Let's talk about sex: uncovering the molecular mechanisms of amphetamine during adolescent development

Christina Popescu

Integrated Program in Neuroscience (IPN)

McGill University

Montréal, Quebec

August 2020

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

©Christina Popescu, 2020

Contents

Abstract

Mesocorticolimbic dopamine (DA) neurons are still growing towards their final target during adolescence; as such, their maturation is vulnerable to environmental influences during this critical developmental time period. Specifically, adolescent experiences – such as drug use – can lead to long-term medial prefrontal cortex (mPFC) dysfunction via the Netrin-1/DCC pathway, which serves as a target recognition system for growing mesocorticolimbic DA neurons. Exposure to the psychostimulant amphetamine (AMPH) in male mice alters the expression of both the guidance cue, Netrin-1, and its receptor, DCC, in early but not mid-adolescence. This altered expression induces targeting errors that reroute some axons to grow into the mPFC, resulting in aberrant mPFC DA innervation and impaired behavioural inhibition in adulthood.

This project investigates whether the biological sexes differ in their sensitivity to the developmental effects of AMPH. To do so, female mice were treated with AMPH during early or mid-adolescence in order to begin investigating these sex-specific effects using a three-pronged approach: molecular (assessing the expression of the guidance cue, Netrin-1, and its receptor, DCC), neuroanatomical (assessing mPFC DA innervation using unbiased stereology in adulthood), and behavioral (assessing mPFC function in adulthood). Results indicate that, unlike males, AMPH exposure in early adolescent females does not result in changes in DCC expression, mPFC innervation abnormalities, or mPFC-dependent behavioural impairments in adulthood. Interestingly, AMPH exposure in mid-adolescence does alter the DCC expression of female mice, suggesting that the timeline of sexual development may demarcate sex-dependent critical periods for the effects of AMPH on mesocorticolimbic DA development. Upon further investigation, however, a compensatory mechanism involving Netrin-1 may serve to protect mid-adolescent female mice from mPFC innervation abnormalities and prominent behavioural impairments in adulthood. To begin assessing the potential role of pubertal timing and gonadal hormones in determining sex differences in the effects of AMPH, the effects of early or mid-adolescent exposure to AMPH on DCC expression were

investigated in a pilot experiment using gonadectomized animals. In conclusion, this work begins to elucidate sexually dimorphic vulnerability to AMPH exposure during adolescence and the potential role that puberty onset and activational sex hormones may play in these events.

Résumé

Au cours de l'adolescence, les neurones dopaminergiques (DA) de la voie mésocorticolimbique continuent leur croissance vers leur cible finale. Durant cette période critique du développement, la maturation des neurones est sensible aux influences environnementales, dont les expériences adolescentes, telle que la prise de drogue. La consommation de certaines drogues peut conduire à un dysfonctionnement du cortex préfrontal (mPFC), via un dérèglement du complexe Nétrine 1/DCC, servant aux neurones dopaminergiques en cours de développement de système de reconnaissance de leur cible finale. L'exposition de souris mâles à une drogue psychostimulante, l'amphétamine (AMPH), altère l'expression de la nétrine 1, et de son récepteur DCC seulement au cours de la phase initiale de l'adolescence. Cette altération induit des erreurs dans le câblage des neurones dopaminergiques, certains de ces neurones étant dirigés vers le mPFC, résultant en une innervation perturbée de celui-ci et à des modifications comportementales chez la souris mâle à l'âge adulte.

Le but de ce projet est de déterminer si les changements observés au cours de l'adolescence, après l'administration d'AMPH, sont spécifiques au sexe de l'individu. Pour répondre à cette question nous avons administré de l'AMPH à des souris femelles, à deux stades de l'adolescence (stade précoce et moyen), en utilisant 3 approches: une moléculaire (mesurant l'expression de la nétrine et de son récepteur DCC), une neuroanatomique (évaluant l'innervation dopaminergique par stéréologie chez l'animal adulte) et une comportementale (évaluant le fonctionnement du mPFC chez l'adulte). Nos résultats montrent que contrairement à la souris mâle, l'administration chez la souris femelle d'AMPH au stade précoce de l'adolescence n'entraîne pas de changement des taux de DCC, de modification de l'innervation du mPFC, ou de détérioration des comportements reliés au mPFC.

Par contre, l'administration d'AMPH chez la femelle au stade moyen de l'adolescence conduit à une modification de l'expression de DCC. Ce résultat suggère que la période à laquelle apparaît la puberté chez la souris femelle modifie la période à laquelle apparaît la phase critique de l'effet de l'AMPH sur le développement du mPFC. Il est aussi possible

qu'un mécanisme compensatoire, médié par la nétrine 1 chez la souris femelle, la protège des anormalités dans l'innervation dopaminergique et de la détérioration des comportements à l'âge adulte.

Afin d'étudier l'impact de la puberté et des hormones sexuelles sur les taux de DCC au cours du temps, suite à l'injection d'AMPH chez les souris mâles et femelles, nous avons réalisé une étude préliminaire chez des souris castrées.

En conclusion, ce travail commence â élucider la vulnérabilité lié au dimorphisme sexuel dans la réponse à l'injection d'AMPH au cours de l'adolescence chez la souris et le rôle potentiel que l'apparition de la puberté et des hormones sexuelles peuvent jouer dans ces événements.

Acknowledgements

I knew this would be the hardest part of this thesis to write – not because it is objectively challenging, but rather because it is difficult to put into words the gratitude I feel for those who helped make this research and thesis possible.

Of course, there are the members of the Flores lab, past and present: a magnificent group of brilliant and passionate scientists – and even better people. Our current lab group as I wrap up this project has provided me with a wonderfully collaborative environment, especially in our little bullpen of an office: Alice Morgunova, Andrea Pantoja-Urbán, and José María Restrepo-Lozano, thank you all for being the best company one could possibly hope for as a graduate student. Also to the wonderful undergrad, Taylor Orsini, thank you for your dedication and contribution all while balancing your classes. For this project, I would like to explicitly thank Dominique Nouel, Giovanni Hernandez, and Michel Giroux for all your incredible work and dedication as well as for your friendship and comradery, I can only hope you learned from me even just a small fraction of what I learned from you. Know that I am grateful beyond words to have been your colleague these past few years and will always think fondly of our long days and late nights and everything you taught me – in terms of lab skills and, more importantly, life skills. If I were to even attempt to put it all into words, it may very well be longer than the thesis itself.

I would also like to thank the lovely Demetra Rodaros for helping me through my "deer-in-the-headlights" phase trying to navigate the world of rodent gonadectomy, it's only with your kindness in taking the time to share your expertise with me (multiple times) that the last, and largest, experiment of this thesis was made possible.

Then there are friends, those who had little idea what I was doing all those hours in the lab but who loved and supported me anyways. I love you all so much.

Annabelle Vlaun, you have made my life better from the moment I met you as a bright-eyed, bushy-tailed first year who couldn't find her assigned group during Discover McGill. I can honestly say that I've never been happier to not find an assigned group in my life;

we've been through a lot since then, but I know you are always there cheering me on and I cannot adequately express how much that means to me. Danijela Michielsen, all these years later and I still think about the time I almost caused you to fail your driver's test because I left the car parked with the e-brake engaged without you realizing it. To think we were connected even then! I suppose our friendship was inevitable... fine by me haha. All joking aside, thank you for your incredible support over the years and for all the crazy experiences we've had together. And Kieth, this is proper "Science!". Florian Hoft, my roommate, my bud, basically the other half of my old married couple lifestyle, thank you for always being just a hug away. Rebecca Cummer, oh, it's been an adventure – I'm so glad you decided to stay in that stats class, I would've been lost without you! I'll never forget the first fact I learned about you and how I thought you were too much of a badass to befriend the likes of me, thanks for proving me wrong. Finally, my dad, my constant pillar of strength, the kindest and most supportive father any child could hope for. Thank you for choosing me, for saving me; thank you for your unwavering faith in me, particularly during the times when I did not believe in myself. I will always do my utmost to make you proud.

And then there are the mentors you are fortunate enough to be embraced by along the way: Santiago Cuesta, who for some reason decided to take a chance on a passionate but inexperienced undergrad, and Lauren Reynolds, who basically did the same with a slightly-less-inexperienced undergrad. Thank you both for taking me under your wing, your guidance showed me what it meant to be a true scientist.

Saving the most vital piece for last, I would like to take a moment to thank the wonderful mentor whose leap of faith led me to where I am today. Dr. Cecilia Flores, you accepted me as a Masters student, perhaps against your better judgement given your knowledge that I was an aspiring physician, a decision that would impact the rest of my life in unimaginable ways. Under your guidance, I learned more than I can even begin to describe and I am eternally grateful for your mentorship, and even more importantly, your friendship. I'm glad my persistence managed to wear you down, eventually.

Author Contributions

Christina Popescu oversaw the project and treated all animals in all experiments contained within this thesis using the 4 mg/kg amphetamine regimen, unless otherwise stated.

Molecular: Michel Giroux and Giovanni Hernandez sliced the flash frozen brains and ran the molecular analyses (namely, qPCR and Western-blot). Both assisted with preliminary data analysis. Further analysis was performed by and graphs were created by Christina Popescu.

Stereological: Dominique Nouel perfused all animals used in stereology experiments, assisted with IHC for some experiments, and provided the stereological counts for the male and female amphetamine experiments in early and mid-adolescence. Christina Popescu performed all the slicing with the vibratome.

Behaviour: Taylor Orsini ran the Go/No-Go task under Christina Popescu's guidance and supervision. Christina Popescu ran the Progressive Ratio and Contingency Degradation task. Dominique Nouel and Christina Popescu collaborated for the CPP experiment. All data analyses and graphs were done by Christina Popescu.

Gonadectomy: Dominique Nouel and Michel Giroux assisted with sham gonadectomy surgeries, supervised and trained by Christina Popescu who conducted all the ovariectomy and orchiectomy surgeries. Dominique Nouel, Giovanni Hernandez, Michel Giroux, and Christina Popescu sacrificed all the animals and flash froze their brains for qPCR analysis. Christina Popescu and Michel Giroux extracted blood plasma for ELISA analysis.

Contribution to Original Knowledge

The work presented in this thesis lays the groundwork for investigating how the exposure to drugs of abuse during various windows of adolescent development impacts the mesocorticolimbic dopamine pathway in a sexually dimorphic manner. It illustrates that sexual dimorphisms not only *exist* at the molecular, neuroanatomical, and behavioural levels, but are in fact very pronounced. The window of vulnerability to non-contingent recreational-like doses of amphetamine seem to differ between the sexes whereby males are vulnerable early in adolescence and protected later, while females are protected early and somewhat vulnerable later – but seem to exhibit a compensatory mechanism which serves to protect the circuit from ectopic axon growth and behavioural impairments. A pilot study is conducted wherein activational sex hormones are investigated as a potential source of the observed sex differences by gonadectomizing early adolescent mice prior to sexual maturation, the first such study to my knowledge.

Introduction and Statement of Problem

Adolescence is a volatile developmental period in life, both psychologically, and biologically, in the body and brain. As such, adolescence is also a time of great vulnerability; in fact, many psychiatric disorders have an adolescent onset, including substance use disorder (Goldstein & Volkow, 2011; Rice et al., 2019; Tan, Callicott, & Weinberger, 2009). As the transitional time between juvenile helplessness/dependence and adult independence, adolescence is remarkably multifaceted with a plethora of developmental changes occurring simultaneously. Puberty often comes to mind as the chief among these changes, yet it is but a piece of a much larger puzzle – the degree to which it serves as a fountainhead, however, remains to be determined. Adolescence is also marked by vast changes in the social domain. From peer pressure to romantic relationships, social aspects are intensified during this period. As many films illustrate and many individuals can attest, the stakes feel extremely high and fitting in essentially becomes the ultimate goal, often resulting in the crazy actions and stories we later wish to forget. Critically for this work, adolescents exhibit a greater propensity for risky behavior – including drug use (Hair, Park, Ling, & Moore, 2009; Steinberg, 2007; Windle, 1994), with amphetamine as one of the top choices alongside marijuana and alcohol (National Institute of Health, 2019).

All in all, adolescent experiences have the capacity to alter and shape the maturation of the brain. For the purposes of this thesis, the focus is specifically on the mesocorticolimbic dopamine pathway. This pathway has largely been studied for its role in pleasure and reward; even colloquially, dopamine is known to be a molecular modulator of "feeling good". Its neurons stem from a midbrain area, the ventral tegmental area (VTA), and terminate in the limbic region, the nucleus accumbens (NAcc), or in the cortex, including the medial prefrontal cortex (mPFC). Work from our lab has shown that segregation between mesolimbic and mesocortical dopamine subservient cells results from axon targeting events occurring in subcortical structures. These events are mediated by the guidance cue, Netrin-1, and its receptor, DCC (deleted in colorectal cancer). Succinctly, there are three main aspects of

this pathway that make it particularly fascinating for my investigation: firstly, it is still undergoing substantial growth and maturation during adolescent development (Reynolds et al., 2018); secondly, it is very vulnerable to environmental influences such as drug use (Cuesta et al., 2018; Reynolds et al., 2015); and thirdly, it appears to be differentially vulnerable between the biological sexes during adolescent development. Moreover, DCC receptors have been shown to be powerfully regulated by the microRNA, miR-218, and both *Dcc* mRNA and miR-218 serve as targets for adolescent exposure to the stimulant amphetamine (Cuesta et al., 2018). Remarkably, increasing evidence from post-mortem and GWAS studies and, soon, computational gene network studies from our lab, find that transcripts for this guidance cue pathway are altered in psychiatric disorders involving mPFC dysfunction that have adolescent onset – particularly major depressive disorder (MDD) (Lee et al., 2019; Li et al., 2020; Ward et al., 2017).

Literature in the field of developmental neuroscience and previous work in the lab has solidified the fundamentality of sex differences both in normal development and in response to environmental influences – particularly adolescent drug use. Despite its ubiquity, adolescent drug use is a predictor of addiction vulnerability, propensity, and severity in later life (Anthony & Petronis, 1995; B. Grant et al., 2003; McCabe, West, Morales, Cranford, & Boyd, 2007; Robins & Przybeck, 1985). Furthermore, the sexes differ with respect to both the type of drug they prefer as well as the amount of time elapsed from first use to compulsive use (Becker & Chartoff, 2019; Becker & Hu, 2008; Hammerslag & Gulley, 2016; McHugh, Votaw, Sugarman, & Greenfield, 2018). Therefore, there is a great need to understand this sexually dimorphic vulnerability from a mechanistic, biological perspective so as to eventually translate this knowledge into new avenues of more targeted, potentially more efficacious patient treatments. The work presented in this thesis endeavours to assess whether and how this experience-dependent maturation in the mesocorticolimbic dopamine pathway differs between the biological sexes and how it is affected by drugs of abuse, as there is a large gap of knowledge about this issue in the field of molecular psychiatry.

Background Information and Rationale

The overarching goal of this project is to understand whether and how adolescent experiences, particularly drug use, affect the developing brain in a sexually dimorphic manner. The following work focuses on amphetamine, a psychostimulant commonly used both clinically and illicitly, at different doses, among adolescents (Degenhardt, Coffey, Moran, Carlin, & Patton, 2007). In low doses, it is utilized clinically to treat conditions such as ADHD, narcolepsy, and obesity (Caye, Swanson, Coghill, & Rohde, 2019; Fleckenstein, Volz, Riddle, Gibb, & Hanson, 2007; Srivastava, O'Hara, & Browne, 2019); in high doses, the psychostimulant is used recreationally for its rewarding effects as well as the enhancement of physical and cognitive capacity – often in the context of sports or academics (Veliz, Boyd, & McCabe, 2013). One of the hallmarks of adolescence, the continued development of the prefrontal cortex – particularly the dopamine circuitry, happens to be part of the mechanism of action for many drugs of abuse. Like many drugs of abuse, amphetamine increases extracellular dopamine levels in the striatum, acting as a competitive inhibitor of dopamine and norepinephrine transporters and increasing cytoplasmic dopamine levels by (1) releasing dopamine-filled vesicles, and (2) promoting the reverse transportation of dopamine-transporter-bound dopamine back into the synaptic cleft (Calipari & Ferris, 2013; Fleckenstein et al., 2007). The excess extracellular dopamine levels results in the rewarding effects of psychostimulants (Calipari & Ferris, 2013).

