inflammatory microglia in the process of nicotine priming of the brain that makes the brain more susceptible to cross sensitization to other drugs of abuse, particularly at adulthood. To achieve this, we sought to address 3 specific aims (SA). The purpose of SA 1 was to investigate changes in the expression patterns of the transcription factor FosB in select areas of the rat brain associated with drug-reward and memory 24 hours following a 10-day schedule of once-daily injections marking the onset of adolescence (PND 35-44). Δ FosB, the spliced isoform of FosB, is often referred to as a ‘sustained molecular switch for addiction’ (Nestler et al. 2001), thus making FosB a suitable protein target for investigating molecular changes in the brain pertaining to sensitization, an ‘important first step’ leading to addiction (Vanderschuren and Pierce 2010). In this study, the rat model system was utilized for testing the molecular and behavioral effects of microglia on the sensitization process, as several studies suggest that microglia may play a crucial role in mediating sensitization and processes involved with addiction such as relapse arising from exposure to drugs of abuse during early adolescence (Chen et al. 2009, Schwarz and Bilbo 2013). For this purpose, subject animals were segregated into 4 groups, namely vehicle, 0.4 mg/kg nicotine, 30 mg/kg minocycline, or 30 mg/kg minocycline 30 minutes prior to each dose of nicotine. Minocycline, the brain-permeable tetracycline antibiotic was used to suppress the pro-inflammatory (M1) activation of microglia (Kobayashi et al. 2013). SA 2 and 3 sought to investigate the impact of these periadolescent treatments at adulthood (PND 80), when the brains of rats are supposedly fully developed. The effect of these treatments on rats’ receptivity to cocaine was measured by using CPP (SA 2), and changes in the levels of FosB and microglia

displaying activated morphology were assessed by immunohistochemistry (IHC) similar to SA1 (SA 3).

5.2.A. Previous studies vs present study

Previous reports involving rats indicate that priming with nicotine during a specific ontological window, namely the onset of adolescence or periadolescence, is crucial in establishing brain-sensitization that is conducive to enhanced reward motivation toward drugs of abuse that is sustained into adulthood (McMillen et al. 2005, James-Walke et al. 2007, Adriani et al. 2003). Studies that utilized operant conditioning or CPP indicate that rats were more anticipatory towards the rewarding effects of submaximal doses of psychoactive substances like nicotine, cocaine, and diazepam when they were primed with nicotine at early adolescence as opposed to later life, an indication that sensitization had developed (Adriani et al. 2003, McMillen et al. 2005, James-Walke et al. 2007). The explanation for this observation, as well as the mechanisms that give rise to this effect were not comprehensively addressed, although one previous study indicated that nicotine exposure at adolescence was able to enhance FosB expression by the inhibition of histone deacetylases to enhance reward sensitivity to cocaine challenge at adulthood (Levine et al. 2011).

Previous studies that investigated molecular mechanisms to explain the establishment of a drug-sensitized brain have uncovered several target candidates that may contribute to this end. Nicotinic receptors appear to be the most obvious candidate in initiating nicotine-induced reward sensitization. Several nicotinic receptor (NACHR) subunits are upregulated in the VTA of the brains of adult rats when the rats were exposed to nicotine during early adolescence (Adriani et al. 2003). Another target candidate to receive recent attention is the IEG transcription factor FosB along with its spliced variant Δ FosB that are enhanced by nicotine as well as other drugs

with addiction liability (Soderstrom et al. 2007, Perrotti et al. 2008). FosB is short-lived compared to Δ FosB that activates downstream genes that effectively regulate dopaminergic (DA) signaling that mediates reward and sensitization (Nestler et al. 2001). Enhanced FosB immunoreactivity (FosB-ir) is achieved in brain areas associated with reward and reward-associated memory not only when rats are exposed to nicotine during the onset of adolescence, but also when nicotine priming occurs at late/post adolescence (PND 60-69) (Soderstrom et al. 2007). Since enhanced cross-sensitization to other drugs of abuse in the adult is a key feature of nicotine priming during the onset of adolescence (Adriani et al. 2003, McMillen et al. 2005), the observed increase in FosB from late adolescent exposure weakens the causal link between FosB induction and the sensitization observed during adult testing. This effect was further highlighted when adult rats showed reduced responsiveness to cocaine or diazepam when primed with nicotine during late adolescence (Nestler et al. 2001, McMillen et al. 2005, James-Walke et al. 2007). Thus, investigation of other mediators that promote this temporal aspect of nicotine-induced reward-sensitization is warranted.

