Adolescent Fragility: Consequences of Social Stress on the Maturing Mesocorticolimbic Dopamine System in Male and Female Mice

Samuel Richer

Integrated Program in Neuroscience

McGill University, Faculty of Medicine

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Contents

Abstract

Adolescence is a highly vulnerable period where many external and environmental factors can greatly impact brain maturation. Experiences of social stress in adolescence, such as bullying, can hamper prefrontal cortex development and impair cognitive control in adulthood. This developmental disruption can have negative consequences on mental health trajectories. In adolescence, dopamine axons undergo targeting events in the nucleus accumbens, with most of them forming local enduring synaptic connections. In contrast, mesocortical dopamine axons continue to grow from the nucleus accumbens all the way to the prefrontal cortex across adolescence, remaining vulnerable to ongoing experiences.

To study long- and short-term effects of social adversity in adolescent C57BL/6 female and male mice and the underlying mechanisms, we modified and adapted an accelerated version of the chronic social defeat stress paradigm used in adult male mice. Our adolescent model, termed accelerated social defeat (AcSD), allows to expose mice to social stress during discrete windows within adolescence. Exposure to AcSD in early adolescent males and females leads to impulse control deficits in adulthood. In males, but not females, these effects associate with alterations in the expression of axonal guidance cues that control adolescent dopamine development.

To explore whether AcSD in adolescence alters the ongoing development of the male and/or female dopamine circuitry, a targeted viral tracing strategy was used to track the growth of dopamine axons to the prefrontal cortex during adolescence. Additionally, longitudinal profiles of gonadal and stress hormones in follicular hair samples were assessed in a different cohort of mice that underwent AcSD or control conditions. The goal of this experiment was to understand *if*, *how*, and *when*, social adversity impacts these endocrine systems.

In males exposed to AcSD, dopamine axons found in the nucleus accumbens during adolescence underwent targeting errors leading to ectopic growth to the prefrontal cortex in adulthood when compared to control counter parts. In female mice, however,

AcSD reduced the number of dopamine axons that grew to the prefrontal cortex. Notably, in control non-stressed male and female mice, significantly more axons were found to grow to the prefrontal cortex in females compared to males. Regarding the profile of follicular hair hormones, corticosterone levels were differently affected by AcSD in males and females: while AcSD-exposed males showed short and long-term elevated corticosterone levels, in females corticosterone levels correlated significantly with social avoidance behavior. These results are the first demonstration that social adversity in adolescence disrupts dopamine long-distance axonal pathfinding and that this effect is opposite in males and females exposed to stress during the same chronological age. Sexually dimorphic molecular mechanisms involving axonal guidance cues and corticosterone may be at play. We propose that adverse experiences in adolescence increases susceptibility to mental illnesses by inducing sex-specific alterations in ongoing dopamine axon targeting and growth.

Résumé

L'adolescence est une période de grande vulnérabilité où de nombreux facteurs externes et environnementaux peuvent avoir un impact important sur la maturation du cerveau. Les expériences de stress social telles que l'intimidation à l'adolescence peuvent entraver le développement du cortex préfrontal et altérer son rôle dans le contrôle cognitif. Cette perturbation du développement peut avoir des conséquences négatives sur le bon développement de la santé mentale. À l'adolescence, les axones dopaminergiques ciblent majoritairement le noyau accumbens, la plupart d'entre eux formant des connexions synaptiques locales durables. En revanche, les axones dopaminergiques mésocorticaux continuent de croître du noyau accumbens jusqu'au cortex préfrontal durant cette période et restent donc très vulnérables aux expériences en cours.

Pour étudier les effets à court et long terme de l'adversité sociale chez les souris adolescentes C57BL/6 mâles et femelles, nous avons adapté une version accélérée du paradigme de stress chronique de défaite sociale pour étudier les effets de l'exposition de ce stress pendant des périodes distinctes de l'adolescence. Nous avons appelé notre modèle d'adolescent "défaite sociale accélérée" (AcSD). L'exposition à la défaite sociale accélérée au début de l'adolescence chez les mâles et les femelles entraîne des déficits du contrôle de l'impulsion à l'âge adulte. Chez les mâles seulement, ces effets sont associés à des altérations de l'expression des signaux de guidage axonal qui contrôlent le développement de la dopamine chez l'adolescent.

Pour déterminer si l'AcSD à l'adolescence modifie le développement du circuit dopaminergique chez les mâles et/ou les femelles, une stratégie de traçage viral a été utilisée pour suivre la croissance des axones dopaminergiques vers le cortex préfrontal au cours de l'adolescence. Dans une autre cohorte de souris, des profils longitudinaux d'hormones gonadiques et de stress ont été étudiés dans des échantillons de poils folliculaires de souris ayant subi l'AcSD, ou de conditions de contrôle. L'objectif de cette expérience était de comprendre *si*, *comment*, et *quand* l'adversité sociale a un impact sur ces systèmes endocriniens.

Il a été constaté que chez les mâles, l'AcSD induit des erreurs de ciblage des axones dopaminergiques dans le noyau accumbens, ce qui déclenche leur croissance ectopique vers le cortex préfrontal. Chez les souris femelles en revanche, l'AcSD réduit le nombre d'axones dopaminergiques qui se migrent normalement vers le cortex préfrontal. Notamment, lorsque l'on suit la croissance des axones dopaminergiques au même âge chronologique à l'adolescence, on constate qu'un nombre significativement plus important d'axones se développent vers le cortex préfrontal chez les femelles que chez les mâles. En ce qui concerne le profil des hormones folliculaires, les niveaux de corticostérone sont différemment affectés par l'AcSD chez les mâles et les femelles: alors que les mâles présentent une corticostérone élevée à court et à long terme, les niveaux chez les femelles sont significativement corrélés avec le comportement d'évitement social. Ces résultats sont la première démonstration que l'adversité sociale à l'adolescence perturbe le cheminement axonal à longue distance de la dopamine. Il est intéressant de noter que ces résultats sont diamétralement opposés chez les mâles et les femelles, ce qui soulève un questionnement des mécanismes moléculaires sexuellement dimorphiques, et le rôle potentiel de la corticostérone à cet égard.

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Author Contributions

This work was conducted by Samuel Richer who performed and coordinated all experiments under the supervision of Dr. Cecilia Flores unless otherwise stated. The following individuals also contributed to the project (in alphabetical order): Andrea Harée Pantoja Urbàn, Aoran Song, Del MacGowan, Fattima Abboud, Giovanni Hernandez, Michel Giroux, Philip Vassilev, Sehar Gul, Tanya Capolicchio.

Importantly, several of the experiments shown in this thesis were conducted in close collaboration with Dr. Andrea Harée Pantoja-Urbàn with whom I shared co-authorship of a manuscript published this year in Biological Psychiatry: Pantoja-Urbán, A. H., Richer, S., Mittermaier, A., Giroux, M., Nouel, D., Hernandez, G., & Flores, C. (2023). Gains and Losses: Resilience to Social Defeat Stress in Adolescent Female Mice. *Biological Psychiatry*, 95:37-47). My contribution in this work is clearly indicated when I described these experiments and the corresponding results.

Experiment #1: Female AcSD Model Validation
Design of the study: Samuel Richer, Andrea Harée Pantoja-Urbàn, Philip Vassilev, Dr. Cecilia Flores
Research Execution: Samuel Richer, Andrea Harée Pantoja-Urbàn, Philip Vassilev, Giovanni Hernandez, Michel Giroux
Data Analysis: Samuel Richer, Andrea Harée Pantoja-Urbàn

Experiment #2: Axonal Mistargeting Experiments
Design of the study: Samuel Richer, Dr. Cecilia Flores
Research Execution: Samuel Richer, Aoran Song, Giovanni Hernandez, Michel Giroux
Data Analysis: Samuel Richer, Del MacGowan

Experiment #3: Hormone Analysis
Design of the study: Samuel Richer, Dr. Cecilia Flores
Research Execution: Samuel Richer, Sehar Gul, Tanya Capolicchio, Giovanni Hernandez, Michel Giroux
Data Analysis: Samuel Richer, Fattima Abboud

Contribution to Original Knowledge

This thesis project explores the effects of adolescent social stress on the developing dopaminergic system in both C57BL/6 female and male mice. This research is the first to our knowledge to adapt the chronic social defeat model such that we can study discrete temporal windows of vulnerability during adolescence in C57BL/6 female mice. It is revealed that following social defeat stress in adolescence, female and male mice display different behavioural phenotypes, opposite alterations in dopamine axon growth to the prefrontal cortex as well as differences in corticosterone responses to stress as measured in follicular hair. Our findings emphasize the critical need to include females in all our research involving organism models. Indeed, our study demonstrates that to learn about enduring responses to a similar experience in adolescence and about the neurobiological mechanistic underpinnings, performing experiments in both males and females is necessary. We propose that it is now the time to start making up for the numerous years the female sex has been neglected in scientific research.

Introduction and Statement of Problem

Adolescence is defined as a very broad period of development where we observe a gradual transition from a juvenile state to independence. During this period, many changes are ongoing including a variety of physiological, hormonal, and behavioral alterations that make it such that we mature into an adult. The field of neuroscience is very keen on studying the adolescent population since this age marks future mental health trajectories (Clark et al., 1997; Hicks et al., 2009; Larsen & Luna, 2018; Lee et al., 2014; Rao et al., 1999; Wills et al., 2001). The age of onset for many different psychopathologies is in fact during the fragile, but plastic, adolescent period (Larsen & Luna, 2018; Paus et al., 2008). Enhanced vulnerability during this age can be explained in part by the fact that the prefrontal cortex (PFC) is still undergoing substantial developmental changes as it is one of the very last brain regions to fully mature (Gogtay et al., 2004).

Via the orchestration of important axonal guidance molecules, such as the Netrin-1/deleted in colorectal cancer (DCC) guidance cue system, dopamine (DA) axons in the mesocortical pathway are still innervating their frontal cortical targets (Hoops et al., 2018; Reynolds, Pokinko, et al., 2018). This gradual increase in the density of DA fibers growing up to the PFC until early adulthood, renders this process highly vulnerable to experiences during adolescence (Kalsbeek et al., 1988; Manitt et al., 2011; Naneix et al., 2012; Rosenberg & Lewis, 1995). In parallel to the maturation of the DA input to the PFC, inhibitory control capacity improves gradually from adolescence to adulthood in rodents, non-human primates, and humans (Luna et al., 2015; Reynolds & Flores, 2021). Since several psychiatric disorders of adolescent onset are characterized by impulse control deficits, this PFC-mediated behaviour can serve as an endophenotype of psychiatric risk (Paus et al., 2008).

