Long-lasting Behavioural and Neuroendocrine Effects of Individual or Combined Adolescent Nicotine and Footshock Exposure.

by

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ABSTRACT

LONG-TERM BEHAVIOURAL AND NEUROENDOCRINE EFFECTS OF ADOLESCENT NICOTINE AND STRESS EXPOSURE

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Adolescent cigarette smoking has declined over the past decade, however nicotine vaping has gained popularity. Adolescence is a critical period for nicotine initiation; early initiation is associated with increased risk of dependence. A bidirectional relationship between stress and nicotine exists as perceived stress is a predictor for, and stress reduction is a commonly reported reason for nicotine use. Here, rats were exposed to high-dose nicotine, footshock, or the combination throughout adolescence and assessed in adulthood on nicotine consumption, anxiety-like behaviour, and stress responsivity. The combination of adolescent nicotine and stress increased nicotine consumption in adulthood without altering baseline anxiety-like behaviour or corticosterone levels. These results indicate that adults with a previous history of adolescent nicotine and stress may be at an increased risk of nicotine use. Given current trends in adolescent nicotine use, preventing a surge in future adult nicotine use and appropriate therapies to treat those individuals is crucial.
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LIST OF ABBREVIATIONS

E-cig – Electronic Cigarette

SES – Socioeconomic Status

**HPA axis** – Hypothalamic-pituitary-adrenal axis

CRH – Corticotropin-releasing hormone

PVN – Paraventricular nucleus of the hypothalamus

**GPCR** – G-protein-coupled receptor

**ACTH** – Adrenocorticotropic hormone

**CORT** – Corticosterone

**AVP** – Arginine vasopressin

OFT – Open field test

**mRNA** – Messenger ribonucleic acid

**NAc** – Nucleus accumbens

**ACh** – Acetylcholine

**nAChR** – Nicotinic acetylcholine receptor

**VTA** – Ventral tegmental area
SN – Substantia nigra

PFC – Prefrontal cortex

SC – Subcutaneous

DA – Dopamine

AChE – Acetylcholinesterase

CP – Conditioned placement

CPP – Conditioned place preference

IVSA – Intravenous self-administration

CTA – Conditioned taste aversion

EPM – Elevated plus maze

LD – Light dark box

FST – Forced swim test

ACE – Adverse childhood experiences

SUD – Substance use disorder

SS – Social stress

ISO – Isolation stress
CSS – Chronic social stress

RS – Restraint stress

GR – Glucocorticoid receptor

IR – Immunoreactivity
Introduction

1.1 Nicotine

Nicotine is the primary reinforcing constituent found in tobacco cigarettes and other smokeless formulations including dipping tobacco and electronic cigarettes (e-cigs), and is the compound that drives dependence (Abuse, 2012). Tobacco cigarette smoking is a global public health concern, with less than half of the Canadian population (only 46.7%) ages 12 and older being lifetime abstainers as of 2017 (Statistics Canada, 2018). Cigarette smoking remains the leading cause of preventable death (World Health Organization, 2017), and life expectancy for smokers is typically reduced by more than 10 years (Jha et al., 2013). Nicotine is extremely addictive, and many individuals struggle to become abstinent. Although a large proportion of individuals will contemplate quitting, and even make an attempt to quit, only approximately 6% of those making a quit attempt are successful in a given year, and relapse rates are still very high after one year of abstinence (Prevention, 2011). Many factors can instigate nicotine use, craving, and relapse. Stress is a common factor that can precipitate nicotine cravings and relapse (Buczek, Lê, Wang, Stewart, & Shaham, 1999; Siqueira, Diab, Bodian, & Rolnitzky, 2000; Woodcock, Stanley, Diwadkar, Khatib, & Greenwald, 2020), and reduction in stress is a commonly reported reason for continued nicotine use (Gilbert, Robinson, Chamberlin, & Spielberger, 1989), particularly self-reported by adolescent females (Nichter, M., Nichter, M., Vuckovic, N., Quintero, G., Ritenbaugh, 1997). Another factor is cue reactivity, which include physiological and psychological responses to the contextual or discrete cues that have been previously paired with tobacco (Chiamulera, 2005; Liu et al., 2007), and visual cues of watching e-cigs being used (Nichols et al., 2016), which may render the individual vulnerable to drug-seeking and relapse. There are sex differences in humans (Sylvestre, Chagnon, Wellman, Dugas, & O’Loughlin,
Nicotine use is particularly popular in adolescence, and early initiation of nicotine use is associated with greater consumption, dependence, and persistent nicotine use in humans (Cullen et al., 2018; Sharapova et al., 2018). Increased future nicotine use and lower probability of quitting are also a result of earlier initiation (J. Chen & Millar, 1998). Though the last decade has seen a steady decline in tobacco use among adolescence, vaporized nicotine consumption via e-cigs and other styles of vaporizers has become extremely popular (Miech, Johnston, O’Malley, Bachman, & Patrick, 2019). The use of these vaporizers allows for fast delivery of nicotine to users via inhalation and provides easier access to nicotine for its users than smoking a traditional cigarette – these devices can be used more discretely, in environments where cigarettes cannot, and have sweet flavours/scents. Thus, novel nicotine consumption patterns can emerge.

Not only do adolescents that use e-cigs have a greater increased prevalence of combustible tobacco product use later on (Leventhal et al., 2015), but a large proportion of individuals who may have otherwise never tried tobacco are more likely to begin to use tobacco following e-cig use (Barrington-Trimis et al., 2016; Cullen et al., 2018). The popular brand JUUL has already been criticized for aggressively targeting young individuals with e-liquids that come in fun, fruity flavours, and ads boasting young, happy, beautiful teenagers superimposed over colourful backgrounds (Jackler & Ramamurthi, 2019). While the health risks associated with tobacco smoking have become generally accepted, leading to changes in legislation
regarding the advertisement of tobacco products, advertisements for e-cigs have not yet had the same restrictions placed on them. There is an emerging drive to do so, along with making sure that product labels are standardized (Jackler & Ramamurthi, 2019). Although e-cigs are presented as less harmful alternatives to cigarettes, there is not enough research looking at the immediate and long-term effects of nicotine vaping.

A recent study compared blood serum nicotine levels in rats following 20 min exposure to the brand name product JUUL, previous generation e-cigs, or Marlboro red cigarettes (P. Rao, Liu, & Springer, 2020) and found that while the previous e-cigs produced similar nicotine levels to cigarette smoke, JUUL had almost a threefold increase in nicotine levels. When compared to other devices on the market, JUUL is the most popular device in adolescence, is associated with increased nicotine use compared to the other devices, and is associated with higher socioeconomic status SES in adolescence (Krishnan-Sarin et al., 2019). In the past, low SES has been associated with increased prevalence of tobacco smoking (Hiscock et al., 2012), and this change in demographics of who is using nicotine allows a larger range of individuals to be targeted for use of these products. Additionally, if individuals from higher SES are using these products, their nicotine use may not be as influenced by funds as those with lower SES, which allows for easier access to nicotine and potentially a larger supply. Due to the popularity of JUUL in adolescence and the novel levels of nicotine consumed as a result of using these products, it is important to investigate the long-lasting effects of high-dose nicotine exposure in adolescence without the additional compounds found in cigarettes.

1.2 Stress

Stressors are conditions, stimuli, or environments that challenge an organism’s homeostasis, or equilibrium point. An organism’s response to a stressor, the stress response, is a
series of autonomic physiological, psychological, and behavioural responses that occur rapidly to maintain homeostasis (Johnson et al., 1992). Following stress exposure, different coping mechanisms can be employed resulting in adaptation to the stressor or distress (Wheaton, B., & Montazer, 2010). The stress signal originates from ‘central command’ populations of neurons that provide dual input to regulate cardiac and adrenal medullary function (Jansen et al., 1995). The stress response is a negative feedback system that includes a complex network of structures, hormones, receptors, and systems. Normal functioning of the stress system is important to mental and physical health, as dysregulation has been linked to numerous pathologies (Charmandari, Tsigos, & Chrousos, 2005; Stratakis & Chrousos, 1995).

### 1.2.1 HPA axis

The hypothalamic-pituitary-adrenal axis (HPA axis) is an important part of the physiological response to stress and includes central and peripheral structures responsible for initiating, producing, and terminating the stress response. Once a threat has been detected, the stress response is initiated by the release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus (PVN), which binds to G-protein-coupled receptors (GPCR’s) type-1 and type-2 CRH receptors in the anterior pituitary gland, where adrenocorticotropic hormone (ACTH) is released. ACTH binds to its receptors in the adrenal cortex and stimulates the release of glucocorticoids including cortisol in humans or corticosterone (CORT) in rodents (Johnson et al., 1992). Increasing CORT levels provide negative feedback ultimately shutting down the stress response by reducing CRH release from the hypothalamus (Charmandari et al., 2005; Stratakis & Chrousos, 1995). Measuring CORT levels is a reliable proxy measure of the stress response and allows for characterization of hormonal responsivity to stressors (Spencer & Deak, 2016).
Acute and chronic stress have different effects on HPA-axis responsivity. When the same stressor is repeatedly encountered in predictable stress procedures, the CORT response to such stressors can habituate over time compared to controls. When stress is more unpredictable or severe there can be an increase in basal glucocorticoid levels, prolonged stress response, increased response to novel stressors, or even hyporesponsiveness (Herman et al., 2016). Interestingly, CORT itself has been demonstrated to function as a reinforcer in oral (Deroche, Piazza, Deminière, Moal, & Simon, 1993) and IVSA paradigms (Piazza et al., 1993) at plasma levels in the range of those evoked by 30 min RS, and CORT also enhances the reinforcing properties of addictive drugs (Piazza et al., 1991). Such findings indicate that there is a motivational component to HPA axis activation, and such motivation may interact with the effects of drugs of abuse.

1.2.2 Nicotine as a stressor

Nicotine can function as a stressor, as it dose-dependently elicits significant increases in plasma CORT levels (Porcu et al., 2003), which is mediated through CRH and arginine vasopressin (AVP) receptors (Lutfy et al., 2012). Nicotine withdrawal, in which nicotine-dependent animals are no longer given nicotine or receive nAChR antagonism, can also function as a stressor and this finding is age- and sex-dependent. Whereas adolescents show fewer nicotine withdrawal symptoms compared to adults, adolescent males also display increased anxiety-like behaviour in an open field test (OFT) and upregulation of CRF messenger ribonucleic acid (mRNA) in the amygdala during both nicotine exposure and withdrawal. During nicotine withdrawal in adults, females have higher levels of anxiety-like behaviour in OFT, plasma CORT, and CRH mRNA expression in the nucleus accumbens (NAc) compared to adult males. However, adult males
exhibit higher levels of CORT and CRF mRNA in the amygdala during nicotine exposure, compared to adult females (Torres, Gentil, Natividad, Carcoba, & O’Dell, 2013).

Similar to what you see with repeated exposure to the same stressor, repeated nicotine exposure via 23 hr/day nicotine IVSA increased CORT and ACTH levels on the first day compared to animals self-administering saline, and this response desensitized by the third day of nicotine IVSA (H. Chen, Fu, & Sharp, 2008). Additionally, chronic nicotine IVSA (20 day; 23 hr/day) sensitized the CORT and ACTH responses to mild footshock (0.6mA; 0.5 s duration; 5 times in 5 min), but not moderate (1.2 mA; 0.5 s duration; 5 times in 5 min), the endotoxin lipopolysaccharide (0.5, or 50µg/kg, IV) or 30 min immobilization stress. This cross-sensitization has been shown with different stressors (Bhatnagar & Dallman, 1998; Ma, Lightman, & Aguiler, 1999), and shows that the intensity of the stimulus is important to cross-sensitization as well.