5.2.B. M1 activation of microglia had impact on FosB, but did not contribute towards sensitization

In this dissertation study, the contribution of microglia in mediating periadolescent nicotine-induced sensitization of the brain was examined as microglia are known to play an active role in brain plasticity along with circuit remodeling during adolescence (Paolicelli et al. 2017). Furthermore, microglia are responsive to the hormonal changes in the brain that are expected in adolescent males (Harry and Kraft 2012) and respond to drugs of abuse from various categories that include alcohol, opioids, and other stimulants that exert their rewarding effects by influencing the mesolimbic pathway (McClain et al. 2011, Schwarz and Bilbo 2013). Alcohol or

morphine exposure induces microglia to shift morphology toward an activated phenotype, with activation of pattern recognition receptors (PRRs) such as Toll-Like Receptors (TLRs) on microglia (Schwarz and Bilbo 2013, McClain et al. 2011, Crews et al. 2017). Interestingly, no studies to our knowledge have investigated microglia's role in periadolescent nicotine-induced sustained reward sensitization. It should also be noted that microglia express receptors sensitive to hormones and other neurotransmitters pertaining to sensitization such as dopamine and estrogens (Färber et al. 2005, Nissen et al. 2017).

In this study, it was observed that nicotine exposure once daily for 10 days at 0.4 mg/kg during the onset of adolescence increased FosB-ir in the NAc, mPFC, and DG of the hippocampus 24 hours following the final set of injections. This result was in harmony with the report by Soderstrom and colleagues (2007). In the present study, the increase in FosB-ir in the NAc was less pronounced than that reported by Soderstrom and colleagues (2007), however an increasing trend was evident for the nicotine treatment group, when compared to vehicle (Fig 4.1.5 C). To accompany the increase in FosB-ir with nicotine treatment, a statistically significant increase was also observed in the density of microglia displaying an 'activated' morphology, implying a positive correlation between microglia activation and FosB-ir. The same trend held in the mPFC, where nicotine enhanced the shift in microglia morphology from ramified to a more activated morphology (Fig 4.1.3 D-F, 4.1.5 B). This was expected as other drugs with abuse liability such as alcohol and opioids encourage microglia polarization that is pro-inflammatory (McClain et al. 2011, Schwarz and Bilbo 2013).

Several *in vitro* studies with isolated microglia indicate that nicotine has an anti-inflammatory effect when the microglia are challenged with lipopolysaccharide (LPS) that functions to polarize microglia to the pro-inflammatory phenotype (Noda and Kobayashi 2017,

Williamson et al. 2011). To our knowledge, there are no studies that demonstrate the activation of microglia in response to nicotine in the context of psychoactive drug-induced reward sensitization. Here we report an increase in the density of microglia displaying activated morphology in the NAc and mPFC following 10 days of once-daily nicotine treatment during periadolescence. Since minocycline is a selective inhibitor of the pro-inflammatory (M1-polarized) microglia, it would be expected that minocycline pretreatment would attenuate this polarization if nicotine induced a shift to the pro-inflammatory phenotype *in vivo* (Kobayashi et al. 2013). During periadolescent testing at PND 45, in the NAc, the minocycline pretreatment reduced the density of Iba1-immunoreactive (Iba1-ir) microglia that displayed the activated morphology significantly. Furthermore, this result suggests that nicotine promotes the shifting of microglia towards the M1 state. Minocycline pretreatment however did not result in a reduction in FosB-ir in the NAc as originally hypothesized, thus weakening the causal link between increased M1-polarized microglia and elevation in FosB-ir. Effect size calculations further suggest that inflammatory microglia activation has a trivial impact on the induction of FosB-ir neurons in the NAc at PND 45 by daily nicotine exposure during the key periadolescent window.