Regardless of gender, one of the most important events in adolescence is socialization and peer interaction (Smetana, 2015). Unfortunately, during this stage of life, stressful incidents of bullying, domestic/sexual violence and navigating one's sexual and gender identity are highly prevalent (Collier et al., 2013; Rijlaarsdam et al., 2021). These

experiences of social stress put individuals at a high risk of developing internalizing and psychiatric disorders (Bowes et al., 2015; Lereya et al., 2015; Oram et al., 2013; Stapinski et al., 2014) due to dysfunctions in PFC-dependent cognitive functions (Clark et al., 1997; Rao et al., 1999; Wills et al., 2001; Wills & Cleary, 1996).

It is essential to note that there are significant individual differences in how adolescents respond to social stress. Some adolescents remain resilient and unaffected by social adversity, while those who are susceptible may experience it in different ways (Notaras & Buuse, 2020; Wood & Bhatnagar, 2015). Whether it be chronological age or biological sex, many different factors contribute to individual differences in resilience and susceptibility to the psychiatric impact of stress exposure in adolescence (Beesdo et al., 2009; Paus et al., 2008). For one, many obvious differences exist between males and females in their onset and prevalence of depression, substance use disorder and other mental illnesses that emerge during adolescence that are tightly linked to stress (Dalsgaard et al., 2020; Pedersen et al., 2014). When compared to males, females exposed to adversity during adolescence have a higher risk of developing mood disorders (Bale & Epperson, 2015; Boyd et al., 2015; Heim et al., 2010; Kessler et al., 2007. It seems that the vulnerability to experiences in an adolescent's environment is highly dependent on both the type of stressor occurring as well as the physiological or behavioral outcomes being examined (Hankin et al., 2007; Kim et al., 2017).

Social stress during this developmental period can be damaging in many ways. Understanding how immediate and enduring consequences of social stress manifest differently in males and females is critical and timely. There is an increasing incidence in peer victimization and depression in youth and therefore there is a pressing need for research data to inform early detection, prevention, and intervention programs. This work was aimed at modeling social stress in adolescent C57BL/6 female rodents and at providing insights regarding the dimorphic sensitivity to adversity at the behavioral, neuroanatomical, and molecular level.

Background and Rationale

Adolescent Dopamine Development in the Maturing Prefrontal Cortex

As is the case in humans, there are no precise boundaries demarcating the beginning and end of adolescence in rodents (Hollenstein & Lougheed, 2013; Sawyer et al., 2018). Our group as well as others suggest that adolescence in C57BL/6 mice spans from postnatal day (PND) 21, when mice are weaned, until the start of adulthood at PND 60 (Reynolds & Flores, 2021; Schneider, 2013). Within this adolescent window of transformation, we can further subdivide this period into what we refer to as a peripubertal "early adolescence" (PND 21-34) and pubertal mid adolescence (PND 35-48). These age ranges encompass discrete DA developmental periods (Kalsbeek et al., 1988; Manitt et al., 2011; Reynolds & Flores, 2019) and distinct behavioural characteristics (Adriani & Laviola, 2004; Makinodan et al., 2012; Spear, 2000; Wheeler et al., 2013).

Our group is interested particularly in the development of the DA system whereby DA neurons originating from the ventral tegmental area (VTA) form the mesolimbic and mesocortical pathways respectively by innervate the nucleus accumbens (NAcc), or the medial prefrontal cortex (mPFC). Both pathways travel together through the medial forebrain bundle (Nieuwenhuys et al., 1982) and then diverge at the NAcc into their separate DA systems serving different brain areas and functions. While most axons remain in the NAcc to form enduring connections (Hoops et al., 2018; Manitt et al., 2011; Reynolds & Flores, 2021) others continue to bypass through this region to reach the mPFC and other frontal cortical regions, including the orbitofrontal cortex (Hoops et al., 2018; Manitt et al., 2011; Reynolds & Flores, 2021). Since collaterals of NAcc DA axons to the mPFC are extremely rare (Beier et al., 2015; J. Fallon, 1981; J. H. Fallon & Loughlin, 1982; Lammel et al., 2008; Reynolds, Yetnikoff, et al., 2018; Swanson, 1982), the NAcc is a decision-making point whereby axons either settle in the NAcc or continue to grow up to the mPFC. Interestingly, these two segregated DA pathways have very different developmental temporal trajectories whereby in rodents, DA axon innervation of the NAcc reaches full maturation by early adolescence (Antonopoulos et al., 1997; Manitt et al., 2011; Voorn et al., 1988) whereas mesocortical DA axons to the mPFC gradually

increase from early adolescence all the way up until adulthood (Benes et al., 1996, 2000; Hoops et al., 2018; Kalsbeek et al., 1988; Leslie et al., 1991; Manitt et al., 2011; Naneix et al., 2012; Willing et al., 2017). This prolonged maturation of the PFC development occurs both in non-human primates (Rosenberg & Lewis, 1995) and most likely in humans too (Padmanabhan & Luna, 2014). By labeling DA axons in the NAcc in early adolescence and tracking their trajectory afterwards, our lab showed that indeed, DA axons still grow from the NAcc to the mPFC across adolescence in mice (Hoops et al., 2018; Reynolds, Yetnikoff, et al., 2018). This demonstration of long-distance axon growth during late postnatal development occurs to the DA system and renders it uniquely vulnerable to environmental disruptions during this prolonged developmental time (Hoops & Flores, 2017). Unlike DA inputs to the mPFC, norepinephrine and serotonergic inputs to the mPFC reach adult density levels much before adolescence by PND 7 and PND 14 respectively (Benes et al., 2000; Levitt & Moore, 1979; Lidov et al., 1980).

Role of the Netrin-1/DCC Guidance Cue System in Dopamine Development

Within the mesocorticolimbic system, several important proteins play a key role in shaping the development of the DA circuitry, such that DA axons end up forming connections at the right place and at the right time. One of these organizers is the Netrin-1/DCC guidance cue system which controls targeting decisions by axons along their growth to their final endpoint. This guidance cue system is expressed in DA systems of rodents, non-human primates, and humans across the lifespan (Cuesta et al., 2018; Manitt et al., 2010, 2011; Osborne et al., 2005; Reyes et al., 2013). The Netrin-1 ligand which is distributed as a gradient across the brain makes it such that axons can either be attracted or repelled depending on the type of receptor they express (Lanoue & Cooper, 2019; Sun et al., 2011). DCC receptors expressed on the growth cone of DA axons mediate their attraction towards sources of Netrin-1 (L. Finci et al., 2015; L. I. Finci et al., 2014).

DCC receptors are highly expressed in VTA DA neurons (Phillips et al., 2022), with levels diminishing from early life to adulthood (Manitt et al., 2010). Interestingly, the level of expression of Netrin-1 and DCC receptors in terminal regions of mesocorticolimbic DA

pathways differ both spatially and temporally. Lower levels of Netrin-1 protein are found in the NAcc and very high levels are found in the mPFC (Manitt et al., 2011), particularly in the inner layers, where there is the densest DA input (Cowan et al., 1994; Eden et al., 1987). In the NAcc, all DA axons express DCC receptors, and in fact this protein is only expressed by these axons (Phillips et al., 2022). However, in the mPFC, very few if any of the DA axons express DCC (Figure 1; Reynolds et al., 2023).

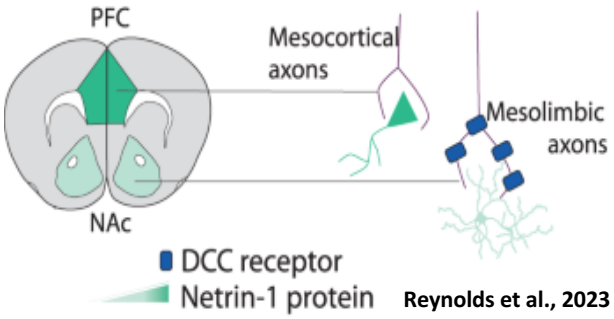


FIGURE 1. Distribution of Netrin-1 and DCC across the mesocorticolimbic system While high levels of Netrin-1 are found in the PFC and very low levels are found in the NAcc, the opposing expression level is seen in DCC whereby there are very high expression levels on mesolimbic axons whereas mesocortical axons express low levels.

The Netrin-1/DCC guidance cue system has crucial roles in shaping the mature mesocorticolimbic DA pathway. When DCC levels are reduced in DA axons that have reached the NAcc by early adolescence, these axons fail to recognize the NAcc as their final target and instead, they grow ectopically to the mPFC (Reynolds, Pokinko, et al., 2018). This rerouting of NAcc axons to the mPFC leads to (i) the presence of DCC+ axons in the mPFC (Manitt et al., 2013; Reynolds, Yetnikoff, et al., 2018), (ii) altered mPFC DA release (Grant et al., 2007; Hernandez et al., 2022) and (iii) a disorganized DA connectivity in the mPFC (Cuesta et al., 2020; Manitt et al., 2011, 2013; Reynolds et al., 2015). DCC is thus a critical receptor for the proper segregation of mesolimbic and mesocortical DA pathways (Reynolds, Pokinko, et al., 2018). Interestingly, during adolescence, the NAcc undergoes abundant changes in its connectivity and activity, including in humans, rendering it a vulnerable target at this developmental period (Antonopoulos et al., 1997; Manitt et al., 2011; Mastwal et al., 2014; McCutcheon et al., 2012; Naneix et al., 2012).

However, variations in DCC not only cause enduring alterations at the circuitry level, but also at the level of behavior and cognitive function. In rodents, ectopic growth

of NAcc DA axons to the mPFC leads to alterations in impulse control in adulthood (Reynolds et al., 2018, 2023). Human work studying adult individuals with *DCC* haploinsufficiency not only show changes in PFC connectivity but also in inhibitory control in adulthood (Vosberg et al., 2018, 2020). An increasing number of studies are also now showing that genetic variations in the Netrin-1/DCC system are tightly linked to disorders that are characterized by deficits in inhibitory control such as major depressive disorder and substance use disorder (Bechara & Martin, 2004; Manitt et al., 2013; Torres-Berrío et al., 2017; Vocci, 2008; Woicik et al., 2011). Dysregulation of these guidance cues during adolescence in the mesocorticolimbic system can perhaps explain mechanistically how experiences during this age lead to enduring impulsivity traits in rodents and humans (Torres-Berrío et al., 2020; Vosberg et al., 2020).