1.3 Adolescence

Adolescence is a transitional period from childhood into adulthood; a transition from being dependent to becoming independent. Though puberty and adolescence overlap, puberty is the attainment of sexual maturity and reproduction, and is a part of adolescence, but not all encompassing (Spear, 2000). Accumulating evidence suggests that this is a sensitive period of development across species, particularly for memory, social stress, and drug use (Fuhrmann, Knoll, & Blakemore, 2015). Increased risk-taking, social play behaviours, and initiation of drug use are associated with the adolescent period (McCutcheon & Marinelli, 2009; Spear, 2000). Such behaviours are all related to the developmental trajectory of the cholinergic system – a major way through which nicotine can exert effects on brain structure and function during development.
1.3.1 Developing CNS and Cholinergic system

Acetylcholine (ACh) is a neurotransmitter involved in critical neural developmental periods and exerts its effects on both metabotropic and ionotropic acetylcholine receptors. Importantly, the nicotinic acetylcholine receptor (nAChR) is also the site of action for nicotine (Dwyer, Mcquown, & Leslie, 2009; Yuan, Cross, Loughlin, & Leslie, 2015). The nAChR is a pentameric ligand-gated ion channel comprised of a variety of possible subunits, including various combinations of α* β* receptors (* indicates the receptor configuration contains that subunit)(Dani, 2015). During the adolescent period the ventral tegmental area (VTA) and substantia nigra (SN) dopaminergic projections to the prefrontal cortex (PFC), NAc, amygdala, and hippocampus—all important for reward processing and associative learning—undergo a critical period of development that co-occurs with an upregulation of nAChRs (Dwyer et al., 2009). Nicotine exposure during adolescence has also been associated with an upregulation of nAChRs (Abreu-Villaça et al., 2003; Trauth, Seidler, Mccook, & Slotkin, 1999). Combined, these results suggest that nAChR upregulation may be a key mechanism through which nicotine can alter the structure and function of the developing brain and increase susceptibility for nicotine use and dependence. Upregulation of nAChRs is evident following short-term adolescent nicotine exposure, with the hippocampus displaying a distinct temporal sensitivity, producing upregulation after only 2 days of treatment (0.6 mg/kg/day, subcutaneous; SC), and the midbrain showing persistent nicotine-induced alternations of nAChRs one month post-treatment (Abreu-Villaça et al., 2003). These effects were noted at doses that produce plasma concentrations of nicotine comparable to those as small as one-tenth of the average levels that are found in regular smokers, which highlights the particular sensitivity of the developing brain to nicotine. Chronic exposure to nicotine in adolescent and adult rats via mini osmotic pumps also results in upregulation of nAChRs, however the regional specificity differs across ages, and the effects persist only when nicotine exposure initiates in
adolescence (Trauth et al., 1999). Adult and adolescent rats also show very different patterns of expression of nAChR subtypes, and differ in their response to chronic nicotine exposure (Doura, Gold, Keller, & Perry, 2008).

Some subunits of the nAChR, or unique configurations of subunits, have been associated with different processes, affective disorders, and vulnerability to addiction. Homomeric nAChR’s comprised of only α7 subunits can be localized pre- and post-synaptically, where they facilitate ACh release and regulation of cognitive function, respectively (Slotkin, Cousins, & Seidler, 2004). Stimulation of the α7 nAChRs in particular may play a role in depression-like behaviours and the increased cholinergic neurotransmission seen in depressed individuals (Mineur et al., 2015). The distinct role of the α7 nAChR has been investigated and is uniquely upregulated in the striatum, only when nicotine exposure occurs in adolescence, whereas when nicotine exposure occurs in adult or neonatal rats, there is no upregulation of α7 receptors in the striatum (Slotkin et al., 2004). The α4β2* receptor is located post-synaptically and is involved in regulation of dopamine (DA) in the NAc (Stahl, 2013). The α4 subunit, has been associated with vulnerability to nicotine addiction in human males, using gene studies (Feng et al., 2004). The α5 and α6 subunits, which are exclusively found on DA containing neurons and facilitate dopaminergic signaling, show an increase in expression following adolescent nicotine preexposure (Exley, Mcintosh, Marks, Maskos, & Cragg, 2012; Klink, De Kerchove D’exaerde, Zoli, & Changeux, 2001). Finally, β2* nAChRs that are also involved in DA release following nicotine exposure (Exley et al., 2012) mediate nicotine conditioned place aversion (CPA) (Jackson, Kota, Martin, & Damaj, 2009).

Manipulation of cholinergic neurotransmission has been associated with anxiety- and depressive-like behaviour (File, Kenny, & Cheeta, 2000; Tucci, Cheeta, Seth, & File, 2003).
Nicotinic receptors have been a suggested target for antidepressants, highlighting the role this receptor and cholinergic neurotransmission play in mood disorders, which are highly comorbid with nicotine use and dependence (Shytle et al., 2002). Perhaps basal differences in nAChR expression and function in adolescence vs. adulthood, and the influence of adolescent nicotine exposure on these receptors, can mediate behavioural outcomes like drug seeking and consumption in a way that interacts with anxiety-like behaviours. Furthermore, decreased activity of the enzyme primarily responsible for breakdown of ACh, acetylcholinesterase (AChE), in the hippocampus increased anxiety- and depression-like behaviours and decreased resiliency to repeated stress exposure in a social defeat paradigm (Mineur et al., 2013). Cholinergic neurotransmission, that can be altered by exogenous nicotine exposure, can impact how adaptive or maladaptive the hormonal and behavioural responses are to stressors in the future.

1.4 Age-dependent Effects of Nicotine on Addiction-Related Phenomena

Nicotine affects a number of behaviours associated with drug seeking and taking in rodents that are useful models of the complex mosaic of substance use disorders in humans, and these effects differ based on the age of exposure. These include drug reward and reinforcement, drug aversion and withdrawal, affective behaviours, locomotor activity, and interactions with the stress response system.

1.4.1 Reward and reinforcement

In rodents, adolescence is a time that is associated with increased sensitivity to the rewarding effects of nicotine. Utilizing a place conditioning (CP) paradigm, the rewarding properties of varying doses of nicotine have been evaluated. Place conditioning with nicotine involves the pairing of the drug with one context, and explicit unpairing with another context. At test, both compartments are available, and the time spent in each is recorded. A drug-paired
preference is presumed to have been learned if the rodent spends increased time on the drug-paired side at test (Bardo & Bevins, 2000; Tzschenkte, 2007). With nicotine, there is a dose-dependent enhancement of conditioned place preference (CPP) in adolescence (P21), and failure to produce CPP in adult rodents (P53-56) at most doses (Ahsan, Chrislean Jun Botanas, Peña, Yu, & Cheong, 2014; Shram Douglas Funk Zhaoxia Li Anh D Lê, 2006). In one study adult male Sprague-Dawley rats nicotine-induced CPP was only evident when nicotine was paired with the initially non-preferred side (Le Foll & Goldberg, 2005). Male Sprague-Dawley rats (~P49; ~225-250g) developed a CPP to nicotine (0.4mg/kg, IP) after 5 pairings in an unbiased design (Wolter, Huff, Speigel, Winters, & Leri, 2019). Male periadolescent (P34-43) and post-adolescent (P60-69) rats that had been pretreated with nicotine (0.4 mg/kg, IP, for 10 days) or saline differed on their CPP scores in adulthood (35 days after preexposure, P78-85 and P104-111, respectively). Adolescent pre-treated animals show CPP to both 0.3 and 0.6 mg/kg nicotine in adulthood, whereas only 0.6 mg/kg nicotine produced CPP in post-adolescent treated rats in adulthood. Notably, that 0.3 mg/kg nicotine dose, along with 0.6 m/kg, was successful in producing CPP in saline pretreatment controls (Adriani, Deroche-Gamonet, Le Moal, Laviola, & Piazza, 2006). This is evidence to suggest a particular vulnerability with adolescent nicotine exposure, compared to post adolescent exposure. Further, early adolescence (P28,29) is a critical developmental period when rodents are particularly sensitive to the rewarding effects of nicotine, as 0.5 mg/kg produces a significant CPP score in that age group, whereas the same dose of nicotine failed to produce significant CPP scores in late adolescent (P38-41) and adult (P90-93) rats (Belluzzi, Lee, Oliff, & Leslie, 2004).

When comparing early (P24-35), middle (P37-48), and late adolescent (P50-61) periods in mice, early adolescence was the only timepoint studied that was able to show a clear and stable preference for nicotine in an oral self-administration paradigm. These early-adolescent mice also
increased consumption when nicotine was slowly reduced from the solution, whereas late adolescent mice tended to avoid the nicotine bottle (Adriani, Macri, Pacifici, & Laviola, 2002). Compared to adults, adolescent rats consume higher quantities of nicotine and have a greater preference for nicotine compared to water, however the same overall proportion of animals, roughly half, from each group ultimately went on to exhibit persistent patterns of nicotine consumption (Nesil, Kanit, Collins, & Pogun, 2011). Higher levels of intravenous self-administration (IVSA), (Levin et al., 2003, 2007) and oral (Nesil et al., 2011) nicotine self-administration are observed in male (Levin et al., 2007; Nesil et al., 2011) and female (Levin et al., 2003; Nesil et al., 2011) adolescent-onset rats compared to adult-onset rats. However, for IVSA, sex differences emerged such that higher intake levels persisted only for females, whereas males declined their intake and approach levels similar to those seen in adult-onset rats after 2 weeks. This observation suggests there may be sex differences in how persistent these effects are. Adolescent-onset rats from the same study also displayed enhanced α4β2* nAChR binding in the midbrain and striatum compared to adult-onset rats (Levin et al., 2007). Rats pretreated with nicotine (0.4 mg/kg, IP, once daily for 10 days) in periadolescence (P34-43) show increased nicotine IVSA in adulthood (5 weeks after exposure), whereas animals with the same pretreatment in post-adolescence (P60-69) failed to show an enhancement of self-administration – again, highlighting the particular vulnerability of the adolescent period (Adriani et al., 2003) consistent with human smoking patterns (Sharapova et al., 2018). Altogether, these data show that adolescents are more sensitive to the rewarding and reinforcing effects of nicotine than adults.

1.4.2 Aversion and withdrawal

Another factor that may make adolescents more vulnerable to the effects of nicotine is that they are less sensitive to the aversive effects of nicotine (Le Foll & Goldberg, 2005; Shram,
Funk, Li, & Lê, 2008; Torres, Tejeda, Natividad, & O’Dell, 2008). Adolescent nicotine preexposure reduces the efficacy of nicotine to elicit CPA in adulthood (P74) at a 1.2 mg/kg dose that is aversive to drug naïve rats in adult (Torres et al., 2008). This finding indicates that adolescent nicotine exposure can alter future perception of the discriminative properties of nicotine in a way that may encourage consumption. Further, conditioned taste aversion (CTA) procedures have shown that adult rats (P53-56) are capable of developing a CTA to saccharin if it was paired with nicotine, while the same exposure in periadolescence (P21) fails to produce CTA (Shram Douglas Funk Zhaoxia Li Anh D Lê, 2006).

Finally, withdrawal from chronic nicotine induces an aversive physical and affective state including slow heart rate, gastrointestinal problems, alterations in appetite, nicotine cravings, depressed and anxious mood, irritability, and difficulty concentrating (Kenny & Markou, 2001). In both spontaneous and mecamylamine-precipitated withdrawal paradigms, adolescent male mice show a reduction in the number of withdrawal symptoms compared to adults, and greater nicotine-induced antinociception measured in a tail-flick test (Kota, Martin, Robinson, & Damaj, 2007). Adolescent male rats also show fewer affective withdrawal symptoms than adults (O’Dell, Torres, Natividad, & Tejeda, 2007). This overall reduction in aversive effects of nicotine in adolescence, or as a result of adolescent nicotine preexposure highlight the increased vulnerability to nicotine use initiating in adolescence.