Amphetamine and the DCC/miR-218 pathway

Previous work done in the lab shows that amphetamine (d-amphetamine sulfate) exposure during early adolescence (post natal day, PND, 22-30$\pm$1) disrupts the dopamine innervation and connectivity in the medial prefrontal cortex (mPFC) in male mice (Reynolds et al., 2015). This effect resulted from repeated 4 mg/kg doses of amphetamine administered non-contingently during early adolescence, once every 2 days in a 9-day period for a total

of 5 injections. Controls animals were treated on the same schedule with the vehicle, 0.9% saline. The aforementioned dose results in peak blood plasma levels similar to those observed with recreational use in humans (Cuesta et al., 2019) and will henceforth be referred to as a recreational-like dose. Non-contingent administration of amphetamine in early adolescence, but not in mid-adolescence or adulthood, results in circuitry abnormalities as well as impairment in a behavioural inhibition task (Reynolds et al., 2015; Reynolds et al., 2019). Interestingly, none of these effects are observed in male mice exposed to the same regimen but using a low dose of amphetamine which results in blood plasma levels similar to those observed in therapeutic settings (Cuesta et al., 2019).

DCC's role in axon guidance in the mesocorticolimbic dopamine pathway

The gene, *Dcc*, encodes a guidance cue receptor that acts as a major regulator of mesocorticolimbic dopamine development during adolescence – the only such gene currently identified to my knowledge. As dopaminergic axons extend from the VTA where their cell bodies lie, they segregate into the mesocortical and mesolimbic pathways via axon targeting events occurring in the NAcc. Mesolimbic dopamine axons recognize their target in the NAcc, while mesocortical dopamine axons continue to grow to the mPFC during adolescence (Reynolds et al., 2018). The Netrin-1 receptor, DCC, plays a vital role in these axon-targeting events. Using a dual-viral technique in DAT Cre male mice, Reynolds et al. (2018) specifically infected dopamine neurons stemming from the VTA and visualized the axons that grew from the NAcc to the mPFC during adolescence (i.e. between PND 21 and adulthood). Moreover, these axonal "decisions" to either remain in the NAcc or continue to grow to the mPFC are DCC-dependent, whereby axons with high levels of DCC recognize the NAcc, an area with a low concentration of the guidance cue Netrin-1, as their final target while neurons expressing low levels of DCC extend past the NAcc and instead recognize the mPFC as their final target, where Netrin-1 expression is greater (Manitt et al., 2011; Reynolds et al., 2018). Ma-

nipulating DCC results in shifted proportions of axons segregating into the aforementioned dopamine pathways, namely mesolimbic and mesocortical. When mesolimbic neurons, those highly expressing the Netrin-1 receptor, are virally manipulated to express less *Dcc* in a circuit-specific haploinsufficient model, they fail to recognize the NAcc as their final target and continue to grow ectopically into the mPFC (Reynolds et al., 2018).

Molecular mechanisms of amphetamine

DCC receptors are highly expressed in both humans and rodents by VTA dopamine across the lifespan (Manitt et al., 2010). The recreational-like dose of 4 mg/kg during early adolescence in male mice downregulates DCC protein and mRNA expression in the VTA, a robust result replicated on numerous occasions (Cuesta et al., 2018; Yetnikoff, Almey, Arvanitogiannis, & Flores, 2011; Yetnikoff, Eng, Benning, & Flores, 2010; Yetnikoff, Labelle-Dumais, & Flores, 2007). The same dose also alters DCC's microRNA regulator, miR-218, by increasing its expression (Cuesta et al., 2018). Of course, given that microRNAs are 22 nucleotide sequences that serve to regulate protein expression post-transcriptionally by attaching to mRNA and effectively inhibiting translation, these findings illustrate a molecular mechanism that is very sensitive to the presence of amphetamine during the vulnerable window of early adolescent development in male mice.

Stereological and behavioural investigations following amphetamine

How do the molecular disruptions (i.e. DCC downregulation and miR-218 upregulation following early adolescent exposure to amphetamine of male mice) affect the neuroanatomy and function of the mPFC? Stereological investigation revealed that amphetamine exposure during early adolescence increased TH-positive fiber innervation across the three subregions of the mPFC: cingulate (Cg1), prelimbic (PrL), and infralimbic (IL) (Reynolds et al., 2015). Stereology is a powerful technique for investigating neuroanatomy closely in an unbiased manner. By counting random areas of a previously-outlined region of interest and extrap-

olating from them to the entire mPFC subregions using serial sections, one can estimate minute aspects of interest in 3-dimentional shapes such as a brain structures; in our case, this involves estimating the volume of cortex innervated by dopaminergic axons, their respective presynaptic sites referred to as varicosities, and the density of varicosities. Interestingly, despite the increased span of dopamine innervation, a reduction in presynaptic sites was observed (Reynolds et al., 2015). In order to visualize this phenomenon using immunohistochemistry, TH (tyrosine hydroxylase) staining is commonly utilized as a marker for dopaminergic cells in the brain due to its enzymatic role in the rate-limiting step of catecholamine synthesis: the conversion of L-tyrosine to L-DOPA, dopamine's precursor (eg. Figure 1 in M. Johnson, Salvatore, Maiolo, and Bobrovskaya (2018)). Behaviourally, the alterations observed in the mPFC after early adolescent exposure to amphetamine results in behavioural inhibition deficits in an mPFC-dependent Go/No-Go task in adulthood (Reynolds et al., 2019). In stark contrast, the same amphetamine regimen provided either in mid-adolescence or adulthood does not differentiate the treatment and control groups in the same Go/No-Go task (Reynolds et al., 2019). These data suggest a critical window of development in which amphetamine acts to disrupt dopamine pathway development and result in long-term mPFC aberrations and dysfunction, in male mice. Importantly, these effects are DCC-dependent.

Interestingly, however, the *way* in which amphetamine affects the DCC/miR-218 pathway is different from simply *downregulating* DCC using viral-mediated strategies. Demonstrated by an elegant series of experiments involving a targeted *Dcc* haploinsufficiency model by Reynolds et al. (2018), downregulating *Dcc* results in an abnormal proportional segregation of mesolimbic and mesocortical DA axons. A greater number of axons grow ectopically to the mPFC than during normal development and alter the structure of layer V pyramidal neurons once there. This is illustrated by an increased volume of eYFP-labelled axons in the mPFC and a decreased number of DA presynaptic sites (varicosities). In this case, however, altering dopamine innervation of the mPFC actually results in better performance in the same Go/No-Go task, as exemplified by decreased commission errors or incorrect responses.

Rationale: what about females?

As usual, females are complicated. Of course, not necessarily because they are, in fact, complicated, but rather because their investigation often stems from the male-as-default-style thought process. Nevertheless, lacking essentially any knowledge specific to females yet having a wealth of mechanistic understanding in males, Reynolds et al. commenced studying female mice under the same conditions (unpublished). Stemming from the null hypothesis that the same amphetamine regimen at the same age would result in similar effects in female mice, qPCR analyses were performed in the VTA one week following early adolescent treatment, as before. These analyses revealed no change between treatment and control groups for neither *Dcc* nor its microRNA regulator, miR-218, in stark contrast to males who were treated with amphetamine in early adolescence. However, the same dose administered just 2 weeks later (namely, during mid-adolescence commencing at PND 35±1) resulted in the same findings as males at PND 21: namely, a significant downregulation of *Dcc* and upregulation of miR-218. Interestingly, females exhibit no differences in behavioural inhibition following early adolescent amphetamine exposure, in stark contrast to the behavioural inhibition impairments observed in males. Despite no differences in task performance between the treatment groups, female mice do seem to require more time in the test phase of a modified Go/No-Go task in order to show significant improvement in task performance (14 days versus 10 days).

For me and for this project, this is where the story begins. Thus far, it seems as though males are vulnerable to the detrimental effects of recreational-like doses of amphetamine during early adolescence while females are protected during that time; however, the tables seem to turn during mid-adolescence whereby male mice are no longer vulnerable, but female mice are – at least at the molecular level. Whether there is a critical window of time for this vulnerability remains to be elucidated as does the mechanism underlying this increased vulnerability during adolescence. To this end, male and female mice treated with non-contingent recreational-like doses of amphetamine are closely analyzed in a three-prong

approach: molecular (via qPCR of mRNA and microRNA, Western blot of Netrin-1 protein), neuroanatomical (via an unbiased stereological approach), and behavioural (via an mPFC-dependent Go/No-Go task).

In summary, this project investigates the molecular, neuroanatomical, and behavioural aspects of how the biological sexes differ in their response to adolescent exposure to non-contingent recreational-like doses of amphetamine. Moreover, it aims to begin elucidating the timing of this vulnerability and the role of puberty and activational sex hormones underlying these phenomena.

Aims

Overarching hypothesis:

The molecular, neuroanatomical, and behavioural effects of non-contingent recreational-like doses of amphetamine during adolescence differ between the biological sexes with females being 'protected' in early adolescence and males being 'protected' in mid-adolescence. The differences in the timing of sensitivity to amphetamine may be a consequence of the difference in the time of onset of puberty and activational sex hormones.

Aims:

1. Determine if the non-contingent administration of amphetamine induces preference in a conditioned place preference (CPP) paradigm in early (PND 21) and mid-adolescence (PND 35) for males and females, respectively.

2. Compare the expression of VTA *Dcc* mRNA, miR-218, and NAcc Netrin-1 protein expression in both males and females following recreational-like doses of amphetamine (1) in early adolescence and (2) in mid-adolescence using quantitative real-time PCR (qPCR) and Western blot.

3. Amphetamine-dependent *Dcc* mRNA downregulation is hypothesized to result in the same aberrant dopamine axon targeting in females as in males. Regardless of the timing of amphetamine's effect, a downregulation of *Dcc* mRNA results in mesolimbic dopamine axons not recognizing the NAcc as their final target and instead ectopically growing into the mPFC. In females, this is hypothesized to occur with amphetamine exposure during mid-adolescence (starting the regimen at PND 35) based on a previous pilot study. With this in mind, the following aims emerge:

   (a) Determine the effects of amphetamine exposure during early and mid-adolescence in males and females stereologically using immunohistochemistry (IHC) to label TH+ axons.

(b) Determine if amphetamine exposure in mid-adolescence resulted in any behavioural impairments/changes for female mice treated in mid-adolescence in the following assays: Go/No-Go task, progressive ratio, and contingency degradation.

4. Determine, in wildtype C57BL/6J mice typically used for our experiments, whether male or female mice undergo puberty first. For the current inquiry, naïve animals were assessed for external signs of puberty: qualified as preputial separation in males and vaginal opening in females.

5. Circulating gonadal hormones may provide a protective effect for female mice during early adolescence (PND 21), but not in later adolescence (PND 35) while having the opposite effect in males. This phenomenon may be altered by gonadectomizing animals at PND 21 prior to sex hormone activation. Male and female mice underwent gonadectomy or sham surgery at PND 21±1 and received amphetamine or saline treatment in either early adolescence (PND 22-30±1) or mid-adolescence (PND 35-43±1). They were analyzed molecularly for VTA *Dcc* mRNA expression one week post treatment.

Methods

Mice were housed in the Neurophenotyping centre at the Douglas Mental Health University Institute on a 12-hour light-dark cycle (light on at 08:00) and had *ad libitum* access to food and water unless otherwise stated. C57BL/6J mice obtained from Charles River Laboratories (Saint-Constant, QC, Canada) were used for all experiments. Mice were segregated in cages of maximum 4 animals by sex. All amphetamine treatments followed the same schedule: 1 injection every 2 days for 9 days for a total of 5 intraperitoneal (i.p.) injections per mouse. The dose utilized was 4 mg/kg of d-amphetamine sulfate salt (Sigma-Aldrich, Dorset, United Kingdom) diluted in 0.9% saline injected at a volume of 0.1 mL/g of bodyweight, resulting in predicted peak plasma levels of 1300±79 ng/mL 5 minutes post-injection (Cuesta et al., 2019). All statistical analyses were performed using Prism Version 8. Reported values are represented as means ± SEM and the significance threshold follows the typical $\alpha = 0.05$. 2- and 3-way ANOVAs were utilized when conditions were met, some planned comparisons were done post hoc as mentioned, and other post hoc multiple comparison tests were used when relevant with a Sidak correction to maintain a familywise $\alpha = 0.05$. All Students' t-tests were two-tailed in this thesis unless otherwise stated.

In order to better differentiate the timing nuances of adolescence, the adolescent period is subdivided into three stages: "early" (PND 21-34), "mid" (PND 33-44) and "late" (PND 45-54) (Hoops & Flores, 2017) with a great focus on mid-adolescence for this project.

Aim 1: Using a conditioned place preference (CPP) paradigm to establish that a 4 mg/kg amphetamine regimen induces preference

To establish that the recreational-like dose used in all prior and following experiments has the potential to induce preference, we utilized a conditioned place preference (CPP) paradigm. Unlike most CPP assays wherein each mouse receives the saline and drug injections on the same day for a certain period of time, the goal here was to demonstrate that the current

treatment regimen of one dose every second day could induce preference at the ages of amphetamine-induced *Dcc* mRNA downregulation – PND 21 for males and PND 35 for females. To that end, mice were exposed to a box consisting of 2 chambers (one striped, one polka-dotted) and neutral area connecting the chambers. On day 1, mice were allowed to freely explore these chambers for 30 min wherein the amount of time spent in each chamber was measured to determine a preference percentage between the chambers for each individual animal. A biased approach was used to decide the drug- vs saline-paired chambers for each mouse so that the less preferred chamber would be paired with the dose of amphetamine, for the animals receiving the drug, or be the first saline dose chamber, for the control animals. A total of 16 animals of each sex were tested – 8 for each condition, drug and saline. Following the pretest day, animals were exclusively exposed to either the drug-paired chamber or the saline chamber post-injection for 30 minutes each day for 9 days, alternating the drug and amphetamine injections each day for the experimental group. After the last day of injections, mice were once again allowed to freely explore the full enclosure for 20 minutes while the time spent in each was measured. A delta preference score was then calculated for each mouse by subtracting the time spent in the originally unpreferred chamber during pretest from the time spent in that same chamber during post-test, see formula below:

$$\Delta\ Place\ Preference = time\ POST - time\ PRE$$

Aim 2: Post-amphetamine molecular analysis in early- and mid-adolescence for both sexes

RNA extraction and quantitative real-time PCR: Mice were sacrificed by decapitation one week post-treatment or at certain developmental timepoints (PND 15, 21, 35, 70) with the brains removed and flash frozen in 2-methylbutane on dry ice. qPCR for *Dcc* mRNA and miR-218 and Western-blot for Netrin-1 is performed as previously (Cuesta et al., 2018; Torres-Berrío et al., 2017). Briefly, brains were sliced in 1-mm slices using a cryostat and

VTA, NAcc, and mPFC punches were taken from the resulting sections. Thereafter, the mRNAeasy Micro Kit (Qiagen) was used to extract VTA total RNA and microRNA and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for the reverse transcription of *Dcc* mRNA. Real-time PCR was performed using the TaqMan assay kit (Applied Biosystems) on a 7900HT RT PCR system (Applied Biosystems). Gapdh was utilized as a reference gene to control for experimental variability. For miR-218, the TaqMan MicroRNA Reverse Transcription Kit alongside the corresponding miRNA TaqMan probes (Applied Biosystems, Foster City, CA) was used for reverse transcription and the expression levels were calculated using the AQ standard curve method. The small nucleolar RNA (snoRNA) RNU6B was used as an endogenous control to normalize miR-218 expression.