Consequences of Social Stress in Adolescent Male Mice

To reproduce aspects of physical and psychological stress experienced by victims of bullying during adolescence, researchers have used the chronic social defeat stress paradigm initially implemented in adult male rodents (Burke et al., 2016; Hasegawa et al., 2018; Huang et al., 2013; Iñiguez et al., 2014, 2016; Kim et al., 2018; Montagud-Romero et al., 2015, 2017; Mouri et al., 2018; Resende et al., 2016; Rodríguez-Arias et al., 2015; Xu et al., 2018; F. Zhang et al., 2016; H. Zhang et al., 2016). In this model, a mouse is subjected to repeated physical attacks from a larger dominant and aggressive mouse. We recently adapted the accelerated social defeat (AcSD) version of the chronic social defeat stress model to expose male mice to social stress during specific adolescent chronological ages (Pantoja-Urbán et al., 2022; Vassilev et al., 2021, 2022) such that we could capture critical windows of vulnerability and assess possible molecular players. In males, it was found that AcSD in early adolescence induced social avoidance, soon after exposure, and had enduring detrimental consequences on impulse control (Vassilev et al., 2021). Although not all males exposed to adolescent AcSD showed impaired social behavior, all defeated mice exhibit impulse control deficits in adulthood. This indicated that a social avoidant phenotype was not a consistent measure of susceptibility to AcSD in adolescence and that there may be a trade-off between protection against social deficits in adolescence and poor inhibitory control in adulthood (Brody et al., 2020; Pantoja-Urbán, Richer, et al., 2023). Indeed, all mice subjected to AcSD showed

reduced expression of the Netirn-1 guidance cue receptor DCC, one week after exposure, when DA axons were undergoing targeting events in the NAcc (Vassilev et al., 2021). As mentioned, DCC receptors in mesolimbic DA neurons controls the extent of the protracted growth of DA axons to the PFC – an event occurring in parallel to the gradual refinement of impulse control (Cuesta et al., 2020; Hoops et al., 2018; Reynolds, Pokinko, et al., 2018).

Rationale and Hypothesis

Because dominance hierarchies in rodents involve males fighting against males, but not females, building a model of adolescent social defeat stress in females was challenging for many groups. Here we overcame this limitation by modifying and adapting our adolescent AcSD male paradigm to female mice. Using a combination of behavioral, molecular, anatomical, and cognitive measures, we were able to assess, for the first time, the short- and long-term impact of social defeat stress in early adolescent female mice and to determine whether sex-specific characteristics emerged.

Because of sex-specific neurodevelopmental trajectories, we hypothesized that social defeat stress during the same adolescent chronological window would affect female mice differently than what was previously reported in males (Vassilev et al., 2021). We anticipated that AcSD would have a different impact in males and females regarding (i) social avoidance patterns, (ii) mPFC DA development, and (iii) hormone levels.

Project Aims

Aim 1: To determine if the AcSD model works for female adolescent mice.

Aim 2: To determine if social stress in early adolescence leads to dopamine axon mistargeting in both male and female mice.

Aim 3: To measure levels of stress and gonadal hormone before and after adolescent AcSD exposure in male and female mice.

Materials & Methods

Animals*

All experiments conducted in this study adhered to the guidelines set forth by the Canadian Council of Animal Care (CCAC) and received approval from the McGill University/Douglas Mental Health University Institute Animal Care Committee. All mice used were housed in a controlled environment at the Douglas's Neurophenotyping Center where a temperature of 21-22°C and humidity level of approximately 60% was maintained. Except for the C57BL/6 *DAT*^{Cre} mice bred in our animal facility specifically for the purpose of the axonal mistargeting experiment (Aim 2), all other C57BL/6J experimental mice were obtained from Jackson Laboratories. For the AcSD paradigm, male CD-1 retired breeder mice were acquired from Charles-River Canada and used as aggressor mice. These single housed CD-1 mice were used for no more than three months and a maximum of three consecutive experiments. All non-experimental C57BL/6 adult and adolescent mice used for the screening and priming during AcSD were also obtained from Charles-River Canada. Throughout the experiments, all mice were given ad libitum access to food and water.

Accelerated Social Defeat*

To replicate aspects of the physical and psychological stress experienced by adolescent victims of bullying, researchers have employed a modified version of the chronic social defeat stress paradigm originally used in adult male rodents (Burke et al., 2016; Hasegawa et al., 2018; Huang et al., 2013; Iñiguez et al., 2014, 2016; Kim et al., 2018; Montagud-Romero et al., 2015, 2017; Mouri et al., 2018; Resende et al., 2016; Rodríguez-Arias et al., 2015; Xu et al., 2018; F. Zhang et al., 2016; H. Zhang et al., 2016). Our lab adapted an accelerated version of this stress model called the Accelerated Social Defeat Stress Model (AcSD) such that both adolescent male and female mice could be subjected to attacks by an aggressive CD-1 mouse during precise periods in adolescence (Figure 1A; Pantoja Urbán, Richer, et al., 2023; Vassilev et al., 2021).

Phase 1- CD-1 Screening: When male CD-1 mice are obtained from Charles River, approximately half of the cohort exhibits aggression towards adolescent C57BL/6 mice, while the other half do not display aggressive behaviour. Due to this variability, it is essential to carefully select aggressor CD-1 mice before undergoing the AcSD experiment. For at least two consecutive days, an adult male C57BL/6 mouse was introduced into the home cage of the CD-1 for 3 minutes or until it experienced 10 attacks, whichever occurred first. From these observations collected, aggressive CD-1 mice were selected to move onto phase 2 based on whether they attacked or not.

Phase 2- CD-1 Priming: The next phase of preparation before starting AcSD is called the priming phase. This phase has the objective of firstly, finding mice that will attack adolescent C57BL/6 mice and secondly, it has the aim of getting CD-1 mice primed and consistently aggressive. Twice a day (9:00 & 14:00) an adult C57BL/6 was introduced to the CD-1's home cage for a brief 30 seconds to stimulate aggressiveness and was immediately replaced by an adolescent C57BL/6 mouse for 5 minutes or until there were 10 attacks. The sex and age (early adolescent PND 21-31) of the priming adolescent mice were matched to that of the experimental mice that would soon follow in the upcoming AcSD phase. This careful matching allowed for the accurate selection of a subset of CD-1 mice aggressive towards experimental mice that would be used in the subsequent step. This was done for 3-4 days until the desired number of CD-1 mice were able to consistently attack for more than one day.

Accelerated Social Defeat Stress: The AcSD set-up consisted of a transparent rat cage with a perforated and see-through central divider separating the area into two mouse housing compartments. Selected aggressive CD-1 mice were housed on one side of the divider 2 days prior to the commencement of the AcSD to allow time to explore the new environment and heighten territorial conduct. Additionally, during these two pre-AcSD days, the priming process used in phase 2 was repeated to ensure the CD-1 mice still displayed aggression towards the adolescent mice.

Once the AcSD phase begun, newly arrived experimental mice were randomized into the AcSD group or the control group. AcSD mice underwent two sessions of physical attacks per day (9:00 & 14:00) for a total of four consecutive days. Each physical stress session consisted of briefly introducing an adult C57BL/6 mouse for 30 seconds to the CD-1 to prime the CD-1 with aggressive behaviour. Immediately after, the adult mouse was replaced by the experimental adolescent C57BL/6 mouse until 10 attacks occurred (see Table 1 for the operational definition of an attack: adapted from (Pantoja-Urbán et al., 2022; Vassilev et al., 2021)) or until 10 minutes had passed. After the physical stress session, the experimental mouse was placed in the adjacent empty compartment beside the CD-1 that had just aggressed it to provoke a form of psychological stress. Every attack session consisted of a novel aggressive CD-1 mouse to allow a variety of attackers.

Table 1: Operational Definition of an "Attack" in Accelerated Social Defeat (AcSD)

1	An attack is defined as "when the CD1 mouse bites the C57BL/6J mouse, and the C57BL/6J mouse moves away in response to the bite."
2	A bite is defined as "when the CD1 mouse places its teeth on any part of the C57BL/6J mouse's body."
3	Moving away is defined as "when the C57BL/6J mouse moves both of its hind paws from the position they were in before the CD1 engaged it."
4	There must be ~2 seconds between separate attacks. If more than one bite occurs <2 s apart, count as one attack.
5	If the CD1 mouse bites more than once in succession without a break (<~2 s apart), gently separate animals.
6	If the CD1 mouse bites and does not let go, gently separate animals.
7	If the C57BL/6J mouse becomes trapped in a corner or is pinned down by the CD1 mouse and cannot move away in response to a bite, gently separate animals with a ruler.
8	In cases 5–7, count as one attack (up to separation).

Control mice were housed in the same dual housing apparatus (rat cage with transparent divider) as the AcSD mice. As opposed to AcSD mice, control counterparts were housed next to an age and sex matched C57BL/6 mouse and did not have any physical contact with any CD-1 nor C57BL/6 mouse. After the final session of AcSD, mice in both control and stress groups were single housed individually until the completion of experiments to avoid aggression amongst the mice.

**The Material and Methods section regarding Animals, Accelerated Social Defeat and Social Interaction were all adapted from (Pantoja-Urbán, Richer, et al., 2023)*

Limited Attacks- "Female-Like" Pattern of Attacks in Males

Based on our operational attack criteria outlined in Table 1, it was observed that during the AcSD, a lower occurrence of attacks was directed to female adolescent cohorts when compared to male adolescent cohorts. In light of this disparity, it was essential to ensure that behavioural, molecular and neuroanatomical outcomes were not a result of these differences in attack patterns. For this reason, a limited attacks AcSD experiment was performed whereby a cohort of male adolescent mice were subjected to a pattern of attacks resembling those that females were exposed to. This was achieved by carefully aligning the number of attacks and the duration of exposure with the CD-1 aggressor to match the recorded values observed in previous female AcSD experiments. Regulating the number of attacks to match the desired number was achieved by using a ruler to maintain separation between the mice.

Social Interaction Test*

To evaluate the potential immediate impact of AcSD on social approach and avoidance behaviour, the Social Interaction Test (SIT) was conducted the day after the final AcSD attack session between 10:00-16:00. This test, widely employed in various social defeat studies, aimed to gauge alterations in social behaviour resulting from adolescent AcSD (Golden et al., 2011). Under redlight conditions, AcSD and control mice were placed in a 42cm x 42cm open field for two consecutive sessions each lasting 2.5 minutes long. The first session consisted of a habituation phase where an unoccupied wire mesh enclosure was centered against one wall of the arena. Immediately following this, a second session followed whereby an unfamiliar CD-1 mouse was placed inside the wire mesh enclosure. To determine social approach/avoidance behaviour, a social interaction zone measuring 14cm x 9cm was delineated surrounding the wire-mesh enclosure. Behaviour was recorded with an overhead video camera such that analysis could be performed with the software TopScanTM 3.0 (Clever Systems Inc.). Using this software, a social interaction ratio was calculated by taking the time spent in the social interaction zone in session 2 when the CD-1 was present divided by the amount of time spent when the CD-1 was not present in session 1. A ratio <1.00 signified that mice did not spend as much time in the social interaction zone when the social target was present,

**The Material and Methods section regarding Animals, Accelerated Social Defeat and Social Interaction were all adapted from (Pantoja-Urbán, Richer, et al., 2023)*

and they were labeled as “susceptible”. Mice with a ratio >1.00 that did spend more time in the social interaction zone when the CD-1 was present were deemed to be “resilient”.