1.4.3 Anxiety- and depression- like effects

In a 10 year longitudinal study in humans, adolescent (14-17 year old) depression and anxiety was associated with increased risk of nicotine dependence in young adulthood (20-24 year old) (McKenzie, Olsson, Jorm, Romaniuk, & Patton, 2010). Adolescent nicotine use is also associated with increased anxiety-like (Hudson et al., 2020; Slaweki, Gilder, Roth, & Ehlers,
2003; Smith et al., 2006) and depressive-like behaviours (Hudson et al., 2020; Iñiguez et al., 2009), which are also risk factors for nicotine use disorder (Laje, Berman, & Glassman, 2001; Morisette, Tull, Bulliver, Kamholz, & Zimering, 2007).

When comparing the immediate effects of nicotine exposure in adolescent and adult rats on anxiety-like behaviour, there are clear age and sex differences, with some protective effects in adolescent males. Nicotine exposure (5 days of 0.1, 0.5, or 1.0 mg/kg/day, SC) during adolescence (P30) produced anxiolytic effects in the elevated plus maze (EPM) (increase in percentage of time spent in open arms) in adolescent males, but anxiogenic (decrease in percentage of time spent in open arms) in adolescent females and adults (P60) of both sexes (Elliott, Faraday, Phillips, & Grunberg, 2004). Adult rats (P65-69) display reduced anxiety compared to adolescents (P33-37) immediately following acute nicotine exposure (0.4, 0.8 mg/kg, SC) in EPM, however there were no differences in the light dark box (LD) (Kupferschmidt, Funk, Erb, & Lê, 2010) – another assay for measuring anxiety-like behaviour which typically shows rats avoiding the light side of the box. Acute IV nicotine administration (0.03 mg/kg/infusion, 2 infusions, 1 min apart) increased center time in OFT in younger adolescence (P28), with more prolonged effects in males than females, and nicotine was anxiolytic in EPM in adolescent males but anxiogenic in females, again, showing some protection from anxiety in adolescent males (Cao et al., 2010).

Nicotine pre-exposure in adolescence produces long-lasting alterations in adult anxiety-like behavior. Increases in anxiety-like behaviours in adults have been observed following nicotine pre-exposure during adolescence, but not following pre-exposure as adults (Lee, Jung, Kim, & Noh, 2018). Adolescent (P31-36) male Sprague-dawley rats pretreated with 5.0 mg/kg/day via a transdermal Nicoderm patch showed increased anxiety-like behaviour in the OFT 2-3 weeks
following the end of nicotine exposure including lower levels of exploratory activity and retreating to the perimeter faster than saline pretreated controls (Slawecki et al., 2003). Adolescents (P28-42) pretreated with chronic nicotine (1 mg/kg/day or 2 mg/kg/day) via a osmotic mini pump for a longer period (15 days) also spent less time in the center of a novel open field when assessed one month following the end of nicotine exposure, however, adults with the same exposure did not differ from controls (Smith et al., 2006).

When adolescent rats (P30-44) were pretreated chronically with nicotine, a depressive-like phenotype emerged in adulthood. These behaviours included decreased sensitivity to natural reward in a sucrose preference test and enhanced sensitivity to stress in EPM and forced swim test (FST) (Iñiguez et al., 2009). The behaviours emerged shortly after nicotine exposure ended (1 day-1 week), with a single exposure to nicotine in adolescence also being sufficient to elicit this phenotype even one month after exposure. This phenotype could be normalized with adult nicotine (0.32 mg/kg, SC) or antidepressant (fluoxetine or bupropion; 10 mg/kg, SC) treatment (Iñiguez et al., 2009). Finally, adolescents (P28-42) that received 1 mg/kg/day nicotine via osmotic mini pump show enhanced acquisition of fear conditioning compared to controls, which persisted during extinction (Smith et al., 2006), and this effect was specific to adolescence, as adults (P85-99) with the same nicotine exposure did not differ.

1.4.4 Locomotion

Baseline activity levels and behavioural sensitization can be assessed by measuring locomotor responses to acute or chronic nicotine (Robinson & Berridge, 1993). The nicotine dose that produces peak locomotion differs across developmental stage and generally follows an inverted U pattern, with low doses evoking little unconditioned locomotor stimulation, and very high doses causing non-specific motor impairment or even ataxia (Matta et al., 2007).
Locomotion for adult rats (P60) peaked with 0.5 mg/kg/SC nicotine whereas adolescents (P30) peaked at 1.0 mg/kg/SC. A dose of 1 mg/kg/SC also elicited locomotor depressant effects in adults on some sessions, whereas adolescents showed no evidence of nicotine-induced locomotor depression (Elliott et al., 2004). Furthermore, male periadolescent (P34-43) nicotine exposed (0.4 mg/kg, IP, 10 days) rats exhibit increased novelty-induced locomotion to nicotine (0.3 and 0.6 mg/kg, IP) later in adulthood (Adriani et al., 2006). Nicotine (2 infusions of 0.03 mg/kg, IV, 1 min apart) increased locomotion compared to saline for the first 5 min post-infusion in early adolescent rats (P28), however, there was no effect on locomotion for mid adolescent rats (P38), and an initial locomotor depressant effect in adult rats (P80) (Cao et al., 2010). Voluntary oral nicotine self-administration (30mg/l) has also been shown to induce hyperactivity only in early adolescents (P24-35), and hypoactivity in late adolescents (P50-61) immediately following a 1hr drinking session (Adriani et al., 2002). Combined, these findings confirm the sensitivity of developmental stage to the psychomotor stimulant effects of nicotine.

1.4.5 Stress

Nicotine and the stress response system interact as a function of developmental stage. Increased Fos positive cells in the PVN, a marker for neural activity and a proxy measure of HPA axis activation, can characterize the severity of the perceived stressor, as a greater number of Fos positive cells in the PVN are seen following a severe acute stressor (immobilization stress) compared to mild (restraint) stress (Mohammad, Chowdhury, Fujioka, & Nakamura, 2000). Acute nicotine exposure (0.5 mg/kg, SC) in adolescence (P30-45) did not result in significant increases in Fos positive cells in the PVN. However, repeated nicotine exposure (0.5 mg/kg, SC) in adolescence resulted in increased Fos expression in the PVN compared to acute nicotine treatment and saline groups (McCormick & Njeri Ibrahim, 2007). While adolescents
displayed elevated CRF mRNA expression levels in the PVN relative to adults in response to nicotine (0.4, 0.8 mg/kg, SC), there was no effect of nicotine on CRF mRNA in the PVN (Kupferschmidt et al., 2010). Acute nicotine exposure (2 infusions 1 min apart, 0.03 mg/kg/infusion) only produced significant increases in CORT levels in adults, and failed to do so at P28 and P38 (Cao et al., 2010). Interestingly, adult (P90-99), but not adolescent rats (P28-35), pretreated with daily injections of nicotine (0.4 mg/kg, SC) for one week displayed nicotine-induced behavioural sensitization and reduced CORT levels after being challenged with 0.4 mg/kg nicotine, SC, 3 days after completion of nicotine exposure (Cruz, DeLucia, & Planeta, 2005), further support for nicotine as a stressor discussed above.

Given that adolescents and adults display clear differences in their sensitivity to the rewarding and aversive properties of nicotine, and the neural mechanisms underlying this sensitivity, the efficacy of smoking cessation therapies may be age-specific, and new therapies that begin targeting neural mechanisms more representative of adolescent neurochemistry should be explored. Investigating differences in adolescent and adult neurochemistry will also be required in order to advance pharmacotherapies in particular. Since adolescence appears to be a vulnerable developmental period for nicotine-induced neuroadaptations, it is important to investigate long-lasting effects of nicotine exposure in adolescence.

### 1.5 Interactive Effect of Adolescent Stress and Nicotine Exposure

In humans, increased night-time cortisol levels in adolescence is associated with initiation and persistence of cigarette smoking when assessed 5 years later (U. Rao, Hammen, London, & Poland, 2009). Stressful life experiences also increased the risk for smoking in depressed and non-depressed adolescents, and smoking was associated with increased depressive episodes at
follow-up. While high nighttime cortisol may be a vulnerability factor for smoking in adolescence, stressful experiences further increase this risk. An online survey by the American Psychological Association found that adolescents reported their stress levels were higher than what they believed to be healthy, and they reported their stress levels to be higher than the stress levels reported by adults (Anderson et al., 2014). Whether teens actually experience more stress or perceive more stress may not matter, as perceived stress is also a predictor of future nicotine consumption, including vaping (Leventhal et al., 2017).

Stress during key developmental periods can alter an organism’s behavioural and neuroendocrine responses, as well as sensitivity to drugs of abuse later in life. This results in early life stressors being associated with an increased vulnerability or susceptibility to adult health risk behaviours and diseases including cigarette smoking, or other nicotine use. Individuals that were exposed to four or more categories of adverse childhood experiences (ACE) had a 2-4 fold increase in smoking (Felitti et al., 1998). They also found a 4-12 fold increased risk for alcohol use disorder (AUD), substance use disorder (SUD), and depression—all highly comorbid with cigarette smoking and nicotine use (Fluharty, Taylor, Grabski, & Munafò, 2017; Kalman, Morissette, & George, 2005). Similarly, familial substance abuse for boys, and sexual assault and depression in girls, were important risk factors for the initiation of smoking (Acierno et al., 2000). While the direction of the relationship between anxiety/depression and smoking behaviours is still unclear, comorbid smoking and psychiatric disorders is extremely prevalent (Fluharty et al., 2017; Kalman et al., 2005). A bidirectional relationship exists between nicotine and stress in adolescence. Stress and perceived stress are predictors of nicotine use (Leventhal et al., 2017), and nicotine use has been shown to relieve stress (Gilbert et al., 1989), when sampled from the general population stress relief was one of
the top two reported reasons, along with enjoyment, and more females reported stress relief than males (Nichter, M., Nichter, M., Vuckovic, N., Quintero, G., Ritenbaugh, 1997). In adolescent females the most commonly reported reason for smoking was stress relief (Fidler & West, 2009). In order to develop treatment and prevention methods to reduce adolescent nicotine use, this relationship needs to be more clearly defined.

1.5.1 Immediate effects of stress and nicotine

McCormick & Njeri Ibrahim, (2006) investigated how nicotine-induced locomotion is affected in both sexes when nicotine exposure occurs 1 day following stress exposure. They also assessed whether this effect was reliant on social instability by including both a social stressor (SS; one-hour isolation and new cage mate daily for 16 days) and an isolation stressor (ISO; daily isolation but same cage mate throughout) group. They found that nicotine-induced locomotion was attenuated in SS animals compared to ISO and non-stressed control rats, that this effect was more exaggerated in females, and the effect of SS in adolescence differed in females depending on how much time had passed since stressor exposure concluded (McCormick & Njeri Ibrahim, 2007; McCormick, Robarts, Gleason, & Kelsey, 2004). They also found that SS males had increased Fos positive cells in the PVN compared to ISO and controls, irrespective of nicotine exposure (McCormick & Njeri Ibrahim, 2006; McCormick et al., 2004). Using a different stressor, Cruz et al., (2008) found that there was no effect of adolescent (P28-37) or adult (P60-69) chronic restraint stress (CRS; 2 hr, once daily for 7 days) on later nicotine-induced locomotion or CORT response. However, when faced with a nicotine challenge (0.4 mg/kg) 3 days following CRS, nicotine increased locomotor activity only in stressed adults, whereas nicotine increased locomotor activity in both stressed and non-stressed adolescence. Nicotine also increased plasma CORT levels in stressed as well as in non-stressed adults and
adolescents (Cruz et al., 2008). The differences in results of these studies could be attributed to a number of factors including the time delay between stressor and nicotine exposure, the age when they received the stressor or nicotine, and the type of stressor (social vs. non-social). Regardless of the differences in patterns of results, the studies all show interactions between stressor and nicotine exposure that are age- and/or sex-dependent.