Whole brains from PND21 and 35 male and female C57BL/6J mice were flash frozen in 2-methylbutane. Bilateral punches from the nucleus accumbens were processed for western blot as before (Alanna Grant et al., 2007; Manitt et al., 2010). Briefly, protein samples (20 μg) were separated on a 10% SDS-PAGE and transferred to a PVDF membrane which was incubated overnight at 4°C with antibodies against Netrin-1 (1:1000, Abcam Inc, Toronto, ON, Canada) and α-Tubulin (1:20000, Cell Signaling, Danvers, MA, USA) for loading control. Protein bands were detected by chemiluminescence (Bio-Rad, Mississauga, ON, Canada) and analysed using Image Lab system software (Bio-Rad, Mississauga, ON, Canada).

In order to accurately compare between the sexes, mice of both sexes were treated and their tissue processed simultaneously so as normalize the samples to the same standard – in our case, a saline-treated male and female. Each individual animal of the group is then normalized using that same factor. 2-way ANOVAs for detecting the main effects of sex and treatment as well as the interaction between sex and treatment. Independent t-tests with Sidak correction are planned to compare saline and amphetamine groups for both male and female mice.

Stereological analysis (neuroanatomy)

Perfusion: Following an overdose intraperitoneal injection of ketamine 50 mg/kg, xylazine 5 mg/kg, and acepromazine 1 mg/kg, mice were perfused intracardially with 50 mL of cooled 0.9% saline followed by 75 mL of chilled 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Brains were extracted and placed in 4% PFA overnight at 4°C before being transferred into PBS and returned to 4°C. Using a Leica vibratome, brains were sliced in coronal sections at a thickness of 35 μm within a maximum of 7 days.

Immunhistochemistry (IHC): Coronal sectionals were processed in a 1:4 series as previously described (Reynolds et al., 2015). In brief, mPFC sections were bathed for 48 hours in a rabbit polyclonal anti-TH primary antibody (Millipore AB152) diluted in blocking solution (2% bovine serum albumin, 0.2% Tween in PBS) 1:1000. The sections were then washed in PBS and conjugated in Alexa Fluor 594 secondary antibody raised in goat (Invitrogen) diluted 1:500 in blocking solution and incubated for one hour before mounting on gel-coated slides with FluoGold mounting medium. Slides were coverslipped, sealed, and left to dry overnight before being placed at 4°C.

Stereological analysis: Stereological quantification of TH-positive fibers in the cingulate 1 (Cg1), prelimbic (PrL), and infralimbic (IL) subregions of the pregenual mPFC was performed as described previously (Manitt et al., 2013; Manitt et al., 2011; Reynolds et al., 2015; Reynolds et al., 2018). The total volume of TH-positive fiber innervation (in cubic micrometers) was assessed using the Cavalieri method using Stereoinvestigator® (MicroBrightField).

12 male and 12 female mice were analyzed blindly following amphetamine or saline treatment (6 animals per treatment) during early adolescence and 12 male and 12 female mice were analyzed blindly following amphetamine or saline treatment during mid-adolescence.

Aim 3: Effects of mid-adolescent amphetamine exposure in female mice on neuroanatomy and behaviour in adulthood

Go/No-Go

One week prior to initiating the behavioural experiment at PND 75 ± 1, mice are food restricted with roughly 1.5 g/day in order to reach and maintain a body weight 85% of their baseline adult weight ad libitum. This weight is maintained throughout the duration of the operant experiments with the animals being weighed daily. The protocol used, developed by Lauren Reynolds (as published in Reynolds et al. (2019)), involves several phases of training in the operant conditioning task before a final test phase is achieved. The training phases involve progressive timing alterations, but essentially work to teach mice to nosepoke upon the activation of a light cue. Each correct nosepoke is rewarded with a chocolate-flavoured dustless pellet (BioServ, Inc., Flemington, NJ). Following the training period, female mice undergo 14 days of the test phase of the task (males underwent 10 days, their performance improved faster despite no differences between the sexes in the early stages of the task). The test phase consists of Go trials and No-Go trials, i.e. a light "go" cue and a light and sound "no-go" cue, respectively. When presented with the 3-second light stimulus alone, mice are required to nosepoke for a reward; conversely, when presented with the light stimulus in tandem with an 80-dB sound stimulus for 3 seconds, mice are required to inhibit their learned nosepoking response in order to receive a reward. Nosepoking in the 3-second no-go trial, a premature response labelled as a "commission error", results in a dark inter-trial time interval and no reward. Sessions last 30 minutes and consist of 30-50 go trials and 30-50 no-go trials that are randomized in a 1:1 ratio. 20 mice were used for this experiment, 10 were randomly assigned to amphetamine treatment and 10 to saline, 1 mouse from the saline group was found dead early during the test phase of the task and was therefore removed from the analysis. Data gathered was analyzed in terms of proportion of commission errors and efficiency (Cuesta et al., 2019) plotted as the mean for each experimental group each day over the entirety of the task.

Previous analyses involved pooling the last couple of days of the task for each treatment group and comparing those means using a Student's t-test, statistically substantiated the aforementioned results; however, this approach only takes a small subset of the analysis into consideration. We have since begun using a sigmoidal analysis technique that essentially assesses 3 aspects of a sigmoidal curve which better utilize the entire dataset: the upper asymptote, the lower asymptote, and the M50. The first refers to the value on the y-axis to which the sigmoid tends at the upper end, the upper limit; in this case, this refers to the proportion of commission errors at the start of the test phase of the task. The second, the lower asymptote, refers to the value on the y-axis to which the sigmoid tends at the lower end or the lower limit, in this case signifying the proportion of commission errors at the end of the test phase of the task. The lattermost aspect, the M50, essentially refers to the value at the midpoint between the 2 asymptotes; within the context of this analysis, the day of the task at which the mice are halfway between their initial and final proportion of commission errors. Related aspects have previously been identified as parameters that can be captured for individual animals in a behavioural task: "In quantifying the appearance of conditioned behavior in individual subjects, we want to know at least three things: (i) how long it took for it to appear [which would be reflected by our M50 measure]; (ii) how abruptly it attained its asymptotic level [not something that is necessarily relevant for us since we standardize the length of time in the test phase of the task]; and (iii) what the asymptotic level was [in our case, both asymptotes provide meaningful information about task performance]" (Gallistel, Fairhurst, & Balsam, 2004). This approach also allows for the easy comparison between the sexes despite that females continue the test phase for a few days longer than males. Each of these measures is calculated for all subjects whose data fits a sigmoidal curve using Origin Version 8. Animals whose data did not fit a sigmoidal curve were excluded from the analysis, resulting in 1-2 animals per experiment. Student's t-tests are then utilized to compare these values between experimental groups and 2-way ANOVAs are utilized to assess sex differences with treatment at each age, early or mid-adolescence.

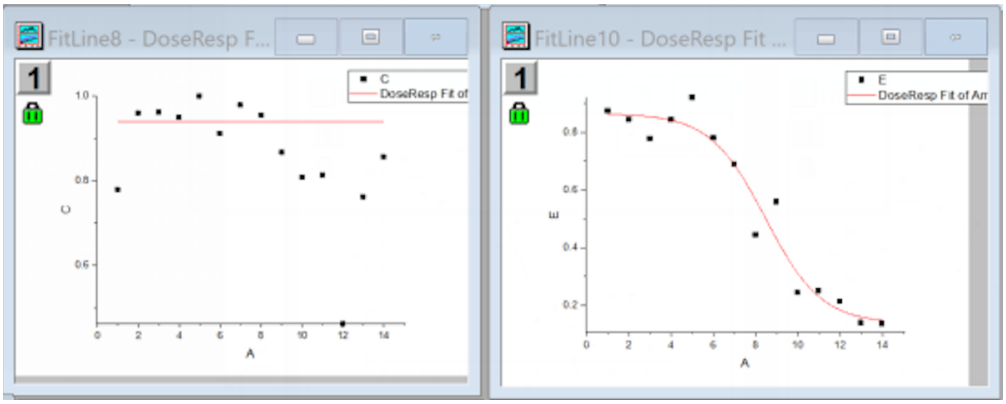

Figure 1: Example AMPH animals: Left failed to fit sigmoid, subject removed from analysis as outlier; right serves as an example of a sigmoidal fit and therefore an animal used in the analysis described above.

Progressive Ratio

A commonly utilized operant paradigm, the overarching aim of a progressive ratio is to measure the strength of a given reward. This is often operationalized as the breakpoint – the number of trained operant behavioural responses with which a subject obtains their final reward – and serves as a proxy for the strength of a given reward, in this case a chocolate pellet. Following a protocol previously used by Lauren Reynolds, animals are trained with several fixed ratio sessions (FR1, FR3, and FR5) over separate days. During the final session of the task, the number of responses required for a reward increases by a power of 2 with each successive reward. The breakpoint of each animal is recorded and a Student's t-test is conducted between the groups treated with amphetamine or saline during mid-adolescence to see if the reward is valued differently.

Contingency Degradation

This task serves as an operationalization of habits versus goal-directed behavior, dissociating between the two – particularly due to the fact that habits can be either advantageous or maladaptive depending on the circumstances. The task involves briefly retraining the animals to respond equally to the two nosepoke holes in the same operant boxes in which they had completed the Go/No-Go task, represented below as the acquisition phase. At this time, both nosepoke holes are illuminated continuously for the duration of the session

and rewards are provided at an FR1 ratio, 1 chocolate pellet per nosepoke. Sessions are 20 minutes in length wherein a max of 30 pellets per nosepoke hole are dispensed. Once performance has stabilized for 5 days (at ≥ 50 rewards, ideally maxing out rewards), animals enter the non-degraded session whereby one nosepoke hole is covered while the other is available and reinforced on a VR2 schedule. From this data, the rate of reward is calculated for each animal and used in the following degraded session. This time, the opposite nosepoke hole is available with the contingent one occluded but the reward is provided non-contingently at the same rate the non-degraded session. On the test day of the task, both nosepoke holes are available, but no rewards are given in the 10-min session. The number of nosepokes in each nosepoke hole is recorded and a difference score is calculated by dividing the degraded by the non-degraded nosepoke hole. These difference scores are then compared between the groups using a Student's t-test. Again, this task follows the protocol used previously by Lauren Reynolds, see below for explanatory diagram borrowed from (Zimmermann, Hsu, & Gourley, 2016).

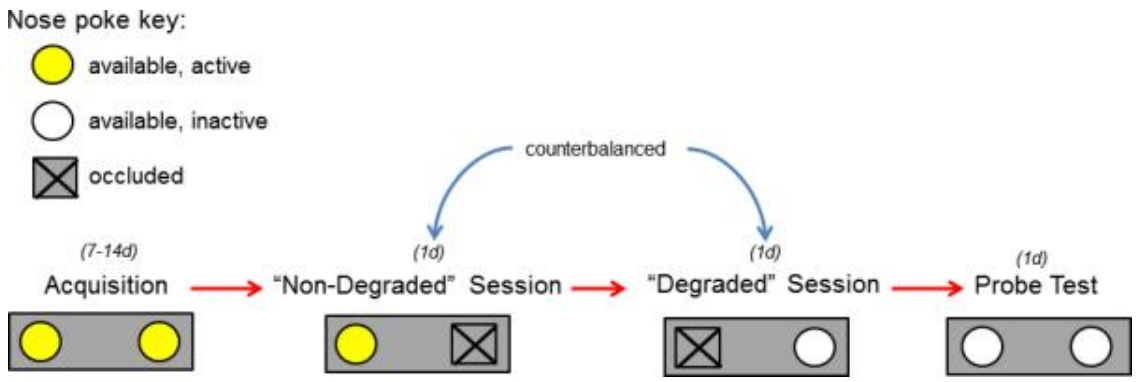

Figure 2: Diagramatic explanation of the contingency degradation task.

Aim 4: Determine puberty timelines for male and female C57BL/6J mice from external signs of puberty

Puberty has long been assessed in rodents as preputial separation in males – when the foreskin can separate fully from the glans penis – and vaginal opening in females – when the vagina is no longer closed and the mouse enters the estrous cycle.[1] Given that there is some disagreement in the field about the timing and order or puberty in mice (Bell, 2018; Walker et al., 2017), I set out to gather a baseline for our own mouse colony before beginning to use external signs of puberty as an outcome measure. To this end, I followed 5 male and 9 female mice at the same time daily from PND 21 to PND 42 (by this time, all the animals had reliably reached puberty). Using Hoffmann (2018) as a visual reference, I developed a scale for the progression of puberty in both males and females and used this codified scale to plot the external evolution of puberty. Ranging from 0-3 for each sex, it follows mice from no preputial separation/vaginal opening whatsoever to full separation/opening. No statistical analyses were planned or carried out; this work was merely to provide a baseline for future work.

Aim 5: Investigate the role of activational sex hormones in sexually dimorphic windows of vulnerability to amphetamine

To explore the effects of activational sex hormones during early and mid-adolescence on the molecular mechanisms affected by amphetamine in a sexually dimorphic manner, I gonadectomized early adolescent male and female mice before any amphetamine treatment. This pilot study involved 4 separate experiments, each consisting of 20 mice. All 80 mice under-

[1]Despite that these measures have been used ubiquitously in the past, it is rather subjective and less reliable for female mice. Moreover, it was found that vaginal opening in mice may occur up to 10 days before the first vaginal cornification and the onset of estrus cycle, but is still highly correlated with cornification (Nelson, Felicio, Randall, Sims, & Finch, 1982; Nelson, Karelus, Felicio, & Johnson, 1990). A newer approach presented in Gaytan et al. (2017) would provide a more precise dating of female puberty and would have been pursued if time had permitted. This approach is simply an extension of the other typical approach of vaginal lavage and staining to determine the stage of the cycle under the microscope.

went either an ovariectomy/orchiectomy or sham surgery at PND 21±1; 2 groups, one with 10 males and one with 10 females underwent amphetamine or saline treatment from PND 22-30±1 while the other 2 groups underwent the same amphetamine or saline treatment between PND 35-43±1. Therefore, there were 4-5 mice per group for each treatment type at each age for each sex. Unfortunately, 2 female mice who had undergone ovariectomy surgery died in the days following the surgery, one in the early adolescent experiment and one in the mid-adolescent experiment. One week post treatment, all animals were sacrificed to collect brain and blood samples, for molecular and hormone analysis, respectively. Mice were sacrificed by decapitation, plasma from trunk blood was collected after centrifugation for 10 minutes at 1000xg and brains were extracted and flash frozen in 2-methylbutane on dry ice. Molecular analyses involve the typical *Dcc* mRNA and miR-218 as before and hormone analyses focus on the primary sex hormones: estradiol, progesterone, and testosterone using an ELISA kit (MilliporeSigma, Burlington, MA, USA). Unfortunately, due to the shutdown of labs during the SARS-CoV-2 pandemic, hormone analyses were not completed as planned.

Results

Aim 1: Using a conditioned place preference (CPP) paradigm to establish that a 4 mg/kg amphetamine regimen induces preference

Previous work has robustly shown that amphetamine during early adolescence for males and mid-adolescence for females downregulates *Dcc* mRNA. What was unknown was whether or not the non-contingent administration of amphetamine as performed in those experiments would induce preference. Using a modified biased Conditioned Place Preference (CPP) paradigm, we demonstrated that the recreational-like dose of amphetamine, 4 mg/kg, induces significant preference at the age of interest for each sex (Figure 3).