Social Interaction Test with an Anesthetized CD-1 Target

To validate the AcSD model in female mice, a separate cohort of mice underwent either AcSD or control conditions during early adolescence and were tested to see whether the state of the social target (awake or asleep) affected proportions of resiliency/susceptibility. The day after the last AcSD session, mice underwent the regular SIT with an awake CD-1 as a social target. The following day, the same mice were tested once again in the SIT but this time with an anesthetized novel CD-1 mouse instead of an awake social target in the second session. Anesthesia was performed using a mixed solution administered intraperitoneally, containing 50 mg/kg of ketamine, 5 mg/kg of xylazine, and 1 mg/kg of acepromazine.

Stereotaxic Surgery

To study Aim 2 and the effect of AcSD on the segregation of NAcc and mPFC DA projections, a dual viral strategy was employed to track DA axon growth in adolescence (Figure 2). Using axon-initiated viral recombination in *DAT*^{Cre} mice, we were able to label specifically VTA DA neurons whose axons had reached the NAcc by early adolescence. At PND 21 (prior to AcSD), *DAT*^{Cre} mice were weaned and anesthetized with isoflurane. Using a Hamilton syringe needle, we injected unilaterally into the NAcc (+1.5 anterior / posterior; +2.6 medial / lateral; -3.84 dorsal/ventral relative to bregma at a 30° angle) 0.5μl of a retrogradely

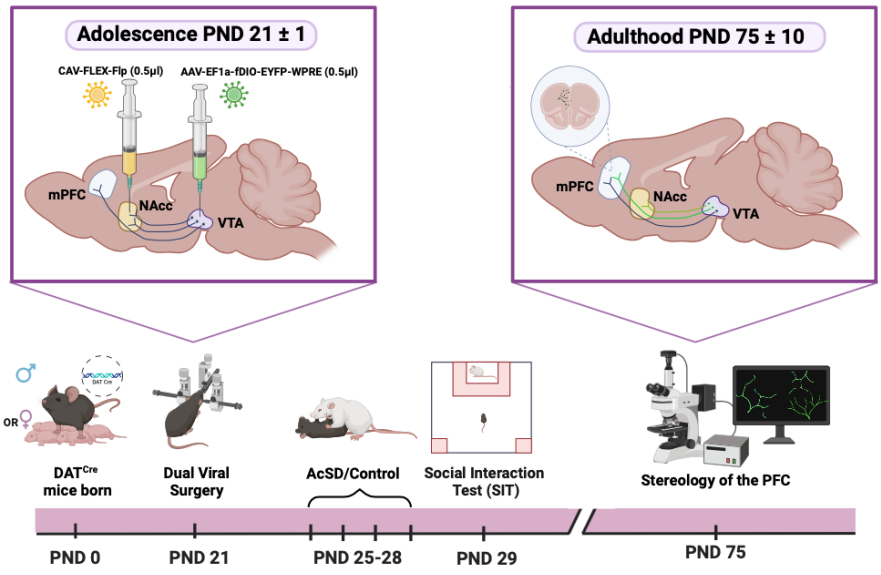


FIGURE 2. Aim 2. Experiment timeline of dopamine axon mistargeting experiment after early adolescent AcSD.

transported virus expressing a Cre-dependent Flp recombinase (CAV-FLEX-Flp, BioCampus Montpellier, Titer: $11x10^{12}$ pp/ml) as in (Reynolds et al., 2023). This design limited expression of the Flp recombinase to DAT-expressing VTA neurons that had reached the NAcc by PND 21. Simultaneously, we injected 0.5μl of Flp-dependent enhanced yellow fluorescent protein (eYFP) virus (pAAV-Ef1a-fDIO-EYFP-WPRE-pA, UNC Vector Core, Titer: $5x10^{12}$ pp/ml) into the ipsilateral VTA (-2.56 anterior/posterior; -0.9 medial/lateral; -4.21 dorsal/ventral relative to bregma at a 4° angel). The delivery of each viral construct spanned 6 minutes followed by a 10-minute waiting period before removing the injector. Mice were then exposed to AcSD or to control conditions from PND 25-28, and tested in the SIT at PND 29. Mice were single housed until they reached adulthood (PND 75 ± 10), when their brains were processed for stereological quantification of eYFP+ fibers in the mPFC.

Perfusion

Axonal mistargeting experiments (Aim 2) required perfusion of mice at PND 75 ± 10. Mice were administered an overdose of ketamine (50 mg/kg), xylazine (5 mg/kg) and acepromazine (1 mg/kg) intraperitoneally. Following this, they were subjected to an intracardial prefusion with 50 ml of 1x phosphate buffered saline (PBS). After clearing all circulating blood, 75ml of chilled 4% paraformaldehyde (PFA) in PBS was intracardial perfused to fix the brain. The brains were carefully removed from the skull and placed in the PFA fixative solution at 4°C for 1 day and then transferred to a 1x PBS solution for 1-2 days. A Leica vibratome was used to section the brains into 35 μm coronal slices where they then underwent immunohistochemistry and mounting onto gelatine coated slides and cover slipped using SlowFade Gold Antifade mounting medium (Invitrogen).

Immunohistochemistry

Brain sections underwent three 10-minute washes in PBS followed by a 1-hour incubation in a blocking solution (2% bovine serum albumin, 0.2% Tween-20, in PBS). Subsequently, these sections were immersed in a polyclonal anti-TH raised in rabbit antibody (1:1000, #AB152; Millipore Bioscience Research Reagents) and a polyclonal anti-GFP raised in chicken antibody (1:1000, antibody #1020, Aves labs) for a period of

48 hours at 4°C. The anti-GFP antibody was raised against whole recombinant GFP so any of the other *Aequorea Victoria* GFP derivatives would be recognized by the antibody as they differ via only a small mutation in a fraction of amino acid substitutions (For more information see: (Lambert, 2024)). For this reason, anti-GFP antibodies are often used to visualize eYFP (Bechelli et al., 2023; Shimizu et al., 2023; O. Singh et al., 2023; U. Singh et al., 2022). Following this, three 10-minute washes in PBS were performed and tissue was then incubated for an hour in the two following secondary antibodies: Alexa Fluor 594 donkey anti-rabbit antibody (1:500, Invitrogen) & Alexa Fluor 488 goat anti-chicken antibody (1:500, Invitrogen). Sections underwent three 10-minute washes in PBS and were then carefully mounted onto gelatin-coated slides and cover-slipped using SlowFade Gold Antifade mounting medium (Invitrogen).

Stereology

Blinded stereological quantification of eYFP+ varicosities in the mPFC was performed using the Stereoinvestigator© (Microbrightfield) software on a Leica DM400B microscope as in (Reynolds et al., 2018; Reynolds et al., 2023). Briefly, eYFP and TH expressing varicosities in the mPFC were identified using the mouse brain atlas (Franklin & Paxinos, 2008) to locate the pre-genual mPFC (plate 14-18, in a 1:4 series). In this region, delineation of cingulate (Cg1), prelimbic (PrL), and infralimbic (IL) subregions were contoured according to the TH-positive innervation using a 5x magnification. The number of co-expressing eYFP+ and TH+ varicosities was quantified at 100x magnification. This was done using an unbiassed counting frame measuring 50 x 50 μm (x = 175 μm, y = 175 μm intervals). An established guard zone of 4μm and optical dissector height 10μm. Following that we averaged all regions to get a measure of the total number of mPFC eYFP+ fibers.

Hormone Analysis

To begin assessing levels of stress and gonadal hormones throughout development in male and female mice exposed to AcSD or control conditions, a protocol to measure corticosterone, testosterone, progesterone from follicular hair was implemented (Figure 3). This method of measuring peripheral hormones is gaining popularity in many human studies and more recently in rodents (Stalder et al., 2017; Steudte-Schmiedgen et al., 2017; Ullmann et al., 2016; Wippert et al., 2014) whereby circulating hormones get incorporated into the growing hair overtime. Concentrations of hormones in follicular hair provide a retrospective reflection of corticosterone/cortisol, testosterone, progesterone, and of other hormone secretions that accumulate over time (Scorrano et al., 2015; Smyth et al., 2016; Uarquin et al., 2016; Walther et al., 2019, 2021; Wang et al., 2015; Weckesser et al., 2021). Although measures of salivary or plasma corticosterone concentrations can accurately detect acute effects of stress as well as variations in hormone levels throughout the day in rodents, many studies have reported that hair glucocorticoid concentrations vary more gradually and better represent the levels of these hormones over prolonged periods of time (Erickson et al., 2017; Scorrano et al., 2015; Uarquin et al., 2016). The following timeline and protocol was used in our laboratory to study levels of follicular hair hormones in adolescent male and female mice exposed to AcSD or to control conditions.

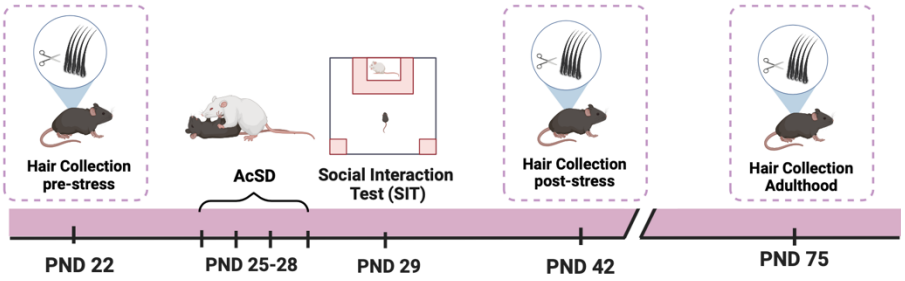


FIGURE 3, Aim 3. Experiment timeline of follicular hair hormone analysis experiment after adolescent AcSD.

Hair Collection #1 - Pre-Stress

At PND21, C57BL/6J male and female mice arrived from the Jackson Laboratory to the Douglas Research Centre Phenotyping Centre group-housed with 3 mice per cage. Mice were given one day to acclimate and at PND 22, they underwent the first shave using a Wahl Peanut clipper. While awake mice were restrained, bilateral flank hair was collected and placed into a folded square of aluminum foil. Hair was kept in at 4°C.

Between each shaving, a can of compressed air was used to clear any remaining hairs on the clipper.

At PND 22 following the first shave, male and female mice were randomly assigned into one of the following groups:

GROUP 1: AcSD- Male and female mice were exposed to the AcSD paradigm from PND 25-28, and on PND29 they underwent the SIT. Mice stayed single housed for the rest of the experiment.