Interactions between nicotine and stressor experience are also evident in shifts in the rewarding and reinforcing value of nicotine following stressor exposure. Exposure to a single 10 min session with intermittent footshock stress in adolescence (P28-42) dose-dependently facilitated acquisition of nicotine-induced CPP (Brielmaier, Mcdonald, & Smith, 2011). This effect was attenuated by pretreatment of the selective CRF-R1 antagonist CP-154,526 (20mg/kg) 30 min prior to footshock exposure. Given that pretreatment with CP-154,526 did not have an effect in non-stressed animals that showed nicotine-induced CPP, the CRF-R1 receptor may play an important role in the enhancement of nicotine reward following stress (Brielmaier et al., 2011). Furthermore, the pharmacological stressor, yohimbine, immediately enhanced nicotine IVSA in both male and female rats (Li et al., 2014). When adolescent rats (P33) that have been trained to self-administer different doses of nicotine (7.5, 15, 30 µg/kg/infusion, IV) are pretreated with yohimbine (0.3, 0.6 mg/kg, IP) in adolescence, nicotine intake and progressive ratio (PR) breakpoints were both increased in male (Brielmaier et al., 2011) and female rats (Li et al., 2014). The dose of nicotine that animals were trained to self-administer was positively associated with yohimbine-induced increases in responding, and though the effect was present in both sexes, females were more sensitive to the stress-induced increase in nicotine self-administration.
1.5.2 Long-lasting effects of stress and nicotine

These age- and sex-dependent interactions between stressor experience and nicotine locomotor, reward, and reinforcement enhancement may be long lasting. However, few studies have assessed prolonged delays between the adolescent exposures and adult assessments. With regards to stressor pre-exposure, adolescent (P33-48) female rats exposed to chronic social stress (CSS) show enhanced locomotor sensitization to nicotine (0.5 mg/kg, SC) in adulthood (3 weeks following CSS exposure), whereas males with the same exposure do not show enhanced locomotor sensitization and no differences were present in rats that never experienced the stress manipulation in adolescence (McCormick et al., 2004). When nicotine pre-exposure is integrated in to the adolescent experience, behavioural sensitization to a challenge dose of nicotine (0.4 mg/kg, SC) 3 days following pre-exposure was evident only in rats that experienced simultaneous nicotine and restraint stress (RS) in adolescence (P28-37) for 7 days, compared to animals that experienced nicotine or RS alone. In adults (P60-67), nicotine resulted in behavioural sensitization regardless of stressor exposure, emphasizing the particular vulnerability during adolescence to the effects of stress on nicotine-induced sensitization (Zago, A., Leao, R.M., Carneiro-de-Oliveira, P.E., MArin, M.T., Cruz, F.C., and Planeta, 2012). Adolescents are more affected by a stressor that is social in nature. Rats (P39) that are socially isolated displayed increased plasma CORT levels 15 minutes following nicotine exposure (0.6 mg/kg) in males and females, but failed to do so in socially housed rats (Pentkowski et al., 2011). Additionally, nicotine produced locomotor sensitization in female, but not male, isolated rats but decreased locomotion in socially housed males and females. Whether being socially housed helps decrease the negative and stress-activating effects of nicotine, or isolation helps facilitate these effects is unclear, however social stressors seem to play an interactive role in the effects of nicotine in adolescence. Consistent with these results, Zou et al., (2014) found that yohimbine increased PR
responding for nicotine in a self-administration paradigm irrespective of nicotine dose or age of animals. They also investigated the effect of additional stressors and found that intermittent footshock was able to moderately attenuate PR responding in adolescence only at the 0.03 mg/kg dose, but neither defeat stress or a defeat-paired olfactory cue affected acquisition of nicotine self-administration (Zou, Funk, Shram, & Lê, 2014).

Adolescent (P31), but not young adult (P47) mice exposed to combination RS (30 min 1 day) and social instability stress for 2 days (isolated for 1 hr then placed with novel same-sex mate) followed by chronic nicotine exposure (P38 or 54; 12 days of 12.6 mg/kg nicotine via osmotic minipump) showed deficits in contextual fear as measured through decreased freezing to the context but not the cue (Holliday, Logue, Oliver, Bangasser, & Gould, 2019). The same results were found when the stressor was instead shipping stress, as shipped mice with chronic nicotine starting at P38 (12 days of 12.6 mg/kg nicotine via osmotic minipump) had significantly lower CORT levels after 1 hr of restraint stress in adulthood (Holliday, Logue, Oliver, Bangasser, & Gould, 2019). The combination of shipping stress and nicotine exposure in adolescence had no effect on CORT levels assessed on the 12th day of chronic nicotine exposure, 24 hr post nicotine (withdrawal), or 30 days after nicotine exposure ended. However, glucocorticoid receptor (GR) immunoreactivity (IR) in the hippocampus varied such that there was an increase in CA1 and CA3 GR IR on the 12th day of nicotine exposure, reduced CA1 GR and decreased CA1 and CA3 CRF receptor IR during 24 hr withdrawal, and a subsequent increase in CA3 GR and CRF IR 30 days after nicotine exposure ends (Holliday, Logue, Oliver, Bangasser, & Gould, 2019).

Notably, to date, no studies have investigated the long-lasting effects of adolescent systemic high-dose nicotine and stressor exposure followed by a period of prolonged abstinence. This may
be a model for adolescent experimentation with a level of nicotine exposure similar to that seen with devices like JUUL providing high nicotine serum levels, followed by temporary cessation before reinitiation in adulthood. Thus, the present set of studies investigated the effects of adolescent nicotine and/or footshock stressor pre-exposure on adult nicotine IVSA across different schedules of reinforcement, anxiety-like behaviour in a drug-free state, and CORT response to adult nicotine and footshock.

1.6 Objectives

1) Assess susceptibility to adult nicotine consumption, motivation for nicotine, and persistent drug-seeking when drug is unavailable as a function of adolescent nicotine, footshock, or the combination of nicotine and footshock exposure followed by a period of abstinence.

2) Investigate the relationship between adolescent nicotine and/or stressor exposure and adult anxiety-like behaviour by assessing activity in a novel open-field test in a drug free state and baseline CORT levels in adult following adolescent pre-exposure (Slawecki et al., 2003; Smith et al., 2006).

3) Assess differences in the magnitude of CORT response, or stressor responsivity, following adult footshock or nicotine as a function of adolescent nicotine, footshock, or combination pre-exposure.
1.7 Hypotheses

1) Adolescent nicotine and footshock exposure followed by a period of abstinence will increase nicotine consumption in adulthood (Adriani et al., 2003; Natividad, Torres, Friedman, & O’Dell, 2013).

2) Adolescent nicotine and footshock will increase anxiety-like behaviour in adulthood (Hudson et al., 2020; Slawecki et al., 2003; Smith et al., 2006), and increased novelty-induced locomotor sensitization in OFT in adulthood (Adriani, Deroche-Gamonet, Le Moal, Laviola, & Piazza, 2006)

3) Adolescent nicotine and footshock stress will result in a blunted CORT response to future adult stressors (Holliday, Logue, Oliver, Bangasser, & Gould, 2019), whereas cross-sensitization may occur for rats receiving individual nicotine or footshock exposure in adolescence and the opposite stressor in adulthood (Bhatnagar & Dallman, 1998; Cam & Bassett, 1984; Ma et al., 1999).

2 Material and Methods

2.1 Subjects

Male Sprague-Dawley rats (Charles River Lab, St. Constant, Quebec, Canada) arrived at the facility on P22 and were pair housed in opaque standard plastic cages throughout the adolescent period. Unless otherwise specified, rats were maintained at ~90% of free-feeding body weight according to the standardized growth chart for this strain provided by Charles River Lab (Envigo, Madison, Wisconsin, Rodent Diet, 18% protein). Water was available ad libitum throughout the experiment. The colony was on a 12:12 light-dark cycle (lights on at 8 a.m.) and
maintained at 21 °C. All animal procedures were approved by the Animal Care Committee of the University of Guelph and adhere to the guidelines set forth by the Canadian Council of Animal Care.

2.2 **Drug Preparation**

For adolescent pre-exposure and adult acute testing, nicotine ditartrate dihydrate (Fisher Scientific, Ottawa, ON, CAN) was dissolved in 0.9% saline at a concentration of 1 mg/ml for subcutaneous SC injections (1 ml/kg) during adolescence (P28-56) (Matta et al., 2007). For adult intravenous self-administration, the nicotine concentration was 0.03 mg base/kg per 0.04 ml/sec intravenous infusion across the average rat weights. Precision infusion pumps automatically adjusted the duration of the infusion for each rat’s individual weight on a given day to keep dosage consistent. All nicotine used was adjusted to a pH of 7.0-7.2 using NaOH and was prepared fresh weekly.

2.3 **Surgery**

Following adolescent exposure procedures, a subset of adult rats (P60) were administered carprofen (5 mg/kg), anaesthetized using isoflurane, and implanted with a jugular catheter. The silastic catheter (RJVR-40; SAI, Lake Villa, IL, USA) was secured into the right jugular vein; the other end was threaded subcutaneously over the shoulder and attached to a back-mount cannula (313-000BM-20-5up/spc; PlasticsOne, Anjou, QC, Canada) exiting between the shoulder blades. Tethers in the operant boxes were attached to the rat via the cannula. Rats were allowed to recover for 7 days and received carprofen (5mg/kg) for 3 days during recovery. Rats were flushed daily with 0.1 ml of flushing solution [heparin (30U/ml)/baytril (5 mg/ml)/saline
(0.9% sterile)] and were single-housed from surgery onward. All surgical drugs were provided by the Ontario Veterinary College at the University of Guelph.

2.4 Apparatus

2.4.1 Adolescent Pre-Exposure

For adolescent nicotine and shock pre-exposure, rats were tested in 8 standard sound- and light-attenuating conditioning chambers with ventilation fans to reduce external noise (Coulbourn Intruments, Allen-town, Pennsylvania, USA). An electrified grid floor was programmed to deliver 0.5 sec duration 0.8mA footshocks. No other chamber-specific stimuli were present during these sessions.

2.4.2 Adult IV nicotine Self-administration

For adult nicotine self-administration, rats were tested in 10 standard sound- and light-attenuating conditioning chambers (Med-Associates, Georgia, VT, USA) with ventilation fans to reduce external noise. Side walls of each chamber (30.5 x 24.1 x 21 cm; l x w x h) were made of aluminum and the front, back, and ceiling were constructed from clear polycarbonate. Located centrally on the right wall was a recessed liquid dipper with retractable levers on either side, and a house light was located at the top of the opposite aluminum wall. Levers were set such that 147 nN of force was required for a successful lever press to be recorded. A white cue light (2.54 cm dia; 28 V, 100 mA) was centered 7 cm above each lever, and a photobeam transected the box 3.5 cm in front of the levers as a measure of locomotion. Outside of each chamber, a motor-driven syringe pump set to a rate of 0.04 ml/sec (adjusted for weight) and fitted with a syringe infused nicotine via PE50 Tygon tubing attached to a liquid swivel and strung through a metal tether that attached to the catheter.
2.4.3 Novel open-field test.