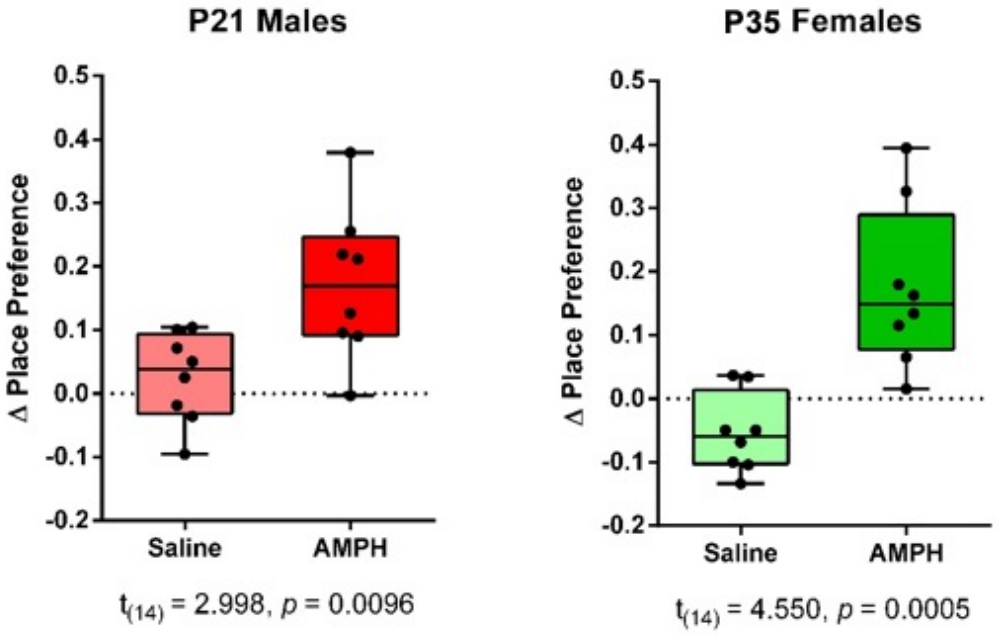

Figure 3: Conditioned place preference (CPP) conducted in early adolescence for males and mid-adolescence for females. Data represented as box plots of the Δ preference scores for each mouse, n = 8 per group.

Aim 2: Post-amphetamine molecular analysis in early- and mid-adolescence for both sexes

Previous work in the lab, like much of the scientific literature in basic science, has focused almost exclusively on male mice. More recently, the same experiments have begun being conducted in female mice, separately; however, in order to address more specific sex differences and make well-founded claims related to those differences, these molecular experiments must be conducted and analyzed in male and female mice simultaneously. This aim endeavours to do just that by analyzing fold changes in Netrin-1 protein, *Dcc* mRNA, and miR-218 one week following amphetamine treatment during either early or mid-adolescence in wild-type male and female mice. Corroborating previous published findings from Cuesta et al. (2018) and unpublished findings from Reynolds et al., *Dcc* in the VTA and Netrin-1 in the NAcc were downregulated following early adolescent exposure to amphetamine in male mice (planned comparisons: $t_{25} = 2.782, p = 0.0101$ and $t_{20} = 3.590, p = 0.0018$, respectively) while miR-218 in the VTA was upregulated ($t_{25} = 3.014, p = 0.0058$), illustrated in Figure 4. This was not the case for female mice treated at the same age: there were no significant differences between the treatment groups for *Dcc*, miR-218, or Netrin-1 (Figure 4). No main effects were observed with either *Dcc* or miR-218, but both revealed significant interactions between sex and treatment ($F_{1,25} = 5.452, p = 0.0279$ for *Dcc* and $F_{1,25} = 9.049, p = 0.0059$ for miR-218). Regression and correlational analyses show a significant relationship between miR-218 and *Dcc* in early adolescent males ($r = -0.594, R^2 = 0.353, p = 0.03$), but not females ($r = -0.003, R^2 = 0.0, p = 0.99$). However, following amphetamine exposure in mid-adolescence (from PND 35-43$\pm$1), the reverse was apparent (Figure 5): there were no significant differences for male mice in any of the above measures, but female mice showed a downregulation of *Dcc* ($t_{32} = 2.072, p = 0.0465$) and an upregulation of miR-218 ($t_{29} = 2.389, p = 0.0471$). At this age, 2-way ANOVAs of *Dcc* and miR-218 fold changes both revealed a main effect of sex ($F_{1,32} = 8.832, p = 0.0056$ and $F_{1,28} = 6.692, p = 0.0152$ for *Dcc* and miR-218, respectively) and a significant interaction between sex and treat-

ment ($F_{1,32} = 4.956, p = 0.0332$ and $F_{1,28} = 10.50, p = 0.0031$, respectively). The reverse relationship was also the case with respect to miR-218 and *Dcc*: mid-adoelscent females exhibited a significant relationship ($r = -0.63, R^2 = 0.38, p = 0.03$), but not males ($r = -0.4, R^2 = 0.16, p = 0.11$).

As for DCC's ligand, Netrin-1, Western blots following amphetamine treatment revealed that the guidance cue is differentially affected by the psychostimulant in each sex *and* at different times. In male mice, it is significantly downregulated in the NAcc after early adolescent treatment, as mentioned previously. In female mice, however, the effect is vastly different: there is no change with respect to saline controls in early adolescence (at PND 21, Figure 4), but a significant upregulation is observed following amphetamine exposure in mid-adolescence (at PND 35, Figure 5) ($t_{20} = 2.838, p = 0.0102$). Whereby previous findings have shown a downregulation of Netrin-1 in the NAcc following recreational-like doses of amphetamine in early adolescent male mice, alongside the downregulation of *Dcc*, the opposite is actually the case following mid-adolescent non-contingent amphetamine exposure in female mice – even though the downregulation in *Dcc* intuitively led to the hypothesis that a downregulation in Netrin-1 may also be expected.

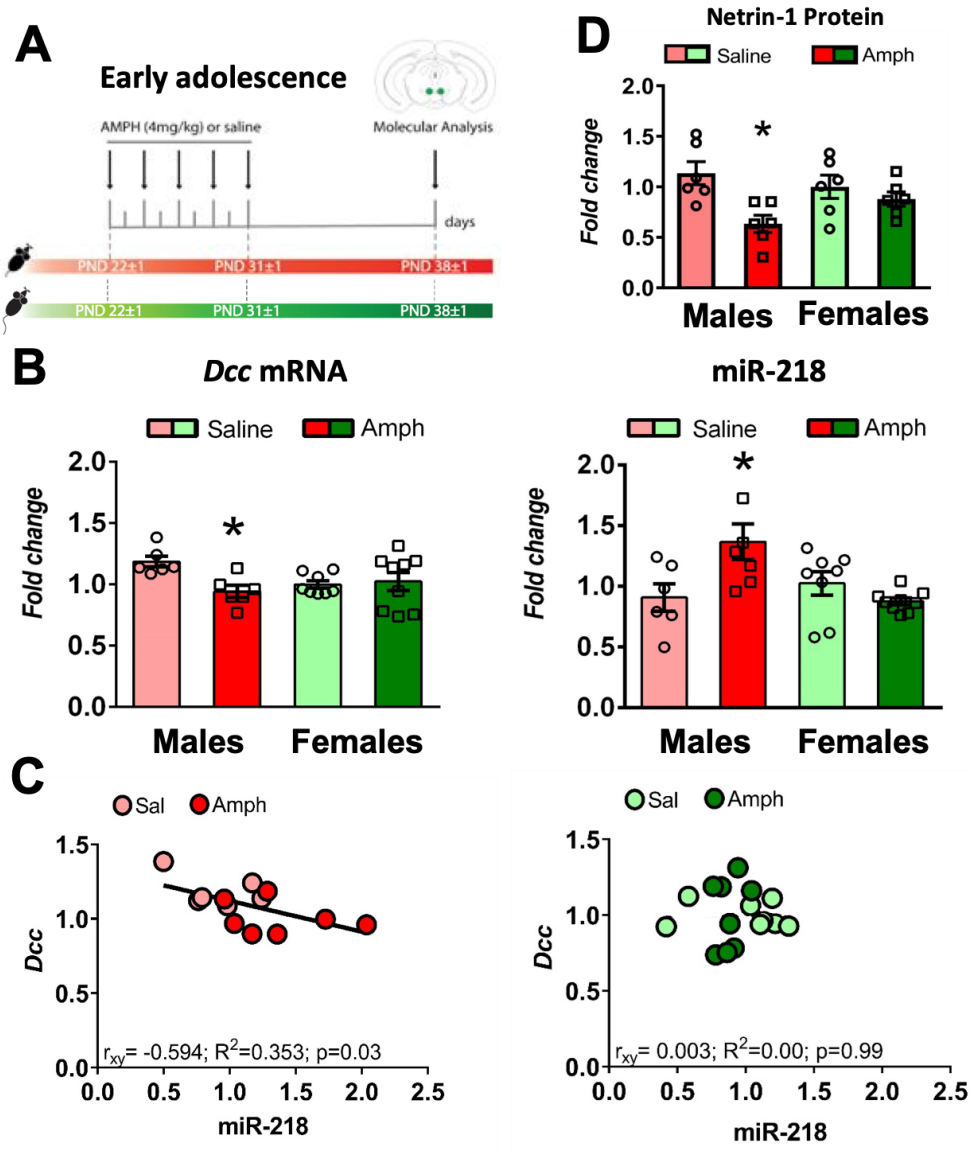

Figure 4: Molecular results represented in fold change (mean ± SEM) following early adolescent amphetamine exposure in male and female mice. (a) Schematic diagram describing experimental design. (b) Bar graph depicting fold change for VTA *Dcc* mRNA and miR-218 in both sexes. (c) Regression lines and values for *Dcc* and miR-218 for males and females. (d) Bar graph depicting NAcc Netrin-1 protein fold change in both sexes. N = 6-8 animals per group. Asterisks denote $p < 0.05$ on post hoc planned comparisons between saline and amphetamine-treated groups.

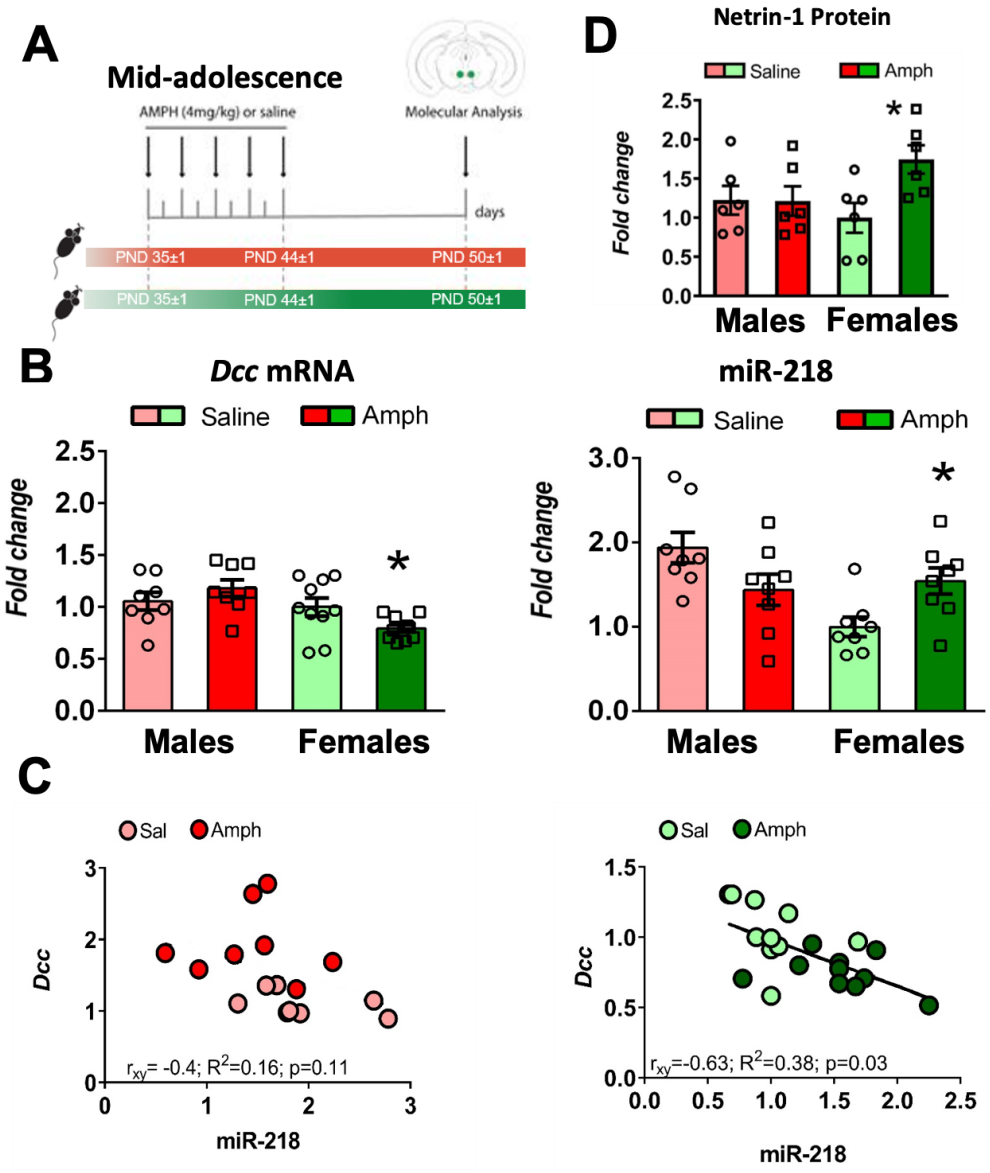

Figure 5: Molecular results represented in fold change (mean ± SEM) following mid-adolescent amphetamine exposure in male and female mice. (a) Schematic diagram describing experimental design. (b) Bar graph depicting fold change for VTA *Dcc* mRNA and miR-218 in both sexes. (c) Regression lines and values for *Dcc* and miR-218 for males and females. (d) Bar graph depicting NAcc Netrin-1 protein fold change in both sexes. N = 8-10 animals per group. Asterisks denote $p < 0.05$ on post hoc planned comparisons between saline and amphetamine-treated groups.

Aim 3a: Determine the effects of amphetamine exposure during early and mid-adolescence stereologically using TH+ axons.

Following the experimental progression of previous work, 3 subregions of the mPFC were investigated stereologically as a means of probing physical perturbations to mesocortical dopamine terminals following the recreational-like doses of amphetamine in early and mid-adolescence in both male and female mice.

For the experiment in early adolescence, I will present some prior published data for males (Reynolds et al., 2015) and unpublished pilot data in females (Reynolds, Popescu, et al.) as we could not finish the stereological counts for all animals before the submission of this thesis due to the closures resulting from the SARS-CoV-2 pandemic. For this reason, the graphs for each sex are separated and no analyses were completed to statistically test for sex differences, male data is presented merely for visual comparison. Figure 6 explores the volume of DA innervation, as operationalized by TH+ fibres, in 6(b); the number of varicosities, an operationalization of the number of synapses with the local circuitry, in 6(c); and the density of varicosities per μm^3 volume in 6(d). As presented in 6(b), a 2-way repeated measures ANOVA revealed a main effect of treatment ($F_{1,6} = 9.965, p = 0.0196$) and subregion ($F_{2,12} = 156.00, p < 0.0001$), indicating that TH+ innervation may occupy a smaller volume in amphetamine-treated animals although this effect seems to be driven by the cingulate and prelimbic subregions but not the infralimbic. This is in contrast to males whose effect seems to be driven mostly from the more ventral subregions – particularly the infralimbic. With respect to the number of varicosities, only subregion remained significant as a main effect ($F_{2,12} = 32.83, p < 0.0001$). Again, in contrast with males who exhibit a pronounced decreased number of varicosities following amphetamine treatment – specifically in the prelimbic and infralimbic subregions. Therefore for female mice, when dividing the number of varicosities by the volume of innervation, it is not surprising that no effects or interactions were observed, as presented in 6(d). In stark contrast, males exhibit a decreased density of TH+ fibres across all subregions.