GROUP 2: Single housed control group- Male and female mice were exposed to the control condition from PND 25-28, and on PND 29 they underwent the SIT. Mice stayed single housed for the rest of the experiment.

GROUP 3: Group housed control group- To assess whether housing conditions were a confound, in this group, mice were kept group housed throughout the entirety of the experiment and were not exposed to any aspect of the AcSD paradigm. The only manipulation done to them was the shaving along with the same frequency of handling as the other two groups.

Hair Collection #2: Post-Stress

As a measure of "post stress" hormone levels, two weeks after the last day of AcSD (PND 42), follicular hair was collected from male and female mice by bilaterally shaving the exact same flank area as was done in *Hair Collection #1* prior to stress. Mice were left undisturbed until they reached adulthood.

Hair Collection #3: Adulthood

In adulthood (PND 90 ± 15), follicular hair was collected from male and female mice by bilaterally shaving the exact same flank area as priorly done in *Hair Collection #1* and *Hair Collection #2*. At this time point, trunk blood and brains were collected, and flash frozen with 2-methylbutane for future molecular analysis.

Follicular Hair Hormone Analysis

The analysis of follicular hair samples was performed in collaboration with Dr. Clemens Kirshbaum at the Technical University of Dresden (Biopsychology Department) was. This lab specializes in the analysis of hormones from specimens of human and non-human origins and offers state-of-the-art steroid hormone analysis from hair samples. A hair sample >5mg was shipped at 4°C and a steroid panel analysis was performed to measure corticosterone, progesterone, and testosterone via Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS).

Statistical Analysis

Statistical analyses were performed using Prism version 9.4.0 (GraphPad Software, La Jolla, CA, USA). A significant threshold value of α <0.05 was used across all experiments. The data are presented as means ± the standard error of the mean (SEM). Depending on the number of factors analyzed, t-tests, 2-way ANOVAs and correlations were utilized to address statistical validation of any data. Planned comparisons as well multiple comparison tests were conducted post hoc when conditions were met and underwent a rigorous correction for the family-wise alpha using a Holm-Sidak or Holm-Bonferroni correction.

Results

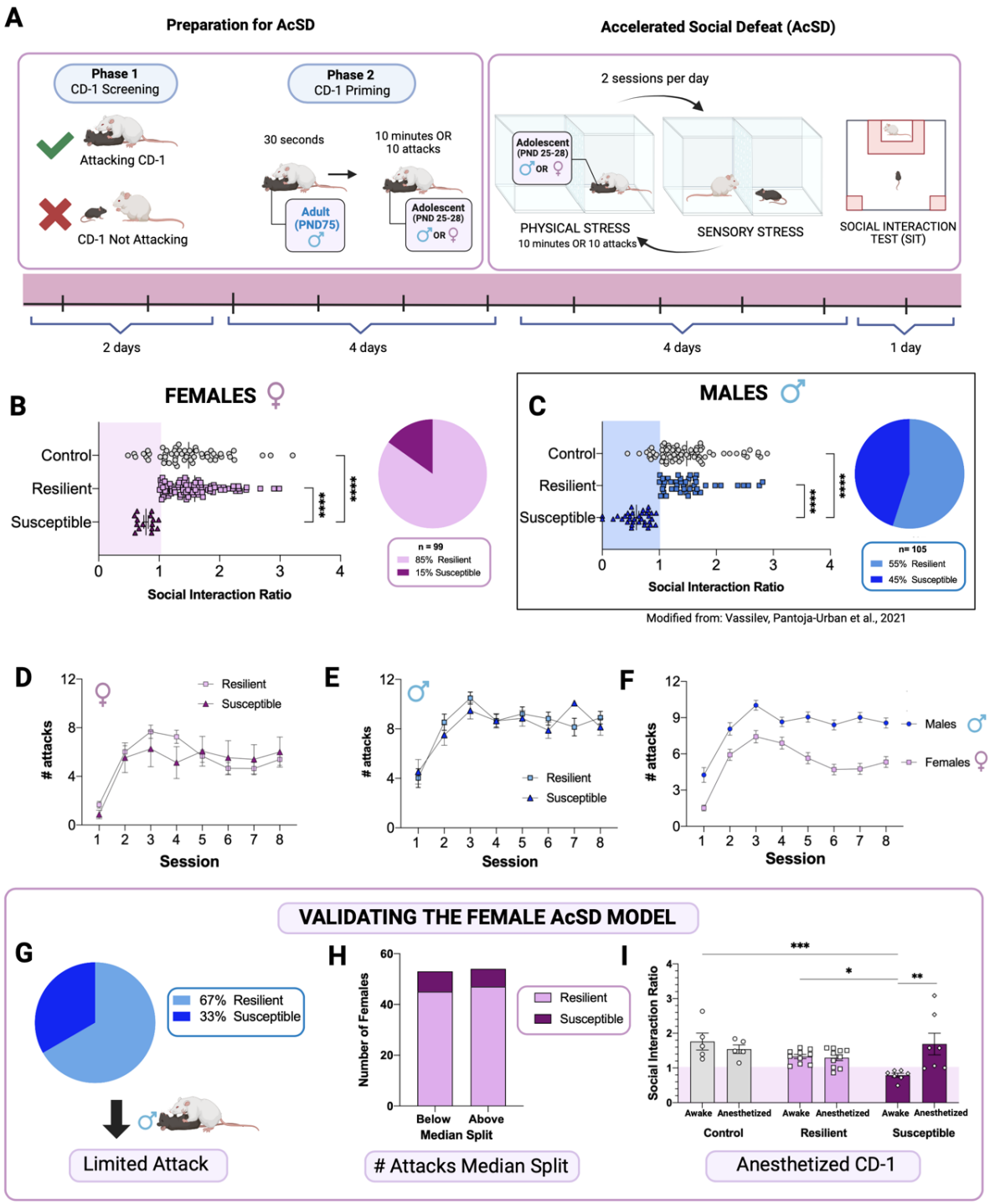
<u>Aim 1:</u> To determine if the AcSD model works for female adolescent mice.

Previous work conducted in our laboratory demonstrated that the AcSD model is an effective and robust model of adolescent social stress in male mice (Pantoja-Urbán et al., 2022; Vassilev et al., 2021, 2022). What was unknown, was whether this AcSD model could also be implemented in female adolescent mice. The modified AcSD model for adolescent females, as shown in Figure 4A, did work (Pantoja-Urbán, Richer, et al., 2023). The great majority (85%) of female mice exposed to AcSD were classified as "resilient" because they did not develop social avoidance in the SIT. In contrast, only a small proportion of females (15%) showed social avoidance and were categorized as "susceptible" (Figure 4B; from (Pantoja-Urbán et al., 2022)).

Compared to the previous reporting in male mice whereby 55% of AcSD mice displayed resilience (Figure 4C; from (Vassilev et al., 2021)), the proportion of resilience in females significantly surpassed the resilience rate reported in males (one-tailed binomial test $p < 0.0001$). Importantly, no difference in the frequency of received attacks was observed amongst resilient and susceptible mice in both the female cohort (Figure 4D, two-way repeated measures ANOVA, $F_{(7, 763)} = 0.84$, $p = 0.56$) and the male cohort (Figure 4E, from (Pantoja-Urbán et al., 2022; Vassilev et al., 2021), two way repeated measures ANOVA, $F_{(7,511)} = 1.321$ $p = 0.2378$).

When we compared the number of attacks between female and all male mice (using previously published data (Vassilev et al., 2021)), we found that females received fewer attacks throughout all defeat sessions (Figure 4F, from (Pantoja-Urbán et al., 2022), two-way repeated measures ANOVA, main effect of session, $F_{(6.82, 1227)} = 27.53$ $p < 0.0001$, main effect of sex, $F_{(1, 180)} = 170.4$, $p < 0.0001$; session × sex interaction $F_{(7, 1260)} = 1.63$, $p = 0.12$). With this concern in mind, to prove the effectiveness of this stress model in adolescent female mice, it was essential to investigate whether reduced physical harm in females could possibly account for their heightened resilience. Firstly, a typical attack pattern previously recorded in female mice was replicated in a cohort of adolescent male

mice through a limited attacks strategy as outlined in the methodology section. It was found that this manipulation did not substantially alter the ratio of resilient (67%) versus susceptible (33%) phenotypes in males when compared to regular AcSD male proportions (Figure 4G, from (Pantoja-Urbán et al., 2022), one-tailed binomial test p = 0.31). Secondly, we retroactively looked back at previous attack numbers in our cohorts and divided the cumulative number of received attacks among all female subjects (n = 107) into two groups: “high number of attacks” and “low number of attacks” based on a median split. When comparing both groups having received high and low attack numbers, the proportion of susceptible and resilient females displayed no notable differences (Figure 4H, from (Pantoja-Urbán et al., 2022), one-tailed binomial test p = 0.45). These two results both demonstrated that female adolescents undergoing AcSD exhibit resilience to social avoidance irrespective of the number of attacks endured. Lastly, to validate the segregation of resilience and susceptibility to a social target in the SIT, it was important to confirm that these phenotypes were specific to an awake and behaving social target as was done in some of the first chronic social defeat studies to prove robustness of the model (Krishnan et al., 2007). We found that susceptible defeated female mice showed avoidance in the presence of an awake, but not anesthetized CD1 social target (Figure 4I, Two-way repeated measures ANOVA, CD-1 state x phenotype interaction $F_{(2,19)}$= 7.411, p=0.004, Holm–Sidak *post hoc* tests: susceptible/awake vs susceptible/anesthetized, p=0.0016; control/awake vs susceptible/awake p=0.009; resilient/awake vs susceptible/awake p=0.0251).



***Adapted from (Pantoja-Urbán, Richer, et al., 2022)**

FIGURE 4. **Validating the AcSD model in adolescent female mice. A**, Experimental timeline of early adolescent AcSD (PND 25-28). **B**, Female SIT results after adolescent AcSD with the proportion of "resilient" & "susceptible" mice. **C**, Male SIT results after adolescent AcSD with the proportion of "resilient" & "susceptible" mice. **D**, Attack number in resilient and susceptible mice was not significantly different in females. **E**, Attack number in resilient and susceptible mice was not significantly different in males. **F,** Attack number received by males was significantly higher than those received by females during AcSD. **G,** Proportions of "resilient" and "susceptible" mice when male adolescent mice underwent a female pattern of attacks. This proportion did not differ from the typical male AcSD protocol. **H,** When performing a median split on the cumulative number of received attacks, the proportion of susceptible or resilient females did not differ between the "low" versus "high" received attack groups. **I,** An awake and behaving CD1 mouse was required to elicit the susceptible phenotype in female mice. All data are shown as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

<u>Aim 2:</u> To determine if social stress in early adolescence leads to dopamine axon mistargeting in both male and female mice.