Anxiety-like behavior was assessed using a novel open field test in a drug-free state using two black opaque open-top chambers (100 x 100 x 30 cm; l x w x h). The floor of the maze was subdivided into 16 equivalent squares. The inner 4 squares constituted the center of the maze, and the external 12 the periphery. Total distance travelled (cm) was recorded along with the percent time spent in the center of the OFT, calculated as follows: (time spent in center/time spent in periphery)*100. Behaviour was automatically recorded using Ethovision (Noldus, Netherlands).

2.5 Procedure

2.5.1 Adolescent preexposure.

Rats received an injection 5 min prior to onset of each daily 20 min session in their assigned conditioning chamber from P28-P55. Saline control rats (SALPRE) received 1 ml/kg saline, SC, every day (28 total injections) with no in-session stimuli. Nicotine pre-exposed (NICPRE) rats received alternating injections of 1 mg/kg nicotine or saline, SC, (14 nicotine and 14 saline injections), and no in-session stimuli. Footshock stress pre-exposed rats (SHOCKPRE) received saline every day and 8 random presentations of footshock (0.8mA;0.5sec) in the operant chamber every other session (14 total sessions with shock). Two groups experienced the combination of both nicotine and shock. Co-occurring nicotine and shock rats (NIC+SHOCK) received their nicotine injections and shock exposure in the same sessions; intermixed sessions involved saline injections and no in-session stimuli. Non-co-occurring nicotine and shock rats (NIC–SHOCK) received the same number of nicotine injections and shock exposures in the chamber, but on alternating days; these stimuli were never experienced together to assess the
effects of the same amount of nicotine and shock as the NIC+SHOCK group, but never at the same time.

2.5.2 IV Self-administration.

Active and inactive levers were counterbalanced across chambers and rats, and there was no lever pretraining. A subset of rats began self-administration on P70 (SALPRE: n=6; SHOCKPRE: n=7; NICPRE: n=7; NIC+SHOCK: n=8; NIC–SHOCK: n=9). The first phase of self-administration was fixed ratio 1 (FR1). On this schedule, a single press of the active lever resulted in a 0.03 mg/kg infusion of nicotine, and initiated a 20-sec time out during which both levers were retracted, the cue light above the active lever was illuminated, and the house light was turned off (Caggiula et al., 2002). Inactive lever presses were not reinforced but were recorded. After 16 1-hr sessions on FR1, the schedule was shifted to fixed interval 1 minute (FI1). On this schedule, a single active lever press was only reinforced at the termination of the 1-min interval. Infusion and time-out stimuli were identical to FR1. After 16 1-hr sessions on FI1, the schedule was shifted to progressive ratio (PR) with a gradual increase in the number of presses necessary to deliver each successive infusion (i.e., 1, 2, 3, 4, etc) for 4 sessions. Finally, rats underwent extinction training with no drug or stimulus presentations for 6 days. Following each schedule, catheter patency was confirmed using a 10% dilution of xylazine (85:15). All animals included in any analyses had catheters that remained patent until the end of all schedules of reinforcement.

2.5.3 Novel open-field test.

On P71, a subset of rats (n=18 per group) were placed on the outer edge of one of two black opaque mazes in a drug-free state and allowed to freely move around the maze for 10 min.
2.5.4 *Corticosterone detection.*

Stress reactivity was assessed the day after the novel OFT. Blood samples (300 µl) were collected in microvivette tubes (Clotting activator, VWR, Radnor, PA) from the saphenous vein immediately before and 15 minutes after stimulus presentation (1.0 mg/kg nicotine, SC, or 4 presentations of 0.8mA; 0.5s, footshock in 2 mins). Samples (n=9 per adolescent treatment group) were centrifuged (10,000g x 5min) 1 hr after collection, and serum was collected and stored at -80° C until processing. An enzyme-linked immunosorbent assay (ELISA) was used (KGE009; R&D Systems) in duplicate to quantify concentrations of CORT. Optical density was determined at 450nm using the EL800 Universal Microplate Reader and KC Junior software package, (BioTek, Winooski, VT). Samples were diluted 1:10 after the pre-treatment step.

2.6 *Statistical analysis*

2.6.1 *IV Self-administration.*

A mixed model repeated measures ANOVA was performed for each schedule of reinforcement and assessed the between subjects effect of Group (5 levels of adolescent pretreatment) by the within subjects factor of session (16; 16; 4; 6 sessions) and included the following concurrently-collected within-subjects factors: Total Infusions, Active Lever Presses, Inactive Lever Presses, and Locomotion. Lever discrimination within each group was assessed using a repeated measures ANOVA that compared lever type (2 levels) across session (16;16;4;6 sessions) for each group. Post hoc Fisher’s LSD was performed for significant interactions. Only animals that remained patent for the entirety of the experiment were used in each analysis.
2.6.2 Novel open-field test.

A one-way ANOVA comparing the between subjects’ effect of group (5 levels of adolescent pretreatment) on total distance travelled (cm) and on percentage of time spent in the center was used.

2.6.3 Corticosterone.

Hormone reactivity measured via CORT levels was assessed using a three-way mixed factor repeated measures ANOVA comparing the within subjects’ factor Timepoint (Pre- vs Post-stressor) across the between subjects effects of Group (5 levels of adolescent pretreatment), and Stressor (Nicotine vs Shock). Significant interactions were followed by post hoc LSD.

3 Results

3.1 Total infusions

3.1.1 Fixed Ratio 1.

For the FR1 phase, all rats increased the number of Total Infusions across Sessions, regardless of Group, with NIC+SHOCK rats earning more infusions compared to NICPRE, SHOCKPRE, and SALPRE and no difference between NIC+SHOCK and NIC—SHOCK, (Fig.1). There was a significant main effect of Session, \( (F_{15,480}=8.876, p<.001, \eta_p^2=.217) \), a main effect of Group, \( (F_{4,32}=3.345, p=.021, \eta_p^2=.295) \), and no significant Session by Group interaction, \( (F_{60,480}=44.704, p=.065, \eta_p^2=.141) \). Post hoc analyses on the main effect of Group revealed that NIC+SHOCK took significantly more infusions compared to NICPRE, SHOCKPRE, and SALPRE. NIC—SHOCK took significantly more total infusions compared to NICPRE, SHOCKPRE, but the difference from SALPRE did not quite reach statistical
significance ($p=.056$). NICPRE, SHOCKPRE and SALPRE controls did not differ from one another.

### 3.1.2 Fixed Interval 1.

For the FI1 phase, all rats increased Total Infusions across Sessions, regardless of Group, (Fig. 1). There was a significant main effect of Session, ($F_{15,480}=11.932$, $p<.001$, $\eta^2=.272$), but no main effect of Group, ($F_{4,32}=2.564$, $p=.057$, $\eta^2=.243$), and no significant Session by Group interaction, ($F_{60,480}=1.066$, $p=.352$, $\eta^2=.118$).

### 3.1.3 Progressive Ratio.

For the PR phase, NIC+SHOCK and NIC−SHOCK rats had a higher breakpoint than NICPRE and SHOCKPRE but not SALPRE, (Fig.1). There was no main effect of Session, ($F_{3,96}=746$, $p=.527$, $\eta^2=.023$), but there was a main effect of Group, ($F_{4,32}=3.899$, $p=.011$, $\eta^2=.328$), though no significant Session by Group interaction, ($F_{12,96}=.770$, $p=.680$, $\eta^2=.088$). Post hoc analyses on the main effect of Group revealed that NIC+SHOCK rats had a significantly higher breakpoint compared to NICPRE and SHOCKPRE, but not SALPRE. NIC−SHOCK had a significantly higher breakpoint than NICPRE and SHOCKPRE, but not SALPRE. NICPRE, SHOCKPRE and SALPRE controls did not differ from one another.

### 3.1.4 Extinction.

For the extinction phase, though total infusions decrease across Sessions, regardless of Group, NIC+SHOCK rats earned more potential infusions than NICPRE and NIC−SHOCK earned more than both NICPRE and SHOCKPRE, but not SALPRE, (Fig.1). There was a main effect of Session, ($F_{5,160}=15.396$, $p<.001$, $\eta^2=.325$), a main effect of Group, ($F_{4,32}=2.895$,
$p=.038$, $\eta^2=.266$), and no Session by Group interaction, $F_{20,160}=.758$, $p=.760$, $\eta^2=.087$). Post hoc analyses on the main effect of Group revealed that NIC+SHOCK maintained higher infusions than NICPRE. NIC–SHOCK was higher than NICPRE and SHOCKPRE, but not SALPRE. NICPRE, SHOCKPRE but not SALPRE controls did not differ from one another.

**Fig. 1** Total infusions (mean SEM) earned during adult nicotine self-administration on FR1, FI1, PR, and EXT schedules of reinforcement. A significant main effect of Group ($$ indicates $p<.05$), representing a significant difference between one of the combination test groups (NIC+SHOCK, NIC–SHOCK) and at least one or all of the control groups (NICPRE, SHOCKPRE, SALPRE) is represented. **Significant between-group post hoc comparisons can be found detailed in the Results of section 1.1.**
3.2 Active lever presses

3.2.1 Fixed Ratio 1.

For the FR1 phase, rats increased the number of active lever presses across Session, regardless of Group, and NIC+SHOCK rats made more drug-seeking responses compared to NICPRE, SHOCKPRE, and SALPRE, (Fig.2). There was a significant main effect of Session for Active Lever Presses ($F_{15,480}=8.341, p<.001, \eta^2=.207$), a main effect of Group, ($F_{4,32}=3.113, p=.028, \eta^2=.280$), but no significant Session by Group interaction, ($F_{60,480}=1.301, p=.073, \eta^2=.140$). Post hoc analyses of the main effect of Group revealed that NIC+SHOCK made significantly more active lever presses than NICPRE, SHOCKPRE, and SALPRE. NIC–SHOCK also had significantly more active lever presses than NICPRE and SHOCKPRE, and the difference from SALPRE did not quite reach statistical significance ($p=.053$). NICPRE, SHOCKPRE and SALPRE controls did not differ from one another.

3.2.2 Fixed Interval 1.

For the FI1 phase, all rats increased active lever presses across Sessions, regardless of Group, (Fig.2). There was a significant main effect of Session, ($F_{15,480}=6.285, p<.001, \eta^2=.164$), but no main effect of Group, ($F_{4,32}=1.529, p=.217, \eta^2=.160$), and no significant Session by Group interaction, ($F_{60,480}=1.108, p=.278, \eta^2=.122$).

3.2.3 Progressive Ratio.

For the PR phase, NIC+SHOCK and NIC–SHOCK rats made more active lever presses than NICPRE and SHOCKPRE but not SALPRE, (Fig.2). There was no main effect of Session, ($F_{3,96}=.717, p=.544, \eta^2=.022$), a main effect of Group, ($F_{4,32}=3.303, p=.023, \eta^2=.292$), and no
significant Session by Group interaction, \((F_{12,96}=.806, p=.643, \eta_p^2=.092)\). Post hoc analyses revealed that NIC+SHOCK made significantly more active lever presses compared to NICPRE and SHOCKPRE but not SALPRE. NIC–SHOCK made more active lever presses compared to NICPRE and SHOCKPRE but not SALPRE. NICPRE, SHOCKPRE and SALPRE controls did not differ from one another.