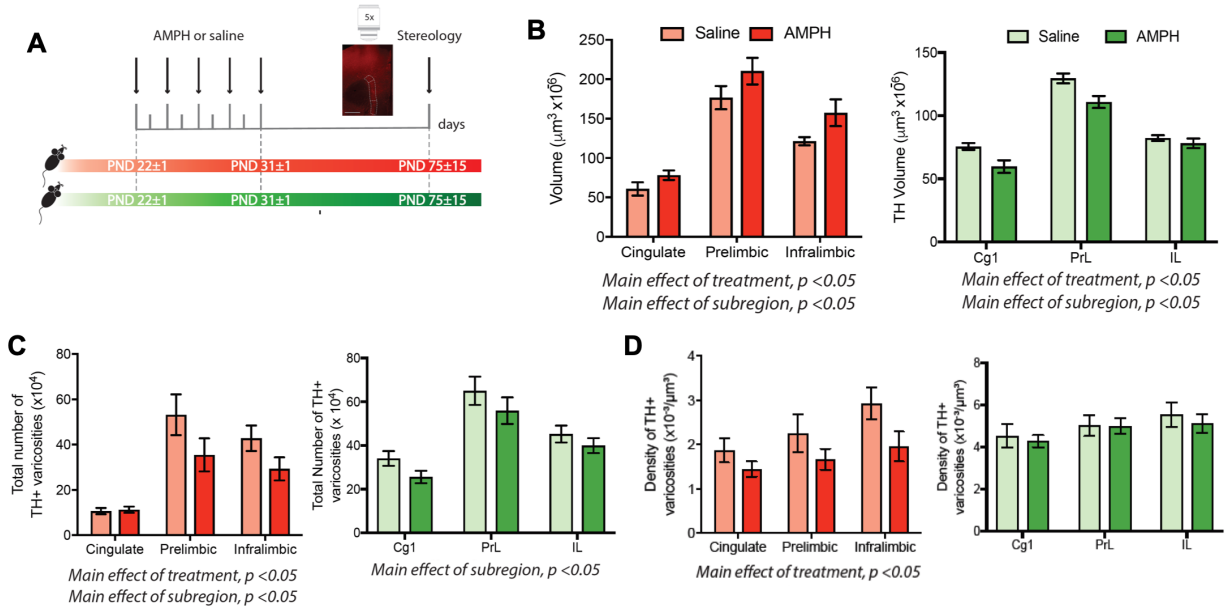

Figure 6: Bar graphs of stereological results for male and female mice treated with amphetamine in early adolescence (mean ± SEM). (a) Schematic diagram describing experimental design. (b) Volume of TH+ innervation per subregion for each sex. (c) Number of varicosities per subregion for each sex. (d) Density of varicosities per subregion for each sex. N = 4-7 animals per group.

As for mid-adolescence, see Figure 7, a main effect of subregion is, of course, apparent in all 3 measures: volume of TH+ innervation, TH+ varicosities, and the density of those varicosities. There was no significant main effect of sex in the volume of dopamine innervation ($F_{1,48} = 2.390, p = 0.1287$), but there was an effect of treatment, $F_{1,48} = 5.1, p = 0.0285$. With respect to the number of dopamine varicosities, however, the reverse was true whereby there was no significant effect of treatment ($F_{1,48} = 1.220, p = 0.2749$), yet there was an effect of sex ($F_{1,48} = 11.31, p = 0.0015$). Finally, combining these two measures into the density of dopaminergic fibre varicosities in the mPFC, a significant main effect of sex emerged whereby female mice treated in mid-adolescence generally exhibited less density than their male counterparts treated at the same age. Interestingly, as with the varicosities, treatment was not significant as a main effect. No interactions were significant in any of the 3-way ANOVA analyses.

Analyzing each subregion by way of 2-way ANOVA, only the cingulate (Cg1) and in-

fralimbic (IL) subregions reveal statistically significant results. The former, the cingulate, revealed significant sex effects in both the number of and density of varicosities ($F_{1,16}$ = 5.885, p = 0.0275 and $F_{1,16}$ = 11.00, p = 0.0044, respectively) as well as a difference in volume between the treatment groups ($F_{1,16}$ = 6.778, p = 0.0192), whereby the latter, the infralimbic, revealed a main effect of sex solely in the number of varicosities ($F_{1,16}$ = 4.944, p = 0.0409) and no significant effect of amphetamine treatment.

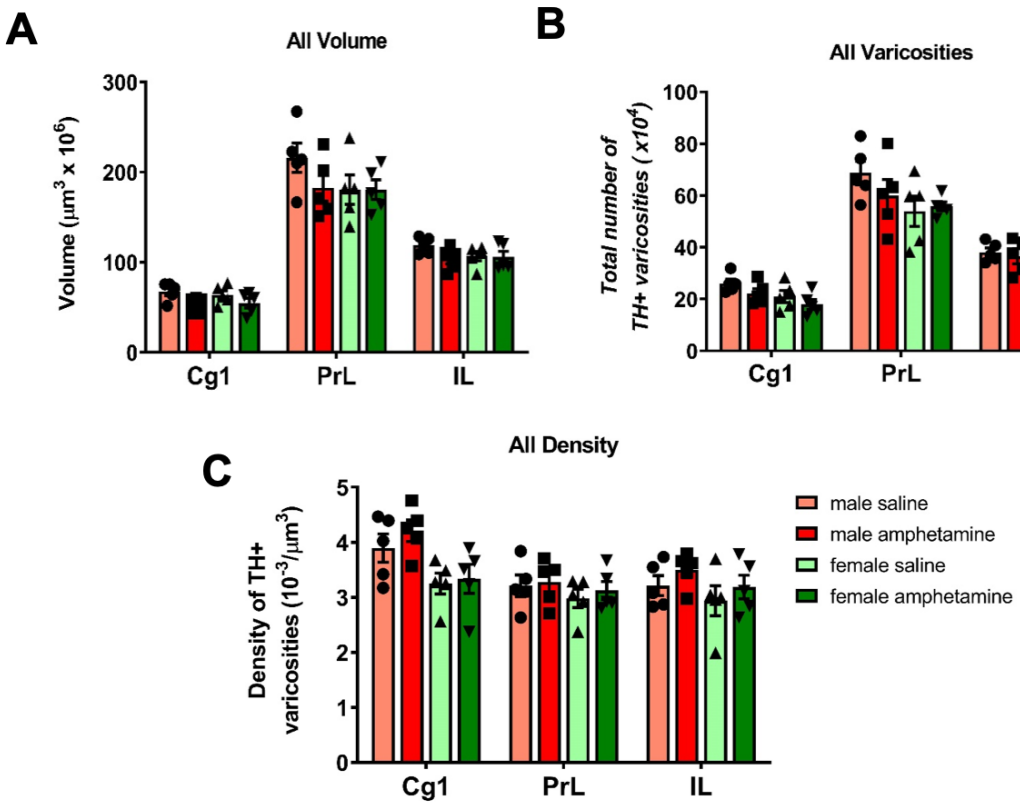

Figure 7: Bar graphs of stereological results for male and female mice simultaneously treated with amphetamine in mid-adolescence (mean ± SEM). (a) Volume of TH+ innervation per subregion for both sexes. (b) Number of varicosities per subregion for both sexes. (c) Density of varicosities per subregion for both sexes. N = 5 animals per group.

Aim 3b: Determine the effects of amphetamine exposure during mid-adolescence on behavioural tasks

Figure 8 explores the summary of Go/No-Go task data proportion commission errors for both sexes following amphetamine exposure at both adolescent timepoints. Male data for both ages was previously published in Reynolds (2019), but are included and re-analyzed here for comparison purposes. Adult males treated with the recreational-like doses of amphetamine during early adolescence show markedly poorer performance in behavioural inhibition compared to their saline counterparts (Student's t-test of lower asymptotes, $t_{13} = 3.309, p = 0.0056$), but this is not the case for males treated in mid-adolescence ($t_9 = 0.1331, p = 0.897$). The female story is a little different: following early adolescent treatment, adult female mice have a significantly smaller proportion commission error lower asymptote ($t_{10} = 4.136, p = 0.002$); interestingly, adult females treated with amphetamine during mid-adolescence show a similar trend whereby amphetamine treated animals exhibit a smaller lower asymptote than saline-treated animals (Welch's t-test due to unequal variances, $t = 1.915, p = 0.05$). A 2-way ANOVA on the lower asymptote data for all animals treated in early adolescence (PND 21-30$\pm$1) revealed both a main effect of sex ($F_{1,23} = 9.333, p = 0.0056$) and an interaction between sex and treatment ($F_{1,23} = 20.02, p = 0.0002$), represented in Figure 8(c). Further post hoc testing for each sex revealed a significant difference for males ($t_{23} = 4.097, p = 0.0009$), but not quite significant difference for females ($t_{23} = 2.295, p = 0.0615$). The same ANOVA could not be run for animals treated in mid-adolescence due to the significant difference in variance; however, the data was analyzed using the nonparametric Kruskal-Wallis test with no significant differences between the 4 groups ($p = 0.2530$). Using Dunn's MCT for each sex did not reveal any significant differences between the treatment groups.

Moreover, there are absolutely no significant differences between the sexes or treatment groups with respect to the upper asymptote for any of the Go/No-Go experiments (P21 males, P35 males, P21 females, P35 females), suggesting all mice start the test phase of the task with roughly the same performance regarding proportion commission errors. The final

aspect of the analysis approach, the M50, allows for the quantification of the time it took to reach the halfway point of performance improvement, operationalized as the midpoint of the reduction in the proportion of commission errors. A 2-way ANOVA revealed that mice treated in early adolescence did not differ in M50, irrespective of both sex and treatment, while mice treated in mid-adolescence exhibit a main effect of sex ($F_{1,22} = 9.062, p = 0.0064$). Taken together with the graphical data, this result suggests that female mice treated in mid-adolescence take longer to improve task performance than male counterparts treated during the same developmental time period, despite no apparent sex differences following amphetamine treatment in early adolescence.

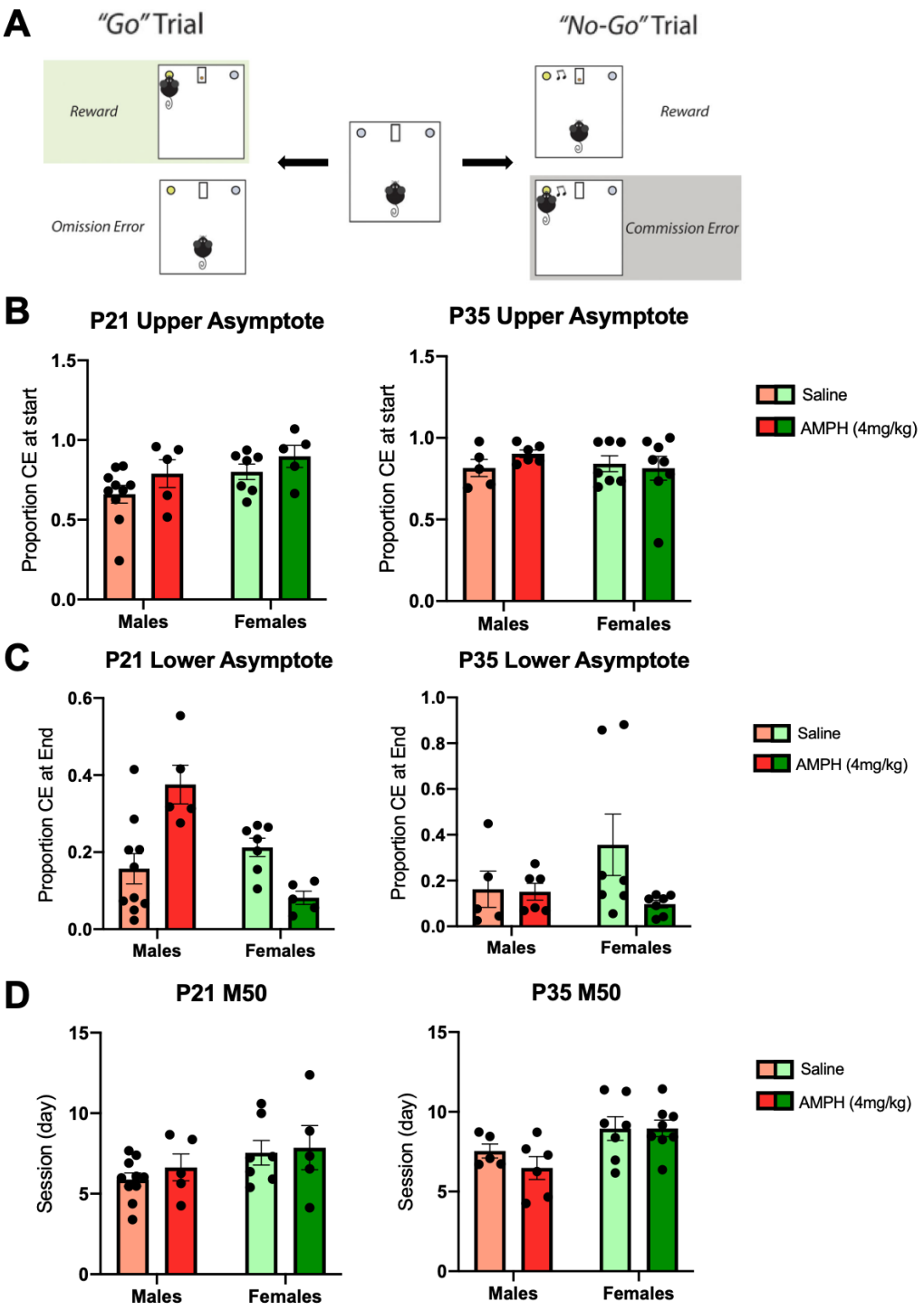

Figure 8: Bar graphs of Go/No-Go performance results for mice treated with amphetamine in early and mid-adolescence (mean ± SEM), P21 and P35, respectively. (a) Schematic diagram describing experimental design of the Go/No-Go task. (b) Comparison of upper asymptotes, the start points of proportional commission errors, for mice of both sexes at each age. (c) Comparison of lower asymptotes, the end points of proportional commission errors, for mice of both sexes at each age. (d) Comparison of M50 measure, the day representing the midpoint of task performance improvement, for mice of both sexes at each age. N = 5-10 animals per group.

Progressive Ratio

To test the strength of the motivation for chocolate pellet reinforcers for mice who had previously undergone the Go/No-Go task, I assessed their breakpoint for nosepoking for a reward, as depicted in Figure 9(a). There was absolutely no difference between the treatment groups using a two-tailed Students' t-test ($t_{17} = 0.8827, p = 0.387$).

Contingency Degradation

Any trained response to a task can simply become a habit, something that happens rather absent-mindedly without directly being a goal-directed behaviour. To dissociate between the more automatized habits versus more intentional goal-directed behavior, I exposed the mice to a contingency degradation task. As per Zimmermann, Hsu, & Gourley (2016), a difference score was created by dividing the response rate (in responses/minute) of the non-degraded nosepoke hole by the degraded nosepoke hole; in other words, a measure that combines both the goal-oriented nosepoke response rate and the habit nosepoke response rate by providing a ratio of the two. A low score (< 1) indicates a preference for the degraded nosepoke, or the habit behaviour, while a high score (>1) indicates a preference for the non-degraded nosepoke, or the goal-oriented behaviour. As depicted in Figure 9(b), amphetamine-treated female mice prefer the non-degraded nosepoke and therefore may exhibit more goal-oriented behaviour than their saline counterparts ($t_{16} = 2.551, p = 0.0213$).

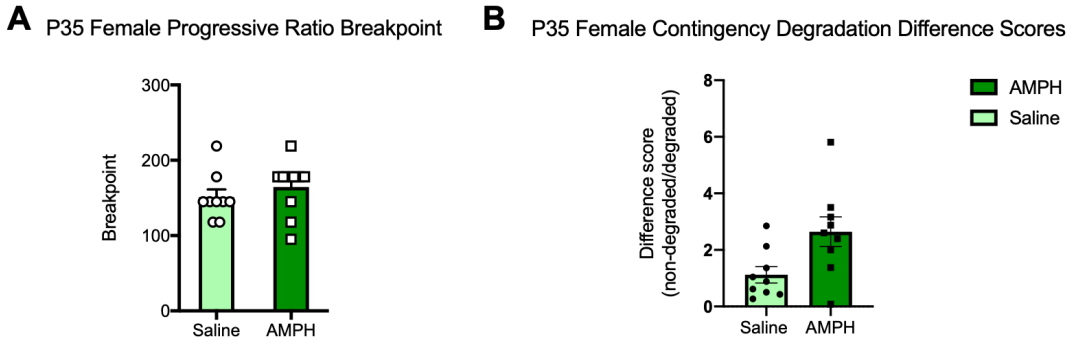

Figure 9: Additional behavioural tests completed with female mice treated with amphetamine in mid-adolescence (mean ± SEM). (a) Breakpoint of nosepokes per chocolate pellet reward in a progressive ratio task. (b) Difference scores of mice in the test day of the contingency degradation paradigm. N = 9 animals per group.