Through a DA specific targeted viral tracing technique using *DAT*^{Cre} mice, we asked whether adolescent AcSD impacts DA axon targeting and growth in adolescent male and female mice (Figure 5A). After undergoing a viral surgery to track axonal growth and adolescent AcSD, the social interaction test was employed to parse out resilient and susceptible male (Figure 5B, one-way ANOVA, $F_{(2, 11)} = 10.47$, $p = 0.003$, Holm–Sidak *post hoc* tests: control vs susceptible, $p = 0.003$; control vs resilient, $p = 0.13$; resilient vs susceptible, $p = 0.024$) and female mice (Figure 5C, one-way ANOVA, $F_{(2, 12)} = 14.93$, $p = 0.0006$, Holm–Sidak *post hoc* tests: control vs susceptible, $p = 0.0005$; control vs resilient, $p = 0.20$; resilient vs susceptible, $p = 0.004$). In adulthood, brains were perfused, sliced, and underwent immunohistochemistry via a dual stain. Anti-TH labeling delineated the mPFC (Figure 5D *top panel*) and anti-GFP staining allowed visualization of the eYFP+ terminals in this outlined region (Figure 5D *bottom panel*). To assess axonal growth from the NAcc to the mPFC after adolescent AcSD or control conditions, stereological quantification was performed to assess the number of eYFP+ varicosities.

We found a strong stress by sex interaction in mPFC eYFP+ fibers (Figure 5E, two-way ANOVA, stress × stress interaction $F_{(2, 24)} = 9.922$, $p = 0.0007$; main effect of stress, $F_{(2, 24)} = 4.679$ $p = 0.019$, main effect of sex, $F_{(1,24)} = 19.04$, $p = 0.0002$). First, we were interested in how resilient and susceptible mice differed in mPFC eYFP+ fibers when compared to controls. We found that male mice showed an increased number of eYFP+ fibers growing to the mPFC in resilient but not susceptible mice when compared to controls (Figure 5E left panel, Holm-Bonferroni *post hoc* tests: control vs resilient, $p = 0.019$; control vs susceptible, $p = 0.238$). Opposite result in females were found whereby a greater number of eYFP+ axons spanned the mPFC of control animals when compared to resilient and susceptible mice (Figure 5E right panel, Holm–Bonferroni *post hoc* tests: control vs resilient, $p = 0.0011$; control vs susceptible, $p = 0.0065$). This remarkable sex-difference indicates that the number of mistargeted axons growing from the NAcc to the mPFC following social stress during adolescence increases in males but decreases in

females. Note that rerouting of NAcc DA axons to the mPFC occurred even though mice displayed resilience against social avoidance in adolescence implying that this social trait did not protect against disruption of DA development. We also wanted to compare how normative control levels of mPFC eYFP+ fibers differed in males and females and found less rerouted fibers to the mPFC in control males versus females (Figure 5E, Holm–Bonferroni *post hoc* tests: control male vs control female, $p < 0.0001$). VTA stereology will be needed to quantify infected DA cells to ensure equal transfection across groups.

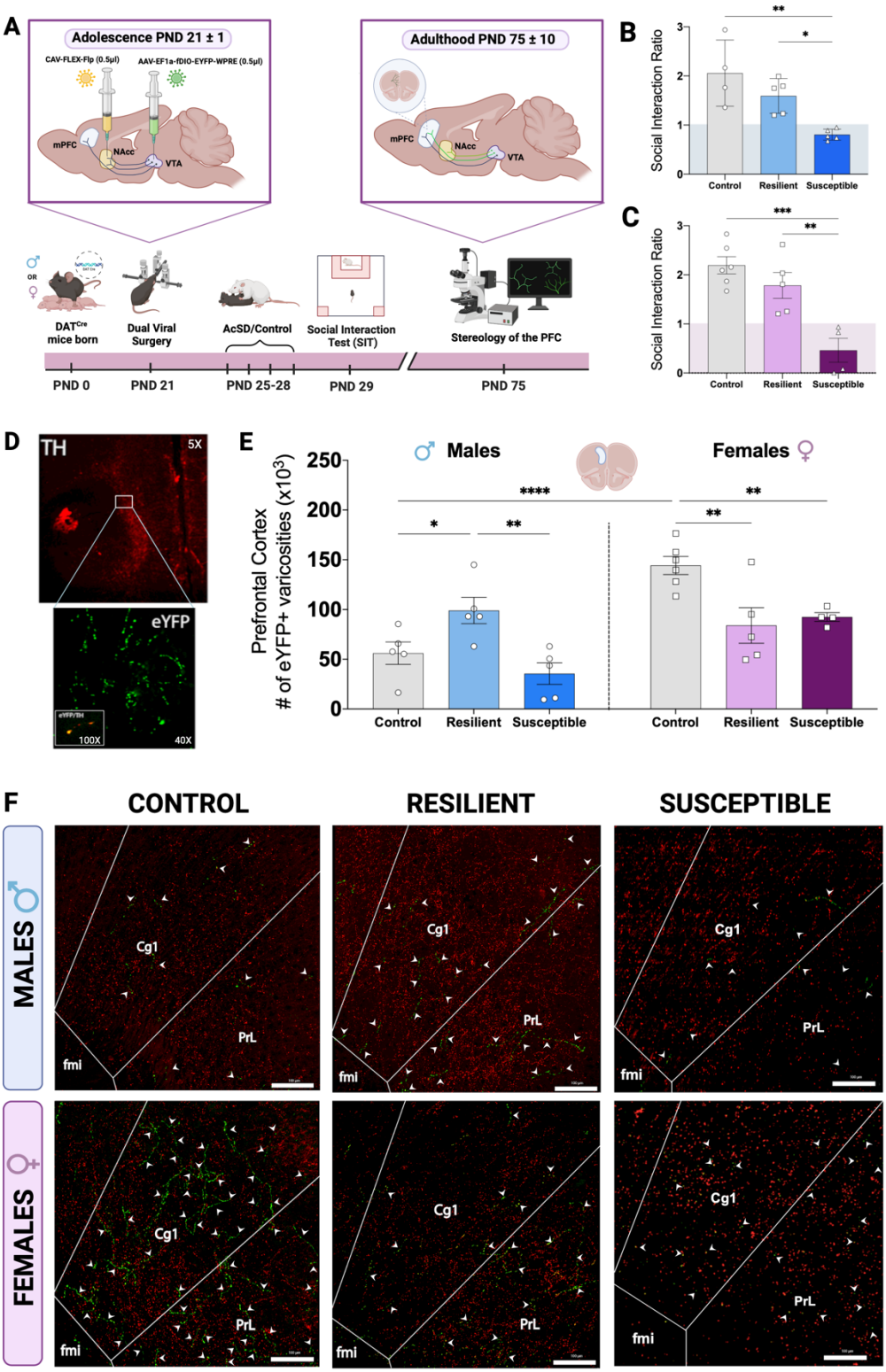


FIGURE 5. **Dopamine axon mistargeting to the mPFC in males and females as a result of AcSD in early adolescence. A**, Experimental timeline of dopamine axon mistargeting experiment after adolescent AcSD. **B,** Social interaction ratio for male mice during SIT. **C,** Social interaction ratio for female mice during SIT. **D**, *Top Panel:* 5X representative micrograph of control TH+ fibers in the mPFC *Bottom Panel:* 40X representative micrograph of control eYFP+ fiber which grew to the mPFC with a 100X zoom in on a representative co-labeled TH+/eYFP+ labeled varicosity. **E,** Stereological quantification revealed that compared to control counterparts, resilient but not susceptible males exposed to adolescent AcSD showed increased number of eYFP+ fibers. Opposingly, when compared to control counterparts, resilient and susceptible females showed a drastic reduction in the number of eYFP+ fibers innervating the mPFC following AcSD in early adolescence. **F,** 40X representative micrographs of TH+/eYFP+ fibers that grew to the mPFC. Data are shown as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001.

<u>Aim 3:</u> To measure levels of stress and gonadal hormone before and after adolescent AcSD exposure in male and female mice.

Follicular hair analysis of corticosterone, testosterone and progesterone levels were quantified in males and females during three developmental periods (Figure 6A). In female mice, the developmental trajectory of follicular hair corticosterone levels increased from early adolescence to adulthood (Figure 6B, two-way repeated measures ANOVA, main effect of time, $F_{(1.68, 38.74)} = 198.6$, $p < 0.0001$). Amongst female mice that underwent AcSD or either control condition, there were no difference in corticosterone levels at any given time (Figure 6B, two-way repeated measures ANOVA, main effect of condition, $F_{(2, 23)} = 2{,}44$ $p = 1.09$, time × condition interaction $F_{(4, 46)} = 1.11$, $p = 0.36$). However, there was a negative and significant correlation in the AcSD group between the time in the interaction zone (with the target during the SIT) and corticosterone post stress (PND 42) (Figure 6C, correlation Pearson's $r_{(9)} = -0.80$, $p= 0.01$, $R^2 = 0.007$). Notably, the correlation was absent in control mice (Figure 6D, correlation Pearson's $r_{(8)} = 0.695$, $p= 0.06$, $R^2 = 0.48$) and importantly, the number of attacks received did not corelate with corticosterone levels (Figure 6E, correlation Pearson's $r_{(9)} = -0.16$, $p= 0.67$, $R^2 = 0.03$).

Developmentally, corticosterone in males also showed increases from early adolescence to adulthood in all three groups (Figure 6F, two-way repeated measures ANOVA, main effect of time, $F_{(2, 45)} = 90.63$, $p < 0.0001$). Unlike females however, male mice that underwent AcSD showed significantly higher levels of corticosterone post stress (PND 42) when compared to their AcSD control group and the group house controls (Figure 6F, two-way repeated measures ANOVA, time × condition interaction, $F_{(4, 45)} = 4.73$, $p < 0.003$, Holm–Sidak *post hoc* tests: AcSD vs AcSD control, $p = 0.02$; AcSD vs group-house control, $p = 0.0017$; AcSD control vs group-house control, $p = 0.48$). Male mice that underwent AcSD also differed from females whereby they exhibited no significant correlation between corticosterone post stress (PND 42) and time in the interaction zone (Figure 6G, correlation Pearson's $r_{(10)} = 0.04$, $p= 0.9$, $R^2 = 0.002$). Additionally, in controls, there existed no correlation between corticosterone levels and time spent in the interaction zone with the social target (Figure 6H, correlation Pearson's $r_{(7)} = 0.05$, $p= 0.92$, $R^2 = 0.002$) nor was there a correlation between corticosterone levels

and number of attacks received by the AcSD group (Figure 6I, correlation Pearson's $r_{(9)}$ = -0.16, p= 0.67, R^2 = 0.03).