### 3.2.4 Extinction.

During the extinction phase, active lever pressing decreased regardless of Group, NIC–SHOCK rats drug-seek more compared to NICPRE, SHOCKPRE, and SALPRE, (Fig.2). There was a main effect of Session, \((F_{5,160}=10.548, p<.001, \eta_p^2=.248)\), a main effect of Group, \((F_{4,32}=2.872, p=.039, \eta_p^2=.264)\), and no Session by Group effect, \((F_{20,160}=1.438, p=.112, \eta_p^2=.152)\). Post hoc analyses on the main effect of Group revealed NIC–SHOCK rats make significantly more active lever presses compared to NICPRE, SHOCKPRE, and SALPRE. NICPRE, SHOCKPRE and SALPRE controls did not differ from one another.
Fig. 2 Active lever presses (mean SEM) made during adult nicotine self-administration on FR1, FI1, PR, and EXT schedules of reinforcement. A significant main effect of Group ($ indicates $p<.05$), representing a significant difference between one of the combination test groups (NIC+SHOCK, NIC–SHOCK) and at least one or all of the control groups (NICPRE, SHOCKPRE, SALPRE) is represented. Significant between-group post hoc comparisons can be found detailed in the Results of section 1.2.

3.3 Inactive Lever Presses

3.3.1 Fixed Ratio 1.

During the FR1 phase, NIC–SHOCK rats made significantly more inactive lever presses compared to all other groups, (Fig.3). There was a significant main effect of Session for Inactive Lever Presses, ($F_{15,480}=1.716, p=.045, \eta^2=.051$), a main effect of Group, ($F_{4,32}=4.572, p=.005, \eta^2=.364$), and no significant Session by Group interaction, ($F_{60,480}=.772, p=.892, \eta^2=.088$). Post hoc analyses on the main effect of Group revealed that NIC–SHOCK made significantly more
inactive lever presses compared to all other groups (NIC+SHOCK, NICPRE, SHOCKPRE, SALPRE). NICPRE, SHOCKPRE and SALPRE controls did not differ from one another.

3.3.2 Fixed Interval 1.

During the FI1 phase, all rats increased inactive lever pressing across Sessions, regardless of Group, (Fig.3). There was a significant main effect of Session, \(F_{15,480}=1.723, p=.043, \eta^2_p=.051\), no main effect of Group, \(F_{4,32}=5.73, p=.684, \eta^2_p=.067\), and no significant Session by Group interaction, \(F_{60,480}=.594, p=.993, \eta^2_p=.069\).

3.3.3 Progressive Ratio

During the PR phase, inactive lever presses did not differ, (Fig.3). There was no main effect of Session, \(F_{3,96}=0.062, p=.979, \eta^2_p=.002\), no main effect of Group, \(F_{4,32}=1.560, p=.209, \eta^2_p=.163\), and no significant Session by Group interaction, \(F_{3,96}=0.926, p=.525, \eta^2_p=.104\).

3.3.4 Extinction.

During the extinction phase, whereas inactive lever pressing decreased across Sessions regardless of Group, NIC+SHOCK and NIC−SHOCK rats continued making more inactive lever presses compared to NICPRE, SHOCKPRE, and SALPRE, (Fig.3). There was a main effect of Session, \(F_{5,160}=3.099, p=.011, \eta^2_p=.088\), a main effect of Group, \(F_{4,32}=2.733, p=.046, \eta^2_p=.255\), and no Session by Group effect, \(F_{20,160}=0.518, p=.956, \eta^2_p=.061\). Post hoc analyses on the main effect of Group revealed that NIC+SHOCK and NIC−SHOCK rats made more inactive lever presses compared to NICPRE, SHOCKPRE, and SALPRE controls. NICPRE, SHOCKPRE and SALPRE controls did not differ from one another.
Fig. 3 Inactive lever presses (mean SEM) made during adult nicotine self-administration on FR1, FI1, PR, and EXT schedules of reinforcement. A significant main effect of Group ($ indicates $p<.05$), representing a significant difference between one of the combination test groups (NIC+SHOCK, NIC–SHOCK) and at least one or all of the control groups (NICPRE, SHOCKPRE, SALPRE) is represented. Significant between-group post hoc comparisons can be found detailed in the Results of section 1.3.

3.4 Locomotion

3.4.1 Fixed Ratio 1.

During the FR1 phase, locomotor activity increased across Session regardless of Group, and NIC–SHOCK rats had greater locomotion compared to all controls, (Fig.4). Significant main effect of Session, ($F_{15,480}=12.256, p<.001, \eta^2=.277$), a main effect of Group, ($F_{4,32}=4.258, p=.007, \eta^2=.347$), and no significant Session by Group interaction, ($F_{60,480}=1.076, p=.333,$
\( \eta^2 = .119 \). Post hoc analyses on the main effect of Group revealed that NIC+SHOCK had significantly greater locomotor activity compared to NICPRE. NIC–SHOCK had significantly greater locomotor activity compared to NICPRE, SHOCKPRE, and SALPRE. NICPRE, SHOCKPRE and SALPRE controls do not significantly differ from one another.

### 3.4.2 Fixed Interval 1.

During the FI1 phase, locomotor activity increased across Session regardless of Group, (Fig.4). There was a significant main effect of Session, \( (F_{15,480} = 7.890, p < .001, \eta^2 = .198) \), but no main effect of Group, \( (F_{4,32} = 1.962, p = .124, \eta^2 = .197) \), and no Session by Group interaction, \( (F_{60,480} = 1.058, p = .365, \eta^2 = .117) \).

### 3.4.3 Progressive Ratio.

During the PR phase, locomotor activity did not differ across Session or Group, (Fig.4). There were no main effects of Session, \( (F_{3,96} = 2.015, p = .117, \eta^2 = .059) \) or Group, \( (F_{4,32} = 2.251, p = .085, \eta^2 = .220) \), and no Session by Group interaction, \( (F_{12,96} = 1.409, p = .175, \eta^2 = .150) \).

### 3.4.4 Extinction.

During the extinction phase, while locomotor activity decreased across Session regardless of Group, NIC+SHOCK and NIC—SHOCK rats had greater locomotor activity compared to NICPRE, (Fig.4). There was a main effect of Session, \( (F_{5,160} = 11.741, p < .001, \eta^2 = .268) \), a main effect of Group, \( (F_{4,32} = 3.865, p = .011, \eta^2 = .326) \), and no Session by Group interaction, \( (F_{20,160} = 7.47, p = .772, \eta^2 = .085) \). Post hoc analyses on the main effect of Group revealed that NIC+SHOCK and NIC–SHOCK maintained greater locomotor activity compared to NICPRE. NICPRE, SHOCKPRE and SALPRE controls did not differ from one another.
3.5 Lever Discrimination

3.5.1 SALPRE Active vs. Inactive.

SALPRE rats learned to discriminate between active and inactive levers, maintained a discrimination across every schedule of reinforcement, and extinguished drug seeking behaviour when nicotine became unavailable, (Fig. 5). During FR1, there was a main effect of Session, \( F_{15,150} = 4.235, p < .001, \eta^2_p = .298 \), a main effect of Lever, \( F_{1,10} = 5.070, p = .048, \eta^2_p = .336 \), and a

Fig. 4 Locomotor activity (number of photobeam breaks; mean SEM) earned during adult nicotine self-administration on FR1, FI1, PR, and EXT schedules of reinforcement. A significant main effect of Group ($ indicates \( p < .05 \), representing a significant difference between at least one of the combination test groups (NIC+SHOCK, NIC–SHOCK) and at least one or all of the control groups (NICPRE, SHOCKPRE, SALPRE) is represented. Significant between-group post hoc comparisons can be found detailed in the Results of section 1.4.
significant Session by Lever type interaction, \( F_{15,150} = 1.785, p=.042, \eta^2=.151 \). Post hoc analyses on the interaction revealed significantly more active lever presses compared to inactive on sessions 7, 9, and 11-16. On FI1, rats continued to discriminate between active and inactive levers. There was a main effect of Session, \( F_{15,150} = 3.299, p<.001, \eta^2=.248 \), a main effect of Lever, \( F_{1,10} = 8.186, p=.017, \eta^2=.450 \), but no significant Session by Lever type interaction, \( F_{15,150}=1551, p=.094, \eta^2=.134 \). Post hoc analyses on the main effect of Lever revealed significantly more active lever presses compared to inactive. On PR, rats continued to press more on the active than inactive lever. There was no effect of Session, \( F_{3,30}=.432, p=.732, \eta^2=.041 \), there was a main effect of Lever type, \( F_{1,10}=5.063, p=.048, \eta^2=.336 \), but no Session by Lever type interaction, \( F_{3,30}=.612, p=.613, \eta^2=.058 \). During extinction, rats gradually decreased active lever pressing to the level of inactive lever pressing. There was a main effect of Session, \( F_{5,50}=6.745, p<.001, \eta^2=.403 \), no main effect of Lever type, \( F_{1,10}=4.717, p=.055, \eta^2=.321 \), and a significant Session by Lever type interaction, \( F_{5,50}=2.891, p=.023, \eta^2=.224 \). Post hoc analyses on the interaction revealed that active lever presses were significantly higher than inactive lever presses on the first 2 extinction sessions.
SHOCKPRE Active vs. Inactive.

SHOCKPRE rats did not significantly discriminate between active and inactive levers across training except on the first 2 extinction sessions when drug became unavailable, (Fig. 6). During FR1, there was no effect of Session, ($F_{15,180}$.980, $p=.478$, $\eta_p^2=.076$), no effect of Lever, ($F_{1,12}$=1.838, $p=.200$, $\eta_p^2=.133$), and no Session by Lever interaction, ($F_{15,180}$.938, $p=.524$, $\eta_p^2=.072$). On FI1, rats increased pressing regardless of lever. There was a main effect of Session, ($F_{15,180}$.1296, p=.208, $\eta_p^2=.098$), but no effect of Lever, ($F_{1,12}$.4.374, p=.058, $\eta_p^2=.267$), or significant Session by Lever interaction, ($F_{15,180}$.510, p=.933, $\eta_p^2=.041$). Similarly, on PR, rats increased responding over sessions regardless of lever. There was a main effect of Session, ($F_{3,36}$.3.895, p=.017, $\eta_p^2=.245$), but no effect of Lever, ($F_{1,12}$.4.689, p=.051, $\eta_p^2=.281$), or Session by Group interaction, ($F_{3,36}$.2.368, p=.087, $\eta_p^2=.165$). During extinction, rats

Fig. 5 Lever discrimination for Saline control rats (SALPRE) across each schedule of reinforcement. Mean (± SEM) active and inactive lever presses. $\$ indicates $p<0.05$, significant main effect of lever; * indicates $p<0.05$, significant difference between lever types after a significant interaction.
demonstrated a discrimination between levers that declined across sessions. There was a main effect of Session, \((F_{5,60}=3.190, p=.013, \eta^2=0.210)\), a main effect of Lever type, \((F_{1,12}=6.338, p=0.027, \eta^2=0.346)\), and a significant Session by Lever type interaction, \((F_{5,60}=2.960, p=0.019, \eta^2=0.198)\). Post hoc analyses on the interaction revealed that active lever presses were significantly higher than inactive on the first 2 sessions.

**Fig. 6** Lever discrimination for Shock control rats (SHOCKPRE) across each schedule of reinforcement. Mean (± SEM) active and inactive lever presses. $ indicates \(p<0.05\), significant main effect of lever; * indicates \(p<0.05\), significant difference between lever types after a significant interaction.