In the mPFC similar patterns emerged between the minocycline pretreatment and nicotine groups, with the exception that the density of FosB-ir neurons is more attenuated in the mPFC in the minocycline pretreatment group. Although this difference is not statistically significant, an effect size of 0.77 suggests that there could be a difference detected if the sample sizes were expanded. In the DG, on the other hand, there was an attenuation in FosB-ir neuronal density in the granular layer with minocycline pretreatment compared to the nicotine group. This was an interesting finding as the presence of microglia in the DG is sparser and less uniform when compared to the other brain nuclei examined. Microglial processes extend well into the granular

layers of the DG and make tactile contact with synapses and neurons in the granular layer.

Although spatial memory tests were not performed in this dissertation study, the reduction in FosB-ir in the DG could have an impact on memory as fluctuations in Δ FosB expression in the DG is demonstrated to affect memory consolidation in rodents (Eagle et al. 2015).

5.2.C. Periadolescent inhibition of inflammatory microglia and consequences at adulthood

To understand the long-term consequences of inflammatory microglia activation during periadolescent nicotine exposure, changes in FosB-ir and microglia activity were tested at adulthood. Since FosB-ir is sustained into adulthood in the brains of rats exposed to nicotine during periadolescence (Soderstrom et al. 2007), we suspected that neuronal expression of FosB and microglia activity in brain areas associated with reward and memory could be impacted if microglia activation during early adolescence due to nicotine had any long-term consequences. Some of the results observed during periadolescence (PND 45) were reversed at the onset of adulthood (PND 80). We initially expected that during adulthood, the densities of microglia displaying activated morphology may reverse due to clearance of minocycline following 35 days, and an increase in microglia proliferation over time. Although our study did not use a maximal or toxic dose of minocycline, it is possible that minocycline prevented proliferation of new-born microglia that retained an activated morphology similar to that reported in a binge alcohol study (McClain et al. 2011), thus leading to a sustained reduction in activated microglia in the minocycline pretreatment group over time (Fig 4.3.5 **A, B**). In harmony with this suggestion, the minocycline-only group resulted in reduced density of activated microglia in both the NAc and mPFC at PND 80 when compared to vehicle, which implies that minocycline is inhibiting the normal progress of microglia to an activated morphology from periadolescence to adulthood as observed in the vehicle group in both the NAc and mPFC (Fig. 4.3.5). From adult

brains, we observe that the difference in FosB-ir between nicotine and vehicle follows the same pattern as that of PND 45 in both the NAc and mPFC (Fig. 4.3.5 C, D) reflecting the report by Soderstrom and colleagues (2007). In the mPFC, both during PND 45 and PND 80, there were reductions in FosB-ir with the minocycline pretreatment, whereas at adulthood, the difference reached statistical significance (Fig. 4.3.5 D) giving the impression that the link between microglia and FosB with periadolescent nicotine exposure is greater and longer lasting in the mPFC than in the NAc. The link between microglia activation and FosB expression in neurons remains unclear but there are mediators that allow microglia to influence neuronal FosB activity. Microglia secrete moderate levels of glial-derived neurotrophic factor (GDNF), and with M1 activation of microglia, as observed with nicotine in this study, the level of GDNF decreases further. Reduced GDNF secretion is associated with increased DA signaling and Δ FosB expression (Airavaara et al. 2004). With minocycline pretreatment, GDNF signaling is expected to not be increased, thus possibly resulting in a lack of change in FosB-ir (NAc), or an attenuation (mPFC) at most. The NAc is the brain nuclei most often associated with drug seeking or incentive salience, and DA functioning in the NAc is implicated in CPP preference (Prus et al. 2009). Thus, the lack of significant change in FosB observed in the NAc both during PND 45 and 80, could be the reason for the lack of change observed in motivation for cocaine as observed with CPP testing.