Aim 4: Determine puberty timelines for male and female C57BL/6J mice from external signs of puberty

The goal of this investigation was simply to assess the external signs of puberty in a lab cohort of mice so as to ascertain the pubertal timeframe specifically in our strain of experimental mice. No statistical tests were planned or performed on this data, but what can be revealed by the data is that, unlike in humans, our cohort of C57BL6/J male mice seem to undergo puberty before their female counterparts, roughly 2-3 days before. Moreover, in males, puberty begins around PND 28-30 and occurs fully around PND 39 whereas in females, puberty begins around PND 30-36 and occurs fully around PND 39 (Figure 10). There are two aspects of this observational pilot study that are worthy of a little extra attention: (1) puberty, like most aspects of development, is a process – not simply a Boolean value whereby the shift from pre- to post-puberty happens instantaneously, and (2) both sexes seem to reach adult external signs around the same developmental time – approximately PND 39. So while the focus is often on the first day of preputial separation or vaginal opening, these results indicate that the sexes are more similar than they may initially seem.

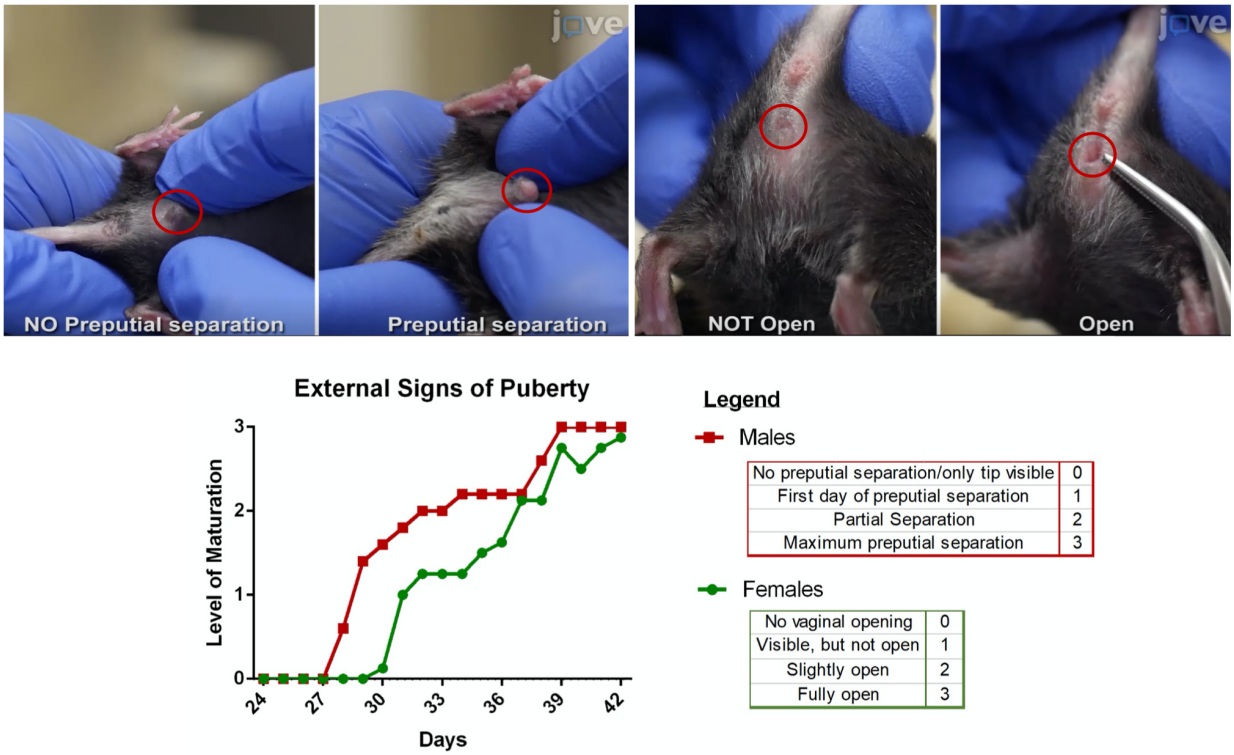

Figure 10: Observational study assessing external signs of puberty in naïve male and female mice. Pictures provide a visual representation for the assessment of sexual maturation – preputial separation in males and vaginal opening in females. Below is a graphical representation of the progression of sexual maturation based on the developmental scale outlined in the legend. N = 5 males and 9 females.

Aim 5: Investigate the role of activational sex hormones in sexually dimorphic windows of vulnerability to amphetamine

To the best of my knowledge, this is the first study of its kind investigating the effects of pubertal hormones on the axon targeting mechanisms of neurons in the mesocorticolimbic dopamine circuit. The interest was particularly with our battery of molecular markers, starting with *Dcc* mRNA in the VTA. Figure 11 shows the fold change of VTA *Dcc* mRNA for animals gonadectomized (or who underwent a sham gonadectomy) in early adolescence and treated in either early or mid-adolescence with recreational-like doses of amphetamine or saline. There were no significant effects of gonadectomy, sex, treatment, or any interactions thereof on *Dcc* levels at either age of interest using a 3-way ANOVA or any multiple comparison tests (MCTs).

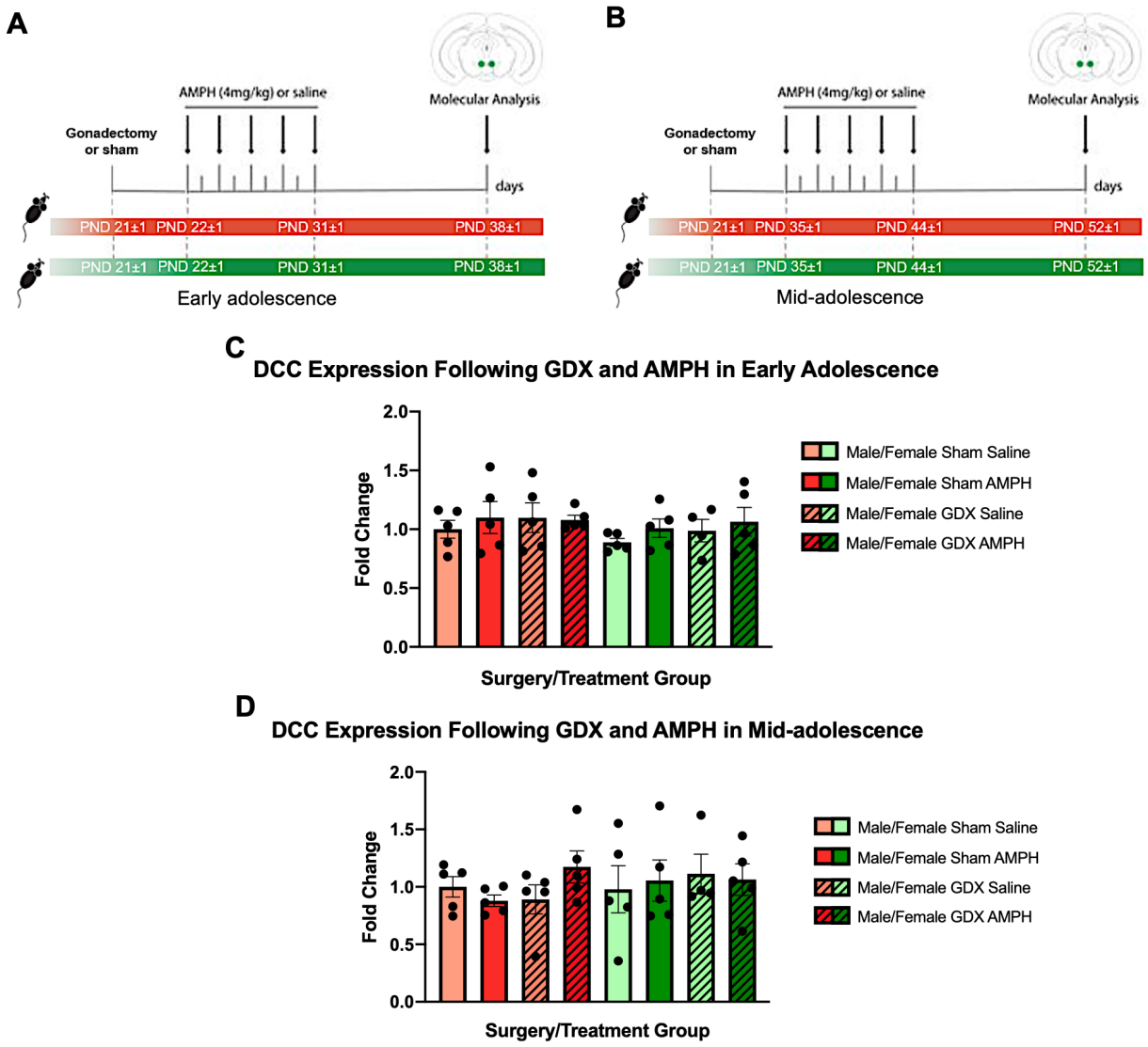

Figure 11: VTA *Dcc* expression (mean ± SEM) following gonadectomy (GDX) or sham and amphetamine treatment for male and female mice. (a) Schematic describing the experimental design for animals treated with amphetamine in early adolescence. (b) Schematic describing the experimental design for animals treated with amphetamine in mid-adolescence. (c) Bar graph depicting *Dcc* expression following early adolescent exposure to amphetamine and GDX or sham. (d) Bar graph depicting *Dcc* expression following mid-adolescent exposure to amphetamine and GDX or sham. N = 4-5 animals per group.

Discussion

Adolescent experimentation with drugs – both legal and illicit – is a common occurrence in North America. As such, it is critical to understand the potential long-term effects of this behaviour: focusing on the mechanisms behind sex differences as well as individual vulnerability and resilience so as to best inform our approach to public policy on the subject. The three-pronged approach inherent in this project, and the larger body of work on which it builds, yields a multifaceted picture of how the various regions of the mesocorticolimbic dopamine system are affected by exposure to recreational-like doses of amphetamine at different times during the turbulent adolescent period. Moreover, the molecular, stereological, and behavioural methods each probe various levels of the circuit under investigation and therefore uniquely contribute to a more nuanced understanding of: (1) how drugs of abuse during adolescence affect the developing brain and (2) how those effects and vulnerabilities differ between the biological sexes. In other words, the molecular prong elucidates key dysregulations following the non-contingent administration of amphetamine and provides a theoretical basis for discerning alterations present in mPFC local circuitry, which are appraised stereologically, which, in turn, serve as a basis for various aspects of cognitive control – mainly operationalized as behavioural inhibition for the purposes of this thesis.

Molecular

Let us follow that progression of thought, beginning with the molecular experiments. Previously published work largely investigated the effects of amphetamine on the molecular mechanisms of the mesocorticolimbic dopamine pathway, specifically involving the guidance cue, Netrin-1, its receptor, DCC, and DCC's microRNA regulator, miR-218, exclusively in male mice. Work done with recreational-like doses of amphetamine, the same dose used in this thesis, resulted in a downregulation of *Dcc* mRNA and Netrin-1 protein as well as an upregulation of miR-218 – but only with amphetamine exposure during adolescence, not

adulthood (Cuesta et al., 2018). Interestingly, these effects were not observed with a low dose, a 0.5 mg/kg dose which induces blood plasma levels similar to those of doses used in therapeutic settings. On the contrary, the effects were trending in the opposite direction: despite there being no change in *Dcc* mRNA, there was an increase in DCC protein without any significant alterations in miR-218 or Netrin-1 (Cuesta et al., 2019). It is therefore the case that the dose of amphetamine during adolescence is a critical variable modulating the long-term developmental effects of exposure.

These fascinating results led me to ponder the effects of amphetamine on the mesocorticolimbic dopamine pathway in female mice and, given my specific interest in understanding developmental effects of recreational-like doses, I endeavoured to specifically explore these effects in female mice. Remarkably, there are very striking sex differences. As depicted in Figures 4 and 5, *Dcc* mRNA is remarkably unaltered following early adolescent exposure to amphetamine, but is downregulated following mid-adolescent exposure. This result is in stark contrast to males where the results are reversed: early exposure results in downregulation while mid-adolescent exposure does not alter *Dcc* levels in the VTA. Similarly, miR-218 is only upregulated by amphetamine in mid-adolescent females but not early adolescent females, in contrast with males who follow the opposite pattern: upregulation in early, but not mid-adolescence. As for the guidance cue itself, Netrin-1, previous male data has illustrated an amphetamine-dependent downregulation of the protein in the NAcc – the region where DA axons are making targeting decisions – again, only during early adolescence. Given this information in tandem with that of *Dcc* and miR-218 presented above would intuitively lead to the following hypothesis: Netrin-1 is also likely to be downregulated in the NAcc of mid-adolescent females treated with amphetamine, thereby painting the picture that amphetamine's effects are similar between the sexes, just delayed in females. Reality, however, paints a far more fascinating picture: not only does Netrin-1 expression not decrease, the reverse occurs: Netrin-1 is upregulated in mid-adolescence while remaining unaltered in early adolescence in female mice. Putting this in the context of axon-targeting

events occurring in the NAcc during the window of adolescent vulnerability, female mice are not only protected from the detrimental molecular effects of amphetamine during early adolescence, but potentially during mid-adolescence as well. Under this presumption, an unknown compensatory mechanism results in an increase in the guidance cue in order to theoretically provide the necessary conditions for typical axon targeting and pathway segregation between the mesocortical and mesolimbic pathways in the presence of recreational-like doses of amphetamine.

Stereology

Transitioning to the stereological approach, there has been a great deal of prior work in assessing the dopaminergic innervation of the PFC following various manipulations. For the purposes of this work, however, I will focus on that which is most relevant: namely, that dopaminergic fibres continue to grow from the striatum to the mPFC during adolescence and that the number of fibre varicosities is altered in the presence of amphetamine. The first is a crucial component of the theoretical backbone for the current work, as described in (Reynolds et al., 2018), whereby a dual-viral approach is used to label dopamine axons stemming from the VTA while simultaneously causing *Dcc* haploinsufficiency specifically in the infected neurons. This approach resulted in two vital morsels of information: (1) dopamine axons that were located in the NAcc during early adolescence (PND 21) are then found in the mPFC in adulthood, and (2) that DCC plays a critical role in the axon targeting events occurring in the NAcc during this critical window of development. When DCC was artificially downregulated in these neurons using viral-mediated induced haploinsufficiency, many axons no longer recognized the NAcc as their target and instead continued growing ectopically into the mPFC, thereby essentially rerouting typically mesolimbic subservient cells to now become part of the mesocortical pathway (Reynolds et al., 2018).

Amphetamine during early adolescence, with the recreational-like dose, similarly reroutes axons into the mPFC by virtue of the downregulation of *Dcc*. The increase in mesocortical

innervation following drug exposure leads to an increase in the volume of TH+ fibre innervation in the mPFC, but a decrease in local synapses (varicosities) and, therefore, an overall decrease in DA fibre density (Reynolds et al., 2015). Preliminary work done in female mice treated in early adolescence revealed that amphetamine resulted in a slight, but significant decrease in both the volume occupied by the TH+ axons as well as the number of varicosities located on those axons (Reynolds, Popescu, et al., unpublished). Taken together, however, there was no overall change in DA fibre density in any of the 3 mPFC subregions. A larger and more complete version of this experiment, including 5-6 animals per group for both sexes, was conducted but not yet finalized by the time this thesis was written due to the unforeseen SARS-CoV-2 pandemic. Nevertheless, the sex differences observed thus far – although not directly comparable – serve as a basis for marked behavioural differences in adulthood that will be discussed in the following section.

Following the patterns revealed in the molecular experiments, stereological observations paint a rather different picture with drug exposure occurring a mere two weeks later, namely during mid-adolescence. Looking in the cingulate, prelimbic, and infralimbic regions of the mPFC in adult male and female mice following concurrent mid-adolescent amphetamine treatment, direct comparisons can be made between the sexes. There is a slight difference between the treatment groups, detected as a main effect of treatment, in the volume of TH+ innervation, likely driven by the differences found in the prelimbic region (see Figure 7). Otherwise there are no apparent differences between the treatments or the sexes. So, despite changes in *Dcc* mRNA and miR-218 in mid-adolescent females, there are no changes in DA innervation to the mPFC, likely by virtue of the increased levels of Netrin-1 preventing the ectopic growth of mesolimbic subservient axons into the mPFC. It would therefore be expected that females treated with amphetamine in mid-adolescence would not differ from their saline counterparts in behavioural tasks such as the Go/No-Go – which is, indeed, what the results suggest.