### 3.5.3 NICPRE Active vs. Inactive.

NICPRE rats learned to discriminate between active and inactive levers, maintained discrimination when an interval schedule was implemented, did not show enhanced motivation to obtain nicotine on PR, and did not extinguish drug-seeking when drug was made unavailable. During FR1, there was a main effect of Session, \((F_{15,180}=5.451, p<0.001, \eta^2=0.312)\), no main effect of Lever, \((F_{1,12}=2.574, p=.135, \eta^2=0.177)\), and a significant Session by Lever interaction,
Post hoc analyses on the interaction revealed significantly greater active vs. inactive lever presses on sessions 11, and 13-16. During FI1, there was a main effect of Session, \(F_{15,180}=2.419, p=.003, \eta^2=.168\), no main effect of Lever, \(F_{1,12}=4.263, p=.061, \eta^2=.262\), and a significant Session by Lever type interaction, \(F_{15,180}=2.078, p=.013, \eta^2=.148\). Post hoc analyses on the interaction revealed that active lever presses were significantly higher than inactive on all sessions. During PR, rats did not significantly discriminate between active and inactive levers. There was no main effect of Session, \(F_{3,36}=.873, p=.464, \eta^2=.068\), no main effect of Lever, \(F_{1,12}=3.032, p=.107, \eta^2=.202\), and no significant Session by Lever interaction, \(F_{3,36}=.790, p=.507, \eta^2=.062\). During extinction, rats made significantly more active lever presses than inactive. There was no effect of Session, \(F_{5,60}=1.339, p=.260, \eta^2=.100\), a main effect of Lever, \(F_{1,12}=5.614, p=.035, \eta^2=.319\), and no significant Session by Lever interaction, \(F_{5,60}=.633, p=.675, \eta^2=.050\).
Fig. 7 Lever discrimination for Nicotine control rats (NICPRE) across each schedule of reinforcement. Mean (± SEM) active and inactive lever presses. $ indicates $p<0.05$, significant main effect of lever; * indicates $p<0.05$, significant difference between lever types after a significant interaction.

3.5.4 **NIC+SHOCK Active vs. Inactive.**

NIC+SHOCK rats learned to discriminate on the very first session, maintained a significant discrimination for the entirety of the experiment, and did not extinguish drug-seeking when nicotine was made unavailable, (Fig.8). During FR1, there was a main effect of Session, $(F_{15,210}=2.543, p=.002, \eta^2=.154)$, a main effect of Lever, $(F_{1,14}=12.220, p=.004, \eta^2=.466)$, and no significant Session by Lever interaction, $(F_{15,210}=1.577, p=.082, \eta^2=.101)$. Active lever presses were significantly higher than inactive lever presses. During FI1, rats maintained a significant discrimination between active and inactive levers. There was a main effect of Session, $(F_{15,210}=1.570, p=.084, \eta^2=.101)$, a main effect of Lever, $(F_{1,14}= 41.273, p<.001, \eta^2=.747)$, and no significant Session by Lever interaction, $(F_{15,210}= 1.284, p=.214, \eta^2=.084)$. Active lever
pressing was significantly higher than inactive lever pressing. During PR, rats again maintained higher active than inactive lever pressing. There was no main effect of Session, ($F_{3,42}=1.254, p=.302, \eta^2_p=.082$), a main effect of Lever type, ($F_{1,14}=29.923, p<.001, \eta^2_p=.681$), and no significant Session by Lever type interaction, ($F_{3,42}=.296, p=.828, \eta^2_p=.021$). Active lever presses are significantly higher than inactive. During extinction, rats significantly discriminated between active and inactive levers and did not extinguish this drug-seeking behaviour. There was a main effect of Session, ($F_{5,70}=4.085, p=.003, \eta^2_p=.226$), a main effect of Lever, ($F_{1,14}=10.753, p=.005, \eta^2_p=.434$), and no significant Session by Lever interaction, ($F_{5,70}=1.619, p=166, \eta^2_p=.104$).

![Figure 8](image-url)  
**Fig. 8** Lever discrimination for simultaneous nicotine and footshock exposure (NIC+SHOCK) rats across each schedule of reinforcement. Mean (± SEM) active and inactive lever presses. $\$$_{<0.05}$ indicates significant main effect of lever; * indicates $p<0.05$, significant difference between lever types after a significant interaction.
3.5.5 NIC–SHOCK Active vs. Inactive.

NIC–SHOCK rats did not learn to significantly discriminate between active and inactive levers during FR1, significantly discriminated when the interval schedule was implemented (FI1) and during PR, and successfully extinguished drug seeking when nicotine became unavailable (Fig. 9). During FR1, rats did not significantly discriminate between active and inactive levers. There was no effect of Session, \((F_{15,240}=1.378, p=.158, \eta^2_p=.079)\), Lever, \((F_{1,16}=.787, p=.388, \eta^2_p=.047)\), or Session by Lever interaction, \((F_{15,240}=.764, p=.716, \eta^2_p=.046)\). On FI1, rats significantly discriminated between active and inactive levers and increased responding over sessions. There was a main effect of Session, \((F_{15,240}=2.652, p=.001, \eta^2_p=.142)\), Lever, \((F_{1,16}=7.042, p=.017, \eta^2_p=.306)\), and a significant Session by Lever interaction \((F_{15,240}=2.595, p=.001, \eta^2_p=.140)\). Post hoc analyses on the interaction revealed that active lever pressing was significantly higher than inactive on all sessions. On PR, rats significantly discriminated between active and inactive levers. There was no effect of Session, \((F_{3,48}=.451, p=.718, \eta^2_p=.027)\), a main effect of Lever, \((F_{1,16}=9.257, p=.008, \eta^2_p=.367)\), and no significant Session by Lever interaction, \((F_{3,48}=.353, p=.787, \eta^2_p=.022)\). During extinction, rats gradually decreased active lever pressing to the level of inactive across sessions. There was a main effect of Session, \((F_{5,80}=5.895, p<.001, \eta^2_p=.269)\), Lever, \((F_{1,16}=8.072, p=.012, \eta^2_p=.335)\), and a significant Session by Lever interaction, \((F_{5,80}=3.858, p=.003, \eta^2_p=.194)\). Post hoc analyses on the interaction revealed that active lever presses were significantly higher than inactive on the first 2 sessions.
Novel open-field test

3.5.6 Total distance travelled.

Adolescent exposure groups did not differ on novelty-induced locomotion in a drug-free state in adulthood, (Fig. 10a). There were no significant differences in total distance travelled (cm), \(F_{4,91} = 2.315, p = .064, \eta^2 = 0.096\). 

3.5.7 Percent time spent in center.

Adolescent exposure groups did not differ on baseline anxiety-like behaviour in a drug-free state, (Fig. 3B). There were no significant differences in the percent time spent in the center of the OFT, \(F_{4,91} = 0.289, p = .884, \eta^2 = 0.013\).
Fig. 10  Baseline anxiety-like behaviour and corticosterone levels. Mean (± SEM) (A) Distance travelled in OFT (B) percent time spent in the center of OFT (C) Baseline adult CORT levels. There are no significant differences in baseline anxiety-like behaviour or CORT levels across groups
3.6 Corticosterone quantification

3.6.1 Baseline CORT.

Adolescent nicotine, shock, or the combination of nicotine and shock exposure did not alter baseline CORT levels in adulthood, (Fig. 10C). All of the rats in the experiment that went on to get either nicotine or shock in adulthood did not differ on baseline CORT levels across adolescent exposure group, \((F_{9,82} = 0.978, p = 0.465, \eta^2 = 0.097)\). Rats that received nicotine in adulthood did not differ from rats that received shock in adulthood on their baseline CORT levels, \((F_{1,90} = .523, p = 0.471, \eta^2 = 0.006)\).

3.6.2 CORT levels in response to adult nicotine and shock across groups.

There was no effect of adolescent preexposure (Group) on stress response to nicotine or shock, however nicotine increased CORT release significantly more than shock, (Fig.11). There was a main effect of Timepoint, \((F_{1,82} = 272.491, p < 0.001, \eta^2 = 0.769)\), and a main effect of Stressor, \((F_{1,82} = 22.995, p < 0.001, \eta^2 = 0.219)\), but no main effect of Group, \((F_{4,82} = 1.024, p = 0.400, \eta^2 = 0.048)\). There was a significant Timepoint by Stressor interaction, \((F_{1,82} = 93.524, p < 0.001, \eta^2 = 0.533)\), but no significant Timepoint by Group interaction, \((F_{4,82} = 1.074, p = 0.375, \eta^2 = 0.050)\). Post hoc analyses reveal that though both nicotine and footshock enhanced CORT levels, nicotine increased CORT levels significantly more than footshock.
Fig. 11 Adult CORT response to nicotine or stress. Mean (± SEM) CORT response to adult nicotine compared to footshock stress. $ indicates $p<0.05$, significant main effect of Timepoint, # indicates $p<0.05$, significant Timepoint x Stressor interaction.
4 Discussion

The synergistic effects of adolescent nicotine and footshock exposure enhanced acquisition of spontaneous adult nicotine IVSA and drug-seeking relative to either of these adolescent experiences independently. This effect occurred without a concomitant enhancement of adult anxiety-like behaviour in a drug-free state or altered CORT responsivity to nicotine or footshock exposure. Receiving alternating sessions of exposure to high-dose nicotine or footshock alone throughout adolescence did not significantly alter adult nicotine IVSA, anxiety-like behaviour, or CORT responsivity. During FR1, SALPRE and NICPRE, rats learned over time to discriminate between active and inactive levers. In the first sessions active and inactive lever pressing did not differ for these rats, but as the FR1 sessions progress, they learned to make significantly more active than inactive lever presses. NIC+SHOCK rats also significantly discriminated between active and inactive levers; however, this discrimination was present from the very first session. This could be due to an increased perceptibility of nicotine in these rats, or enhanced learning for this task. While NIC–SHOCK rats took more infusions during FR1 compared to shock and nicotine controls, they did not significantly discriminate between the two levers. This could be due to an overall increased excitability evidenced by increased locomotor activity, and a sudden increase in inactive lever presses from session 7 onward. Finally, the SHOCKPRE group did not learn to significantly discriminate between active and inactive levers. When a 1 min delay was implemented during FI1, all rats successfully learned to discriminate between active and inactive levers except for SHOCKPRE rats again. When the schedule is again shifted to a PR, which requires more responses for each successive infusion, SHOCKPRE rats still failed to significantly discriminate, along with NICPRE rats. Finally, when nicotine is made unavailable during extinction, SALPRE rats significantly discriminate on the first 2 sessions, and then successfully extinguish their drug-seeking behaviour. SHOCKPRE rats show
the same pattern as SALPRE rats during extinction – evidence that SHOCKPRE rats knew the contingency throughout self-administration, since the first 2 sessions of extinction were not reinforced. It may be that the SHOCKPRE rats had increased sensitivity to the reinforcing effects of nicotine resulting in less nicotine required to achieve optimal titration, or increased sensitivity to the aversive effects of nicotine and reduced propensity to self-administer. NIC–SHOCK rats also show significant discrimination on the first 2 sessions of extinction, and then extinguish their drug-seeking. Interestingly, both NICPRE and NIC+SHOCK rats do not extinguish this drug-seeking behaviour across extinction sessions and continue to make significantly more active than inactive lever presses throughout all extinction sessions. The same dose of nicotine used in this study spread out over the course of the day (1 mg/kg/day via mini osmotic pump) for 15 days resulted in enhanced acquisition of fear conditioning and failure to extinguish the learned behaviour (Smith et al., 2006). Though this paradigm does not use fear conditioning, it is possible that this effect can generalize to different types of learning and promote perseverative behaviours.