Following the transition from periadolescence to adulthood, the magnitude of the differences in densities of activated microglia between the vehicle and nicotine groups are attenuated to the point where the difference was no longer statistically significant at PND 80, indicating that nicotine may not have had a long-lasting effect on keeping microglia in an activated state, or that over time there was an increase in the density of activated microglia in the vehicle group, a

phenomenon that is common with aging in adult rats (Lynch 2009). A significant difference was only present between the nicotine and minocycline-only groups. Furthermore, it was noted that overall microglia density in the minocycline-only group was significantly reduced compared to the nicotine group. This was interesting, as it appears that minocycline discouraged microglia proliferation which may have manifested as lower microglia density at adulthood. In the minocycline pretreatment group, nicotine appears to have promoted microglia proliferation or stability, whereas minocycline by itself discouraged microglia proliferation in the long term (Fig. 4.3.5 **A, B**; 4.3.7 **A, B**). The results from the CPP experiments were interesting, in that minocycline pretreatment prior to each nicotine exposure during periadolescence failed to inhibit anticipation for cocaine as initially hypothesized due to the enhanced sensitization from periadolescent nicotine (Fig 4.2.1). Submaximal doses of cocaine were used to test sensitization in this study as cocaine is a CNS stimulant that has its rewarding actions mediated via mesolimbic reward pathway and is commonly used in studies investigating reward sensitization (McMillen et al. 2005). Furthermore, nicotine priming in mice prior to cocaine enhances CPP for cocaine as well as locomotor responsiveness possibly through inhibition of histone deacetylase activity (Levine et al. 2011). The important caveat in our study compared to other studies testing for locomotor responsiveness to cocaine challenge such as that by Chen and colleagues (2009) is that the animals in this study were not subjected to the test drug on the day of testing. Twenty-four hours following final vehicle injection, rats were placed back into the CPP apparatus for 15 minutes and chamber preference and entries were determined. Also, prior to conditioning the rats displayed similar baseline preferences for the chamber that was to be paired with cocaine among all the 4 groups (Fig. 4.2.1 **A**), indicating that the different exposures at periadolescence had not altered the baseline exploration or locomotion of the adult rats. As

expected, the periadolescent nicotine treatment group did show increased responsiveness, in this case seeking behavior, to cocaine as compared to vehicle in harmony with previous studies (McMillen et al. 2005). The minocycline-only treatment group showed a trend similar to vehicle, indicating that suppression of M1 microglia by minocycline at the onset of adolescence did not have an impact on the rats' responsivity to cocaine at adulthood, neither an increase nor an inhibition of response, which in itself is an interesting find. Minocycline pretreatment to nicotine at periadolescence did not alter nicotine's ability to enhance response for cocaine, and the rats thus exhibited a response for the cocaine-paired chamber similar to that of the nicotine group. This result suggests that M1 polarization of microglia during periadolescence, when nicotine exerts its sensitizing effect, does not appear to be the cause for nicotine's ability to sensitize the brain to the rewarding effects of cocaine. Although, this study did not measure the expression levels of cytokines characteristic of M1 polarization such as IL-1 β , TNF-alpha, or certain TLRs, staining with antibodies selective for Iba1 during periadolescence revealed fewer microglia displaying morphology characteristic of activation both during periadolescence and adulthood (Fig 4.1.5; 4.3.5). This reduction in the density of microglia displaying an activated morphology indicates that the dose of minocycline (30 mg/kg) used was effective towards this purpose.

Place preference in the CPP paradigm is typically attributed to enhanced DA signaling stimulated by the rewarding substance (Prus et al. 2009). In this study, we observed that the difference in density of microglia displaying the activated morphology between the minocycline pretreatment group and the nicotine group at PND 80 was reversed compared to PND 45. This suggests that the effects of the minocycline pretreatment were dissipating with age. Furthermore, minocycline pretreatment did not impact FosB-ir in the NAc despite the age of the

subject. This could explain why at adulthood no changes in cocaine seeking were observed with CPP testing. If a reduction in preference for cocaine was accompanied with reduced FosB-ir at adulthood from the periadolescent minocycline pretreatment, then it could be implied that both FosB and M1 activation of microglia contribute to sensitization at adulthood in the rats previously primed with nicotine. This however was not the case, as our data indicate that neither FosB-ir in the NAc nor cocaine seeking were hindered with minocycline pretreatment at the onset of adolescence, thus making a case that neither FosB-ir, nor inflammatory microglia are involved in mediating the sensitization that is observed with periadolescent nicotine exposure.