Behaviour

Finally, the behavioural approach chiefly examines the function of the mPFC in adult mice following adolescent amphetamine treatment. This is primarily assessed by a modified version of the Go/No-Go task which probes the capacity for behavioural inhibition by requiring animals to inhibit a learned response when the original conditioned cue is paired with an additional unconditioned auditory cue. From previous experience with the task in male and female mice treated with amphetamine in early adolescence, we know that female mice tend to take longer to improve their performance in the task. With this in mind, the female experiment conducted for this thesis was also extended to 14 days instead of the 10 that were typically used for males (including the experiment involving males who were treated with amphetamine in mid-adolescence). However, despite our firsthand experiences, an analysis of the M50, a measure of the day in which an individual mouse's performance has improved to halfway between the start (upper asymptote) and end (lower asymptote) of proportion commission errors (proportion CE), revealed no significant main effect of sex for animals treated in early adolescence. For animals treated in mid-adolescence, however, a main effect of sex was observed whereby females took approximately two days longer to improve task performance on average. Importantly, there are no sex or treatment differences at the start of the test phase of the task between animals treated with amphetamine during either early or mid-adolescence. All animals have a similar performance across all experiments thereby providing a rather equal baseline measure for all groups and adding greater meaning to other differences that are found.

For example, in prior experiments with males and females treated in early adolescence (Reynolds et al., 2019; Reynolds, Popescu, et al., unpublished), amphetamine-treated males have a much higher number of proportion commission errors than their saline-treated counterparts, indicating poor task mastery, while females, if anything, show the opposite trend leading to a strong main effect of sex and an even stronger interaction between sex and treatment. These results provide strong evidence supporting the idea that early adolescent

males are vulnerable to the long-term effects of amphetamine while early adolescent females are protected from amphetamine-dependent deficits in behavioural inhibition.

Remarkably, the same dose provided in mid-adolescence for both sexes does not seem to result in any task-related impairments. Once again, it is important to note that training for the task begins in adulthood – long after the end of the amphetamine treatment regimen so effects seen are presumably long term effects due to adolescent drug exposure. Also, given that this is a prefrontal cortex-mediated behavioural inhibition task, these effects are the functional consequences of the perturbations observed stereologically in the dopaminergic innervation of mPFC. In the case of mid-adolescence, males were protected from the detrimental effects of amphetamine while females compensated for those effects with an increase in Netrin-1 and therefore are not susceptible to the same impairments as males were when treated in early adolescence. This said, it is noteworthy that saline females treated in mid-adolescence exhibited a much larger variance in the number of proportion commission errors than their amphetamine-treated counterparts and mid-adolescent males which made a 2-way ANOVA impossible. However, for a direct comparison between the female treatment groups, I conducted a Welch's t-test which yielded a borderline significant result ($t_{12} = 1.92, p = 0.05$) suggesting that amphetamine-treated females may perform slightly better than saline females. I also tried a different approach: I used a log function to transform the data and, after ascertaining that the variances are no longer statistically significant, I ran a Student's t-test which similarly yielded a slightly significant result ($t_{12} = 2.243, p = 0.0445$). Given that this data is now transformed, it becomes more difficult to interpret. Nevertheless, taken together, both approaches tend to the same result, but I would hazard to put much value on these analyses as they seem to be solely driven by two data points which – while not strictly falling into the category of outliers by any method – are so vastly different from the other data points that there are likely no meaningful differences after all.

In summary, at this stage of adolescence, males seem to be entirely protected from the amphetamine-dependent perturbations observed a mere a couple of weeks earlier in all as-

pects: molecular (no statistically significant changes in *Dcc*, miR-218, or Netrin-1), stereological (no differences in volume, number of varicosities, or density of DA innervation), and behavioural (no differences with respect to the upper or lower asymptotes or the M50 measure). Females, on the other hand, exhibit stark molecular perturbations in *Dcc* and miR-218, but not stereological differences or behavioural differences in the Go/No-Go task.

I also performed other behavioural tests soon after the Go/No-Go task, namely progressive ratio and contingency degradation. The former revealed no differences between the treatment groups which is intuitively in line with the Go/No-Go results since there are no differences to try and explain, but it is an important test to have completed nonetheless. The results of the latter, the contingency degradation task, were counterintuitive for reasons I do not currently understand. Typically, control animals prefer the non-degraded option due to the regularity of the reward output, but the control animals in this particular experiment seem to have no preference. In addition, amphetamine-treated animals who have previously performed the Go/No-Go task in past experiments in the lab have either matched the preference of the saline animals (who preferred the non-degraded option) or preferred the non-degraded option less, but did not tend to prefer the degraded option. Either way, the difference score for the amphetamine-treated animals is higher than anticipated so I am unsure of how much weight to put on these results as a means of further enlightening the inner workings of habit vs goal-oriented behaviours in these subjects.

Activational sex hormones and puberty

There are two main effects of sex hormones: organizational, typically in gestational development or early in life, and activational, typically in adolescence leading further development and differentiation. The latter may serve as the basis for the sexually dimorphic effects of amphetamine during adolescence and, as such, are especially important to consider for the current investigation. It is well known that the ovarian sex hormones, estradiol and progesterone, act on DA receptors in the striatum and modulate the response to cocaine and

amphetamine (Becker, 1990; Becker & Hu, 2008; Hu & Becker, 2003; A. R. Johnson et al., 2019; Justice & de Wit, 1999; Parylak, Caster, Walker, & Kuhn, 2008). As such, female rodents are known to have enhanced responses to stimulant drugs (Satta, Certa, He, & Lasek, 2018; Van Swearingen, Walker, & Kuhn, 2013) – a finding that parallels nicely with human data which suggests that adult women are more vulnerable to psychostimulant abuse due to being more sensitive to the reinforcing effects of psychostimulants and, thus, progress more rapidly from initial to compulsive use (Anker & Carroll, 2010; Becker & Hu, 2008; Lynch, Roth, & Carroll, 2002). Interestingly, despite this commonly reported fact, men are typically reported to abuse psychostimulants, among other drugs of abuse, more than women (Tetrault et al., 2008), although this may be due more to opportunity rather than vulnerability. Furthermore, gonadectomy in rodents reduces the response to psychostimulants more in females than males (Becker, 1999; Becker & Hu, 2008). Overall, the evidence seems to paint the picture that estrogen enhances addiction-like behaviour in rodents, progesterone works to suppress these behaviours, and testosterone slightly suppresses them or is rather inactive (Kuhn et al., 2010).

Therefore, to casually dissect the role of pubertal timing and activational hormones in determining the presented effects of amphetamine on DA development, I first endeavoured to demarcate puberty from non-invasive external signs in male and female mice as a baseline measure in our mouse colony. I then investigated the effects of amphetamine in early or mid-adolescence in gonadectomized animals.

Determining puberty via external signs has a long tradition in rodent research. Yet, despite having a clear demarcation – preputial separation in males and vaginal opening in females (see Figure 10) – there is no consensus on exactly when this occurs or which sex undergoes puberty first. Of course, the timing of external signs of puberty is vastly dependent on strain of the mice under observation, but even then, there is no unanimity in the literature (Bell, 2018; Nelson et al., 1990; Safranski, Lamberso, & Keisler, 1993; Walker et al., 2017). It was therefore necessary to create a baseline measurement specifically for

the colony utilized in the experiments described. To my surprise, it was not a clear flip-of-a-switch-style demarcation as was alluded to in the literature; on the contrary, I observed a progression of external sexual maturity. I documented this progression using a stepwise 4-point legend which, remarkably, applied similarly to both sexes. In my case, it was clear that male mice underwent puberty first, but that both sexes seemed to achieve the final phase of sexual maturity around the same time. I think it was imperative to learn how to spot external signs of puberty and determine puberty status, but would be cautious about using this as an outcome measure in the future – particularly in female mice where the measure is less reliable and there are more accurate methods (Gaytan et al., 2017).

The interesting experiment that begins to causally dissect the role of peripubertal sex hormones as mediating factors for the effects of amphetamine on the mesocorticolimbic DA circuitry involves gonadectomizing mice in early adolescence and then exposing them to amphetamine during the two periods of interest. In this pilot, mice were gonadectomized at PND 21 were then treated with amphetamine either in early or mid-adolescence and sacrificed one week post treatment. Most studies in the literature are conducted with mice gonadectomized around 8 weeks, but this would be far too late for the insurgence of sex hormones that occurs during puberty (Bell, 2018). Even the typically accepted age for pre-pubertal gonadectomy of PND 25 (Delevich, Hall, Piekarski, Zhang, & Wilbrecht, 2020; Kercmar, Snoj, Tobet, & Majdic, 2014) may be slightly later than the start of sex hormone increases. Given that our interest was in the early and mid-adolescent periods, it was imperative that we perform our gonadectomy surgeries on young mice as early as feasibly possible post-weaning. After some expert training and painstaking practice, we completed 80 successful surgeries on PND 21 animals, 2 of the them unfortunately died in the days following surgery.

It was difficult to anticipate the results from this experiment, although I had hypothesized the possibility of a widening window of vulnerability to amphetamine-dependent effects to encompass mid-adolescence for male mice. Intuitively, if testosterone levels did not increase peri-pubertally – during mid-adolescence – then perhaps male mice would no longer be

protected against amphetamine's effects during that time. I was unsure of the expected results for female mice; I did not anticipate any significant differences for early adolescent animals as I could not foresee a reason for those results to differ, but I believed there was a possibility that the Netrin-1 compensatory mechanism may have been regulated, in part, by the increase in sex hormones. Despite no statistically significant results, some potential trends merit brief discussion.

There seems to be slight sex difference in the trend of variances for groups of the same sex at each age of treatment: there is a greater variance within the females of the mid-adolescent exposure groups (irrespective of treatment or type of surgery) compared to those of the early adolescent exposure groups whereas the males are either similar between the adolescent periods in question or seem to exhibit the opposing pattern. The surprising finding here is the difference between saline and amphetamine-treated sham male animals in early adolescence or, rather, the lack thereof. Amphetamine did not seem to downregulate *Dcc* in line with previous findings. It is uncertain why this is the case, but I would hypothesize that stress is playing an important role. In fact, there is some work providing evidence for the ides that shipping stress during adolescence results in "enduring, negative influences on behavioral responses to estradiol and progesterone in females and to testosterone in males, and it induces changes in response of the hypothalamic-pituitary-adrenal axis" (Laroche, Gasbarro, Herman, & Blaustein, 2009). In light of this evidence, the effects of shipping stress may have strongly confounded the experiment. All of the experimental mice, whether treated with amphetamine in early or mid-adolescence, arrive at PND 20 and only had one day to acclimate before having to undergo surgery; the stress of surgery could be adding to the stress of travel. It will be important to attempt at least one aspect of this study with mice bred locally in order to put this intuitive hypothesis to the test and provide better experimental conditions to test for the interplay between pubertal hormones and sexually dimorphic time- and amphetamine-dependent DA alterations.

Future Directions

The project is, of course, much larger than what is presented in this thesis and there are many other important aspects that require careful consideration. The larger story requires several more experiments in order to be rounded out. Among them would be utilizing the dual-virus approach (Reynolds et al., 2018) in both male and female mice with early and mid-adolescent exposure to amphetamine to: (1) see if early and mid-adolescent females are truly protected from the ectopic growth observed with early adolescent males, and (2) see if mid-adolescent males are also protected from this ectopic growth as molecular results would suggest. These experiments would provide additional evidence for the critical role of the DCC/miR-218 pathway in determining axon targeting decisions during adolescent development and how they may be perturbed in the presence of recreational-like doses of amphetamine. These experiments are currently underway. In this line of thought and in light of the unexpected upregulation of Netrin-1 in mid-adolescent female mice, it is necessary to downregulate Netrin-1 (using viruses that have already been employed previously in the lab), provide the usual amphetamine treatment regimen, and observe: (1) the ectopic growth of DA fibres from the NAcc to the mPFC using the dual-virus technique, and (2) if the first is true, then observe the performance in the Go/No-Go task in adulthood. This has yet to be pursued. One more important avenue of investigation – one that is currently in the works – is that of utilizing the CRISPR/Cas9 system to upregulate DCC in the NAcc and observe the subsequent effect. DCC is a large gene, measuring 45.24 cM (http://www.informatics.jax.org/allele/MGI:6316289), and so cannot be easily targeted for upregulation via other methods. This approach may also be used to potentially protect vulnerable early adolescent male mice to the detrimental effects of amphetamine exposure.

Altogether, the results presented in this thesis suggest that the timeline of sexual development may demarcate sex-dependent critical periods for dopamine development and vulnerability. Fitting the results from this work into the larger puzzle of how amphetamine affects the developing brain and particularly the mesocorticolimbic DA pathway is a difficult

task. For instance, the guidance cue, Netrin-1 is merely a part of the larger netrin family of canonical guidance cues which, in turn, is a member of the laminin family; moreover, DCC, responsible for chemoattraction when bound by Netrin-1 in the process of axon guidance, is only one of the 4 receptors for Netrin-1 – the others being DSCAM, netrin G ligands (NGLs), and members of the UNC5 family (Boyer & Gupton, 2018; Sun, Correia, & Kennedy, 2011). Therefore, we are looking at the interaction between several pieces of a much larger puzzle. How these other pieces are simultaneously affected by drugs of abuse during adolescence is still unknown – not to mention how the effects may be sexually dimorphic. Needless to say, the possible routes of investigation are endless, and endless fascinating.

Conclusion and Expected Contributions

Sex differences with respect to drugs of abuse have been known for some time (Becker & Hu, 2008); however, the molecular mechanisms underlying these differences remain largely unknown or poorly understood. This work aims to investigate some of these mechanisms during various stages of adolescence and begin teasing apart the influence of puberty and activational sex hormones to improve our understanding of the interplay between biological sex, brain development, and addiction mechanisms.

The work from this project contributes to a growing body of literature addressing sex differences during development and, furthermore, with relation to environmental factors – particularly adolescent exposure to the psychostimulant amphetamine. It is fascinating to observe the juxtaposition of the same mechanism responding very differently to amphetamine at different stages of development in each sex. In males, amphetamine disrupts dopamine growth and development resulting in long-term mPFC dysfunction – but only in early adolescence. By mid-adolescence, this vulnerability seems to disappear and the circuit and related behaviour are somehow protected. Females, however, seem protected against amphetamine-dependent disruption in early adolescence overall: early adolescent exposure does not significantly modify the DCC/miR-218 pathway or adult behaviour. This protection seems to continue into mid-adolescence whereby mid-adolescent exposure does not result in long-term alternations to mPFC structure or function, despite VTA *Dcc* downregulation. A protective mechanism seems to be at play, one which causes the significant upregulation of Netrin-1 expression in the NAcc and therefore prevents the ectopic growth of mesolimbic dopamine axons into the mPFC.

The work presented here provides substantial preliminary evidence that mPFC dopamine development occurs over a different time course in each biological sex and is therefore differentially vulnerable. Moreover, the work demonstrates that the mesocorticolimbic dopamine circuitry is affected by stimulant drugs in a sexually dimorphic manner, illustrating a sex specificity in the molecular mechanisms underlying the vulnerability of developing psychi-

atric disorders later in life. While still considered basic science, the impact of this work is not to be underestimated – particularly when considering the sex differences in the progression from recreational to compulsive use and the increasing prevalence of drug abuse in adolescent and adult women (Becker & Chartoff, 2019; Cotto et al., 2010; McHugh et al., 2018). The identification of sex differences in the underlying mechanisms of both the adolescent development of mesocorticolimbic dopamine circuits and the effects of drugs of abuse on the events occurring in these circuits during normal development provides critical foundational knowledge. Gaining a nuanced understanding of the processes mediating the effects of drugs of abuse on the developing brain will hopefully contribute to evidence-based prevention and intervention strategies (perhaps even offer new avenues of possible treatments) targeted specifically at adolescent boys and girls. In the age of a growing drug epidemic in North America, understanding the biological basis of long-term effects resulting from illicit drug use is critical – especially if they are complicated by being sexually dimorphic in nature.