During adult spontaneous acquisition of nicotine IVSA on an FR1 schedule of reinforcement, rats exposed to co-occurring nicotine and shock every other day (NIC+SHOCK) showed enhanced adult nicotine IVSA compared to shock pre-exposed, nicotine pre-exposed, and saline control rats. Interestingly, rats with the same amount of nicotine and footshock in adolescence, but never co-occurring (NIC–SHOCK), also showed this enhancement of nicotine self-administration compared to shock and nicotine controls – evidence that these two experiences did not have to occur simultaneously in order to exert their synergistic effects. Rats that received nicotine and footshock never co-occurring (NIC–SHOCK), and thus received a CORT-releasing stimulus every day, also showed increased inactive lever pressing and
locomotion during acquisition (FR1) and enhanced locomotion and drug-seeking during extinction when nicotine became unavailable. These animals may have been displaying an overall increased excitability that drove this increased inactive lever pressing and locomotor activity (Cole et al., 2019). Increased inactive lever pressing during PR in adult following adult and adolescent nicotine pretreatment has been previously observed in mice, with this effect being more pronounced in mice that had adolescent nicotine pretreatment. This may be due to non-specific behavioural activation as opposed to generalization between the two levers that can be observed in earlier stages of instrumental conditioning and may be indicative of increased impulsivity and deficits in reward-based decision making (Cole et al., 2019). In addition, male and female adolescent (P33) rats showed an increase in inactive lever pressing on a PR schedule when the pharmacological stressor yohimbine was administered (0, 0.3, 0.6mg/kg yohimbine, IP) (Li et al., 2014). This increased excitability was specific to the IVSA environment, where drug was present, and was not evident in a novel open field test in a drug-free state, where they never encountered any drug or drug-paired cues.

The key difference between these two experimental groups is that NIC+SHOCK rats receive a CORT-stimulating stimulus every other day, whereas NIC–SHOCK rats experience a stimulus every single day – these subtle differences in exposure patterns may underlie differences between these groups in persistent drug-seeking during extinction and locomotion. Another potential explanation for the subtle differences between these two groups is that nicotine has been shown to have antinociceptive properties (Tripathi, Martin, & Aceto, 1982), and stressors have been shown to potentiate the rewarding effects of nicotine in adolescence (Li et al., 2014; Pentkowski et al., 2011; Zou et al., 2014). Taken together, it is possible that for the NIC+SHOCK group, concurrent nicotine and shock exposure results in nicotine exerting an
antinociceptive effect on the shock stressor, while the shock stressor is simultaneously enhancing the rewarding value of the nicotine. This unique experience in adolescence results in increased acquisition of nicotine self-administration, increased motivation to obtain nicotine compared to individual nicotine or shock pre-exposure, without the additional increase in inactive lever presses seen from NIC–SHOCK rats during FR1 or drug-seeking during extinction.

Across FR1 training, responding in control rats approached levels of the combined nicotine and shock pre-exposed rats, and there were no group differences once the 1-min delay was implemented between each possible nicotine infusion (FI1). All groups continued to increase nicotine consumption across those sessions. This indicates that nicotine and footshock stressor in adolescence may predispose individuals to acquire self-administration faster, but not necessarily to take more nicotine in the long-term. During PR, both of the nicotine and shock combination groups worked harder to obtain nicotine compared to nicotine- or shock pre-exposed rats, and when drug availability stopped during extinction, combination alternating adolescent nicotine and shock pre-exposed rats (NIC–SHOCK) persisted in drug-seeking to a greater extent than nicotine, shock, and saline controls. Furthermore, both of the combination adolescent nicotine and shock groups also showed greater inactive lever pressing compared to controls during extinction, evidence of extinction-evoked increases in response variability (Neuringer, Kornell, & Olufs, 2001), and indicative of greater levels of extinction-induced frustration (Amsel, 1958; Dudley & Papini, 1997).

Of note, none of the three control groups, nicotine pre-exposure (NICPRE), shock pre-exposure (SHOCKPRE), or no-nicotine/no-shock (SALPRE) ever differed from each other on any of the dependent measures assessed herein. This finding is in contrast to some existing literature showing that adolescent nicotine exposure increases adult nicotine self-administration.
Specifically, previous studies have shown increased adult nicotine self-administration following adolescent nicotine exposure (Adriani et al., 2003; Cole et al., 2019; Natividad et al., 2013). However, procedural differences in studies may account for differences in findings. Rats in the current study received 1 mg/kg nicotine or shock every other day throughout adolescence, whereas studies that found increased nicotine IVSA administration used 0.4 mg/kg, IP, once per day for 10 days from P34-43 (Adriani et al., 2003), 4.7 mg/kg/day for 14 days via mini osmotic pump beginning on P32-34 (Natividad et al., 2013), and 3 mg/kg/day for 2 weeks (P33-35) or 6.3 mg/kg/day (P61-63) for 2 weeks via osmotic minipumps (Cole et al., 2019). Therefore, the differences are a higher bolus dosage and more spread out exposure for our study compared to previous literature. Furthermore, a recent study from our lab exposed rats starting at the same age to the same number of injections and same dose as this current study but for 14 days in a row (P28-41), instead of every other day, and found a significant increase in spontaneous adult nicotine IVSA under a FI1 schedule of reinforcement at 0.02 mg/kg/infusion instead of the current 0.03 mg/kg/infusion. Those training conditions were such that the saline pre-exposed rats did not develop reliable self-administration (Renda et al., 2020). It is possible that daily nicotine exposure, as opposed to intermittent or alternating exposure, may have had a more profound of an effect during a critical developmental stage. In stark contrast, one study reported a reduction in adult nicotine self-administration following adolescent nicotine exposure of 3.2 mg/kg/day nicotine from P28-34 compared to drug naïve rats (de la Peña et al., 2014), however this was the only study that lever trained rats for pellets prior to IVSA, and the reinforcer switch may have been more disruptive to the nicotine pre-exposed rats. Adolescent mice (P33-35) exposed to nicotine (3mg/kg/day for 2 weeks via osmotic minipump) had a greater preference for saccharin solution in adulthood (P90) compared to controls. However, if this adolescent nicotine exposure
is followed two weeks later with adult (P61-63) nicotine re-exposure (6.3mg/kg/day for 2 weeks via osmotic minipump) there is a preferential enhancement of nicotine reinforcement (Cole et al., 2019). Given that rats clearly demonstrate spontaneous nicotine IVSA without pretraining, it is preferred to avoid any confounding effects of training and non-drug reward.

The stress exposure in the current study prevented SHOCKPRE rats from learning to significantly discriminate between the active and inactive levers, while SALPRE controls did learn to discriminate and increased their intake across sessions and schedules of reinforcement. Albeit speculative, the effects of intermittent mild footshock exposure in adolescence may have resulted in some resilience to nicotine intake in adulthood. Conversely, perhaps these rats were more sensitive to the effects of nicotine in adult and thus a smaller dose was able to elicit the desired effect. When adolescent (P28-37) rats are exposed to 2 hr daily RS for 7 days followed by a challenge of nicotine 3 days later (0.4mg/kg, SC), behavioural sensitization was observed, compared to non-stressed controls. Though not significant, on average SHOCKPRE rats have higher locomotor scores during the first IVSA sessions compared to SALPRE and NICPRE controls, although the means for their nicotine infusions and lever pressing were not higher on average. Future studies will look at the potential for behavioural sensitization in rats with this type of adolescent preexposure with more precise dosing and measures, and plasma nicotine levels can also be assessed in these rats.

Previous studies have found anxiety-like behaviour in OFT and CP procedures that differ from the results of the current experiment. Decreased time spent in the center of a novel OFT has been shown 1 month after adolescent (P28-42) nicotine exposure (1, 2mg/kg/day via osmotic minipump for 15 days)(Smith et al., 2006). Decreased exploratory activity in novel OFT has also been shown 2-3 weeks after adolescent (P31-36) nicotine exposure (5 days of 5.0mg/kg/day via
Nicoderm CQ patches) (C. Slawecki, 2003). Additionally, increased novelty-induced locomotion in CP is observed 5 weeks after adolescent (P34-43) nicotine exposure (0.4 mg/kg, IP, 10 days daily) (Adriani et al., 2006). Similar to how nicotine or shock preexposure alone in the present study was not able to enhance adult nicotine IVSA, there was no change in anxiety-like behaviour in adulthood. Previous studies utilize nicotine preexposure methods that result in consistent nicotine exposure for days at a time, whereas the present study administered nicotine in a high-dose bolus once every other day. It is conceivable that this difference in pre exposure may underlie differences in adult novelty-induced locomotion and anxiety-like behaviour.

Adolescent combination nicotine and footshock exposure used in this study was able to alter adult IVSA behaviour in nicotine intake and seeking without altering adult stress responsivity to nicotine or footshock. Indeed, CORT concentrations across adolescent preexposure groups did not differ for nicotine or shock exposure in adulthood. Similarly, while adolescent nicotine and shipping stress altered hippocampal GR and CRF receptor IR on the 12th day of nicotine exposure, after 24 hr withdrawal, and after 30 days abstinence, there was no associated alterations in CORT levels compared to controls. However, the interaction between adolescent shipment stress and nicotine exposure did reduce adult CORT response to RS in adulthood (Holliday et al., 2019). Perhaps the amount of CORT released was not affected, but the incentive value of the CORT that was released may have been affected. CORT is a reinforcer (Deroche et al., 1993; Piazza et al., 1993) and can alter the reinforcing effects of other behaviours/drugs (Piazza et al., 1991), so perhaps this adolescent preexposure did not alter the way the HPA axis functions, but rather, it altered the way the reward circuit processes that release of CORT. Additionally, perhaps there are differences in GR and CRF receptor distribution in key brain structures (Holliday et al., 2019) that are involved in the enhancement of
adult nicotine reinforcement seen in this study. Future studies will assess the value of CORT in animals with this type of adolescent preexposure by, for example, measuring DA levels in the nucleus accumbens following exposure to a CORT releasing stimulus or CORT administration.

We show here that intermittent adolescent nicotine and footshock stress exposure function synergistically to enhance vulnerability to adult nicotine consumption and nicotine-seeking during unavailability without altering baseline anxiety or stress responsivity. The current rates of adolescent nicotine vaping are extremely high and continue to rise yearly with each set of updated statistics. In 2019, about 12% of 12th graders self-reported vaping nicotine daily, while approximately a quarter of them reported use in the last month. Nicotine vaping in 8th graders in 2019 was almost as high as the prevalence of nicotine vaping in 12th graders in 2017 (Miech et al., 2019). The increase in nicotine vaping from 2017-2018 was the largest one-year jump ever tracked in the 45-year history of the ‘Monitoring the Future’ survey for any substance in 8th, 10th, and 12th graders (Miech et al., 2019). The brand name product JUUL is the most popular nicotine vaping device in adolescence (Krishnan-Sarin et al., 2019), produces high plasma nicotine content – almost threefold the levels of a cigarette (P. Rao, Liu, & Springer, 2020), and is associated with higher SES (Krishnan-Sarin et al., 2019) compared to low SES previously (Hiscock et al., 2012). Furthermore, adolescents will inevitably face stress or perceived stress – as this thesis is currently being written, the COVID-19 pandemic is occurring and constitutes a major source of stress for everyone, including adolescents. Given that adolescent nicotine and stress exposure in the current study was able to increase adult nicotine reinforcement and vulnerability to relapse, and there are high rates of adolescent vaping and adolescent stress or perceived stress (Leventhal et al., 2017), there may be a surge in adult nicotine use in the next decade. It is well known that adolescent initiation of nicotine use does
increase the risk of adult use and dependence (J. Chen & Millar, 1998; Cullen et al., 2018; Sharapova et al., 2018), however the range and number of adolescents that are using these products today differ from what is known about the demographic of cigarette smoking. An emphasis on improving prevention methods, understanding the interaction between nicotine and stress in adolescence, and advancing smoking cessation therapies now may help avoid a new rise in adult nicotine use and the negative health outcomes that accompany this use.
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