This study also investigated the impact of microglia on FosB-ir in the mPFC, as the NAc is increasingly regulated by neurotransmission from the mPFC as the subject ages. As discussed in Chapter 2, the DA system is less mature during adolescence, but as the subject ages, the connections between NAc and mPFC become more integrated. This leads to the possibility that nicotine, during early adolescence, via expression and accumulation of Δ FosB may cause long-lasting changes in DA neurotransmission in the NAc that is not possible with late adolescent or adult exposure, in other words, a synergistic link between DA and Δ FosB that is temporally dependent. In this study, we demonstrated that at PND 45, minocycline pretreatment did not significantly reduce FosB-ir compared to the nicotine group in the mPFC, but at adulthood, the reduction in FosB-ir becomes significant with the minocycline pretreatment, which implies that minocycline may have a more time-dependent impact on FosB-ir in the mPFC as compared to the NAc. Furthermore, this correlated with a reduction in density of microglia with activated morphology that almost reached statistical significance on comparison with nicotine group (Fig. 4.3.5 **B**). FosB gene products activate the transcription factor cdk5 that phosphorylates DARPP-32 at residue Thr-75. This phosphorylation at Thr-75 is reported to mediate the psychoactive

effect of many drugs of abuse such as cocaine, amphetamine, and nicotine (Svenningsson et al. 2005). In our study, at PND 45, there was no significant reduction in FosB-ir in the NAc or mPFC between nicotine and minocycline pretreatment, although at adulthood the difference became more pronounced in the mPFC.

The lack of change in FosB-ir in the NAc observed during adulthood (Fig. 4.3.5 C) in the minocycline pretreatment group is expected to translate to a lack of substantial change in phosphorylation of DARPP32 in the NAc, as DARPP32 is downstream of FosB (Norrholm et al. 2003). This could further explain why the cocaine seeking behavior during adult testing was similar to the nicotine group. Even though nicotine is a stimulant similar to several varieties of drugs with abuse liability, studies with nicotine as a priming substance is expected to yield results different from other drugs with abuse liability such as cocaine, amphetamines, morphine, and alcohol as different drugs act on their targets differently. Most drugs of abuse enhance reward sensitivity via the mesocorticolimbic reward pathway that includes the VTA and NAc with inputs from other nuclei such as the hippocampus and mPFC. Unlike other drugs with abuse liability such as THC and opiates that stimulate G-coupled receptors on medium spiny neurons (MSN) of the NAc, nicotine activates the nicotinic receptors on VTA glutamatergic inputs to open ligand-operated ion channels that effectively enhance extracellular DA in the NAc and at the post-synaptic neurons (Pierce and Kumaresan 2006). The pulsatile flow of DA into the NAc due to exposure to low or high concentrations of nicotine also plays a critical role in nicotine self-administration and CPP. DA's actions on NAc DA receptors can vary greatly with the dose of nicotine (high vs low), with higher nicotine concentrations being conducive to increased self-administration and CPP (Laviolette and van der Kooy 2004). How nicotine and microglia impact DA to modulate reinforcement in the context of reward -sensitization from

periadolescent nicotine exposure needs to be further explored. In this study, we demonstrated that inflammatory microglia polarization due to nicotine during a small window marking the onset of adolescence, does not contribute to enhancing the rat's sensitivity to cocaine at adulthood. Rather than simply testing for behavioral sensitization, we further demonstrated that in the NAc, pretreatment with minocycline did not alter nicotine's ability to enhance FosB, an IEG transcription factor whose truncated isoform is referred to as 'a sustained molecular switch for addiction' (Nestler et al. 2001).

Nicotine and microglia function differently based on the physiological or pathological context of nicotine acting on microglia. Studies in Parkinson's disease as well as other neuroinflammatory models suggest that nicotine plays an anti-inflammatory role by engaging α -7 nicotinic receptors on microglia and inhibiting the actions of microglial proton currents mediated by NADPH oxidase (Noda and Kobayashi 2017). Neuroinflammatory diseases related to aging are contextually different from that of microglia mediating the gateway actions of psychoactive drugs. Our study indicates that nicotine during periadolescence promotes inflammatory microglia activation in the NAc and mPFC that is sustained into adulthood in the context of nicotine-induced reward sensitization. The role of inflammatory microglia in mediating altered DA neurotransmission in the context of drug reinforcement is unclear, although studies suggest that inflammatory microglia are involved in morphine reinstatement in rats and mediation of locomotor sensitization from chronic cocaine exposure in mice following cocaine stimulation, both being processes that involve DA neurotransmission (Schwarz and Bilbo 2013, Chen et al. 2009, Prus et al. 2009). The context in which microglia are stimulated is also key in the extent that minocycline would influence reward perception. In a chronic pain study involving rodents where inflammatory microglia were stimulated by chronic pain that