References

Anker, J. J., & Carroll, M. E. (2010). Females are more vulnerable to drug abuse than males: Evidence from preclinical studies and the role of ovarian hormones. In *Biological basis of sex differences in psychopharmacology* (pp. 73–96). Springer.

Anthony, J. C., & Petronis, K. R. (1995). Early-onset drug use and risk of later drug problems. *Drug and alcohol dependence*, *40*(1), 9–15.

Becker, J. B. (1990). Estrogen rapidly potentiates amphetamine-induced striatal dopamine release and rotational behavior during microdialysis.

Becker, J. B. (1999). Gender differences in dopaminergic function in striatum and nucleus accumbens. *Pharmacology Biochemistry and behavior*, *64*(4), 803–812.

Becker, J. B., & Chartoff, E. (2019). Sex differences in neural mechanisms mediating reward and addiction. *Neuropsychopharmacology*, *44*(1), 166–183.

Becker, J. B., & Hu, M. (2008). Sex differences in drug abuse. *Frontiers in neuroendocrinology*, *29*(1), 36–47.

Bell, M. R. (2018). Comparing postnatal development of gonadal hormones and associated social behaviors in rats, mice, and humans. *Endocrinology*, *159*(7), 2596–2613.

Boyer, N. P., & Gupton, S. L. (2018). Revisiting netrin-1: One who guides (axons). *Frontiers in cellular neuroscience*, *12*, 221.

Calipari, E. S., & Ferris, M. J. (2013). Amphetamine mechanisms and actions at the dopamine terminal revisited. *Journal of Neuroscience*, *33*(21), 8923–8925.

Caye, A., Swanson, J. M., Coghill, D., & Rohde, L. A. (2019). Treatment strategies for adhd: An evidence-based guide to select optimal treatment. *Molecular Psychiatry*, *24*(3), 390–408.

Cotto, J. H., Davis, E., Dowling, G. J., Elcano, J. C., Staton, A. B., & Weiss, S. R. (2010). Gender effects on drug use, abuse, and dependence: A special analysis of results from the national survey on drug use and health. *Gender medicine*, *7*(5), 402–413.

Cuesta, S., Restrepo-Lozano, J. M., Popescu, C., He, S., Reynolds, L. M., Israel, S., ... Flores, C. (2019). Dcc-related developmental effects of abused-versus therapeutic-like amphetamine doses in adolescence. *Addiction biology*, *25*(4), e12791.

Cuesta, S., Restrepo-Lozano, J. M., Silvestrin, S., Nouel, D., Torres-Berrío, A., Reynolds, L. M., ... Flores, C. (2018). Non-contingent exposure to amphetamine in adolescence recruits mir-218 to regulate dcc expression in the vta. *Neuropsychopharmacology*, *43*(4), 900–911.

Degenhardt, L., Coffey, C., Moran, P., Carlin, J. B., & Patton, G. C. (2007). The predictors and consequences of adolescent amphetamine use: Findings from the victoria adolescent health cohort study. *Addiction*, *102*(7), 1076–1084.

Delevich, K., Hall, C. D., Piekarski, D., Zhang, Y., & Wilbrecht, L. (2020). Prepubertal gonadectomy reveals sex differences in approach-avoidance behavior in adult mice. *Hormones and Behavior*, *118*, 104641.

Fleckenstein, A. E., Volz, T. J., Riddle, E. L., Gibb, J. W., & Hanson, G. R. (2007). New insights into the mechanism of action of amphetamines. *Annu. Rev. Pharmacol. Toxicol.*, *47*, 681–698.

Gallistel, C. R., Fairhurst, S., & Balsam, P. (2004). The learning curve: Implications of a quantitative analysis. *Proceedings of the National Academy of Sciences*, *101*(36), 13124–13131.

Gaytan, F., Morales, C., Leon, S., Heras, V., Barroso, A., Avendaño, M. S., ... Tena-Sempere, M. (2017). Development and validation of a method for precise dating of

female puberty in laboratory rodents: The puberty ovarian maturation score (pubscore). *Scientific reports*, *7*, 46381.

Goldstein, R. Z., & Volkow, N. D. (2011). Dysfunction of the prefrontal cortex in addiction: Neuroimaging findings and clinical implications. *Nature reviews neuroscience*, *12*(11), 652–669.

Grant, A. [Alanna], Hoops, D., Labelle-Dumais, C., Prévost, M., Rajabi, H., Kolb, B., ... Flores, C. (2007). Netrin-1 receptor-deficient mice show enhanced mesocortical dopamine transmission and blunted behavioural responses to amphetamine. *European Journal of Neuroscience*, *26*(11), 3215–3228.

Grant, B., Dawson, D. A., Stinson, F. S., Chou, P. S., Kay, W., & Pickering, R. (2003). The alcohol use disorder and associated disabilities interview schedule-iv (audadis-iv): Reliability of alcohol consumption, tobacco use, family history of depression and psychiatric diagnostic modules in a general population sample. *Drug and alcohol dependence*, *71*(1), 7–16.

Hair, E. C., Park, M. J., Ling, T. J., & Moore, K. A. (2009). Risky behaviors in late adolescence: Co-occurrence, predictors, and consequences. *Journal of Adolescent Health*, *45*(3), 253–261.

Hammerslag, L. R., & Gulley, J. M. (2016). Sex differences in behavior and neural development and their role in adolescent vulnerability to substance use. *Behavioural brain research*, *298*, 15–26.

Hoffmann, H. M. (2018). Determination of reproductive competence by confirming pubertal onset and performing a fertility assay in mice and rats. *JoVE (Journal of Visualized Experiments)*, (140), e58352.

Hoops, D., & Flores, C. (2017). Making dopamine connections in adolescence. *Trends in neurosciences*, *40*(12), 709–719.

Hu, M., & Becker, J. B. (2003). Effects of sex and estrogen on behavioral sensitization to cocaine in rats. *Journal of Neuroscience*, *23*(2), 693–699.

Johnson, A. R., Thibeault, K. C., Lopez, A. J., Peck, E. G., Sands, L. P., Sanders, C. M., ... Calipari, E. S. (2019). Cues play a critical role in estrous cycle-dependent enhancement of cocaine reinforcement. *Neuropsychopharmacology*, *44*(7), 1189–1197.

Johnson, M., Salvatore, M., Maiolo, S., & Bobrovskaya, L. (2018). Tyrosine hydroxylase as a sentinel for central and peripheral tissue responses in parkinson's progression: Evidence from clinical studies and neurotoxin models. *Progress in Neurobiology*, *165*, 1–25.

Justice, A. J., & de Wit, H. (1999). Acute effects of d-amphetamine during the follicular and luteal phases of the menstrual cycle in women. *Psychopharmacology*, *145*(1), 67–75.

Kercmar, J., Snoj, T., Tobet, S. A., & Majdic, G. (2014). Gonadectomy prior to puberty decreases normal parental behavior in adult mice. *Hormones and behavior*, *66*(4), 667–673.

Kuhn, C., Johnson, M., Thomae, A., Luo, B., Simon, S. A., Zhou, G., & Walker, Q. D. (2010). The emergence of gonadal hormone influences on dopaminergic function during puberty. *Hormones and Behavior*, *58*(1), 122–137.

Laroche, J., Gasbarro, L., Herman, J. P., & Blaustein, J. D. (2009). Reduced behavioral response to gonadal hormones in mice shipped during the peripubertal/adolescent period. *Endocrinology*, *150*(5), 2351–2358.

Lee, P. H., Anttila, V., Won, H., Feng, Y.-C. A., Rosenthal, J., Zhu, Z., ... Posthuma, D., et al. (2019). Genomic relationships, novel loci, and pleiotropic mechanisms across eight psychiatric disorders. *Cell*, *179*(7), 1469–1482.

Li, H.-J., Qu, N., Hui, L., Cai, X., Zhang, C.-Y., Zhong, B.-L., ... Wang, L., et al. (2020). Further confirmation of netrin 1 receptor (dcc) as a depression risk gene via integrations of multi-omics data. *Translational psychiatry*, *10*(1), 1–15.

Lynch, W. J., Roth, M. E., & Carroll, M. E. (2002). Biological basis of sex differences in drug abuse: Preclinical and clinical studies. *Psychopharmacology*, *164*(2), 121–137.

Manitt, C., Eng, C., Pokinko, M., Ryan, R., Torres-Berrio, A., Lopez, J., ... Schmidt, E., et al. (2013). Dcc orchestrates the development of the prefrontal cortex during adolescence and is altered in psychiatric patients. *Translational psychiatry*, *3*(12), e338–e338.

Manitt, C., Labelle-Dumais, C., Eng, C., Grant, A., Mimee, A., Stroh, T., & Flores, C. (2010). Peri-pubertal emergence of unc-5 homologue expression by dopamine neurons in rodents. *PLoS One*, *5*(7), e11463.

Manitt, C., Mimee, A., Eng, C., Pokinko, M., Stroh, T., Cooper, H. M., ... Flores, C. (2011). The netrin receptor dcc is required in the pubertal organization of mesocortical dopamine circuitry. *Journal of Neuroscience*, *31*(23), 8381–8394.

McCabe, S. E., West, B. T., Morales, M., Cranford, J. A., & Boyd, C. J. (2007). Does early onset of non-medical use of prescription drugs predict subsequent prescription drug abuse and dependence? results from a national study. *Addiction*, *102*(12), 1920–1930.

McHugh, R. K., Votaw, V. R., Sugarman, D. E., & Greenfield, S. F. (2018). Sex and gender differences in substance use disorders. *Clinical psychology review*, *66*, 12–23.

National Institute of Health. (2019). Monitoring the future study: Trends in prevalence of various drugs. Retrieved April 29, 2020, from. https://www.drugabuse.gov/trends-statistics/monitoring-future/monitoring-future-study-trends-in-prevalence-various-drugs.

Nelson, J. F., Felicio, L. S., Randall, P. K., Sims, C., & Finch, C. E. (1982). A longitudinal study of estrous cyclicity in aging c57bl/6j mice: I. cycle frequency, length and vaginal cytology. *Biology of reproduction*, *27*(2), 327–339.

Nelson, J. F., Karelus, K., Felicio, L. S., & Johnson, T. E. (1990). Genetic influences on the timing of puberty in mice. *Biology of reproduction*, *42*(4), 649–655.

Parylak, S. L., Caster, J. M., Walker, Q. D., & Kuhn, C. M. (2008). Gonadal steroids mediate the opposite changes in cocaine-induced locomotion across adolescence in male and female rats. *Pharmacology Biochemistry and Behavior*, *89*(3), 314–323.

Reynolds, L. M., Makowski, C. S., Yogendran, S. V., Kiessling, S., Cermakian, N., & Flores, C. (2015). Amphetamine in adolescence disrupts the development of medial prefrontal

cortex dopamine connectivity in a dcc-dependent manner. *Neuropsychopharmacology*, *40*(5), 1101–1112.

Reynolds, L. M., Pokinko, M. [Matthew], Torres-Berrío, A., Cuesta, S., Lambert, L. C., Pellitero, E. D. C., ... Kolb, B., et al. (2018). Dcc receptors drive prefrontal cortex maturation by determining dopamine axon targeting in adolescence. *Biological psychiatry*, *83*(2), 181–192.

Reynolds, L. M., Yetnikoff, L., Pokinko, M., Wodzinski, M., Epelbaum, J. G., Lambert, L. C., ... Flores, C. (2019). Early adolescence is a critical period for the maturation of inhibitory behavior. *Cerebral Cortex*, *29*(9), 3676–3686.

Rice, F., Riglin, L., Thapar, A. K., Heron, J., Anney, R., O'Donovan, M. C., & Thapar, A. (2019). Characterizing developmental trajectories and the role of neuropsychiatric genetic risk variants in early-onset depression. *JAMA psychiatry*, *76*(3), 306–313.

Robins, L. N., & Przybeck, T. R. (1985). Age of onset of drug use as a factor in drug and other disorders. *NIDA Res Monogr*, *56*(1), 178–192.

Safranski, T., Lamberso, W. R., & Keisler, D. H. (1993). Correlations among three measures of puberty in mice and relationships with estradiol concentration and ovulation. *Biology of reproduction*, *48*(3), 669–673.

Satta, R., Certa, B., He, D., & Lasek, A. W. (2018). Estrogen receptor β in the nucleus accumbens regulates the rewarding properties of cocaine in female mice. *International Journal of Neuropsychopharmacology*, *21*(4), 382–392.

Srivastava, G., O'Hara, V., & Browne, N. (2019). Use of lisdexamfetamine to treat obesity in an adolescent with severe obesity and binge eating. *Children*, *6*(2), 22.

Steinberg, L. (2007). Risk taking in adolescence: New perspectives from brain and behavioral science. *Current directions in psychological science*, *16*(2), 55–59.

Sun, K. L. W., Correia, J. P., & Kennedy, T. E. (2011). Netrins: Versatile extracellular cues with diverse functions. *Development*, *138*(11), 2153–2169.

Tan, H.-Y., Callicott, J. H., & Weinberger, D. R. (2009). Prefrontal cognitive systems in schizophrenia: Towards human genetic brain mechanisms. *Cognitive neuropsychiatry*, *14*(4-5), 277–298.

Tetrault, J. M., Desai, R. A., Becker, W. C., Fiellin, D. A., Concato, J., & Sullivan, L. E. (2008). Gender and non-medical use of prescription opioids: Results from a national us survey. *Addiction*, *103*(2), 258–268.

Torres-Berrío, A., Lopez, J. P. [Juan Pablo], Bagot, R. C., Nouel, D., Dal Bo, G., Cuesta, S., ... Cooper, H. M., et al. (2017). Dcc confers susceptibility to depression-like behaviors in humans and mice and is regulated by mir-218. *Biological psychiatry*, *81*(4), 306–315.

Van Swearingen, A. E., Walker, Q. D., & Kuhn, C. M. (2013). Sex differences in novelty- and psychostimulant-induced behaviors of c57bl/6 mice. *Psychopharmacology*, *225*(3), 707–718.

Veliz, P., Boyd, C., & McCabe, S. E. (2013). Adolescent athletic participation and nonmedical adderall use: An exploratory analysis of a performance-enhancing drug. *Journal of studies on alcohol and drugs*, *74*(5), 714–719.

Walker, D. M., Bell, M. R., Flores, C., Gulley, J. M., Willing, J., & Paul, M. J. (2017). Adolescence and reward: Making sense of neural and behavioral changes amid the chaos. *Journal of Neuroscience*, *37*(45), 10855–10866.

Ward, J., Strawbridge, R. J., Bailey, M. E., Graham, N., Ferguson, A., Lyall, D. M., ... Mackay, D. F., et al. (2017). Genome-wide analysis in uk biobank identifies four loci associated with mood instability and genetic correlation with mdd, anxiety disorder and schizophrenia. *Translational Psychiatry*, *7*.

Windle, M. (1994). Substance use, risky behaviors, and victimization among a us national adolescent sample. *Addiction*, *89*(2), 175–182.

Yetnikoff, L., Almey, A., Arvanitogiannis, A., & Flores, C. (2011). Abolition of the behavioral phenotype of adult netrin-1 receptor deficient mice by exposure to amphetamine during the juvenile period. *Psychopharmacology*, *217*(4), 505–514.

Yetnikoff, L., Eng, C., Benning, S., & Flores, C. (2010). Netrin-1 receptor in the ventral tegmental area is required for sensitization to amphetamine. *European Journal of Neuroscience*, *31*(7), 1292–1302.

Yetnikoff, L., Labelle-Dumais, C., & Flores, C. (2007). Regulation of netrin-1 receptors by amphetamine in the adult brain. *Neuroscience*, *150*(4), 764–773.

Zimmermann, K. S., Hsu, C.-C., & Gourley, S. L. (2016). Strain commonalities and differences in response-outcome decision making in mice. *Neurobiology of learning and memory*, *131*, 101–108.