Overall, when comparing male and female mice, striking differences arise. Firstly, during normative development, both males and females showed increased corticosterone levels from adolescence to adulthood, with female controls displaying much higher corticosterone levels compared to male control counterparts during the mid-adolescent/pubertal age of PND42 (Figure 6J, two-way repeated measures ANOVA, time × sex interaction $F_{(2, 62)}$ = 18.13, p < 0.0001, *post hoc* test: PND42 male control vs PND42 female control, p = 0.0001). As previously mentioned, at PND 42, females display no changes in corticosterone after undergoing AcSD whereas a trend of elevated corticosterone in males was seen (Figure 6J, two-way repeated measures ANOVA, time x condition interaction $F_{(2, 19)}$ = 8.27, p = 0.003 *post hoc* test: PND42 female control vs PND42 female AcSD, *p =0.997;* PND42 male control vs PND42 male AcSD, *p =0.068*). Later in adulthood (PND 75), corticosterone levels were similar between control and AcSD mice in both males (Figure 6J, two-way repeated measures ANOVA, time x condition interaction $F_{(2, 19)}$ = 8.27, p = 0.003 *post hoc* test: PND75 male control vs PND75 male AcSD, *p =0.67*) and females (Figure 6J, two-way repeated measures ANOVA, time x condition interaction $F_{(2, 19)}$ = 8.27, p = 0.003 *post hoc* test: PND75 female control vs PND75 female AcSD, *p =0.9998*).

Additionally, when studying gonadal hormones across development, we found increased male testosterone levels (Figure 6K, two-way repeated measures ANOVA, main effect of time $F_{(1.22, 42.8)}$ = 276, p < 0.0001) and female progesterone levels (Figure 6L, two-way repeated measures ANOVA, main effect of time $F_{(1.76, 39.7)}$ = 163, p < 0.0001) across development. However, when comparing mice having undergone adolescent AcSD or control conditions, there was no significant difference in male testosterone (Figure 6K, two-way repeated measures ANOVA, time × condition interaction $F_{(2, 70)}$ = 0.66, p = 0.52, main effect of condition $F_{(1, 70)}$ = 0.65, p = 0.42) nor in female progesterone levels (Figure 6L, two-way repeated measures ANOVA, time × condition interaction $F_{(2, 45)}$ = 0.33, p = 0.72, main effect of condition $F_{(1, 24)}$ = 0.47, p = 0.50) amongst these groups.

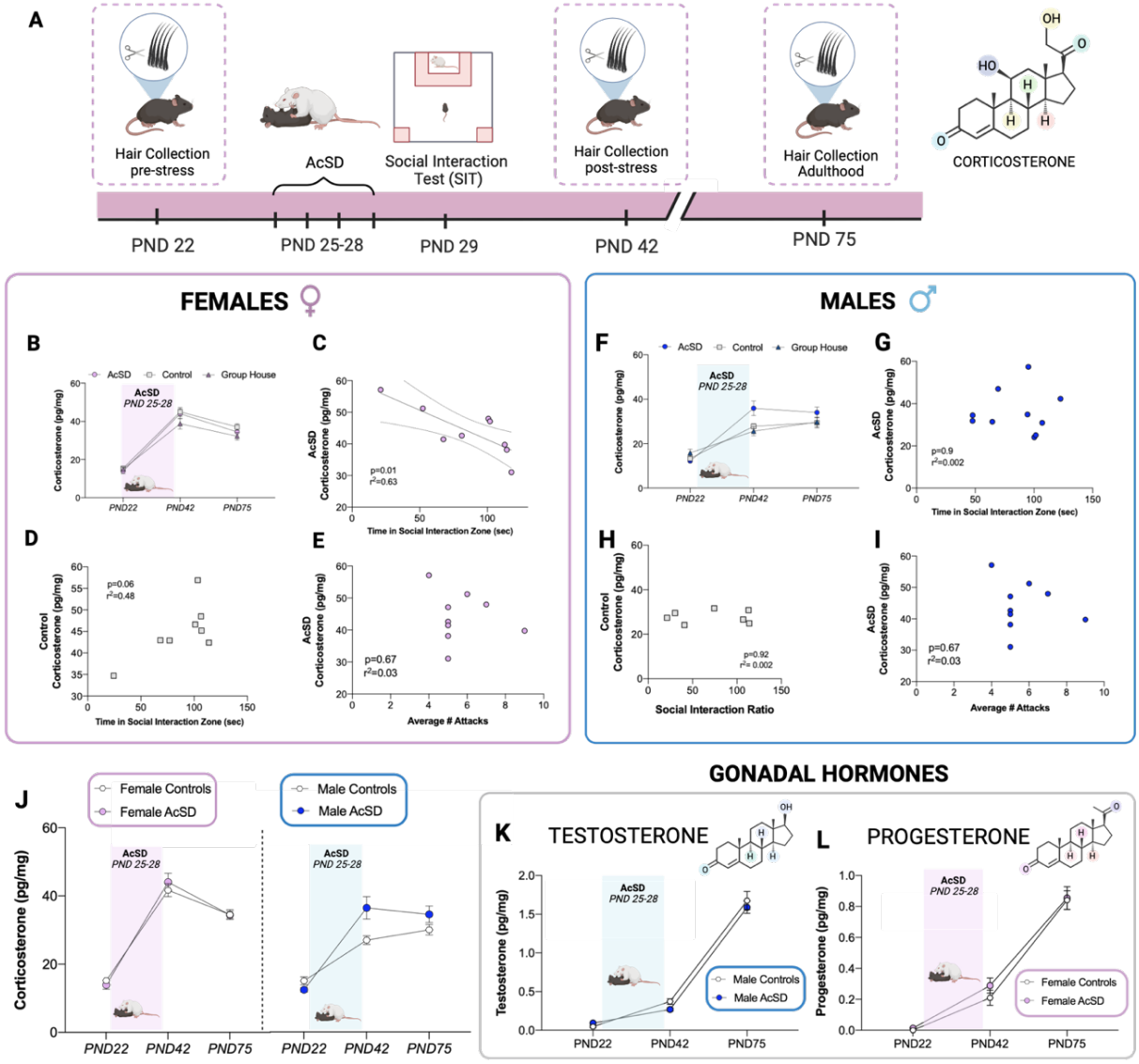


FIGURE 6. **Follicular hair hormone analysis in adolescent mice exposed to AcSD or control conditions.** **A**, Experimental timeline of adolescent AcSD and hair collection. **B**, Female levels of corticosterone increased from adolescence to adulthood with no differences in corticosterone levels at each timepoint between female mice having undergone AcSD, AcSD control or group house control. **C**, Female corticosterone levels post stress (PND 42) correlated negatively with social interaction in the AcSD group. **D,** Female corticosterone levels post stress (PND 42) did not correlate with social interaction in the AcSD control group. **E,** In females, the number of attacks received by the AcSD group did not correlate with corticosterone levels. **F,** Male levels of corticosterone increased from adolescence to adulthood. After undergoing AcSD stress in adolescence, male mice displayed significantly higher levels of corticosterone post stress (PND 42) when compared to the AcSD control and group house control mice. **G,** Male corticosterone levels post stress (PND 42) correlated negatively with social interaction in the AcSD group. **H,** Male corticosterone levels post stress (PND 42) did not correlate with social interaction in the AcSD control group. **I,** In males, the number of attacks received by the AcSD group did not correlate with corticosterone levels. **J,** Male and female mice showed normative corticosterone differences and only AcSD male mice show elevated corticosterone post stress (PND 42). **K,** Male mice showed increased testosterone levels throughout development but no differences amongst control or AcSD groups. **L,** Female mice showed increased progesterone levels throughout development but no differences amongst control or AcSD groups. All data are shown as mean ± SEM.

Discussion

Paradigms of adolescent social defeat stress have long served as valuable models to reproduce certain physical and psychological aspects experienced by victims of bullying and domestic violence (Bourke & Neigh, 2011; Burke et al., 2016; Harris et al., 2018; Hoeve et al., 2013; Huang et al., 2013; Iñiguez et al., 2014; Montagud-Romero et al., 2015). Constructing such models in female adolescent mice has proven challenging for numerous research teams, as dominance hierarchies in rodents primarily entail male-male aggressive behaviour. Here, for the first time, we were able to assess the immediate and future consequences of social defeat stress during early adolescence in female C57BL/6 mice using the modified AcSD paradigm (Pantoja-Urbán et al., 2022; Vassilev et al., 2021). We found that social defeat stress in adolescence induced robust targeting errors by DA axons with completely opposite effects in males versus females. Resilient male mice that underwent AcSD in adolescence showed a greater number of eYFP+ axons in the adult mPFC when compared to control and susceptible mice. Contrastingly, all female mice that underwent AcSD showed a decrease in eYFP+ axons growing to the mPFC when compared to their control counterparts. Lastly, our findings highlight the presence of sex differences in endocrine response to AcSD as corticosterone levels are influenced differently in males and females: AcSD males exhibited both short and long-term elevations in corticosterone when compared to controls whereas in females, these levels were significantly associated with social avoidance behaviour. These findings revealed for the first time that an adverse social experience in adolescence could significantly disrupt ongoing long-distance dopamine axon pathfinding in adolescence, with opposite changes in males versus females. Our results suggest that sex-specific alterations in brain development and in corticosterone levels following AcSD may be at play. Overall, this work allows us to better understand the mechanistic underpinnings that might be at play in the sexually dimorphic development of the dopaminergic system in adolescence and how it is influenced by adverse experiences that are common during this age.

The early adolescent AcSD model leads to a large majority of "resilient" females that do not display social avoidance

When exposed to the AcSD stress paradigm during early adolescence, both males and females received consistent attacks from the CD-1 aggressor mouse across all 8 sessions, yet, they showed very different social avoidance behaviour as a result of this experience. Unlike in male cohorts that typically displayed around 50% susceptibility to AcSD-induced social avoidance, very few females (~15%) consistently and repeatedly exhibited resilience to social deficits as measured by the SIT. While the origin of this disparity remains unknown, sexually dimorphic trajectories of adolescent social behaviour may help explain how males and females distinctively adapt to such an experience during this vulnerable period (Burke et al., 2011; Panksepp et al., 2007). During adolescent development, rodents exhibit heightened social behaviour more than at any other age (Burke et al., 2017; Kopec et al., 2018). Rodent males and females have shown divergence in the timing of when behaviours such as social play or social exploration occur (Kopec et al., 2018). Perhaps protective mechanisms are in place aimed at preserving social behaviour relevant to males or females at this critical developmental stage and could be important to understand brain maturation and vulnerability. Interestingly, when observing proportions in adulthood social defeat models, numerous studies have reported that both male (Krishnan et al., 2007; Vassilev et al., 2021) and female rodents (Greenberg et al., 2015; Harris et al., 2018; Hoeve et al., 2013; Steinman & Trainor, 2017; Trainor et al., 2011) display markedly greater proportions of susceptibility when compared to those seen in our adolescent AcSD model emphasizing the critical importance of studying age and sex. Numerous social defeat studies have emphasized resiliency and susceptibility based on only the SIT outcomes however in this study we emphasize the need for prudence when using such terms as we have learnt that susceptibility is age, sex, and domain-specific depending on which behavioural outcome is being measured.