further inhibited DA neurotransmission by opiates in the NAc, minocycline reversed the attenuation of DA neurotransmission leading to an amelioration of reward perception and mood (Taylor et al. 2015). Our study indicated that minocycline pretreatment prior to nicotine at periadolescence did not inhibit the desire for cocaine in the adult rat via minocycline's action on inflammatory microglia. However, in our study the testing with cocaine was more than 35 days removed from the last dose of minocycline, thus rendering our study difficult for comparison with results from others.

Results from the minocycline pretreatment group at PND 80 indicate that microglia revert to the activated morphology following maturation into adulthood. To expand upon the role of microglia at adulthood in promoting sensitization, treatment with minocycline at adulthood prior to cocaine conditioning would indicate if adult microglia are inducing sensitization or facilitating the enhanced response to cocaine during conditioning. Also, to understand microglia's effect on dopamine neurotransmission, certain nicotinic receptor subunits such as the $\beta 2$ subunit in the VTA, along with dopamine transporter (DAT) in the NAc, can be probed both during periadolescence and adulthood. Alternatively, extracellular DA concentration in the NAc can be measured using microdialysis.

5.3. Conclusions.

Although this study did observe an increase in microglia displaying the activated morphology (putatively M1 polarized microglia) due to nicotine exposure at periadolescence, we did not attempt to measure changes in the levels of inflammatory-cytokines typical of microglial activation cascades. Additionally, expression levels of microglial PAMPs such as TLRs along with cytokines characteristic of M1 polarization could be useful biomarkers for assessing the extent of inflammation resulting from the treatments used in this study.

The aim of this study was to extend the current knowledge of the age-related Gateway effect of nicotine. The reasons behind the observation that nicotine priming at the onset of adolescence enhances sensitivity to drugs of abuse from different categories at adulthood remains a mystery. This study contributed toward solving that mystery by investigating if inflammatory microglia, because of nicotine exposure at periadolescence, play a role in this process. To test for this, minocycline was used to suppress the inflammatory activation of microglia during periadolescent nicotine exposure, and molecular and behavioral effects were assessed by measuring changes in FosB-ir in brain areas relevant to drug-reward and memory, along with CPP to the test responsiveness to cocaine at adulthood.

Several novel findings emerged from this study. The daily injections of nicotine resulted in an increase of M1 type polarization of microglia. Minocycline did prevent nicotine's ability to activate microglia and this effect further persisted into adulthood, although the differences between nicotine and minocycline pretreatment groups were not as robust at adulthood. Minocycline pretreatment did have a variable effect on expression of FosB-ir at the molecular level, but at the behavior level, minocycline pretreatment did not prevent the sensitization to cocaine as originally hypothesized. The conclusion is that although there may be an interaction between expression of FosB and inflammatory activation of microglia, neither appears to be important for nicotine-induced behavioral sensitization in the early adolescent rat.

Recent National Youth Tobacco Survey results indicate a 10-fold increase in e-cigarettes use by middle and high school students between the years 2011-2015 (Singh et al. 2016). The results obtained from our study suggest that nicotine use during early adolescence, when youth are attending middle and high school, will result in a neuroinflammatory response in the brains of these students. Our study further suggests that suppressing the activation of microglia

associated with inflammation is not adequate in attenuating preference and responsiveness to other drugs with abuse potential in the future. Thus, it is best if exposure to nicotine-laced products is minimized in such you

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Appendix: IRB APPROVAL LETTER



**Animal Care and
Use Committee**

212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

December 15, 2014

Brian McMillen, Ph.D.
Department of Pharmacology
Brody 6S-10
ECU Brody School of Medicine

Dear Dr. McMillen:

Your Animal Use Protocol entitled, "Drug-Induced Sensitization of the Adolescent Rat to Drugs of Abuse" (AUP #W241) was reviewed by this institution's Animal Care and Use Committee on 12/15/14. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

A handwritten signature in black ink that reads 'S. McRae'.

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