To confidently assess the robustness of this model, we report that there was no discernible difference in the number of received attacks between resilient and susceptible mice. Additionally, we show that the susceptible phenotype is truly due to social

interaction since an anesthetized CD-1 does not induce susceptibility. While females experience slightly fewer attacks than males due to the nature of aggressive behaviour between males, we show that proportions are not a result of the number of attacks. We do so by exposing male mice to a “female” attack pattern and showing similar proportions of susceptibility. When ranking mice based on the cumulative number of attacks they received and performing a medium split, we show that the proportions of susceptibility are the same in the mice subjected to a higher cumulative number of attacks when contrasted to those subjected to a lower cumulative number of attacks. As was shown in our study, other groups studying adult chronic social defeat models in females report variable amounts of aggression by the attacker yet still show the stress induced physiological increases in corticosterone as well as behavioural manifestations of a stress response (Harris et al., 2018). The fact that an aggressive CD-1 mouse attacks a female rodent differently than its male counterpart should not be a reason to halt the development and use of social defeat models to advance our knowledge on female specific impacts of stress and our understanding of psychiatric vulnerability in females.

AcSD in adolescence significantly disrupts ongoing dopamine axon growth to the PFC with opposite effects in males and females

It has long been known that the cortical and striatal dopaminergic pathways exhibit markedly different temporal trajectories during development. In rodents, the mesolimbic system attains matured axonal growth in the NAcc by prepubertal adolescence (Antonopoulos et al., 1997; Manitt et al., 2011; Voorn et al., 1988) whereas mesocortical DA axon projections to the PFC experience a gradual increase up until early adulthood (Benes et al., 1996, 2000; Hoops & Flores, 2017; Kalsbeek et al., 1988; Leslie et al., 1991; Manitt et al., 2011; Naneix et al., 2012; Willing et al., 2017). Previous work has shown that altering guidance cue levels, specifically reducing levels of DCC receptors in DA axons that have reached the NAcc during adolescence, causes them to reroute towards the mPFC and to recognize this region as their final target (Manitt et al., 2013; Reynolds, Pokinko, et al., 2018). Furthermore, this misrouting event leads to aberrant DA metabolisms and release in this region (Grant et al., 2007; Hernandez et al., 2022) and to impaired impulse control in adulthood (Reynolds et al., 2018).

The NAcc is an important intermediate target for axons to pass through along their way to the cortex. Indeed, this region undergoes substantial dynamic changes in activity and connectivity during adolescence rendering it highly vulnerable to external experiences (Antonopoulos et al., 1997; Manitt et al., 2011; Mastwal et al., 2014; McCutcheon et al., 2012; Naneix et al., 2012). Amphetamine administration in adolescent male, but not female mice has been shown to induce significant alterations in the ongoing growth of DA axons to the mPFC when compared to saline-treated counterparts (Reynolds et al., 2023). Here, for the first time, we assess how a social adverse experience in adolescence can induce axonal targeting errors in DA axons in both males and females. In male mice we observed an increase of eYFP+ fibers in the mPFC of resilient AcSD exposed mice compared to control and susceptible mice. This notable discovery suggests that resilience to social stress during early adolescence redirects DA axons, originally intended to innervate the NAcc, all the way up to the mPFC in males. Critically, it is important to note that these findings of rerouted axons from the NAcc to the mPFC occurred in mice that exhibited resilience against social avoidance in adolescence as measured by the SIT. This displays once again that this social trait does not provide protection against the disruption of DA development. Understanding the molecular mechanisms that dictate ectopic axonal growth to the PFC and how susceptible mice avoid this circuitry change remains an area to be further studied.

To our surprise, resilient and susceptible females undergoing AcSD in early adolescence showed the exact opposite pattern: they had a decrease in eYFP+ fibers in the mPFC compared to their control counterparts, indicating reduced mesocortical dopamine axon growth in adolescence. The fate of the DA axons that were initially destined to innervate the mPFC remains to be investigated to understand where they go instead and why they innervate elsewhere. These findings reveal that sexually dimorphic mechanisms may mediate the effects of adolescent AcSD on the maturation of the dopamine system and of impulse control (Vassilev et al., 2021; Pantoja-Urbán, Richer, et al., 2023).

It is also interesting to see that when looking at normative control development, there was a greater number of eYFP+ fibers in female control mice when compared to male control mice. This may be explained by several studies suggesting that the dynamic period of DA activity and connectivity occurs at an earlier chronological age in females compared to males (Drzewiecki et al., 2016; Mastwal et al., 2014; McCutcheon et al., 2012; Willing & Juraska, 2015). Another scenario that could possibly account for this discrepancy amongst male and female controls is that some studies report a higher proportion of VTA DA neurons projecting to the mPFC in females (>50%) compared to males (~30%) (Kritzer & Creutz, 2008).

While in males the changes in dopamine axon growth are associated with stress-induced reductions in DCC receptor levels, in females this guidance cue system is not altered (Pantoja-Urbán, Richer, et al., 2023). We are currently conducting RNAseq experiments in male and female mice exposed to AcSD or to control conditions to investigate guidance cue alterations in dopamine regions.

Normative corticosterone levels are sexually dimorphic and are divergently altered in male and female mice exposed to early adolescent AcSD

Many studies have demonstrated a prominent interaction between gonadal and HPA hormones which makes it critical to consider corticosterone when looking at the developmental trajectories of adolescent male and female mice. As reported in other rodent studies, we have corroborated that female mice have much higher normative levels of corticosterone in comparison to males both during adolescence (Martínez-Mota et al., 2011; McCormick et al., 2005) and during adulthood (Weinstock et al., 1998) which could potentially explain the many sexually dimorphisms outlined throughout this thesis. Overall, it seems that although females displayed much higher levels of corticosterone during normative development compared to males, only males displayed corticosterone elevations in response to AcSD in adolescence. This intriguing finding leads us to question whether the high basal level of female corticosterone makes it such that no experiences could further increase such levels via a ceiling effect (Kokras et al., 2019).

Additionally, we did not find differences in the levels of testosterone in males nor in female progesterone levels measured in the follicular hair analysis between the AcSD and control groups. Perhaps repeating this experiment with AcSD during a mid-adolescent period when the activation effects of sex hormones ramp up, would allow us to better address the interaction between these two systems. Many studies have found that testosterone inhibits the HPA axis (Handa et al., 1994), while estrogens have an opposing stimulatory effect due to the estrogen receptor's role in blunting the normal negative feedback regulation of the HPA axis' corticosterone secretion (Weiser & Handa, 2009). Such changes in the female stress response have even been noted in the estrous cycle whereby basal corticosterone is much higher at periods of the cycle that report higher estrogen levels (Carey et al., 1995). While many studies convey that estrogens can protect adolescent females from anxiety-like behaviours after social stress (McCormick et al., 2008), many groups show the same trade off we report whereby estrogens contribute to the impaired PFC functioning during stress (Shansky et al., 2004). It is clear that the opposing effects sex hormones have on the HPA axis influence the effects of social stress in a sex-specific manner and may be an important mechanism in understanding the human sex-differences that exist in onset and prevalence of many psychiatric disorders.

Future Directions

While these findings have served as great insight into the sex and age dependent mechanisms responsible in AcSD induced perturbations of the developing dopamine system, many questions remain to be addressed. We have shown that the phenomenon of axonal mistargeting occurs in dopamine axons whereby AcSD in adolescence induces changes to the correct target that DA axons normally grow to. In males, it seems that the coordinated effects of DCC and Netrin-1 may explain the mechanisms of axonal mistargeting as well as explain the deficits in inhibitory control however, functional analyses must be conducted to assess whether these proteins are causally implicated in this pathway (Vassilev et al., 2021). Additionally, these guidance cues are not at all altered in females (Pantoja-Urbán, Richer, et al., 2023), yet we observe the opposite trend whereby AcSD induces less axons rerouting to the mPFC. This finding highlights that

other guidance cues must be responsible in dictating axonal growth including the potentially critical UNC5C receptor which repels axons from Netrin-1. Moreover, studying whether postsynaptic changes in dopamine axons (ie: spine density in the MSN) during adolescence can help further explain mechanisms of rerouting would be very interesting to understand.

Lastly, reproducing all studies (axonal mistargeting, guidance cue expression levels and follicular hair hormones) with AcSD at a mid-adolescent time point will be crucial to observe the effects that puberty and gonadal hormones have on the developing DA system. The gonadectomising of male and female mice to blunt the antinational effects of sex-hormones will provide many insights into the interaction between sex and the response of the HPA axis.

Conclusion and Expected Contributions

The experiments presented throughout this study have been essential in shaping our understanding of male and female dopaminergic development and the possible mechanisms that could be at play in orchestrating its growth to the right targets. The first objective of this study was to validate the robustness of the AcSD model in assessing the impacts of social stress in females. To our knowledge, this is the first model used to study social defeat stress in early adolescent female C57BL/6 mice. Using this model, our axonal tracking viral strategy showed that males and females differed in the number of fibers that grew to the mPFC. Moreover, early adolescent AcSD produced sexually dimorphic outcomes whereby resilient males showed more axons in the mPFC because of adolescent social stress whereas AcSD females showed less fibers reaching the cortex. Lastly, we started to explore the potential effects of stress and gonadal hormones and how they may be important players in the stress response. We found that as a result of AcSD, corticosterone levels differed in a sex-dependent manner with males displaying elevated corticosterone immediately after AcSD as well as in adulthood when compared to controls. Interestingly, this is not seen in females however, levels correlated significantly with social avoidance behavior.

Adolescence is a highly vulnerable period for developing mental health trajectories. Psychiatric susceptibility to experiences such as stress is heightened during this developmental window and social stress during adolescence increases vulnerability to psychopathology. Studies pertaining to this age group can help us discover the mechanisms of early life vulnerability and can help shape early intervention treatment for patients suffering from psychiatric illness. This is the first demonstration that exposure to physical/psychosocial harm in adolescence can deviate DA axons from their intended target, inducing their input into off-target regions, and likely altering adult cognitive processing. Most importantly, this project studies the many sex differences that arise as a result of social defeat stress in rodents and uncovers potential cellular underpinnings critical to help prevent, protect and treat both male and female youth form the harmful effects of peer victimization at this vulnerable age.

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